Heparinized chitosan stabilizes the bioactivity of BMP-2 and potentiates the osteogenic efficacy of demineralized bone matrix

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Abstract

Background: Demineralized bone matrix (DBM), an allograft bone processed to better expose osteoinductive factors such as bone morphogenetic proteins (BMPs), is increasingly used for clinical bone repair. However, more extensive use of DBM is limited by its unpredictable osteoinductivity and low bone formation capacity. Commercial DBM products often employ polymeric carriers to enhance handling properties but such carriers generally do not possess bioactive functions. Heparin is a highly sulfated polysaccharide and is shown to form a stable complex with growth factors to enhance their bioactivities. In this study, a new heparinized synthetic carrier for DBM is developed based on photocrosslinking of methacylated glycol chitosan and heparin conjugation.

Results: Heparinized chitosan exerts protective effects on BMP bioactivity against physiological stressors related to bone fracture healing. It also enhances the potency of BMPs by inhibiting the activity of BMP antagonist, noggin. Moreover, heparinized chitosan is effective to deliver bone marrow stromal cells and DBM for enhanced osteogenesis by sequestering and localizing the cell-produced or DBM-released BMPs.

Conclusions: This research suggests an essential approach of developing a new hydrogel carrier to stabilize the bioactivity of BMPs and improve the clinical efficacy of current bone graft therapeutics for accelerated bone repair.

Keywords: Heparin, Hydrogel, Bone morphogenetic protein, Demineralized bone matrix, Osteogenesis

Background

Autologous bone grafts have been considered the "gold standard" for bone regeneration. However, bone grafts are limited by donor sites morbidity, extended operative time, and autogenous supply [1]. Demineralized bone matrix (DBM) is processed to reduce immunogenicity and has been used widely in the orthopedic industry [2,3]. During processing, the mineral content of DBM is removed to better expose the osteoinductive factors present in DBM particularly bone morphogenetic proteins (BMPs), powerful regulators for bone formation [4]. However, the processing also eliminates stem cells and the osteoinductivity of DBM can be variable, and clinical failure in orthopedic applications is reported high compared to autograft bone [5]. Moreover, the released osteoinductive cytokines will easily lose their biological function due to their inherent proteolytic and chemical hydrolysis in vivo [6]. Commercially available DBM products often use liquid carriers but they have poor irrigation resistance following implantation, diminishing a localized effect of osteogenic components at the bone defects [7]. Exogenous recombinant BMPs...
can be employed to improve osteogenic effects of DBM [8]. However, their clinical applications require a supra-
physiological concentrations potentially leading to vari-
side effects [9] such as undesired ectopic bone
development [10], cyst formation [11], osteoclastic res-
orption [12], or adipogenesis [13]. Therefore, there is a
need to develop an alternative biological carrier that can
enhance the potency of BMPs present in DBM and bet-
ter guide the osteogenic response at the defect site.

Heparin is a highly sulfated polysaccharide in extracel-
lar matrix and has been widely studied as a delivery
carrier of growth factor proteins including BMPs [14–
16] due to its high binding affinity to the proteins. It has
also been shown to maintain or enhance the biological
activities of growth factors by forming a stable complex
[17]. Heparin can act as a catalyst to form the signaling
complexes of BMPs by recruiting type II receptor sub-
units, even though it has no direct activation role [18].
Heparin is also known to regulate BMP functions by
interacting with BMP antagonists such as noggin. Hep-
arin has been shown to regulate the diffusion of noggin
in vivo and form the gradients of BMP-2 activity by inhi-
biting its binding to BMP [19–21].

Chitosan is a biocompatible and biodegradable polysac-
charide [22] that has been well studied in bone tissue engi-
neering applications [23, 24]. Chitosan can be easily modified
with different functional groups and readily forms three-
dimensional networks by simple processing such as thermo-
gelling [25] or photocrosslinking [26]. Recent studies report
the use of chitosan as composites with other polymers or
ceramics for improved mechanical properties [27].

In this work, we report a novel heparinized synthetic car-
rier for DBM that can stabilize and enhance the bioactivity
of BMPs for increased osteoinduction. We previously re-
ported a light-inducible hydrogel system composed of
methacrylated glycol chitosan (MeGC) as a photocrosslink-
able polymer and riboflavin as a photoinitiator [26]. The
MeGC system is highly biocompatible to support the
growth of encapsulated cells and can be readily modified
with different functionalities such as cell-adhesive-motifs
[23] or matrix-degradable-unit [28]. Herein, we immobi-
lized heparin to MeGC by simple chemical conjugation and
investigated whether the heparinized MeGC can have pro-
tective effects on BMP-2 for osteogenesis under physio-
logical stressors found under bone fracture healing or in
the presence of BMP antagonist, noggin. We also demon-
strated the potential of heparinized MeGC as biological car-
riers for DBM to enhance osteogenic efficacy.

