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**Research Article**

# INCORPORATION OF A BMP-2 DELIVERY SYSTEM INTOCHITOSAN-BASED SCAFFOLDS FOR OSTEOGENESIS AND BONE HEALING

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**ABSTRACT**

It is advantageous to incorporate controlled growth factor delivery into tissue engineering strategies. The aim of this study was to develop a 3-D construct carrying an inherent sequential growth factor delivery system.BMP-2 was encapsulated in silk chitosan particles that were produced by a simple and very mild processing method. The dose–response of BMP-2-loaded chitosan particles was examined in C2C12 cells, after5 days of culture. The BMP-2 retained most of its activity as observed by the increase in alkaline phosphatase activity, which was much higher when BMP-2 was encapsulated into the particles rather than just surface adsorbed. After 2 weeks of culture, increased mineralization was observed with BMP-2-loaded particles in comparison to soluble added growth factor. No significant cytotoxicity was detected. When implanted in a ratectopic model, bone formation was observed by in vivo micro-computed tomography after 2 and 4 weeks post-implantation, with particles loaded with 5 mg BMP-2. An increase in bone density was observed over time. Our findings show that chitosanmicroparticles may present an interesting option for future clinical applications in the bone tissue engineering field.

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## INTRODUCTION

Various bioactive factors that induce chemotaxis, proliferation, differentiation and matrix synthesis are essential in natural tissue/organ development and wound healing.[1] Owing to the rapid advances in recombinant technology and the availability of large scale manufacturing of cytokines and growth factors, many recent tissue engineering strategies have turned to the use of specific growth factors to stimulate cellular activity in vitro and for improved functional new tissue formation in vivo.[2] Importantly, to achieve the optimal biological function of these factors, it is highly desired to develop anappropriate delivery system.

Bone morphogenetic proteins (BMPs) are a group of cytokines from the transforming growth factor-beta (TGF-b) superfamily, which have been used as power fulosteo-inductive components in several late-stage tissue engineering products for bone grafting.[3-5]Currently, two devices using BMPs have been approved by the Food and Drug Administration for human clinical application based on the use of collagen sponges.[6] However, as collagen poses several drawbacks such as suboptimal handling conditions and the risk of disease transmission, there is still a need for an optimized BMP delivery system.

The search for efficient, simple, and cheap delivery systems for drug targeting has lead to a great investment in the area of nano- and micro particles for drug delivery. In tissue engineering, these systems are excellent choices for growth factor delivery, because they can be easily prepared and sterilized. They can be processed into inject able systems allowing an easy and noninvasive implantation in thepatient.[7]

Diverse synthetic and natural origin polymers have been suggested as alternatives for the delivery of BMPs in bone tissue engineering. Chitosan(CS) is a hydrophilic polysaccharide, which has been widely used for the controlled delivery of polypeptides and proteins in the format of microspheres or nano/microspheres.[8] The release kinetics can be modulated by adjusting the factor loading amount,[9] chitosan molecular weight and degree of deacetylation,[10] and preparationmethods.[11] Notably, the bioactivity of released factors can be largelymaintained during the encapsulation process and upon release.[12]

In this study, we examined the activity of BMP-2 released from Chitosannano/microparticles in vitro in C2C12 cells, by quantification of the alkaline phosphatase (ALP) activity and calciummineralization, and in a rat ectopic bone formation model, using microcomputer tomography (mCT) and histological analysis

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## MATERIALS AND METHODS

### *Materials*

Low molecular weight chitosan (deacetylation degree 90.85%, i.v. 185 cps for1% in 1% acetic acid) was purchased from Aldrich Sigma (USA). Recombinant human BMP-2 from Ray Biotech (USA) were used as the growth factors. For cell experiments, Dulbecco's modified Eagle medium (DMEM), fetalbovine serum (FBS), trypsin/EDTA, penicillin/ streptomycin (P/S), and phosphatebuffered saline (PBS, pH 7.4) were purchased from Gibco BRL (Carlsbad, CA, USA).Cell Counting kit-8 (CCK-8) was purchased from Dojindo Co. Ltd (Kumamoto, Japan). Alkaline phosphatase (ALP) Lab Assay kit was purchased from Wako Chemicals Inc.(USA). PKH26 red-fluorescent cell linker mini kit was obtained from AldrichSigma. Balb/c mice and Sprague dawley (SD) rats were supplied from Nara Biotech (Seoul, Korea).

