



## Development of novel gene carrier using modified nano hydroxyapatite derived from equine bone for osteogenic differentiation of dental pulp stem cells

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### ABSTRACT

Hydroxyapatite (HA) is a representative substance that induces bone regeneration. Our research team extracted nanohydroxyapatite (EH) from natural resources, especially equine bones, and developed it as a molecular biological tool. Polyethylenimine (PEI) was used to coat the EH to develop a gene carrier. To verify that PEI is well coated in the EH, we first observed the morphology and dispersity of PEI-coated EH (pEH) by electron microscopy. The pEH particles were well distributed, while only the EH particles were not distributed and aggregated. Then, the existence of nitrogen elements of PEI on the surface of the pEH was confirmed by EDS, calcium concentration measurement and fourier transform infrared spectroscopy (FT-IR). Additionally, the pEH was confirmed to have a more positive charge than the 25 kD PEI by comparing the zeta potentials. As a result of pGL3 transfection, pEH was better able to transport genes to cells than 25 kD PEI. After verification as a gene carrier for pEH, we induced osteogenic differentiation of DPSCs by loading the BMP-2 gene in pEH (BMP-2/pEH) and delivering it to the cells. As a result, it was confirmed that osteogenic differentiation was promoted by showing that the expression of osteopontin (OPN), osteocalcin (OCN), and runt-related transcription factor 2 (RUNX2) was significantly increased in the group treated with BMP-2/pEH.

In conclusion, we have not only developed a novel nonviral gene carrier that is better performing and less toxic than 25 kD PEI by modifying natural HA (the agricultural byproduct) but also proved that bone differentiation can be effectively promoted by delivering BMP-2 with pEH to stem cells.

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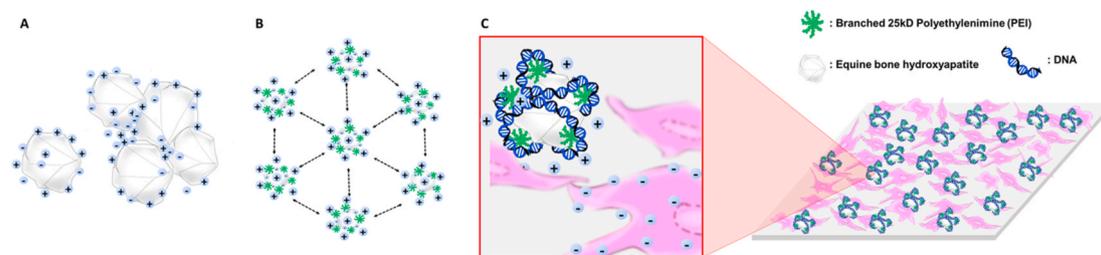
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**Fig. 1.** Strategy of this study. (A) Hydroxyapatite, which is derived from equine bone, tends to collect together in water. (B) By coating the equine bone with PEI, each particle is well distributed. (C) PEI-coated equine hydroxyapatite (pEH) holds genes and makes it easier to enter cells.

## 1. Introduction

Hydroxyapatite (HA) is a mineral substance made of calcium phosphate and is a major component of bone [1,2]. This material has been studied in tissue engineering for bone regeneration [3–7]. HA can synthesize a combination of calcium and phosphate molecules, or it can be obtained from natural sources. In a previous study, our research team showed no significant difference between synthetic and natural HA from the bones of a porcine or equine [8,9]. In general, HA is used as a material for bone regeneration, but we tried to modify natural HA to a gene carrier, a molecular biological tool, and at the same time tried to promote bone differentiation using the novel carrier with a specific gene for osteogenic differentiation.

The methods of gene transfection are usually categorized as using a viral vector or using a nonviral vector [10]. Viral vectors are more effective in introducing genes than other methods. However, there is a potential danger of using the virus, which can cause unintended immune responses or unwanted gene expression [11]. Therefore, many researchers are studying ways to replace viral methods. There are two main ways to use nonviral methods. One is physical methods, such as electroporation, electrospray, sonoporation, laser and gene gun [12–17]. In such a method, a physical impact is applied to the outside of the cell so that the gene is introduced. Another method is chemical methods. Calcium phosphate, cationic lipids and cationic polymers, such as polyamidoamine (PAMAM), polyethylenimine (PEI), dendrimers and poly(L-lysine) (PLL), are widely used as typical chemical methods for gene transfection [18–26]. These materials can be combined with or contain the target DNA and pass through the cell membrane. These methods have the advantage of low potential risk, but they have the disadvantage that the gene transfer efficiency is low. Therefore, many research teams are developing a nonviral carrier that is efficient in delivering genes to cells [27–31].

Several growth factors, such as bone morphogenetic protein 2 (BMP-2), transforming growth factor beta 1 (TGF- $\beta$ 1), and basic fibroblast growth factor (bFGF), have been studied as important factors in promoting bone regeneration [32,33]. Among them, BMP-2, which was discovered by Urist in 1965, was approved by the U.S. Food and Drug Administration and proved to have an excellent effect on bone regeneration [34,35]. Thus, many studies have been conducted on the treatment of bone defects using BMP-2 protein [36–38]. In addition, gene therapy for bone regeneration by delivering the BMP-2 gene to the bone defect site has also been studied. As a result, recovery of the bone defect was observed [39–42]. Researchers have used cationic polymers, adenoviruses, and plasmids to deliver the BMP-2 gene.

In this study, we attempted not only to develop a novel gene carrier using a complex of the most representative polymer, 25 kD polyethylenimine (PEI), and an equine bone extract, natural hydroxyapatite (EH) but also to induce osteogenic differentiation using a BMP-2 plasmid with our gene carrier. Fig. 1 shows the strategy of this study. EH powder is aggregated in the water and sunk because it has high crystallinity. However, we found that EH was well distributed when we coated it with PEI, which is used as a dispersant [43,44]. Thus, we hypothesized that the complex of EH coated with several PEI molecules would have a

stronger positive charge than PEI alone. If so, then the complex would be able to better capture and to deliver target genes than PEI. Furthermore, it was hypothesized that using this carrier to deliver the BMP-2 gene to stem cells could induce osteogenic differentiation more than using PEI.