Results

Characterization of heparinized chitosan
Hep-MeGC was prepared by conjugation of heparin into
MeGC via EDC chemistry and a hydrogel was fabricated by
visible blue light crosslinking with a riboflavin initiator (Fig. 1a). The successful modification of heparin was
confirmed by 1H NMR that showed the increment of sul-
fonate peaks (Fig. 1b). The FTIR spectra of Hep-MeGC ex-
hibited the peak of SO$^-$ at 1200 cm$^{-1}$, confirming the
successful conjugation of heparin (Fig. 1c). The stable
and homogenous functionalization of heparin in a chitosan
hydrogel was verified by toluidine blue that stained the
negatively charged sulfonate groups (Fig. 1d). Heparinization
enhanced the osteogenic efficacy of hydrogels as demon-
strated by intensified staining of ALP and alizarin red S. The
ability of heparinized chitosan to sequester endogenous
BMP-2 secreted by the encapsulated BMSCs was further de-
tected by immunofluorescence staining (Fig. 1e). These re-
results suggested that the improved osteogenesis by heparin
modification was potentially induced by BMP-2 sequester-
ing of hydrogels.

Heparinized chitosan stabilizing BMP-2 bioactivity
The BMSCs were used to evaluate the bioactivity of
BMP-2 associated with heparinized chitosan after the ex-
posure to different stressed conditions (Fig. 2a). The
BMP-2 was incubated with PBS, MeGC, or Hep-MeGC
in physiological pH for 1 week at 37 °C or in MMP-9 so-
lution for 16 h at 37 °C to mimic protease-enriched bone
healing environments. The ALP staining and activity
tests were performed after the treatment of with BMP-2
incubated for 4 days to verify the bioactivity of BMP-2
after exposure to the stressors. The groups without
BMP-2 and with freshly prepared BMP-2 were used as a
negative and a positive control, respectively. The ALP
activity was gradually enhanced with the addition of
MeGC and Hep-MeGC. This is possibly due to the in-
creased half-life of BMP-2 with the addition of chitosan
or heparinized chitosan in comparison to PBS group
(Fig. 2b) under both prolonged and MMP conditions.
The bioactivity of treated BMP-2 dropped significantly
in all groups but Hep-MeGC showed the higher bio-
activity compared with MeGC and PBS. Hep-MeGC
demonstrated higher protective effects compared with
MeGC and PBS in both prolonged and MMP-9 treated
conditions.

Heparinized chitosan protecting BMP-2 from noggin
Noggin is a BMP antagonist and inhibits BMPs signal-
ing. The BMSCs were cultured with BMP-2 (100 ng mL$^{-1}$)
and various concentrations of noggin (0, 30, 100, and
300 ng mL$^{-1}$). The ALP expression started to decrease
with the addition of 30 ng mL$^{-1}$ noggin in PBS group
(Fig. 3a and Fig. S1). The BMSCs incubated with MeGC
exhibited higher ALP expression compared with PBS
only group with 30 ng mL$^{-1}$ noggin. Furthermore,
BMSCs incubated with Hep-MeGC have more intensi-
fied staining up to the addition of 100 ng mL$^{-1}$ noggin.
Then, we investigated whether the expression level of
noggin as an early response gene of BMP-2 [29] maintained with the addition of MeGC or Hep-MeGC. After 1 h of stimulation of BMP-2, noggin mRNA expressions of BMSCs were increased regardless of chitosan supplement (Fig. 3b). The level of noggin expression after 72 h decreased for BMP only and MeGC groups but did not drop for Hep-MeGC group. These results indicated that heparinized chitosan enhanced BMP signaling by protecting it from noggin.

Characterization of hydrogel-DBM composite

DBM was incorporated in heparinized chitosan to form osteoinductive hydrogel-DBM composites. The chitosan hydrogel was mixed with various ratio of DBM and the incorporated DBM was homogeneously distributed in the hydrogel-DBM composite (Fig. 4a). Compressive modulus of the composites increased with the addition of DBM in a dose-dependent manner (Fig. 4b). Mechanical strength of the composites also increased with the addition of DBM but was not significantly different based on hydrogel types (MeGC or Hep-MeGC). Water content of the composites decreased with the addition of DBM but was not affected by hydrogel types (Fig. 4c). From these results, physical properties of the composites were not significantly affected by hydrogel types but the incorporation of DBM.

