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# Enhanced bioactivity of bone morphogenetic protein-2 with low dose of 2-*N*, 6-*O*-sulfated chitosan *in vitro* and *in vivo*

Huanjun Zhou [a,b], Jiangchao Qian [a], Jing Wang [b], Wantong Yao [c], Changsheng Liu [a,b,*], Jianguo Chen [c], Xuehua Cao [c]

[a] The State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, PR China
[b] Key Laboratory for Ultrafine Materials of Ministry of Education, East China University of Science and Technology, Shanghai 200237, PR China
[c] Engineering Research Center for Biomedical Materials of Ministry of Education, East China University of Science and Technology, Shanghai 200237, PR China

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ABSTRACT

Bone morphogenetic protein-2 (BMP-2) has been widely used as an effective growth factor in bone tissue engineering. However, large amounts of BMP-2 are required to induce new bone and the resulting side effects limit its clinical application. Sulfated polysaccharides, such as native heparin, and heparan sulfate have been found to modulate BMP-2 bioactivity and play pivotal roles in bone metabolism. Whereas the direct role of chitosan modified with sulfate group in BMP-2 signaling has not been reported till now. In the present study, several sulfated chitosans with different positions were synthesized by regioselective reactions firstly. Using C2C12 myoblast cells as *in vitro* models, the enhanced bioactivity of BMP-2 was attributed primarily to the stimulation from 6-*O*-sulfated chitosan (6SCS), while 2-*N*-sulfate was subsidiary group with less activation. Low dose of 2-*N*, 6-*O*-sulfated chitosan (26SCS) showed significant enhancement on the alkaline phosphatase (ALP) activity and the mineralization formation induced by BMP-2, as well as the expression of ALP and osteocalcin mRNA. Moreover, increased chain-length and further sulfation on 26SCS also resulted in a higher ALP activity. Dose-dependent effects on BMP-2 bioactivity were observed in both sulfated chitosan and heparin. Compared with native heparin, 26SCS showed much stronger simultaneous effects on the BMP-2 bioactivity at low dose. Stimulated secreted Noggin protein failed to block the function of BMP-2 in the presence of 26SCS. The BMP-2 ligand bound to its receptor was enhanced by low dose of 26SCS, whereas weakened by the increasing amounts of 26SCS. Furthermore, simultaneous administration of BMP-2 and 26SCS *in vivo* dose-dependently induced larger amounts of ectopic bone formation compared with BMP-2 alone. These findings clearly indicate that 26SCS is a more potent enhancer for BMP-2 bioactivity to induce osteoblastic differentiation *in vitro* and *in vivo* by promoting BMP-2 signaling pathway, suggesting that 26SCS could be used as the synergistic factor of BMP-2 for bone regeneration.



## 1. Introduction

Bone morphogenetic protein-2 (BMP-2), which is a member of the transforming growth factor-β (TGF-β) superfamily of multifunctional cytokines, has remarkable ability to induce bone formation and bone tissue reconstruction, is secreted in adult vertebrates, and plays critical roles in osteogenesis and bone metabolism [1–5]. However, it was reported that half of BMP-2 was degraded within 1 h *in vitro* [6]. And more than 100-fold larger amounts of BMP-2 (up to milligram) are required to induce new bone formation in higher animals, such as monkeys and humans than in rodents [7]. In addition, high dose for implantation may also be associated with potential side effects such as the stimulation of bone resorption, excessive bone formation and nerve cell reactions in unintended areas [8–11]. To overcome these shortcomings, carriers, such as scaffolds or microspheres, have been established to sustain the release of BMP-2 in local bone defect areas [7,12–15]. Considerable researches have been attempted to identify specific agents or materials to enhance the bioactivity of BMP-2 *in vitro* and *in vivo* as a direct approach. In recent years, several sulfated polysaccharides such as heparin and heparin sulfate were reported to stimulate the biological activity of BMP-2 *in vitro* [16]. In the

* Corresponding author. The State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, PR China. Tel.: +86 21 6425 1308; fax: +86 21 6425 1358.
*E-mail address:* csliu@sh163.net (C. Liu).

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presence of heparin, half-life of BMP-2 in the culture medium was prolonged by nearly 20-fold and larger amount of bone formation was observed in the *in vivo* model [6]. However, some conflict viewpoints have also been published recently, which shows that exogenous heparin inhibits BMP-2 osteogenic bioactivity and reduces BMP-2 signaling [17–19]. On the other hand, from the well known clinical observation, short-term use of heparin after bone fracture can delay bone healing and long-term treatment with heparin can increase the risk of osteoporosis [20–23]. These drawbacks might be critical obstructions for the usage of heparin on clinic.

From the uncertain bioactivity of heparin and clinical requirements on enhancing BMP-2 bioactivity, it's rather important to seek for such kind of agents which can synergistically work with BMP-2. Over the years, chemical modification on sulfated chitosan has attracted great interest because it promotes the structure similarity to that of heparin and achieves higher degree of sulfation than any natural sulfated polysaccharides. In fact, sulfated chitosans have been applied as effective blood anticoagulant (a typical clinical function for heparin) [24]. On the other hand, low cytotoxicity raises its feasibility of application on developing specific anti-HIV agents [25]. However, the direct role of sulfated chitosans on BMP-2 activity has not been reported till now. In the present study, chitosans modified with different sulfation were synthesized, and we describe for the first time that dose-dependent effects of 2-*N*, 6-*O*-sulfated chitosan on the bioactivity of BMP-2 were observed *in vitro* and *in vivo*. The availability of sulfated chitosan to enhance the bioactivity of BMP-2 was demonstrated, which provided a direct and effective approach to reduce costs and risks of BMP-2 implantation.

## 2. Materials and methods

### 2.1. Materials

All reagents used are available from commercial sources, used as received, unless otherwise noted. Two kinds of highly deacetylated (>90%) chitosans with $M_w$ of $20–30 \times 10^4$ and $5–8 \times 10^4$ were purchased from Carbo and Weikang Biologics (Shanghai, China), respectively. Heparin derived from porcine intestine was purchased from Shanghai Yuanju Biotechnology Co. Ltd. Chlorosulfonic acid was from Sinopharm Chemical Reagent Co. (Shanghai, China), pyridine-sulfur trioxide ($SO_3$-Pyd) from Acros Chem. (New Jersey, USA). Recombinant human BMP-2 was generous gift from Shanghai Rebone Biomaterials Co. Ltd. (Shanghai, China). Water used in all experiments was purified using a Millipore Synergy 185 System (≥18 MΩ resistance).