First, we obtained nanoscale EH through several processes from equine bone, and then we obtained PEI-coated equine bone hydroxyapatite (pEH) through an ultrasonic vibration process with PEI and EH. The pEH was found to be well distributed in D.W by scanning electron microscopy (SEM) and transmission electron microscopy (TEM), and to be well coated with PEI by energy dispersive spectrometry (EDS), calcium concentration measurement and FRIR. For verification as a gene carrier, the zeta potential of pEH was measured, and the transfection efficiency was confirmed through luciferase expression. Furthermore, a GFP (green fluorescent protein)-tagged BMP-2 plasmid was delivered with various carriers to dental pulp stem cells (DPSCs). Finally, osteogenic differentiation was induced by delivering pEH with the BMP-2 plasmid to stem cells, and bone differentiation was confirmed through alizarin red staining and western blotting.

## 2. Materials and methods

### 2.1. Preparation of natural HA from equine bone

The general preparation process of making HA from animal bone was carried out in our previous study (Jang et al., Lim et al.). Equine bone originated from the slaughterhouse (Jeju, Republic of Korea), and it was initially cut into pieces of approximately 2–3 cm to be put into the crucible. Next, the bone pieces were soaked in distilled water for 48 hr to drain blood, and the surface flesh was cut out. These equine bone samples were immersed in H<sub>2</sub>O<sub>2</sub> (#7722-84-81, Duksan Chemicals Co., Korea) for 48 hr and dried. After drying, the horse bone was sintered in an electric furnace (UP350E, Yokogawa Co, Japan) at 900 °C for 2 h. Then, the sintered bone was ground into powder using a miller (A10, IKA, Germany), and the fabricated equine bone powders were sintered twice as described before. The sample powder was classified by particle size using 5  $\mu$ m sieves (Daihan Scientific, Korea). Then, to obtain nanoscale particles derived from equine bone, a high-energy ball mill, Planetary Mono Mill PULVERISSETTE 6 classic line (Fritsch, Germany), was used.

### 2.2. Characteristics of natural HA from equine bone

By XRD and XRF, the chemical composition of synthetic HA (289396, Sigma-Aldrich, USA) and natural EH from equine bone was compared and analyzed.

The XRD patterns were recorded with a powder X-ray diffractometer (D8 ADVANCE with DAVINCI, Bruker, Germany) with Cu-K $\alpha$  radiation ( $\lambda = 0.154$  nm) at 40 kV and 40 mA. Scans were obtained from 20 to 60° 2 $\theta$  in 1°/min. The crystalline phase compositions were identified with reference to standard joint committee on powder diffraction standard (JCPDS) cards available in the system software. XRF (S4 PIONEER, Bruker AXS, Germany) was used to obtain the elemental chemical composition of synthetic HA and EH from equine bone.

### 2.3. Preparation of pEH

After mixing EH and 1.3 kD branched PEI (482595-100 ML, Sigma-Aldrich, USA) or 25 kD branched PEI (408727-100 ML, Sigma-Aldrich, USA) at 1:1, 1:2, 1:5, 1:10, 1:50, and 1:100 concentration ratios (w/w), sonication treatment was performed for 30 min using a probe ultrasonic processor (VCX130, Sonics & Materials Inc., USA). Each mixture was dialyzed with a Spectra/Por membrane (MW cutoff 25 kD) against distilled water at 4 °C for 24 hr to remove the remaining PEI.

### 2.4. Comparison of characteristics of EH and pEH

The dispersity and particle sizes of EH and pEH fabricated in this study were observed using a field emission scanning electron microscope (FE-SEM; SUPRA 55 VP, Carl Zeiss, Germany). All samples were coated using a BALTEC SCD005 sputter coater for 250 s at 15 mA. The effect of the annealing temperature on the microstructural change of the samples was examined. Chemical analysis was also conducted using EDX spectroscopy at 30.0 kV. The morphology and size of each sample were confirmed at a high magnification by EF-TEM (LIBRA 120, Carl Zeiss, Germany). Each sample was loaded on a carbon grid, dried for 2 h, stained with 1% uranyl acetate for 10 s, washed with distilled water, and dried for an additional 10 min. Samples were then analyzed under an electron microscope.

### 2.5. Calcium concentration measurement

The calcium concentrations of the EH and pEH solutions were measured after sonication using a QuantiChrom™ Calcium Assay Kit (DICA-500, BioAssay Systems, USA). The detailed experimental process was performed according to the manual provided by the company.

### 2.6. Zeta potential measurement

Zetasizer Nano ZS (Malvern Instruments Ltd, UK) was used to measure the zeta potential of the EH, DNA and pEH complexes in comparison to the PEI 25 kD. Then, the results were analyzed using specific Malvern software (Zetasizer software 7.12, Malvern Instruments Ltd, UK).

### 2.7. Cell preparation

NIH-3T3 (ATCC, USA) cells were cultured in a T75 flask (NUNC, USA) for 2 days with Dulbecco's modified Eagle's medium (DMEM, Welgene Inc., Republic of Korea) containing antibiotics (Welgene Inc., Republic of Korea) and 10% fetal bovine serum (FBS, Welgene Inc., Republic of Korea). NIH-3T3 cells were seeded at a density of  $2 \times 10^6$  cells mL<sup>-1</sup>. DPSCs (Intellectual Biointerface Engineering Center, Dental Research Institute, Seoul National University, SNUDH\_IRB No. CRI05004) were cultured in a 90 mm dish (NUNC, USA) for 2 days with  $\alpha$ -minimum essential medium ( $\alpha$ -MEM, Welgene Inc., Republic of Korea) containing 10% fetal bovine serum (FBS, Welgene Inc., Republic of Korea) and antibiotics (Welgene Inc., Republic of Korea). DPSCs were seeded at a density of  $1 \times 10^6$  cells mL<sup>-1</sup>. All cells were cultured at 37 °C and 5% CO<sub>2</sub> in an incubator (Steri-Cycle 370 Incubator, Thermo Fisher Scientific, USA).

### 2.8. Luciferase assay

Luciferase reporter, pGL3-vector with SV-40 promoter, and enhancer encoding firefly (*Photinus pyralis*) luciferase were obtained from Promega (Madison, WI, USA). pEH/pGL3 (4  $\mu$ g) polyplexes were used at 10, 15, 20, and 25 N/P ratios of 1:1, 1:2, 1:5, 1:10, 1:50, and 1:100 (w/w). After transfection, cells were cultured for 48 hr in a CO<sub>2</sub> incubator. The luciferase assay was then performed according to the manufacturer's protocol. A multiple plate reader (Victor3, PerkinElmer, USA)

was used to measure relative light units (RLUs) (normalized by protein concentration) in the cell extract estimated using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA).