Proliferative potential of cells in hydrogel-DBM composite

The live/dead images of BMSCs in composites were monitored for 2 weeks. High cell viability over 90% was observed for all groups at day 0. There was no significant change in cell viability over the culture period while cell viability decreased to 85% for DBM-free groups by day 14 (Fig. S2). The morphology of cells was significantly changed with the addition of DBM which showed extended spreading alongside of DBM (Fig. 5a). The cell spreading in the composites also influenced the overall growth as confirmed by the alamarBlue result (Fig. 5b). The proliferative potential of cells increased with the incorporation of DBM in a dose dependent manner. In order to observe the interaction of DBM with the encapsulated BMSCs in composites, H&E staining was performed on the histological section (Fig. 5c). An intense purple color was observed in the DBM hydrogel.
composite, indicating proliferation of encapsulated cells, while cell-free constructs stained with light- or dark-pink (Fig. S3).

**Osteogenic effect of hydrogel-DBM composite**

The hydrogel-DBM composites provided osteoinductive environment to the encapsulated BMSCs by sequestering
the cell-produced or DBM-released BMPs. The encapsulated BMSCs in composites were stained with ALP and alizarin red S at day 7 and day 21, respectively (Fig. 6a). The strengthened ALP production and activity were observed with the addition of DBM, and the trend was more escalated when heparinized chitosan was used. The mineralization result verified the augmented calcium deposition with the incorporation of DBM as well as heparin functionalization. The DBM and heparinization also upregulated the osteogenic gene expression (Fig. 6b). The Runx2, a transcription factor associated with osteoblast, significantly enhanced at day 4 in MeGC-DBM 2% and heparinized groups (Hep-MeGC, Hep-MeGC-DBM 1%, and Hep-MeGC-DBM 2%). The ALP expression was more dynamically increased with the addition of DBM as well as heparinization. The late osteogenic marker, OCN, also confirmed the enhanced osteogenesis with heparin and DBM incorporation.

Discussion

Although its clear utility is reflected in its market position for skeletal reconstruction, more extensive use of DBM is hampered by its unpredictable osteoinductivity and low bone formation rate due to source- or process-related variations [30, 31]. Current DBM products often employ polymeric carriers for better handling properties but such excipients generally have no bioactive function [7]. In this work, we demonstrated that chemically functionalized chitosan with heparin (Hep-MeGC) can stabilize BMP-2 and increase its biological activity for osteogenesis. Moreover, Hep-MeGC can augment endogenous BMP activity by sequestering and localizing the cell-produced BMPs that should synergistically stimulate osteogenesis induced by DBM.

In order to investigate the underlying mechanism of heparinized chitosan to stabilize BMP bioactivity, we incubated rhBMP-2 with MeGC or Hep-MeGC under
therapeutically related stressors. First of all, Hep-MeGC maintained BMP-2 bioactivity in the prolonged incubation condition, but the bioactivities in PBS and MeGC groups were reduced significantly (Fig. 2a). This was possibly due to the increased half-life of BMP-2 with Hep-MeGC which was verified by quantification of BMP-2 at both prolonged and MMP-treated conditions (Fig. 2b). BMP-2 quantification near cell layer area were also measured to verify that BMP-2 was not bound near cell layer but degraded (Fig. S4). The addition of heparinized chitosan maintained the higher concentration of BMP-2 in culture medium compared with chitosan only or nontreated groups. We also assessed the amount of BMP-2 in cell layers which exhibited less than 5% in the presence or in the absence of heparinized chitosan. This suggested the vanishing of BMP-2 was not a result of accumulation on the cell layer but a result of degradation [21]. The observed stabilizing effect of unmodified MeGC could be attributed to intermolecular interactions between MeGC and BMP-2 to form chitosan-BMP complexes, preventing protein denaturation and aggregation, thus conferring greater stability.

Fig. 4 Characterization of hydrogel-DBM composites. a The images of composites with various concentration of DBM weight ratio, 0, 0.5, 1, 2, and 4% (w/v) in 2% hydrogel. b Compressive modulus of MeGC-DBM and Hep-MeGC-DBM composites. c Water content of composites. *p < 0.05, **p < 0.01, and ***p < 0.001

Kim et al. Journal of Biological Engineering (2020) 14:6
Many proteolytic enzymes such as MMP-2, MMP-9, or cathepsin-K were involved in bone remodeling process [32], and MMPs were key degrading enzyme expressed in either physiological [33] or pathological conditions including osteoarthritis [34], and osteoporosis [35]. The concentration of MMP-2 increased around two times, and MMP-9 increased around ten times under bone formation and disease environment [36–38]. In this MMP-9 enriched condition, we observed all PBS, MeGC, and Hep-MeGC groups suffered decrement of BMP-2 bioactivity (Fig. 2a). However, BMP-2 activity in Hep-MeGC was significantly higher than PBS and MeGC groups, indicating the most protective effects of Hep-MeGC under enzymatic stressors. MeGC only group had a minimal
protective effect, suggesting interaction through heparin is essential to stabilize BMP-2 bioactivity rather than interacting with chitosan chains.