### 2.2. Synthesis and characterization of sulfated chitosan

#### 2.2.1. 2-N-Sulfated chitosan (2SCS)

Low molecular weight chitosan ($5–8 \times 10^4$, 1 g) was dispersed in 75 mL of water under gentle agitation. Then, 2 g of $Na_2CO_3$ was added to maintain alkaline environment (pH>9). 3 g $SO_3$-Pyd was added to this viscous solution to start the reaction. The mixture was maintained at 60 °C for 24 h under argon atmosphere to form a yellow mixture [26].

#### 2.2.2. 6-O-Sulfated chitosan (6SCS)

6-*O*-Sulfated chitosan was prepared according to Nishimura's method [25] with minor modifications. Briefly, high molecular weight chitosan ($20–30 \times 10^4$, 2 g) was added to 50 mL of formamide, 5 mL of concentrated sulfuric acid (98%) was added to maintain high acidity reaction environment (pH < 1). With the protection of argon, 10 mL of $HClSO_3$ was added dropwise with mechanical stirring and cooling. The reaction was run at 0–4 °C for 3 h to obtain a pale yellow mixture.

#### 2.2.3. 2-N, 6-O-Sulfated chitosan (26SCS)

2-*N*, 6-*O*-Sulfated chitosan was prepared using the similar method as reported [27]. In short, sulfating reagent was prepared firstly, $HClSO_3$ was added dropwise to 50 mL of *N*,*N*-dimethylformamide (DMF) cooled at 0–4 °C, the mixture was stirred for stabilization for 15 min and then transferred to a three-necked bottomed flask containing 2.5 g chitosan ($20–30 \times 10^4$) in 50 mL of formamide and 2 mL of formic acid. Under mechanical agitation and argon atmosphere, the reaction was maintained at 45–55 °C for 2 h, a homogeneous pale yellow solution was obtained finally.

After the three reactions described above, the following post-processing treatments were performed: 500–1000 mL of EtOH was added to precipitate the products. The precipitation was filtered under vacuum, washed with EtOH for at least 3 times, and then redissolved in water. The pH value was adjusted to 7–8 with 1 N HCl or 1 N NaOH. The mixture with undissolved substance was centrifuged at 5000 rpm for 15 min, the supernatant was obtained and dialyzed against water for 3 d with a 14 000 Da $M_w$ cut-off dialysis membrane. Consequently, all kinds of sulfated chitosans were then obtained by lyophilization.

#### 2.2.4. Characterization of sulfated chitosan

The FTIR spectrum of sulfated chitosan was measured by ATR on Nicolet 380 (Thermo, USA). Laser light scattering was carried out to measure molecular weight on Dawn Heleos light scattering instrument (ALV/CGS-5022F). As all the sulfated chitosans obtained in this paper were water-soluble, 0.1 N $NaNO_3$ aqueous solution was used as solvent at a flow rate of 0.5 mL/min. Sulfur content (%) was measured by Elementar (Vario Macro, Germany). The sulfur content of heparin used in this paper is 10.23%.

### 2.3. Evaluations of sulfated chitosan on the bioactivity of BMP-2 in vitro

#### 2.3.1. Cell culture

C2C12, a myoblastic precursor cell with osteoblastic potential, was purchased from the American Type Culture Collection (ATCC). C2C12 cells were cultured in 37.5 $cm^2$ flasks with growth medium (Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) containing 10% fetal calf serum (Sijiqing, Hangzhou, China), antibiotics (100 U/mL penicillin-G, and 100 μg/mL streptomycin)) at 37 °C in humidified atmosphere of 5% $CO_2$/95% air until confluence, then detached with 0.25% trypsin/0.03% ethylenediamine tetraacetic acid (EDTA) and the cell density was calculated and used at the desired density in later experiments.

#### 2.3.2. Determination of alkaline phosphatase (ALP) activity

To detect the ALP activity induced by BMP-2, C2C12 cells were seeded at a density of $10 \times 10^4$/mL to 96-well plates. After 24 h incubation in growth medium, cells were washed with PBS twice and refreshed with DMEM containing 2% fetal calf serum (called maintenance medium) in the presence of BMP-2 with or without sulfated chitosan or heparin. At the end of 72 h incubation, 50 μL 1% Nonidet P-40 (NP-40) solution was added to each well at room temperature (RT) for 1 h to obtain cell lysate, to which 50 μL of 1 mg/mL *p*-nitrophenylphosphate (Sangon, Shanghai, China) substrate solution (pH = 9) composed of 0.1 mol/L glycine, 1 mmol/L $MgCl_2 \cdot 6H_2O$ was added and incubated for 15 min at RT. The reaction was quenched by adding 100 μL of 0.1 N NaOH, the absorbance of ALP was quantified at the wavelength of 405 nm using a microplate reader (SPECTRAmax 384, Molecular Devices, USA). To determine the ALP activity histochemically, cells were fixed with 1% glutaraldehyde for 10 min on ice, washed with PBS, and incubated in a mixture of 0.5 mg/mL naphthol AS-BI phosphate (Sigma, St. Louis, USA), 2% (V/V) DMF, 0.05 mol/L $MgCl_2$, 0.01 mol/L levamisole, and 1 mg/mL fast blue BB salt (Yuanju, Shanghai, China) in 0.2 mol/L Tris–HCl (pH = 9.0) for 30 min at RT. Stained cells were visualized and photographed with inverted light microscope (TE2000U, Nikon Corp., Japan).

#### 2.3.3. Semi-quantitative reverse transcription polymerase chain reaction

Cells were cultured to 80% confluency in 10-cm Corning® dishes with 0.8 μg/mL BMP-2 in the presence of indicated concentrations of 26SCS for 48 h. Total RNAs were extracted from the cells using the mRNA Purification Kit (Shanghai Shenergy Biocolor Bioscience & Technology Company, China) according to the manufacturer's guidelines. The concentrations of RNA were determined by $A_{260nm}/A_{280nm}$. Complementary first strand DNA (cDNA) was synthesized from 2 μg of total RNA using the reverse transcriptase MMLV (Promega, USA) and Oligo (dT) (Takara, Japan) according to the manufacturer's protocol. One tenth of the cDNA products were used for PCR amplification with targeted primers designed as reported to amplify fragments corresponding to ALP, osteocalcin and Noggin mRNAs [16]. PCR was carried out as follows: an initial denaturation of 3 min at 94 °C was followed by 32 cycles of 45 s at 94 °C, 45 s at 52 °C, and 1 min at 72 °C, followed by 10 min of final elongation at 72 °C. Control PCR amplifications were performed with β-actin specific primers which were purchased from Waston Biotech (Shanghai, China). Oligonucleotide sequences for primers used in this study are listed in Table 1. Amplification products were analyzed through 1% agarose gel electrophoresis and following ethidium bromide staining. For an estimation of the relative expression, integrated optical densities of the bands were scanned and quantified by Total 2.01 (Nonlinear Dynamics Ltd, USA), and normalized by that of β-actin.