### 2.9. Cell viability assay

Prior to the viability assay, NIH 3T3 cells and DPSCs were seeded on 24-well plates at a density of  $5 \times 10^4$  cells per well. After 24 hr, complexes of pEH and 25 kD PEI with DNA (4  $\mu$ g) at a 20 N/P ratio were added to each well with media without FBS. After 3 h, the medium without FBS was changed to fresh medium with FBS. Cell viability was measured by WST-1 (EZ3000, Daeilab Service Co., Korea) assays at 3 days following cell seeding.

### 2.10. Induction of osteogenic differentiation by delivering BMP-2 plasmid/pEH

DPSCs were seeded on 24-well plates at a density of  $5 \times 10^4$  cells per well for the alizarin red staining assay and on 90 mm dishes at a density of  $1 \times 10^6$  cells on plates for the Western blot assay after osteogenic differentiation. The GFP-tagged bone morphogenetic protein 2 (BMP-2) plasmid was obtained from ORIGENE (USA). pEH/BMP-2 (4 g) polyplexes were used at a 20 N/P ratio. After transfection, cells were cultured for 48 hr in a CO<sub>2</sub> incubator. Then, the media were exchanged with proliferation media without osteoinductive components every two days for the next two weeks.

### 2.11. Alizarin red staining

Two weeks after transfection, the samples were washed twice with phosphate-buffered saline (PBS, Welgene Inc., Republic of Korea). Then, the samples were fixed at 4 °C for 1 h with 4% paraformaldehyde (Sigma, USA) and washed again with PBS 3 times. Next, samples were stained with an alizarin red S solution (Sigma, USA) at room temperature for 30 min and washed 3 times with D. W, and observed.

### 2.12. Western blot

Two weeks after transfection, cells were harvested and lysed in 1x RIPA soluble buffer (Millipore, USA). Protein concentrations were measured using the BCA protein assay kit (Thermo Fisher Scientific, USA). The same amount of protein (25 mg) from each sample was separated by a Novex NuPAGE 4–12% SDS-PAGE gel (Life Technologies, USA), transferred to nitrocellulose membranes using an iBlot Gel Transfer Device (Invitrogen, USA), and blocked with 5% skim milk for 1 h at room temperature. The membrane was washed with 1X PBST and treated with antibodies (at a 1:1000 dilution) against RUNX2 (Abcam, UK), OCN (Abcam, UK), OPN (Abcam, UK) and  $\beta$ -actin (AbFrontier, Seoul, Korea) overnight. The membrane was then incubated with a secondary antibody (at a 1:2000 dilution) conjugated to horseradish peroxidase (HRP) (Invitrogen, USA). Protein bands were visualized using the ChemiDoc XRS + Imaging System (Bio-Rad, USA). The intensity of each band was quantitatively analyzed using Image J software (NIH, USA), and the average pixel values were plotted.

### 2.13. Statistical data analysis

The statistical analysis was performed using R v3.2.1 software (The R Foundation, <http://www.r-project.org>). The least significant difference (LSD) method, Duncan's test, and one-way ANOVA were used to compare the property means of the samples. The level of significance was  $p < 0.05$ . The data are reported as the mean  $\pm$  standard deviation,  $n = 3$  or  $n = 5$ .

**Table 1**

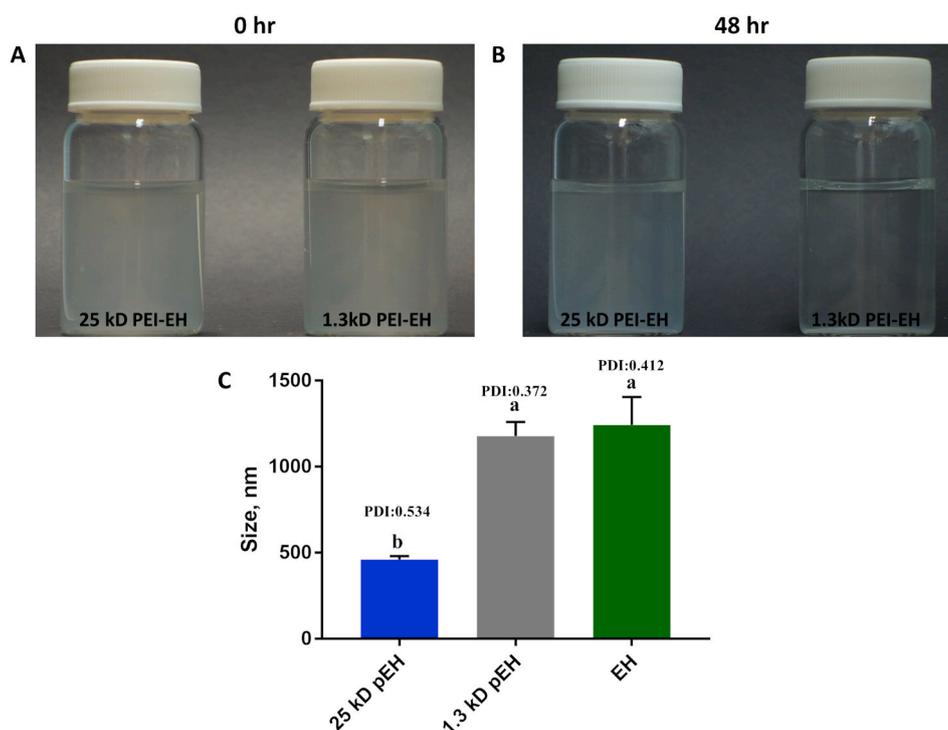
Elemental composition of synthetic hydroxyapatite and natural hydroxyapatite derived from Equine bone.