Hep-MeGC may also interact with BMP antagonists to regulate BMP functions. Noggin is a specific antagonist of BMP-2 and restricts its interaction with receptors to regulate increased levels of BMP stimuli [29]. Noggin is also known to bind S domains of heparan sulfate like BMP-2 [39, 40] and its interaction with heparan sulfate proteoglycan can form the activity gradients of BMP-2 by modulating the diffusion of noggin [20]. As such, heparin enabled BMPs to preserve longer in the culture medium indeed in the presence of noggin (Fig. 3aa and Fig. S1) [21].

Lastly, we evaluated whether heparinized chitosan can enhance the osteogenic efficacy of the incorporated DBM. The compressive modulus of hydrogel-DBM composite significantly increased with the incorporation of 1% (w/v) DBM and the water content significantly decreased with the addition of 2% (w/v) DBM (Fig. 4b, c). Therefore, we selected two DBM concentration (1 and 2%) to fabricate hydrogel-DBM composite for further studies. Both ALP and mineral productions were enhanced with DBM incorporation as well as heparin functionalization (Fig. 6a). In particular, expression of *Runx2* was significantly enhanced in dual modification group (Hep-MeGC-DBM 2%), indicating that DBM synergistically enhanced osteogenesis with heparin (Fig. 6b). We also examined whether DBM-hydrogel composite can sequester BMP-2 released from DBM. BMP-2 immunostaining results showed that heparinized chitosan was able to sequester BMP-2 not only secreted from the encapsulated cells, but also released from DBM (Fig. S5).

In addition, DBM incorporation provided a cell-favorable environment in the hydrogels by increasing cell spreading and proliferation (Fig. 5). The main component of DBM mass was a type I collagen which contributed to
physical and biological properties to the matrix [41]. In our previous studies, the incorporation of type I collagen or its derivatives into MeGC could enhance the spreading, proliferation, and osteogenic differentiation of the encapsulated cells [23, 42]. Herein, the observed increase in cell spreading and proliferation in the composites were possibly due to the incorporation of collagen matrix through DBM. Cell spreading become evident adjacent to DBM at 14 days of culture, while most cells remained round morphology in DBM-free hydrogels (MeGC-DBM 0% and Hep-MeGC-DBM 0%) over the culture period. Cell spreading in this study appeared to be slower and spatially non-uniform in comparison to our previous chitosan hydrogels modified with collagen or RGD peptides possibly due to the heterogeneous distribution of large DBM particles varied from 200 to 850 μm throughout the hydrogel. The spreading and proliferation of the encapsulated cells were dominantly mediated by DBM incorporation rather than heparinization.

This study proposed a novel heparinized hydrogel carrier which stabilized BMP-2 bioactivity and improved DBM osteogenic efficacy. Bone healing capacity of DBM has not been successful in clinical situations where large quantity of bone regeneration is required. We will demonstrate the efficacy of DBM hydrogel composites for bone repair in more challenging healing environments such as in large segmental defects in comparison to currently available DBM products. Additionally, future study will employ additional approaches to abrogate BMP antagonism such as utilizing an RNAi strategy to downregulate noggin expression. These strategies could maximize activity of BMP pathway in DBM-mediated osteogenesis and may increase the utility of a bone graft approach for improved bone repair. In addition, this heparinized platform could be utilized as functional polymeric carriers to deliver various proteins with heparin binding ability.

Conclusions
Heparinized chitosan was designed by simple conjugation and photopolymerization to provide a hydrogel surface that stabilized the bioactivity of BMP-2 and potentiated the osteogenic efficacy of DBM. Heparinization preserved BMP-2 bioactivity under stressors conditions mimicking a bone healing environment and protected BMP-2 from its antagonist, noggin. The heparinized hydrogel promoted endogenous BMP activity by sequestering the cell-produced BMPS. The heparinized system was effective to deliver DBM as composite hydrogels and further enhanced osteogenic differentiation of encapsulated cells induced by DBM. This study suggests a promising strategy to increase effectiveness of BMPS and utility of current bone graft approaches for improved bone regeneration in conjunction with polymeric carrier systems.