#### 2.3.4. Immunofluorescence assay of BMP-2 localized on the surface of C2C12 cells

To observe the interactions of BMP-2 and receptors on cell layers, cells were precultured on chamber slides with 0.01% poly-L-lysine (PLL) for attachment for 24 h. Medium was supplemented with 0.8 μg/mL BMP-2 and/or 26SCS. After 4 h incubation, cells were placed on ice for 5 min, washed twice with ice cold PBS. Cells were fixed with 1% glutaraldehyde for 15 min at 4 °C. 1% BSA solution was

**Table 1**
Oligonucleotide primers utilized for RT-PCR amplification.

| Target | Forward primer sequence | Reverse primer sequence |
|---|---|---|
| ALP | 5′-GAT CAT TCC CAC GTT TTC AC-3′ | 5′-TGC GGG CTT GTG GGA CCT GC-3′ |
| Osteocalcin | 5′-CAA GTC CCA CAC AGC AGC TT-3′ | 5′-AAA GCC GAG CTG CCA GAG TT-3′ |
| Noggin | 5′-ATG GAG CGC TGC CCC AGC CT-3′ | 5′-CTA GCA GGA ACA CTT ACA CT-3′ |
| β-Actin | 5′-GAT TCC TAT GTG GGC GAC GAG-3′ | 5′ CCA TCT CTT GCT GGA AGT CC-3′ |

added as the blocking buffer for 1 h at 37 °C. After washing with PBS twice, cells were incubated with anti-BMP-2 antibody (R&D systems Inc., Minneapolis, USA) for 1 h at 37 °C and the cultures were placed at 4 °C for 1 h. For BMP-2 staining, cells were incubated with a dilution of FITC-conjugated goat-anti-mouse IgG (Sigma, St. Louis, USA) for 1 h at RT. 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI, Beyotime Biotech, Jiangsu, China) solution was added to stain cell nucleus for 5 min. After washing with PBS for 5 times with 3 min interval, slides were covered with 50% glycerol in water. BMP-2 and cell nucleus were monitored with fluorescence microscope inspired by blue and UV light, respectively.

#### 2.3.5. Mineralization assay

Cells were cultured in growth medium, with the supplement of 10 mM ascorbic acid (Boyun Biotech, Shanghai, China), 5 mM β-glycerophosphate (Boyun Biotech, Shanghai, China), and 0.8 μg/mL BMP-2 with or without 26SCS for 1 or 2 weeks, the culture medium was replaced every 3 days. At the end of incubation, cells were washed with PBS at RT and fixed with 1% glutaraldehyde for 10 min. Fixed cells were stained with 1% Alizarin Red (Major Biotech, Shanghai, China) (pH = 4.2) for 10 min. Then the cells were rinsed with water, and staining was viewed with inverted light microscope.

### 2.4. Ectopic bone formation study in vivo

The effects of 26SCS on ectopic bone formation induced by BMP-2 were studied in mice. 100 μg of BMP-2 and 0, 2, 20, 50, 100, 500 μg 26SCS were mixed well and blotted onto a gelatin sheet (6 × 20 mm in diameter, 5 mm thickness) cut from commercially available absorbable gelatin sponge (Jinling Med, Nanjing, China), freeze dried, sealed in bottles and stored at −20 °C until used. All procedures were carried out under aseptic conditions. Eighteen 4-week female mice (Swiss Mouse, supplied by animal center in Shanghai Jiaotong University) were anesthetized by diethyl ether inhalation. The gelatin pellets prepared before were surgically implanted into right leg muscle pouches for 6 groups (3 mice per group). At 4 weeks after surgery, the mice were sacrificed, and implants were harvested. The weights of all these samples were obtained at wet and ash contains (burned at 800 °C for 4 h). Entities were pictured as contradistinctions among groups of fresh ectopic bone formed. Sections of one sample from each group were stained with hematoxylin/eosin after decalcification and viewed under a light microscope.

### 2.5. Statistical analysis

All numerical data were expressed as the mean ± standard deviation, with similar results obtained in each experiment. Statistical analysis was performed with one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Synthesis and characterization of sulfated chitosan

2-*N*-Sulfated chitosan (2SCS), 6-*O*-Sulfated chitosan (6SCS) and 2-*N*, 6-*O*-Sulfated chitosan (26SCS) were synthesized successfully according to the methods described in "Materials and methods" (Fig. 1). By controlling synthesis conditions (alkaline [26] and acid atmosphere [25]), 2SCS and 6SCS were substituted by only one sulfate group, respectively. Compared with 2SCS and 6SCS, 26SCS has the best similarity with heparin. The sulfate positions in different polymers were characterized with two absorption peaks at ~1230 $cm^{-1}$ derived from S=O and ~800 $cm^{-1}$ for C–O–S (not existed in 2SCS) bond stretching, respectively (Fig. 2).

The reaction temperature and sulfating agent amount could be adjusted to obtain 26SCS with different $M_w$ and sulfate content. An increase in reaction temperature caused a significant decrease in molecular weight and slight decrease in sulfur substitution, which might indicate that the saccharide chain on chitosan degradation