### *Preparation of BMP-2 loaded nanocapsules*

Chitosan microspheres were fabricated utilizing an established emulsion-ionic cross-linking technique as described previously.[13] Briefly, 250 mg of chitosan was dissolved in 10 mL of 2% (v/v) aqueousacetic acid solution till the solution was transparent. 10 mg of BMP-2 was then added to theabove solution. The mixture was dropped into 100 mL of liquid paraffin containing 2% (w/v) of surfactant span 80 and agitated mechanically for2 h at room temperature. 25 mL of 5% (w/v) tripolyphosphate (TPP) solution was further added drop wise to the emulsion and stirred for another 2 h to stabilize the microspheres through the electrostatic interaction with TPP. The microspheres, precipitated in the mixturesolvent, were repeatedly washed with excess amounts of petroleum ether and isopropyl alcohol, prior to lyophilization.

### *Bioactivity in C2C12 cells*

C2C12 cells have been used for screening the osteogenic activity of BMPs in a variety ofworks.[14, 15] These cells do not express significant amounts of endogenous BMPs, thus making them an effective model for testing the activity of the released BMP-2. C2C12 cells were seeded at $10^5$ cells/mL per well in a 24-well plate, attached in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1% (v/v) fetal bovine serum, at 37℃ and 5% $CO_2$, in a humidified environment. The cells were incubated with unloaded particles or with particles containing BMP-2. BMP-2 was added as apositive control to the culture medium at 0.1 mg/mL. As another control, unloaded particles were also added to the cells; these particles were added in the same volumes as the particles with encapsulated growth factor were added to the cultures.

### *Alkaline phosphatase activity assay*

ALP enzymatic activity was measured according to standard procedures, after 5 days in C2C12 cultures. The activity recovery of the encapsulated BMP-2 was estimated by comparing the ALP activity induced by a specific amount of growth factor released from the chitosan particles to the activity induced by the same amount of growth factor added t the culture medium as a positive control.

### *Micro-computed tomography*

Mean porosity and porosity distribution of the 3-D scaffolds were assessed by using micro-computed tomography (m-CT 20, SCANCO Medicals, Switzerland). Scanner settings were 40 keV and 248 mA. Entire scaffolds were scanned in slices of7 mm thickness. CT Analyser and CT Vol Realistic 3-D Visualization (SkyScan, Belgium) softwares were used for image processing in CT reconstructions, and increation and visualization of the 3-D representations.

### *In situ release studies*

Release from the particle incorporated constructs was simulated by using BSA as a model molecule to represent growth factors. Protein release was determined spectrophotometrically by using Coomassie Plus Assay (Pierce, USA).

### *Scanning electron microscopy*

The morphology of the obtained chitosan microspheres was examined by a scanning electron microscopy (SEM). For the measurement, the microspheres were dispersed in ethylalcohol, and the dispersion was dropped on aluminum foil and dried atambient atmosphere. The different freeze-dried scaffolds were fractured in liquid nitrogen using a razor blade. Then, the samples were attached to metal stubs, sputter coated with gold under vacuum, and observed using a LEO Gemini 1530 Field Emission Gun SEM (Germany).

### *Statistical analysis*

Experiments were run in triplicate per sample and all data were expressed as means±standard deviation (SD). Statistical analyses of the data were performed using student t-test. The difference was regarded statistically significant when pb0.05.

## RESULTS

### *Characterization of chitosan microspheres*

The chitosan microspheres loaded with BSA (a model protein) and BMP-2 were prepared by the emulsion method in the presence of TPP, carrying five negative charges which allows the electrostatic interaction with protonated chitosan in anaqueous acidic solution.[13] The microspheres were spherical inshape, and had a regular surface without any cracks (Fig. 1).

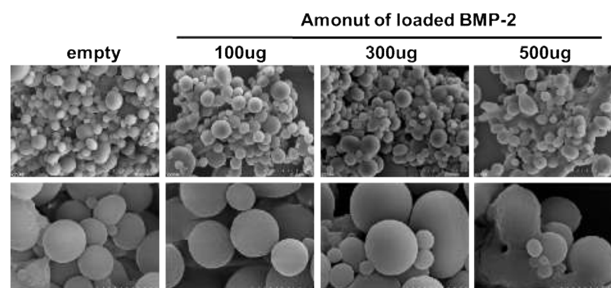


**Fig 1** SEM micrographs of chitosan microspheres loaded with BMP-2 with different protein contents.

The particle size distribution was in the range of 0.2 –6μm based on SEM observation. In this study, the chitosan concentration is crucial for the morphology of obtained microspheres. To prepare spherical microspheres with smooth

surface, the chitosan concentration should be larger than20 g/L; otherwise, only fragile microspheres with irregular surface were obtained.