Materials and methods
Materials
Glycol chitosan (~100 kDa) was purchased from Wako Chemical USA, Inc. (Richmond, VA). Glycidyl methacrylate, heparin sodium salt from porcine intestinal mucosa (heparin, ~18 kDa), and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) were supplied from Sigma Aldrich (St. Louis, MO). Recombinant human bone morphogenetic protein-2 was supplied from GenScript (Piscataway, NJ). Recombinant human matrix metalloproteinase-9 (MMP-9) was purchased from abcam (Cambridge, MA) and recombinant mouse noggin/Fc chimera (Noggin) was purchased from R&D Systems (Minneapolis, MN). Demineralized Bone Matrix (DBM; Demineralized Cortical Powder, 0.212–0.850 mm) was provided from MTF Biologics (Edison, NJ). Mouse bone marrow stromal cells (BMSCs, D1 ORL UVA, CRL-12424) was obtained from American Type Culture Collection (ATCC, Manassas, VA). All reagents were used without further purification.

Preparation of heparinized chitosan and hydrogel-DBM composite
Visible-light inducible methacrylated glycol chitosan (MeGC) was prepared by the previously published methods [26, 43]. Heparin conjugated glycidyl methacrylated chitosan (Hep-MeGC) was prepared by EDC activated reaction of heparin and MeGC. EDC solution was prepared as 23 mg in 1 mL distilled water and reacted with 8 mg of heparin for 30 min. Then, the reacted solution was mixed with 5 mL of 1% (w/v) MeGC in PBS for 16 h, dialyzed with 50 kDa tubes for 16 h and lyophilized. Hydrogel-DBM composites incorporated hydrogel and DBM in various weight ratio, 0, 0.5, 1, 2, and 4% (w/v), based on 2% (w/v) hydrogel concentration. All hydrogels were fabricated by 40 s irradiation of hydrogel prepolymer and 6 μM riboflavin initiator (100:0.5 volume ratio) under visible blue light (400–500 nm, 300 mW cm⁻²) irradiation.

Characterization of heparinized chitosan and hydrogel-DBM composite
Heparin conjugation was analyzed by 1H nuclear magnetic resonance spectroscopy (NMR) in D₂O (Bruker ARX400 spectrometer). It was quantified by the integration of the peaks at 2.7–3.2 ppm (CHNHSO3⁻, CHOSO₃⁻, CH₂OSO₃⁻) and compared with methacrylate peak at 5.6–6.1 ppm. The degree of heparin substitution was reported as 6.76%. Heparinized chitosan was also characterized by fourier transform infrared spectrophotometer (FTIR, Jasco 420) to verify the heparin conjugation. The incorporation of heparin over time was examined by toluidine blue (Sigma-Aldrich, MO) staining that the hydrogels were stained with 1% (w/v) toluidine blue in PBS and washed with PBS for 1 h.
Mechanical properties of composites were measured by compressive modulus and water content. The compressive modulus was evaluated by 1.6 mm diameter flat-ended indentation test via Instron Electro- Mechanical Testing Machines (Instron, Model 5564, Norwood, MA) using a Poisson’s ratio of 0.25 [26, 43]. Water content was calculated by the following equation where $W_{\text{wet}}$ and $W_{\text{dry}}$ referred wet and dry weight of hydrogels respectively.

$$\text{Water content} \%(\%) = \frac{W_{\text{wet}} - W_{\text{dry}}}{W_{\text{wet}}} \times 100$$

Alkaline phosphatase staining and activity
Bioactivity of BMP-2 and osteogenic efficacy of MeGC, Hep-MeGC, and composites were evaluated by alkaline phosphatase (ALP) staining and activity. First of all, 100 ng mL$^{-1}$ of rhBMP-2 was incubated in the presence of 10 μL of MeGC or Hep-MeGC under different stressed conditions of 37 °C for 7 days or 200 ng mL$^{-1}$ of MMP-9 for 16 h. Then, 100 ng mL$^{-1}$ of incubated rhBMP-2 was treated to BMSCs in osteogenic media (OM) including high glucose Dulbecco’s Modified Eagle’s Medium, 10% fetal bovine serum, 1% antibiotic-antimycotic, 10 mM β-glycerophosphate, 50 mg mL$^{-1}$ L-ascorbic acid, and 100 nM dexamethasone for 4 days. Second, 100 ng mL$^{-1}$ of rhBMP-2 was incubated in the presence of 10 μL of MeGC or Hep-MeGC with noggin (0, 30, 100, and 300 ng mL$^{-1}$) for 4 days. After all the culture, BMSCs were fixed with 10% formalin and incubated in solution containing nitro blue tetrazolium, 5-bromo-4-chloro-3-indoxylphosphate, 100 mM Tris (pH 8.5), 50 mM MgCl$_2$, and 100 mM NaCl for 3 h. ALP activity was evaluated by colorimetric analysis using p-nitrophenol phosphate as a substrate. The value was read at 405 nm and normalized by total protein expression from BCA assay [23, 44].