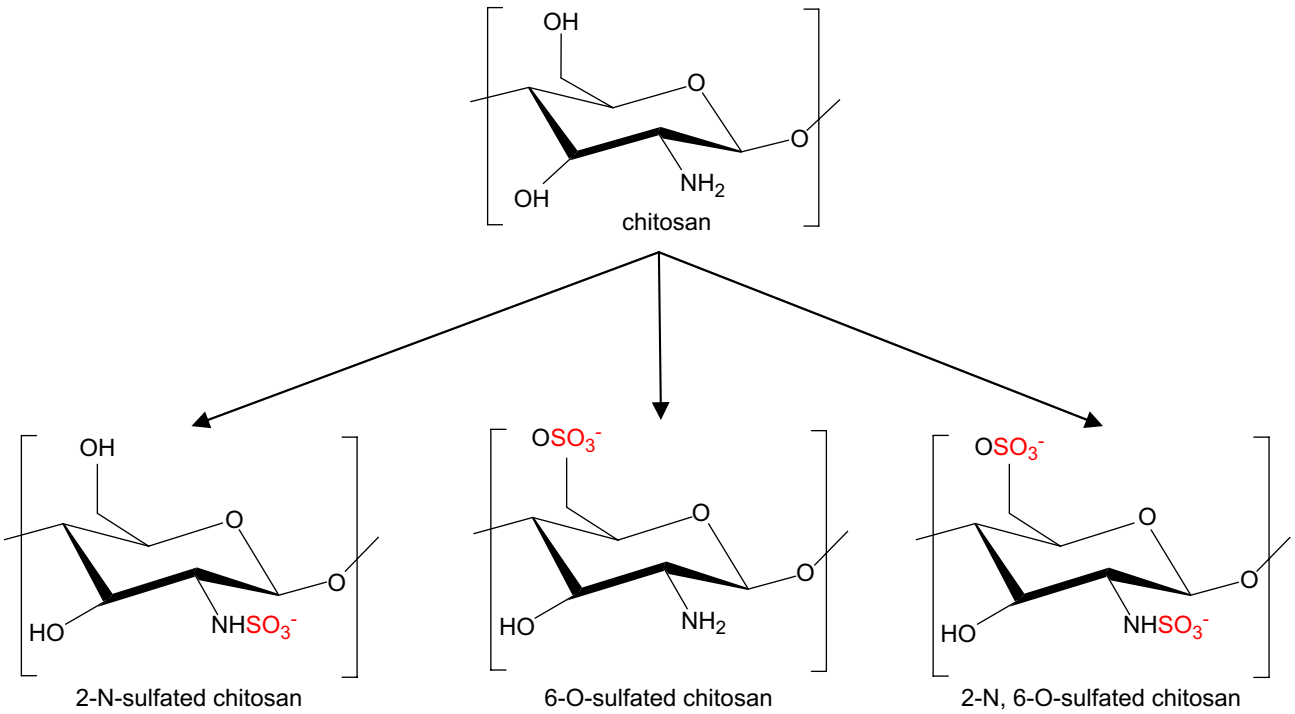


**Fig. 1.** Chemical structures of chitosan modified with different sulfate groups. 2-*N*-Sulfated chitosan, 2SCS; 6-*O*-Sulfated chitosan, 6SCS; 2-*N*, 6-*O*-Sulfated chitosan, 26SCS.

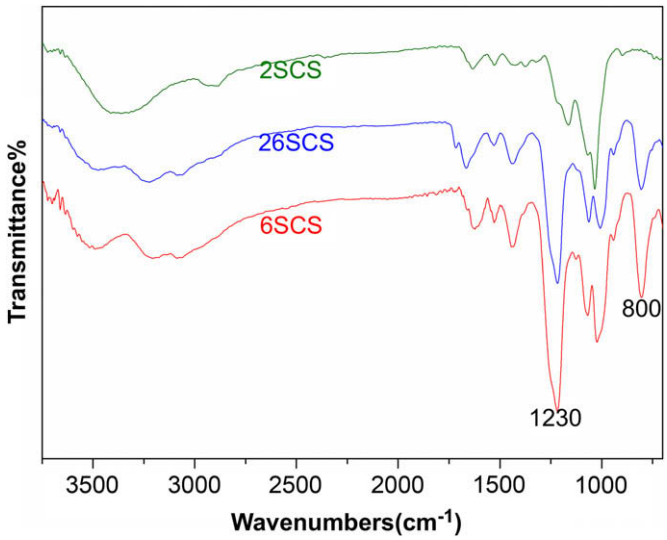


**Fig. 2.** FTIR spectra of different sulfated chitosans. 2-*N*-Sulfated chitosan, 2SCS; 6-*O*-Sulfated chitosan, 6SCS; 2-*N*, 6-*O*-Sulfated chitosan, 26SCS.

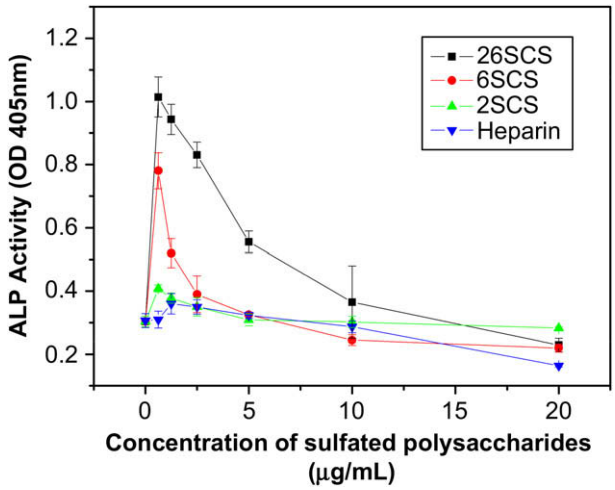


**Fig. 3.** Sulfated polysaccharides at low dose enhanced the bioactivity of BMP-2 in C2C12 cells. Cells were cultured for 3 days with graded concentrations of different sulfated polysaccharides (26SCS, 6SCS, 2SCS and heparin) in the presence of 0.4 μg/mL BMP-2. ALP was measured at day 3 using soluble substrate *p*-nitrophenylphosphate. The values represent the mean ± standard deviation ($n = 4$).

and desulfur reactions happened. Adding more sulfating reagent ($HClSO_3$) to the reaction would increase sulfate content in the product. 26SCS was obtained with sulfur content of 12.89–14.86%, which corresponds to a degree of sulfation of 1.96–2.25 per glucosamine unit. Actually, C-3 hydroxyl groups might be sulfated in 26SCS, but only little part participated in the reaction due to steric hindrance limitations [27]. The detailed parameters of sulfated chitosan used in later experiments were listed in Table 2.

### 3.2. Effect of sulfated polysaccharides on BMP-2-induced cell differentiation

BMP-2 can convert the differentiation pathway of C2C12 myoblast cells into osteoblast lineage [28]. It has been shown that inhibition of myoblastic differentiation and induction by BMP-2 of osteoblast differentiation by BMP-2 involve the BMP receptor-1A and the transducers Smad1 and Smad5. BMP-2 induction of the transdifferentiation of C2C12 myoblasts to osteoblasts provides an excellent model to investigate mechanisms regulating commitment to the osteoblast phenotype [29]. As all the sulfated chitosans were designed to promote the BMP-2 activity, their effects on the BMP-2-induced osteoblast differentiation were evaluated by ALP activity measurement in C2C12 cells stimulated with both sulfated polysaccharides and BMP-2. These different experiments were performed at the same initial seeding density ($1 \times 10^4$/well). In the preliminary experiments, we found that the enhancement of ALP activity could be observed when the BMP-2 concentration was above 0.4 μg/mL. On the other side, to ensure that the measured absorbance value did not exceed the linear limit (<1.5), the BMP-2 concentration of 0.4 or 0.8 μg/mL was used in our experiments. The results in Fig. 3 showed that all the synthetic sulfated chitosans were superior to heparin as higher ALP activity could be obtained within the concentrations used. In the presence of BMP-2 alone, the ALP value is about 0.3, whereas in the range of the material concentrations tested, the values were elevated in a dose-dependent manner: the highest ALP activity was reached to the range of 0.4–1.0 with respect to different sulfated polysaccharides at the concentration about 0.625–1.25 μg/mL, then the enhancement to ALP activity was decreased and even became to inhibition at the concentration of 20 μg/mL. Among the three sulfated chitosans, 26SCS was the most potent BMP-2 enhancer, the highest ALP activity was more than 2 times higher than that of heparin, while 2SCS showed the weakest enhancement. These results suggested that 6-*O*-sulfate was critical for the positive stimulation and 2-*N*-sulfate was a subsidiary group. This was consistent with previous study that heparin bound to BMP-7 and its binding property was specific for heparin structure, such as *N*- and 6-*O*-sulfates [30]. Native heparin has little positive effect on the bioactivity of BMP-2, and higher dose would inhibit the ALP activity induced by BMP-2. Differently, even though 26SCS has similarity with heparin on chemical structure, the enhancements of 26SCS on the ALP activity by BMP-2 were significantly higher than those of heparin at a relative low dose, which might be attribute to much higher sulfur content than native heparin and different polysaccharide structures.