***ALP and osteogenic mineralization in C2C12 cells***

The bioactivity of BMP-2 released from the chitosanparticles was studied by determining the ability to induce ALP enzymatic activity over basal levels in C2C12 cells(negative controls) after 5 days of culture, and by comparing the cell response to a BMP-2 directly added to the culture medium (positive control). The BMP-2released from the chitosan particles was able to induce a significant increase in the ALP activity over basal levels after 5 days ($p<0.01$; Fig. 2). Cells were observed to differentiate into osteoblast morphology (data not shown). Unloaded particles did not induce any increase in ALP activity or osteoblast morphology.

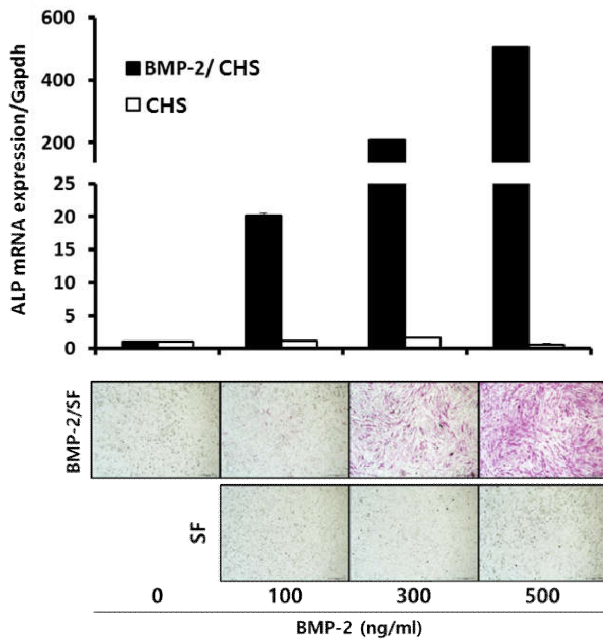


**Fig 2** Alkaline phosphatase (ALP) activity of C2C12cells after 5 days of culture. Particles were added insimilar amounts to the cell cultures containing encapsulated BMP-2 (0.1, 0.3 or0.5 mg, respectively). ALP activity is reported as nmol/min/mg of total protein.

***Cell viability***

The silk chitosanmicroparticles did not show any significant evidence of cytotoxicity in human osteosarcoma cells, after 1, 3, or 5 days of cell culture (Fig. 3).

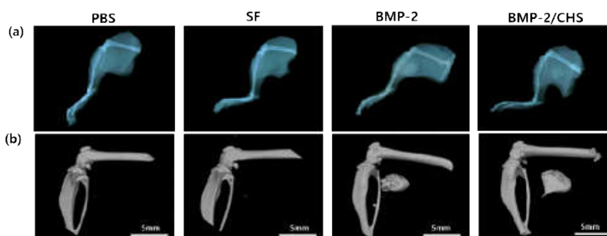


**Figure 3** Ectopic bone formation induced by BMP-2/chitosan particle (CHS). A) soft radiograph images of ectopic bone formation. B) Detailed mCT imagesof ectopic bone formation inducedby chitosan particles.

The cells were able to proliferate normally during this time period. MTT is aviability/proliferation test, and an relationship of toxicity to cells can be assumed. Data correlated with the morphological observations of the cells by optical microscopy (data not shown).

***In vivo osteogenesis a mice ectopic model***

All animals showed no complications in wound healing, during the 4-week follow-up. After 2 and 4 weeks, all sites of chitosan particle implantation were easily identified and retrieved for analysis. The mCT reconstructions (Figs. 4) clearly showed ectopic calcifications in sites where BMP-2-loaded particles were implanted. In Group II (unloaded particle), no ectopic bone could be detected. However, In Group III (BMP2; positive control) and Group IV (BMP-2-loaded particle), ectopic bone was clearly visible having a bone volume of 2.55 $\pm 0.5 mm^3$ and 2.28 $\pm 0.74 mm^3$, respectively.