Formula	Concentration(%)	
	Hydroxyapatite	EH
Ca	38.11	42.29
O	40.7	39.2
P	19.24	16.72
Others	1.95	1.79

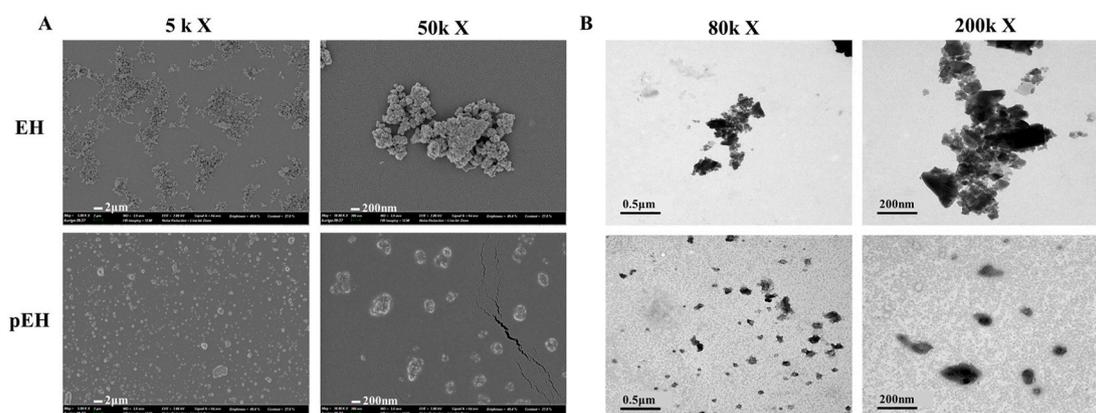
### 3. Results & discussion

#### 3.1. Characteristics of pEH

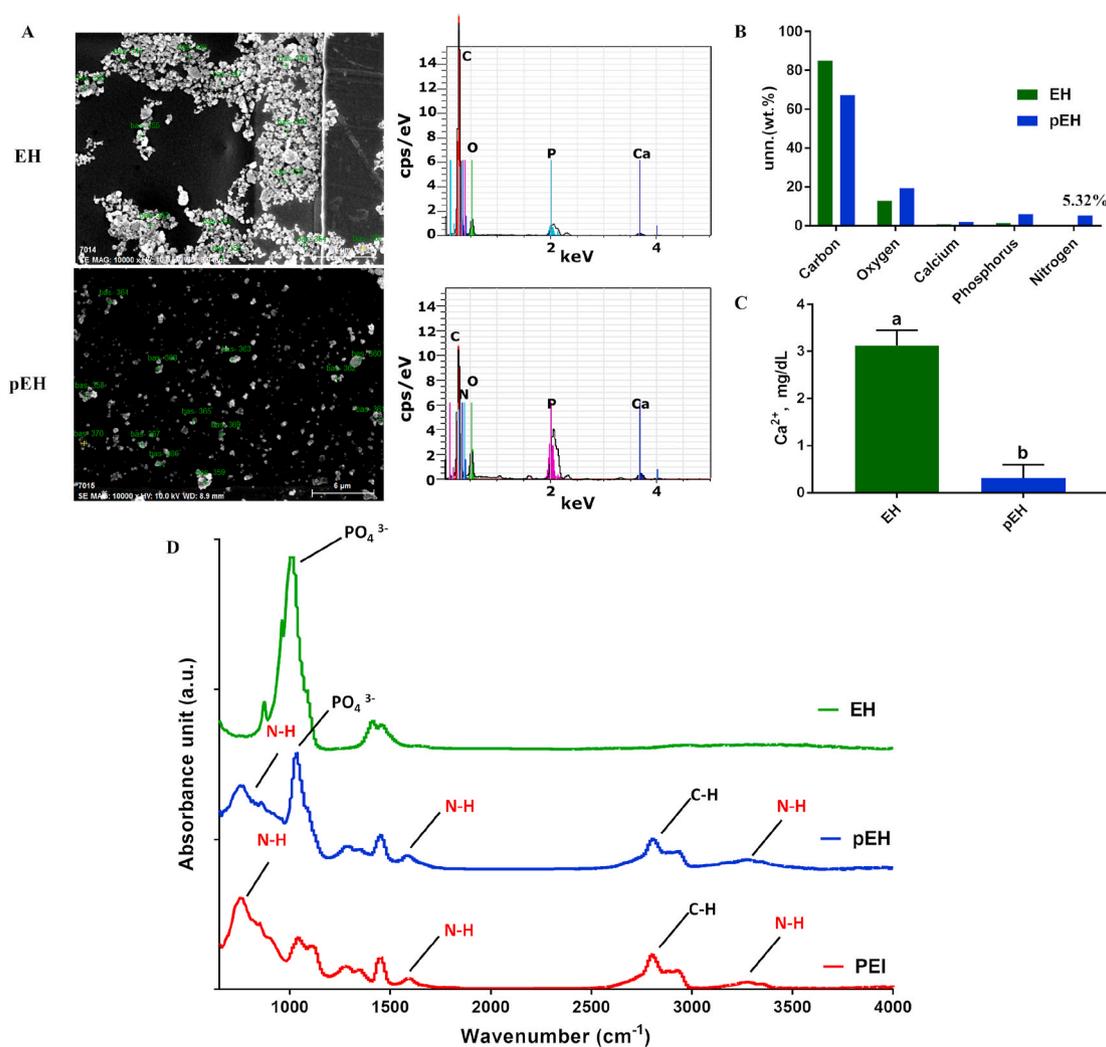
We extracted EH from equine bone by previous studies. The composition of EH we obtained was not different compared to those of synthetic HA by XRF and XRD (Table 1, Fig. S1). Similar to synthetic HA, the composition of EH derived from equine bone consists of calcium and phosphate groups. Therefore, EH in the water is aggregated and sunk because of the charge of calcium and phosphate groups. We assumed to find a different application of positively charged EH as distributing each particle well if we could coat nanoscale EH with the cationic polymer PEI. The process of coating the EH with PEI is simple. First, the EH was functionalized noncovalently with two types of PEI (1.3 kD and 25 kD branched PEI) by sonication. Then, 25 kD PEI-coated EH was well



**Fig. 2.** Observation of the dispersivity of 25 kD PEI-coated EH and 1.3 kD PEI-coated EH. (A, B) Comparison of the dispersivity of 25 kD PEI-EH and 1.3 kD PEI-EH at 0 hr and 48 hr. (C) The particle size of 25 kD PEI-EH and 1.3 kD PEI-EH ( $n = 3$ ,  $p < 0.05$ . Columns with different letters are significantly different according to one-way ANOVA and Duncan's test). The results showed that the EHs coated with 25 kD PEI were well dispersed into smaller sizes.