Real-time reverse transcription polymerase chain reaction (qRT-PCR)
The induced noggin expression by MeGC or Hep-MeGC and osteogenic efficacy of composites were examined by qRT-PCR. The RNAs were extracted from BMSCs by TRIzol (Invitrogen, CA) and RNeasy mini kit (Qiagen, CA), and then reverse-transcribed by SuperScript III kit (Invitrogen, CA). The cDNA products were amplified for 45 cycles with SYBR green in LightCycler 480 PCR system (Indianapolis, IN). The value was normalized with GAPDH expression and the primer sequences are: GAPDH (AGGTCCGTGTGAACGGATTGG and TGTAGACCATGTTGAGGCTCA), Noggin (GCCAGCCTATCTACACATCC and GGCTTCGGTCAAGATCTTTCT), Runx2 (GGCTTCCCTTCCAGGATGTT and CCTCCGCCCACTGACCATCAC), ALP (GTTGCCAAGCTGGGAAGAAC and CCCACCACGCTATTCCAACAC), and OCN (GGGAGACACAGGGAC and CAGGCTTCTGCCAGTACCT). All experiments were triplicated.

Proliferation of cells in hydrogel-DBM composite
The BMSCs were encapsulated in composites with concentration of 2 × 10$^6$ cells mL$^{-1}$. The base hydrogels, MeGC and Hep-MeGC, were mixed with various DBM ratio, 0, 1, and 2% (w/v) in 2% (w/v) hydrogel. The BMSCs encapsulated in hydrogels were cultured in culture media for 2 weeks at 37 °C. The morphology of BMSCs were monitored by Live/Dead staining kit (Invitrogen, CA) at day 0, 7, and 14 and proliferative potential was measured by alamarBlue (Thermo Fisher Scientific, CA) assay at day 0, 1, 7, and 14. To investigate the further morphology change of cells near DBM, hydrogels at day 14 were fixed with 10% formalin for overnight, embedded in paraffin, and cut into 5 μm section. The sections were stained with hematoxylin and eosin (H&E) after deparaffinizing.

Osteogenic differentiation of cells in hydrogel-DBM composite
The BMSCs encapsulated in composites were incubated in OM for 21 days to induce osteogenesis. The samples were collected at day 7 for ALP staining and activity test. At day 21, the mineral deposition in the samples were measured by alizarin red S staining after fixation. The hydrogels were incubated in 2% alizarin red S solution for 5 min and washed with PBS for overnight. All stained hydrogels were visualized by Olympus SZX16 stereo microscope. Calcium quantification was carried out by acetic acid extraction of alizarin red S stained hydrogels [45].

Statistical analysis
All experiments were executed three times and represented as values with error bars which were the average and the standard deviation. One- or two-way analysis of variance with Tukey’s post hoc test was used for statistical analysis. A value of $p < 0.05$ was considered as significant.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s13036-020-0231-y.
Abbreviations
ALP: Alkaline phosphatase; BMP: Bone morphogenic proteins; BMSCs: Bone marrow stromal cells; DBM: Demineralized bone matrix; EDC: 1-Ethyl-3-(3-dimethylamino-propyl)-carbodiimide; FTIR: Fourier transform infrared spectrophotometer; Hep-MeGC: Heparin conjugated glycidyl methacrylated chitosan; MeGC: Methacrylated glycol chitosan; MPP-9: Matrix metalloproteinase-9; NMR: Nuclear magnetic resonance spectroscopy; OM: Osteogenic media; qRT-PCR: Real-time reverse transcription polymerase chain reaction