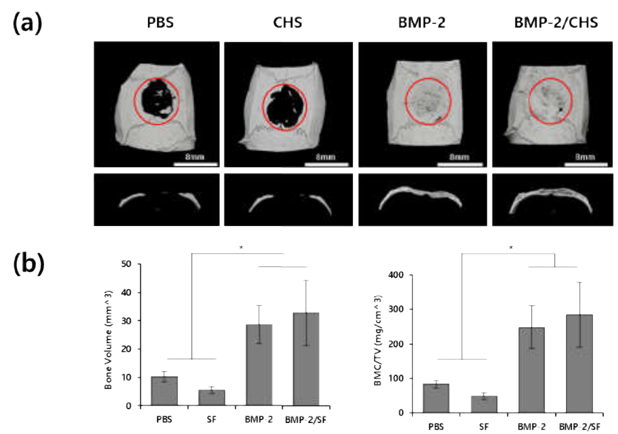


**Figure 4** Effect of BMP-2/CHS microparticle on orthotopic bone formation in the rat calvarial defect model

***In vivo bone regeneration in a calvarial defect model***

A calvarial bone defect model was used to examine the effect of immobilized BMP-2 on bone regeneration.8 mm critical-sized defects were created on the rat skull, and were successfully filled with BMP-2-loaded particle (BMP2/CHS group). A representative top view of the 3D reconstructed bone tissue is shown for each group (Fig. 5). As expected, limited bone regeneration was observed in the defect-only groups. By 8 weeks, all experimental groups exhibited significantly greater qualitative one density than the control group. Specifically, upon quantification, the defect area for the BMP2/CHS group was nearly regenerated after 8 weeks. Similarly, with regards to the volume of newly-formed bone, that of the BMP2/CHS group was significantly higher than all other groups; no statistical difference, however, was observed between the CHS particle and BMP2/CHS group.

## DISCUSSION

The need for an efficient delivery system for BMPs as away of providing effective and sustained stimulation of bone formation, has been well recognized. The main role of a carrier is to retain these growth factors at the site of injury for a prolonged timeframe, protecting the immobilized drugs from degradation and maintaining its bioactivity, while releasing the protein in a time- and site-controlled way to promote the formation of new bone at the treatment site.

Chitosan biomaterials have been explored as novel alternatives for drug delivery and in tissue engineering, as a result of their

biocompatibility, biodegradability, mechanical strength, and versatility of formats into which the material could be processed. In our studies, we have demonstrated that CHS particle not only provide physical cues in triggering the differentiation of osteoblasts but also serves as a natural reservoir of various cell differentiation. The naturalchitosan polymer particle scaffolds demonstrate good porosity and biocompatibility, and are easy to handle.

The basis for evaluating the activity of the BMP released by the chitosan particles was to test initially the particles loaded with growth factor in C2C12 cell line and then using aectopic bone formation model. C2C12 murine premyoblastcells are well defined by their ability to rapidly differentiate into osteoblasts when cultured in the presence of BMPs. In these cells, chitosan particles loaded withBMP-2 were able to induce a significant increase in ALP activity (after 5 days of culture), thereby confirming that the growth factor was loaded into the particles, retaining its bioactive state. Similar cases were reported but using different methodologies. In one report, chitosan particles retained the activity of loaded horseradishperoxidase, using lipid vesicles as particle templates, followed by methanol treatment. In our study, the activity recovery was estimated as 88±6.1%, whereas no such results were presented in the former works using particles as carriers for BMP-2 delivery.

To monitor the dose effect of BMP-2 in our system, we employeda calvarial bone defect model. Previous research by Palaez *et al*. using BMP-2-loaded collagen sponge carriers in a rat calvarial defect model, exhibited minimal difference of BMP-2 doses ranging from 1.25 to 20.0 mg. Therefore, a BMP-2 dose of less than 1 mg was chosen as the maximum loading concentration in our system. Micro radiographic studies after 8 weeks posttreatmentindicate that the most active bone regeneration in the calvarial defects was found in the BMP-2/CHS group. Furthermore, 3D reconstruction of the micro-CT scansdisplay near-complete healing compared to that observed in the other experimental groups. Interestingly, there was no statistical difference in the newly regenerated bone volume between CHS particle and BMP2 group, but significance difference between the control and unload particle group, strongly indicating a positive role of CHS particle itself in the bone regeneration.

## CONCLUSION

In the present study, a chitosan particle scaffold capable of immobilizing BMP-2 and releasing in a controlled manner was developed. We demonstrated that the BMP-2-loadedCHS particle allows for the differentiation and growth of preosteoblasts in addition to the provision of a suitable microenvironment for their osteogenic differentiation. We also demonstrated enhanced ectopic mineralization upon subcutaneous transplantation of the BMP-2-loaded particle system into mice. Finally, we prepared a rat calvarial defect model to show a significant increase of newly regenerated bone when using the CHS particle/BMP-2 particle scaffold. We envision that our CHS particle could be used to mimic the biophysical microenvironment, and when combined with BMP-2, act as a synergisticosteoconductive platform for bone tissue engineering applications.

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