**Fig. 3.** Image analysis of electron microscopes. (A) Scanning electron microscopy images of EH and pEH (scale bars = 2 μm and 200 nm). (B) Transmission electron microscopy images of EH and pEH (scale bars = 0.5 μm and 200 nm). These images show a noticeable difference in the particle size of the EH and pEH. In the case of pEH, most particles are less than 500 nm in size, and many smaller than 200 nm have been found. However, most particles are clustered together in the case of EH.



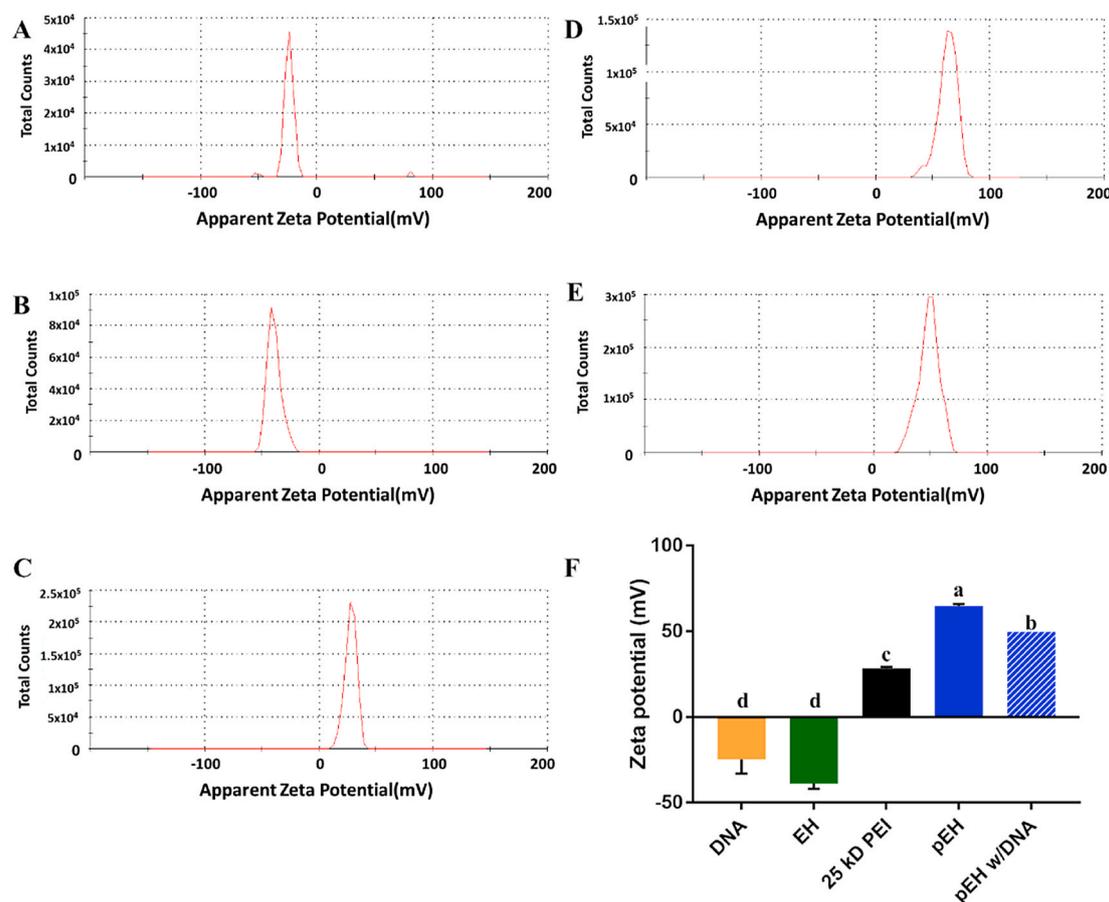
**Fig. 4.** Chemical composition analysis of EH and pEH. (A) The results of energy dispersive spectrometry (EDS) for EH and pEH. (B) The plot of elemental content inside EH and pEH. Although no nitrogen was detected on the surface of EH, it was detected on the surface of pEH (5.32%). (C) Calcium concentration comparison of EH and pEH. The calcium ion elution of pEH was significantly less than that of EH. ( $n = 5$ ,  $p < 0.05$ . Columns with different letters are significantly different according to one-way ANOVA and Duncan's test). (D) FT-IR analysis of EH, 25 kD PEI and pEH. These results also showed that pEH had amine groups. This indirectly showed that pEH was well coated with 25 kD PEI.

dispersed in D. W over time while most 1.3 kD PEI-treated EH settled down in D. W (Fig. 2A and B). Additionally, comparing the particle size of each group, the EH coated with 1.3 kD PEI had little difference in size from only the EH. However, the particle size of EH coated with 25 kD PEI was greatly reduced (Fig. 2C). Thus, we confirmed that EH can be coated with a cation polymer and selected 25 kD branched PEI to disperse EH. Then, we observed pEH by electron microscopy (SEM and TEM) (Fig. 3). The results were very interesting. Fig. 3A shows the SEM results, and Fig. 3B shows the TEM results for EH and pEH. The nanolevel EH that was treated with sonication was not distributed at all and was aggregated. However, pEH was not only well distributed but also showed that most of them were less than 500 nm in size. Even pEH, which is less than 200 nm long, had been observed and seemed to be surrounded by something (Fig. 3B). Thus, we tried to ensure that 25 kD PEI was right to wrap up EH particles and to help them disperse. Three methods were used to confirm that process. The first method was EDS. It was assumed that nitrogen would exist on the surface if the EH was coated with 25 kD PEI with many amine groups. By the EDS results, nitrogen elements were not found on the surface of the EH. However, nitrogen elements (5.32%) were found on the surface of pEH (Fig. 4A and B). By this experiment, we confirmed that the EH was indirectly coated with 25 kD PEI. The second method was the measurement of calcium concentration changes after

sonication for each sample. Generally, a hydroxyapatite crystal structure has calcium ions inside and phosphate groups outside [45]. Therefore, if phosphate groups of the EH combined with amine groups of PEI, it would not only be possible to cover the entire EH particle but also calcium ions would not be released after sonication. Fig. 4C shows the results of the measurement of the released calcium ion concentration for the EH and pEH. There was a significant difference in the results for released calcium ions between the two groups. As expected, there was less calcium ion release in the pEH group. As a third method, Fourier transform infrared spectroscopy (FT-IR) data were obtained to confirm the composition of the samples (Fig. 4D). Compared with all samples, only pEH was found to have both  $\text{PO}_4^{3-}$  and amine functional groups. By these experiments, we confirmed that pEH was well coated with 25 kD PEI.