**Table 2**
Reaction conditions and parameters of synthesized sulfated chitosan.

| Groups | Denomination | Reaction conditions | | | | $M_w$ | Sulfur content (%) |
|---|---|---|---|---|---|---|---|
| | | Chitosan (g) | $HClSO_3$ (mL)/$SO_3$-Pyd (g) | Temperature (°C) | Time (h) | | |
| 1 | 2SCS | 1 | 3 | 50 | 24 | $5.565 \times 10^4$ | 7.951 |
| 2 | 6SCS | 2 | 10 | 0–4 | 3 | $2.885 \times 10^5$ | 9.04 |
| 3.1 | 26SCS | 2.5 | 5 | 45 | 2 | $8.765 \times 10^4$ | 13.36 |
| 3.2 | 26SCS | 2.5 | 5 | 55 | 2 | $2.884 \times 10^4$ | 13.32 |
| 4.1 | 26SCS | 2.5 | 5 | 50 | 2 | $4.781 \times 10^4$ | 12.89 |
| 4.2 | 26SCS | 2.5 | 8 | 50 | 2 | $5.034 \times 10^4$ | 14.86 |

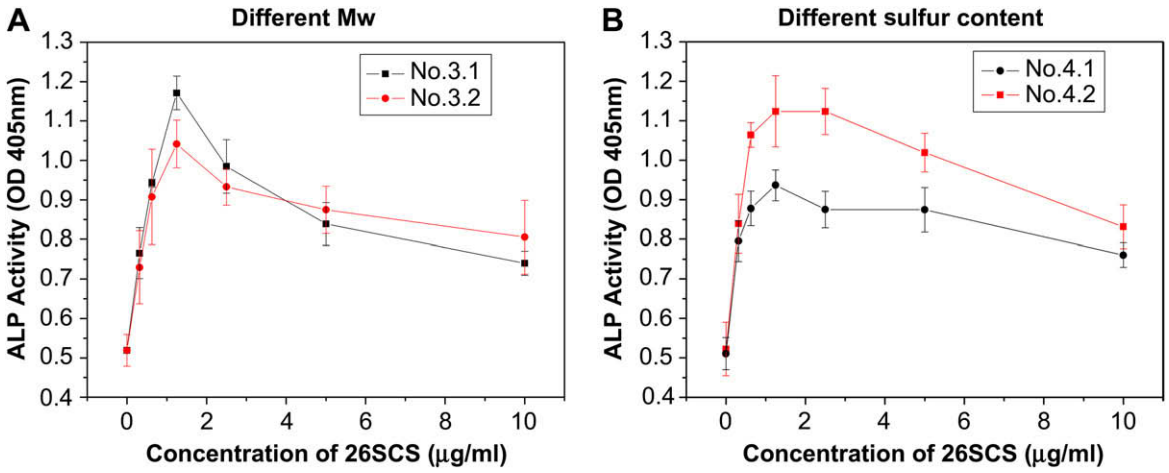


**Fig. 4.** 26SCS with different $M_w$ and sulfur content enhanced the bioactivity of BMP-2 dose-dependently. Cells were cultured for 3 days with graded concentrations of 26SCS with different $M_w$ (No. 3.1, $8.765 \times 10^4$; No. 3.2, $2.884 \times 10^4$) (A) and different sulfur content (No. 4.1, 12.89%; No. 4.2, 14.86%) (B) in the presence of 0.8 μg/mL BMP-2. ALP was measured at day 3 using soluble substrate *p*-nitrophenylphosphate. The values represent the mean ± standard deviation ($n = 4$).

Sulfated polysaccharide (2SCS,6SCS,26SCS or heparin) alone failed to stimulate C2C12 cells to secrete alkaline phosphatase in the absence of BMP-2, which means that these sulfated polysaccharides interacted with BMP-2 to indirectly influence C2C12 cells osteoblastic differentiation, in addition, treatment of cells with these sulfated polysaccharides and BMP-2 under the same concentrations did not have any significant effect on cell proliferation rate or cell viability, as demonstrated by a cell proliferation assay (data not shown).

During the synthesis, 26SCS with different $M_w$ and sulfur content were obtained. As listed in Table 2, 4 types of 26SCS in 2 groups were selected to compare the effects of $M_w$ and sulfur content on the enhancement of BMP-2 activity at 0.8 μg/mL of BMP-2. With the similar sulfur content, No. 3.1 had a higher $M_w$ and showed a stronger enhancement as higher ALP activity. As higher ALP activity was obtained in the concentration range from 0.313 μg/mL to 2.5 μg/mL (Fig. 4A). Meanwhile, for group 4, No. 4.1 and No. 4.2 had a similar $M_w$. But No. 4.2 had higher sulfur content and showed a stronger BMP-2 enhancement under all the concentrations tested (Fig. 4B). Therefore, from our data, 26SCS with a higher molecular weight and sulfur content would be better enhancer for BMP-2 activity. And the No. 4.2 26SCS was selected and used in our experiments unless otherwise noticed.

To further confirm the enhancement of 26SCS on the BMP-2-induced ALP activity, naphthol AS-BI phosphate and fast blue BB salt were employed for histochemical analysis (Fig. 5). Under the concentrations of 26SCS used as before, incubation with 0.625–2.5 μg/mL 26SCS and 0.8 μg/mL BMP-2 for 3 days strikingly induced ALP-positive cells to differentiation. The extent of stained ALP-positive cells with 26SCS >5 μg/mL was almost equal to control (BMP-2 only). The results obtained with histochemical staining were consistent with experiments on ALP activity before. Therefore, we could conclude that 26SCS at relative low or high concentrations showed dose-dependent effects on the bioactivity of BMP-2 in C2C12 cells.