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Authors’ contributions
SK conducted the experiments, participated in data analysis and discussion, and drafted the manuscript. JF and CL participated in discussion and revised the manuscript. CC and KS carried out DBM experiments. ML designed the experiments and drafted the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References
1. Pape HC, Evans A, Kobbe P. Autologous bone graft: properties and techniques. J Orthop Trauma. 2010;24(Suppl 1):S36–40.
2. Ladd AL, Pilam NB. Use of bone-graft substitutes in distal radius fractures. J Am Acad Orthop Surg. 1999;7:279–90.
3. Kühls R, Werner-Rustner M, Küchler I, Soost F. Human demineralised bone matrix as a bone substitute for reconstruction of cystic defects of the lower jaw. Cell Tissue Bank. 2001;2:143–53.
4. Wildemann B, Kadow-Romacker A, Haas NP, Schmidmaier G. Quantification of various growth factors in different demineralized bone matrix preparations. J Biomed Mater Res A. 2007;81:437–42.
5. Pieske O, Wittmann A, Zapfel J, Löffler T, Treintsch H, et al. Autologous bone graft versus demineralized bone matrix in internal fixation of united long bones. J Trauma Manag Outcomes. 2009;5:11.
6. Cheesn KD, Branger J, Wertheim H, Scarbrough N. Healing response to various forms of human demineralized bone matrix in athymic rat cranial defects. J Oral Maxillofac Surg. 1998;56:685–693.
7. Shehadi JA, Elzein SM. Review of commercially available demineralized bone matrix products for spinal fusions: a selection paradigm. Surg Neurol Int. 2017;8:203.
8. Francis CS, Mobin SS, Lyypka MA, Rommer E, Yen S, Urata MM, et al. rhBMP-2 with a demineralized bone matrix scaffold versus autologous iliac crest bone graft for alveolar cleft reconstruction. Plast Reconstr Surg. 2013;131:1071–1075.
9. James AW, LaChaud G, Shen J, Ashtian G, Nguyen V, Zhang XL, et al. A review of the clinical side effects of bone morphogenetic Protein-2. Tissue Engineering Part B Reviews. 2016;22:284–97.
10. Tang TT, Xu XL, Dai KR, Yu CF, Yue B, Lou JR. Ectopic bone formation of human bone morphogenetic protein-2 gene transfected goat bone marrow-derived mesenchymal stem cells in nude mice. Chin J Traumatol. 2005;8:3–7.
11. Choudhry OJ, Christiano LD, Singh R, Golden BM, Liu JK. Bone morphogenetic protein-induced inflammatory cytokine formation after lumbar fusion causing nerve root compression case report. J Neurosurg Spine. 2012;16:296–301.
12. Tachi K, Takami M, Zhao B, Mochizuki A, Yamada A, Miyamoto Y, et al. Bone morphogenetic protein 2 enhances mouse osteoclast differentiation via increased levels of receptor activator of NF-κappa B ligand expression in osteoblasts. Cell Tissue Res. 2010;342:213–20.
13. Sottile V, Seuw K. Bone morphogenetic protein-2 stimulates adipogenic differentiation of mesenchymal precursor cells in synergy with BRL 49653 (rosiglitazone). FEBS Lett. 2000;475:201–4.
14. Jean O, Song SJ, Yang HS, Bang SH, Kang SW, Sung MA, et al. Long-term delivery enhances in vivo osteogenic efficacy of bone morphogenic protein-2 compared to short-term delivery. Biochem Biophys Res Commun. 2008;367:774–80.
15. Rider CC. Heparin/heparan sulphate binding in the TGF-beta cytokine superfamily. Biochem Soc Trans. 2006;34:458–60.
16. Ruppert R, Hoffmann E, Sebald W. Human bone morphogenetic protein 2 contains a heparin-binding site which modifies its biological activity. Eur J Biochem. 1996;237:295–302.
17. Takada T, Katagiri T, Ifuku M, Morimura N, Kobayashi M, Hasegawa K, et al. Sulfated polysaccharides enhance the biological activities of bone morphogenetic proteins. J Biol Chem. 2003;278:43229–35.
18. Kuo WJ, Digman MA, Lander AD. Heparan sulfate acts as a bone morphogenetic protein coreceptor by facilitating ligand-induced receptor hetero-oligomerization. Mol Cell Biol. 2010;30:4028–41.
19. Groppe J, Greenwald J, Wiater E, Rodriguez-Leon J, Economides AN, Kwiatkowski W, et al. Structural basis of BMP signalling inhibition by the cystine knot protein noggin. Nature. 2002;420:36–42.
20. Paine-Saunders B, Viviano BL, Economides AN, Saunders S. Heparan sulfate proteoglycans retain noggin at the cell surface: a potential mechanism for shaping bone morphogenic protein gradients. J Biol Chem. 2002;277:2089–96.
21. Zhao BH, Katagiri T, Toyoda H, Takada T, Yanai T, Fukuda T, et al. Heparin potentiates the in vivo ectopic bone formation induced by bone morphogenetic protein-2. J Biol Chem. 2006;281:23246–53.
22. Kumar M, Muzzarelli RAA, Muzzarelli C, Sashihara H, Domb AJ. Chitosan chemistry and pharmaceutical perspectives. Chem Rev. 2004;104:6017–84.
23. Kim S, Cui ZK, Fan JB, Fastash A, Aghaloo T, Lee M. Photocrosslinkable chitosan hydrogels functionalized with the RGD peptide and Phosphoserine to enhance Osteogenesis. J Mater Chem B. 2016;4:5289–98.
24. Gao LY, Werkmeister JA, Wang J, Glattauer V, McLean KM, Liu CS. Bone regeneration using photocrosslinked hydrogel incorporating rhBMP-2 loaded 2-N,6-O-sulfated chitosan nanoparticles. Biomaterials. 2014;35:2730–9.
25. Tian M, Yang Z, Kuvahara K, Nimmi ME, Wan C, Han B. Delivery of demineralized bone matrix powder using a thermogelling chitosan carrier. Acta Biomater. 2012;8:753–62.
26. Hu J, Hsu Y, Park H, Choi B, Hsu S, Chung A, et al. Visible light Crosslinkable chitosan hydrogels for tissue engineering. Acta Biomater. 2012;8:1730–8.
27. Logith Kumar R, KeshavNarayan A, Dhiya S, Chawla A, Saravanan S, Selvamurugan N. A review of chitosan and its derivatives in bone tissue engineering. Carbohydr Polym. 2016;151:752–62.
28. Kim S, Cui ZK, Koo B, Zhang J, Aghaloo T, Lee M. Chitosan-lysozyme conjugates for enzyme-triggered hydrogel degradation in tissue engineering applications. ACS Appl Mater Interfaces. 2018;10:138–45.
29. Gazzero E, Gangji V, Canalis E. Bone morphogenetic proteins induce the expression of noggin, which limits their activity in cultured rat osteoblasts. J Clin Invest. 1998;102:2106–14.
30. Kenley R, Marden L, Turek T, Jin L, Ron E, Hollinger JO. Osseous regeneration in the rat CALVARUM using novel delivery systems for...
31. Marden LJ, Hollinger JO, Chaudhari A, Turek T, Schaub RG, Ron E. Recombinant human bone morphogenetic protein-2 is superior to demineralized bone matrix in repairing craniotomy defects in rats. J Biomed Mater Res. 1994;28:1127–38.