### 3.2. Verification of pEH as a gene carrier

When introducing genes into the cells using a cationic polymer, the proper positive charge of carriers is important to hold onto the negatively charged DNA and to pass the negatively charged cell wall. Then, we measured the zeta potential of EH and pEH in various combinations of concentrations (Fig. 5, Fig. S2). The results were very interesting. As



**Fig. 5.** Physicochemical characterization of pEH/DNA complexes. (A)–(E) zeta potential of several materials. (A) DNA ( $-24.6 \pm 8.3$  mV), (B) EH ( $-38.7 \pm 3.1$  mV), (C) 25 kD PEI ( $28.4 \pm 0.9$  mV), (D) pEH ( $64.7 \pm 1.2$  mV), and (E) pEH with DNA ( $49.7 \pm 2.5$  mV). (F) Quantitative analysis of zeta potential ( $n = 3$ , columns with different letters are significantly different, according to the Duncan test). Although EH alone has a negative charge, pEH has a stronger positive charge than 25 kD PEI only.

expected, only the gene and EH had negative charges, while the 25 kD PEI had positive charges. When EH existed alone, it had a stronger negative charge than the gene regardless of its concentration. However, EH coated with 25 kD PEI showed a much stronger positive charge than 25 kD PEI alone. The zeta potential difference between the two groups was approximately twice. The pEH combined with genes had a slightly lower zeta potential but still had a higher potential than 25 kD PEI. By these results, we assumed that pEH has more cationic branches than 25 kD PEI because the pEH is a complex of EH and several PEI polymers resulting in that a larger electrical field can be charged to it. We also wondered whether pEH with a stronger positive charge than 25 kD PEI would be able to deliver genes or to show better performance than 25 kD PEI.

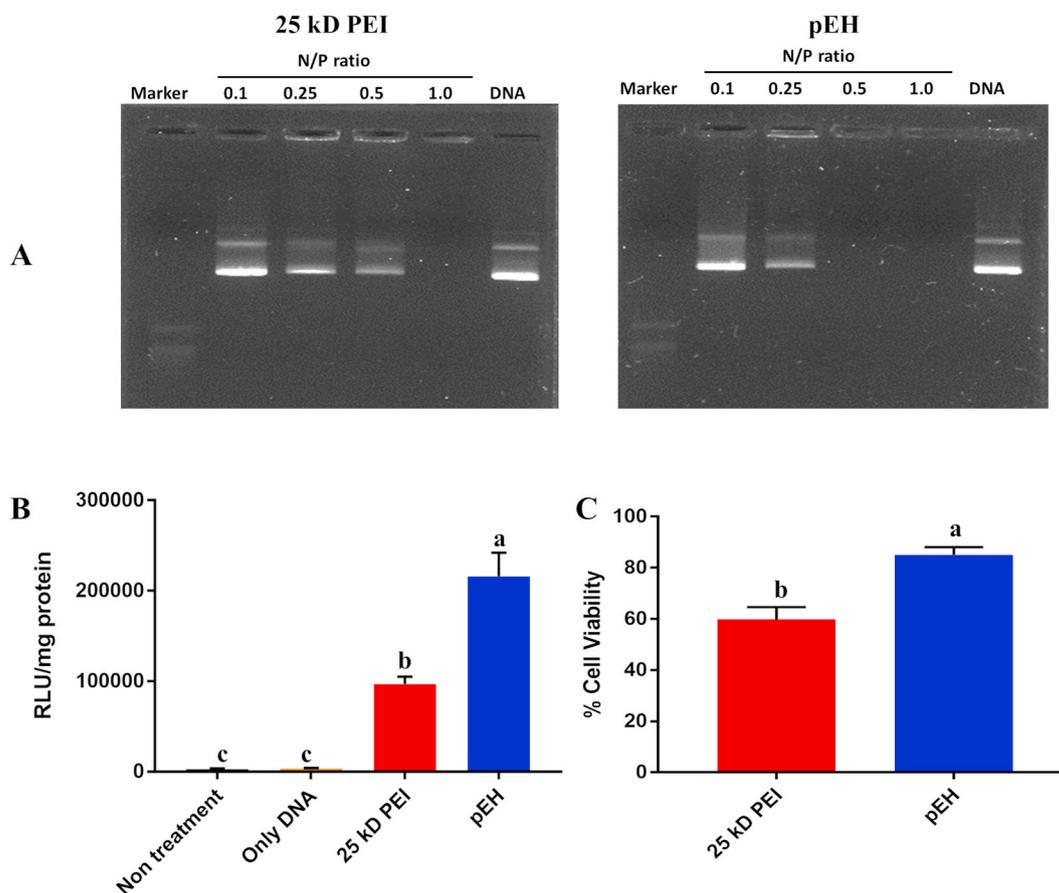
First, DNA retardation analysis was performed on an agarose gel at various N/P ratios to prove that pEH condenses DNA well to form a complex. As a result, it was shown that pEH formed a complex with DNA very well at a 0.5 N/P ratio and showed that it could form a complex better than 25 kD PEI (Fig. 6A). It means that the gene loading efficiency of pEH is higher than that of PEI.