### *3.3. Effect of 26SCS on the expression of genes related to the induction of cell differentiation by BMP-2*

We further examined the changes in the expression levels of ALP and osteocalcin (OC) (Fig. 6) in our system. Previous studies showed that the expression of ALP is earlier than osteocalcin in osteoblastic

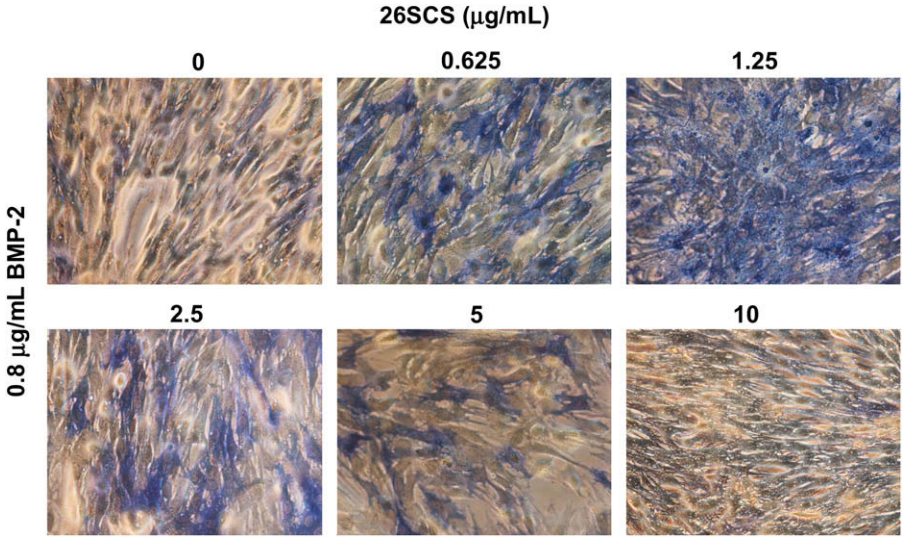


**Fig. 5.** 26SCS enhanced the ALP activity dose-dependently. Cells were cultured for 3 days with 0, 0.625, 1.25, 2.5, 5 or 10 μg/mL of 26SCS (No. 4.2) in the presence of 0.8 μg/mL BMP-2. Alkaline phosphatase activity was stained with naphthol AS-BI phosphate and fast blue BB salt (200×).

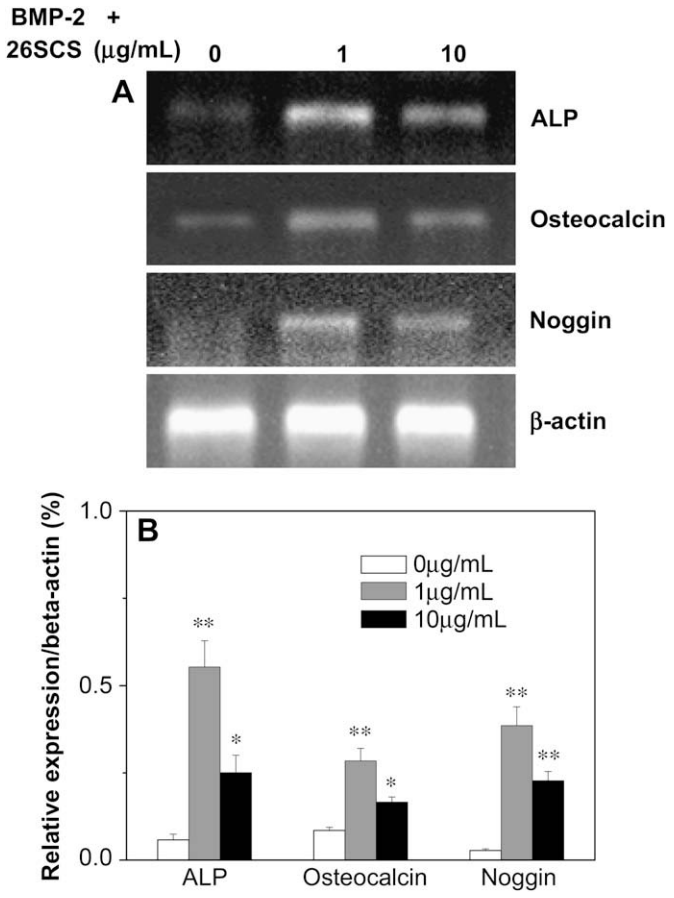


**Fig. 6.** Effect of 26SCS on gene expression levels induced by BMP-2. C2C12 cells were treated with 0, 1 or 10 μg/mL of 26SCS (No. 4.2) in the presence of 0.8 μg/mL BMP-2. Total mRNAs were obtained after 48 h incubation, and reverse transcription-PCR was performed to analyze mRNA of ALP, osteocalcin, and Noggin. β-Actin was used as an internal control. (A) Representative profiles from 3 independent experiments by RT-PCR. (B) Integrated optical data analyzed by TotalLab 2.01, $^{*}p < 0.01$; $^{**}p < 0.001$, compared with group without 26SCS.

differentiation and mineralization, and these two genes were constantly expressed after 12 h stimulation [31]. In our preliminary experiments, 26SCS under the concentrations used alone could not affect the expression of ALP, OC and Noggin. After 48 h stimulation, expression of ALP and OC was induced by BMP-2 (0.8 μg/mL) and further stimulated by adding 1 μg/mL and 10 μg/mL 26SCS. From the semi-quantitative analysis, the extent of induction of ALP and OC at 1 μg/mL 26SCS was 9.5-fold and 3.3-fold higher than that with BMP-2 alone, respectively. The expression levels of ALP and OC at 1 μg/mL 26SCS were higher than those at 10 μg/mL. In addition, the mRNA level of Noggin, the antagonist of BMP-2 was also investigated. The extent of the expression of Noggin was enlarged 13.9-fold in the presence of 26SCS (1 μg/mL), whereas almost undetectable with only BMP-2 treatment, suggesting that while the osteoblastic differentiation was enhanced by 26SCS, negative feedback of cells was also activated. Apparently, the increased expression of Noggin failed to block the bioactivity of BMP-2, suggesting the inhibition from antagonists might be protected by 26SCS.