32. Ortega N, Behonick D, Stickens D, Werb Z. How proteases regulate bone morphogenesis. Ann N Y Acad Sci. 2003;995:109–16.

33. Vu TH, Werb Z. Matrix metalloproteinases: effectors of development and normal physiology. Genes Dev. 2000;14:2123–33.

34. Tchetverikov I, Lohmander LS, Verzijl N, Huzinga TW, TeKoppele JM, Hanemaaijer R, et al. MMP protein and activity levels in synovial fluid from patients with joint injury, inflammatory arthritis, and osteoarthritis. Ann Rheum Dis. 2005;64:694–8.

35. Kochetkova EA, Ugal LG, Malistrovskii IV, Buria KA, Nevzorova VA. Role of matrix metalloproteinase-9 in the pathogenesis of osteoporosis in patients with chronic obstructive pulmonary disease. Ter Arkh. 2012;84:37–40.

36. Thrailkill K, Cockrell G, Simpson P, Moreau C, Fowlkes J, Bunn RC. Physiological matrix metalloproteinase (MMP) concentrations: comparison of serum and plasma specimens. Clin Chem Lab Med. 2006;44:503–4.

37. Masuhara K, Nakai T, Yamauchi K, Yamasaki S, Sasaguri Y. Significant increases in serum and plasma concentrations of matrix metalloproteinases 3 and 9 in patients with rapidly destructive osteoarthritis of the hip. Arthritis Rheum. 2002;46:2625–31.

38. Hu Z, Ma C, Liang Y, Zou S, Liu X. Osteoclasts in bone regeneration under type 2 diabetes mellitus. Acta Biomater. 2019;84:402–13.

39. Abe E, Yamamoto M, Taguchi Y, Lecka-Czernik B, O’Brien CA, Economides AN, et al. Essential requirement of BMPs-2/4 for both osteoblast and osteoclast formation in murine bone marrow cultures from adult mice: antagonism by noggin. J Bone Miner Res. 2000;15:663–73.

40. Viviano BL, Paine-Saunders S, Gasiunas N, Gallagher J, Saunders S. Domain-specific modification of heparan sulfate by OsuFl modulates the binding of the bone morphogenetic protein antagonist noggin. J Biol Chem. 2004;279:5604–11.

41. Holt DJ, Grainger DW. Demineralized bone matrix as a vehicle for delivering endogenous and exogenous therapeutics in bone repair. Adv Drug Deliv Rev. 2012;64:1123–8.

42. Arakawa C, Ng R, Tan S, Kim S, Wu B, Lee M. Photopolymerizable chitosan-collagen hydrogels for bone tissue engineering. J Tissue Eng Regen Med. 2017;11:164–74.

43. Amsden BG, Sukarto A, Knight DK, Shapka SN. Methacycilated glycol chitosan as a Photopolymerizable biomaterial. Biomacromolecules. 2007;8:3758–66.

44. Sabolzbar A, Millett PJ, Myer B, Rushton N. A rapid, quantitative assay for measuring alkaline phosphatase activity in osteoblastic cells in vitro. Bone Miner. 1994;27:57–67.

45. Cui Z-K, Kim S, Baljon JJ, Wu BM, Aghaloo T, Lee M. Microporous methacyclated glycol chitosan-montmorillonite nanocomposite hydrogel for bone tissue engineering. Nat Commun. 2019;10:3523.

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