Next, to confirm the gene transfer ability of pEH, a luciferase analysis method was used in NIH3t3 cells, which are a suitable transfection cell line [46]. Surprisingly, pEH showed excellent gene transfer capability compared to 25 kD PEI (Fig. S3). In the results of the luciferase assay, pEH, which consists of a 1:5 concentration ratio (EH:PEI), showed four times more efficiency than PEI in delivering genes. We also found one interesting thing. There is a similarity between the luciferase assay results and the zeta potential measurement results. Based on the results of the zeta potential measurement, the sample blended with EH and PEI at

a 1:5 concentration ratio (w/w) showed the highest potential. Similarly, the same group showed the highest gene expression in the results of the luciferase assay. Therefore, we confirmed that groups with high potential zeta potential showed a high gene transfer capability, and pEH, which blends HA and PEI at a 1:5 concentration ratio, had the best gene transfer capability at a 20 N/P ratio (Fig. S2, S3). After establishing the conditions for gene delivery using pEH, the luciferase assay was performed in DPSCs again. As a result, luciferase was expressed with almost twice the efficiency of 25 kD PEI in the group treated with pEH/pGL3 (Fig. 6B). Additionally, as a result of FACS analysis, the rate of transfected cells was 13.6% at pEH treated group and 8.16% at 25 kD PEI treated group (Fig. S4). Since pEH has more cationic branches than 25 kD PEI, it is expected that it can deliver more DNA at once. Fig. S5 showed that more pEH than the 25 kD PEI is attached to the cell wall or is moving closer to the nucleus.

Generally, the endocytosis occurs in four ways (micropinocytosis:  $\geq 1 \mu\text{m}$ , clathrin-dependent endocytosis:  $\sim 200$  nm, clathrin-independent endocytosis:  $\sim 90$  nm, caveolae-dependent endocytosis:  $\sim 60$  nm), depending on the size of the polyplex of cationic polymer and genes [57]. In the case of pEH, it is expected to occur in three pathways of endocytosis excluding caveolae-dependent endocytosis since the size is less than 500 nm on average.

Next, we compared the cytotoxicity of pEH and 25 kD PEI (Fig. 6C). The 25 kD PEI-treated group had more cytotoxicity than the group treated with pEH after transfection. Generally, the concentration is 1 mg/ml to deliver genes using 25 kD PEI. However, pEH contains 0.5 mg/ml PEI in the case of blending HA and PEI at a 1:5 concentration ratio. In other words, pEH consisted of a final concentration of 25 kD PEI that



**Fig. 6.** Results of transfection using pEH and cytotoxicity assays. (A) Gel retardation assay. (B) Luciferase assay results after transfection using pEH ( $n = 3$ , columns with different letters are significantly different, according to the Duncan test). (C) Cell viability analysis after treatment with 25 kD PEI and pEH ( $n = 5$ , columns with different letters are significantly different, according to the Duncan test). pEH has lower cytotoxicity to DPSCs and better gene transfection ability than 25 kD PEI.

made the toxicity half that of the control group. Thus, pEH is less toxic to cells than 25 kD PEI alone. These experiments suggested an interesting conclusion. That is, pEH has lower cytotoxicity to cells and better gene transfection ability than 25 kD PEI.

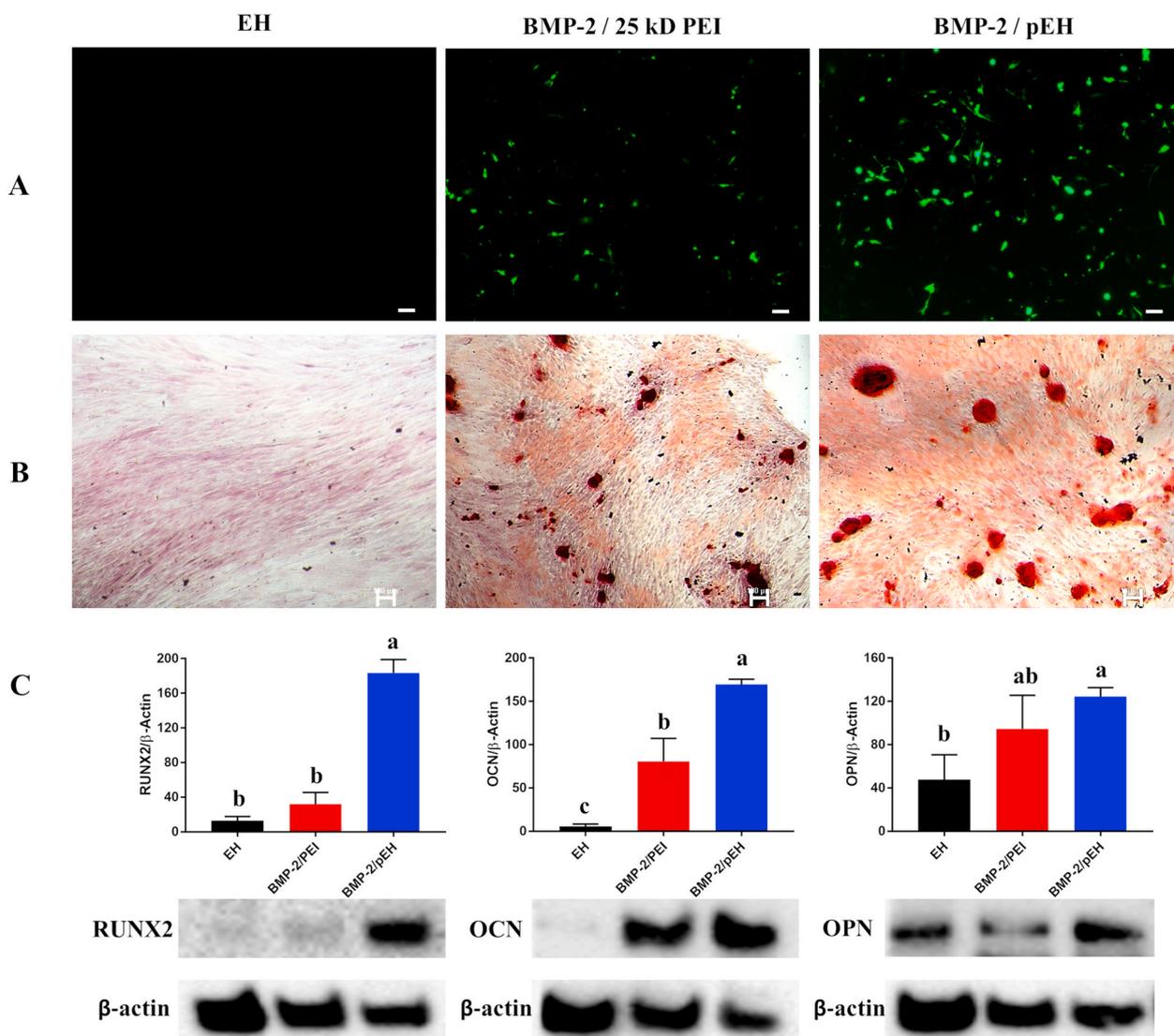
### 3.3. Induction of osteogenic differentiation using pEH with BMP-2 plasmid