### 3.4. Effect of 26SCS on the BMP-2s binding to cell surface

In order to investigate the dose-dependent effect of 26SCS on BMP-2 activity, we examined the effects of 26SCS on the binding efficiency between BMP-2 and its receptor which is deposited on cell surface. Hence, the detectable amount of BMP-2 localized on cell layer reflects BMP-2 signal intensity directly. BMP-2 was detected by immunofluorescence staining via anti-BMP-2 primary-antibody and FITC-conjugated goat-anti-mouse IgG. Cell nucleus was stained with DAPI served for cell localization. Fig. 7 showed that BMP-2 was distributed on cell membrane uniformly. In the presence of 1 μg/mL 26SCS, BMP-2 signaling was intensively enhanced and spindled. With the addition of 10 μg/mL 26SCS, the detected BMP-2 signaling was weakened. The result suggested that low dose of 26SCS would increase the binding efficiency between BMP-2 and its receptor, 26SCS at higher dose would impair BMP-2 and its receptor binding.

### 3.5. Effect of 26SCS on the BMP-2-induced mineralization and ectopic bone formation

Matrix bound mineral was detected by Alizarin Red-S to further characterize the effect of 26SCS on the bioactivity of BMP-2 *in vitro* (Fig. 8). Within 1 week, compared with control (BMP-2 only), no obvious enhancement or inhibition on mineral content treated with different dose of 26SCS could be observed. At 2 weeks, strongly staining and connected mineral-containing structures were formed in all the cultures regardless of the presence of 26SCS. However, treated with 1 μg/mL 26SCS promoted mineral formation mostly. The result suggested that long-term treatment with low dose of 26SCS and BMP-2 would accelerate bone mineralization.

We further examined the enhancing effect of 26SCS on BMP-2 activity *in vivo*. Within 4 weeks implantation, 26SCS increased the

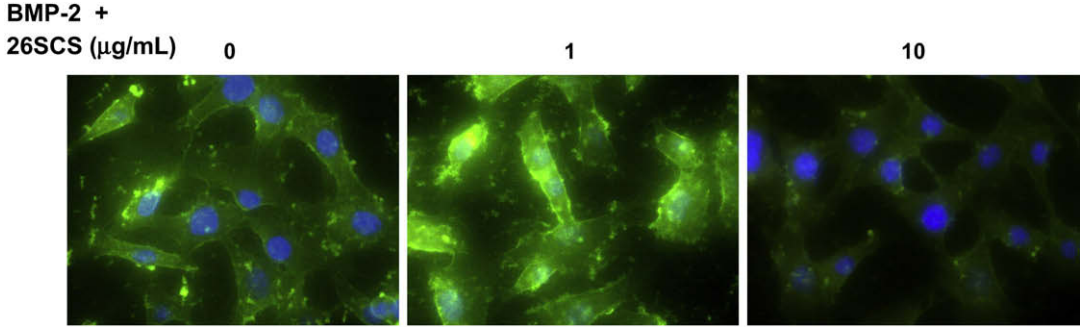


**Fig. 7.** Effect of 26SCS on localization of BMP-2 on cell layers. C2C12 cells were pretreated with 0, 1 or 10 μg/mL 26SCS (No. 4.2) in the presence of 0.8 μg/mL BMP-2 in culture medium for 4 h and fixed. BMP-2 was detected with anti-BMP-2 antibody and FITC labeled anti-mouse IgG (green) and cell nuclei were stained with DAPI (blue) for cell orientation (400×).

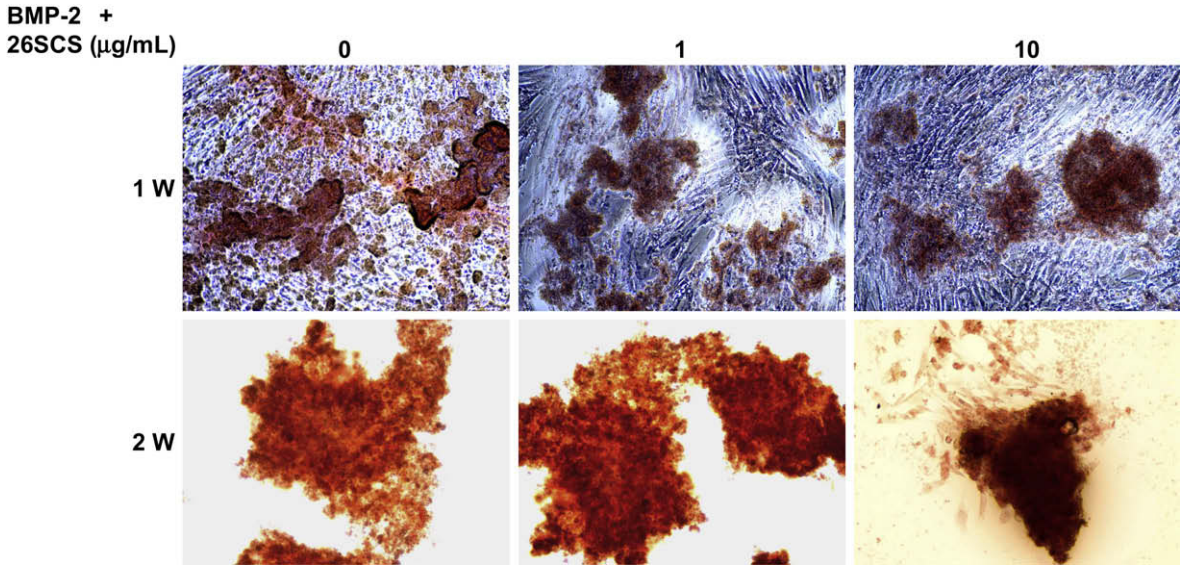


**Fig. 8.** Effects of 26SCS on C2C12 cells mineralization induced by BMP-2. C2C12 cells were treated with 0, 1 or 10 μg/mL 26SCS in the presence of 0.8 μg/mL BMP-2 for 1 week (upper panel) or 2 weeks (lower panel). Cells were stained with 1% Alizarin Red-S to detect mineralization and examined under a magnification of 100×.

size of new bone induced by BMP-2 in a dose-dependent manner (Fig. 9A). No ectopic bone formed without the induction of BMP-2 (data not shown). From the data of the harvested implants (Fig. 9B), bone wet and ash weight in the control group (only BMP-2 implanted) was ~0.11 g and 0.012 g, respectively. Along with the addition of 26SCS, bone wet and ash weights were increased. In the presence of 100 μg 26SCS, the new bone weight reached a peak of 173% (wet) and 155% (ash)-fold higher than control group ($p < 0.05$). Then the new bone weight decreased with larger amount of 26SCS implantation (500 μg).

For histological evaluations of the samples from each group, sections of the implants were stained with hematoxylin/eosin and representative data were showed (Fig. 9C). At 4 weeks, there was no ectopic bone formed without BMP-2 treatment in mice. However, bone tissues could be observed in the group treated with BMP-2 alone. With the addition of 100 μg 26SCS, not only bone marrow but also a large amount of trabecular bone was observed in the pellets. The amounts of trabecular bone and bone marrow decreased with BMP-2 and 500 μg 26SCS. From the results obtained *in vivo*, we concluded that similar to the experiments *in vitro*, appropriate amount of 26SCS could enhance the capability of ectopic bone formation induced by BMP-2 significantly, while its excessive usage would cause negative effect on new bone formation.