After verification of pEH as a gene carrier, we analyzed whether osteogenic differentiation was induced when the BMP-2 gene was delivered to dental pulp stem cells (DPSCs). Fig. 7A shows the results after GFP-tagged BMP-2 plasmid/pEH delivery. The results showed that pEH delivered the BMP-2 gene much better to cells than 25 kD PEI. Two weeks after transfection, alizarin red staining analysis was performed. The cells in the BMP-2/pEH-treated group were most clearly stained red (Fig. 7B). To confirm more accurately, the expression of osteogenic differentiation markers was analyzed by Western blot. Osteopontin (OPN) and osteocalcin (OCN) are major markers expressed by osteoblasts when stem cells differentiate into bone cells [47,48]. Runt-related transcription factor 2 (RUNX2) is an important transcription factor associated with differentiation into osteoblasts [49], and its expression can be regulated by BMP-2 [50]. Surprisingly, in the BMP-2/pEH-treated group, RUNX2 increased by more than 170%, OCN increased by more than 160%, and OPN increased by more than 70% compared with the EH-treated group. In addition, every expressed marker of the BMP-2/pEH-treated group was significantly higher than that of the BMP-2/25 kD PEI-treated group (Fig. 7C). In particular, delivery of BMP-2 plasmid using pEH showed a difference of approximately 4 times in the amount of expression of RUNX2 compared to when

using 25kD PEI. This means that the BMP-2 protein was increased by BMP-2/pEH, and as a result, the expression of RUNX2 was induced, and other markers induced by RUNX2 were serially expressed.

Through these results, it was confirmed that when the BMP-2 gene was delivered to stem cells using pEH, osteogenic differentiation was very well induced.

Our research has two important meanings. First, it was the first time that our team developed modified natural HA from equine bone into a nonviral gene carrier. Several research teams have reported the development of modified synthetic HA for gene delivery. Dr. Wang reported that chitosan-coated hydroxyapatite (CS-Hap) is better than lipofectamine in delivering genes [51]. Khan et al. showed that poly(L-lysine)-coated calcium phosphate has a positive effect on transfection [52]. Sun's team used 10 kD PEI-coated synthetic HA to deliver genes to neuronal cells [53]. In addition, gene delivery was attempted by HA itself to cancer cells [54] or by glucose-decorated HA to endothelial cells [55] and using calcium phosphate coated with strontium to bone cells [56]. However, we are the only ones to create a nonviral gene carrier using natural nano HA, which has been made from the bones of living creatures. Just as each team's carrier showed better transfection efficiency compared to products (Lipofectamine, Jet Prime, and PEI), we showed that pEH was almost twice as effective as the positive control, 25 kD PEI, representative of the cations polymer for gene delivery, and which is less toxic to cells than it. Of course, it is difficult to make the size of pEH completely uniform. Thus, we will conduct additional research to compensate for these shortcomings. Second, we showed the possibility of gene therapy for bone regeneration using a novel gene carrier with a BMP-2 plasmid. The pEH is a superior gene carrier than 25 kD PEI. It induced osteogenic differentiation by delivering the BMP-2 plasmid to



**Fig. 7.** Osteogenic differentiation using the BMP-2 plasmid loaded with pEH. (A) GFP-tagged BMP-2 expression results after transfection using pEH (scale bars = 100  $\mu$ m). (B) Alizarin red staining results after 2 weeks of BMP-2/pEH treatment. (scale bars = 100  $\mu$ m). (C) Western blot results after 2 weeks of BMP-2/pEH treatment (n = 3, columns with different letters are significantly different, according to Duncan's test). The BMP-2/pEH treatment group induced osteogenic differentiation better than the other groups.

DPSCs without the aid of bone differentiation medium. BMP-2 plasmid delivered to stem cells promoted signaling downstream of osteogenic differentiation. In addition, a core of pEH, hydroxyapatite, which is well known to promote bone differentiation, is expected to have helped osteogenic differentiation. Thus, pEH could be used as an optimal gene carrier to induce osteogenic differentiation of stem cells and expected to be applicable to in vivo studies.

#### 4. Conclusion

In summary, we developed a novel nonviral gene carrier using natural HA from animal bones. When coating EH with 25 kD branched PEI, it was observed that the particles were well distributed in D. W without sinking. The results of electron microscopy observations showed that each particle of pEH is well distributed and not more than 500 nm in size. In addition, we indirectly confirmed that the surface of pEH has a 25 kD PEI by EDS and calcium ion concentration measurements. Next, we confirmed that pEH, which consists of a 1:5 concentration ratio (EH: PEI), had a higher positive charge and was not only more efficient in gene transfer than 25 kD PEI but also the cytotoxicity of pEH was lower than 25 kD PEI. Finally, by delivering the BMP-2 plasmid/pEH to stem

cells, it was proven that bone differentiation was promoted very well.

Our developed carrier has the advantage of recycling discarded agricultural byproducts. In addition, we hope that applied research to new fields using livestock waste that is being discarded will be conducted more actively. Our work is the first study of a gene delivery system using modified hydroxyapatite derived from equine bone, and it is a very innovative study to induce bone differentiation using this approach. Our team will try tooth regeneration using this system that successfully induces bone differentiation by DPSCs, and plans to study deeply the use of new renewable biomaterials.

#### CRediT authorship contribution statement

**Myung Chul Lee:** Conceptualization, Investigation, Methodology, Formal analysis, Data curation, Writing - original draft. **Hoon Seonwoo:** Conceptualization, Methodology, Writing - review & editing, Funding acquisition. **Kyoung Je Jang:** Conceptualization, Resources, Validation. **Shambhavi Pandey:** Data curation, Investigation. **Jaewoon Lim:** Investigation, Visualization. **Sangbae Park:** Investigation, Data curation. **Jae Eun Kim:** Investigation, Data curation. **Yun-Hoon Chung:** Supervision, Writing - review & editing. **Pankaj Garg:** Supervision,

Writing - review & editing, Funding acquisition. **Jong Hoon Chung:** Supervision, Writing - review & editing, Funding acquisition.

### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioactmat.2021.01.020>.

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