## 4. Discussion

As we mentioned before, a major obstacle limiting the widespread adoption of BMPs is the high cost and potential side effects caused by the large amount of this protein required to promote new bone formation. In this paper, the effects of synthetic sulfated chitosans with different sulfate groups (2SCS, 6SCS and 26SCS) on BMP-2 activity were investigated. Interestingly, dose-dependent effects of sulfated chitosan at low doses and high doses on ALP activity induced by BMP-2 were observed. We have also demonstrated that low dose of 26SCS enhanced the BMP-2-induced mineralization *in vitro*. And the similar effect was also observed when the expression levels of genes related to bone differentiation (ALP, osteocalcin) were analyzed. Low dose of 26SCS enhanced ectopic bone formation induced by BMP-2 *in vivo* as well. In the presence of relative low amount of 26SCS (100 μg/implantation), BMP-2 induced larger amounts of mineralized bone tissues within 4 weeks in mice.

Within sulfated chitosans we synthesized, 6-*O*-sulfate was critical for the enhancement of ALP activity induced by BMP-2, and 2-*N*-sulfate was subsidiary, this result was similar to the anticoagulant activity by sulfated chitosan [24], and 26SCS showed the greatest stimulation. At low dose, 26SCS with the increased polysaccharide chain size and further sulfation showed stronger enhancement to the bioactivity of BMP-2 positively.

Several studies have suggested that native heparin contains a BMP-2 binding site [32]. The structure of BMP-2 showed that it had prominent positive charged cavities [33], heparin and heparin sulfate have been shown to interact directly with BMP-2 via highly negative charged polysaccharide chains [16]. It is noticed that at low concentrations, exogenous heparin had little effect on BMP-2 induction of ALP [17]. From our results, the potency of 26SCS to enhance the bioactivity of BMP-2 is much higher than that of native heparin (Fig. 4), and the break-over point (enhancing to inhibiting) was also higher. Taking these findings into account, 26SCS has great structure similarity with native heparin and extremely high sulfur content, which indicated that BMP-2 might recognize specific sulfation motifs in 26SCS chains and interact with 26SCS more intensively than heparin. More precise control on the synthesis of sulfated chitosan might be carried out and the relationship between $M_w$ or sulfur content of sulfated chitosan and the interactions with BMP-2 might be elucidated in later experiments.

From our results, dose-dependent effects of 26SCS on BMP-2 activity were observed not only *in vitro* but also *in vivo*. Therefore, with the introduction of highly sulfated polysaccharides, the mechanism involved in BMP-2 signal transduction should be rather complicate. To date, as we mentioned before, many researchers have focused on the function of heparin on BMP-2 activity, and conflicting results on this point have been established [6,16–19]. Under their independent experiments, conclusions that adding exogenous heparin either enhanced or inhibited BMP-2 bioactivity have been made. Apparently, different amount of heparin employed might lead to the

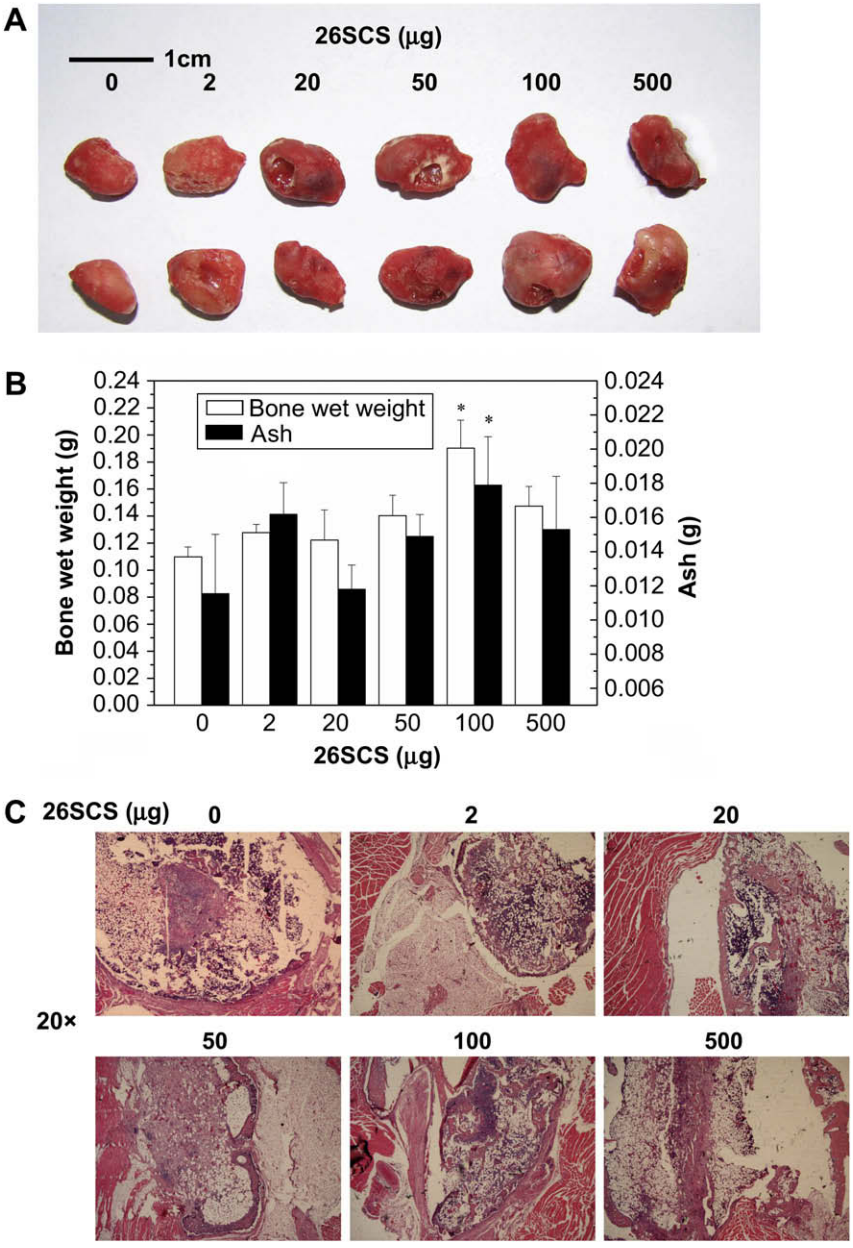


**Fig. 9.** Effect of 26SCS on ectopic bone formation induced by BMP-2 *in vivo*. 100 μg BMP-2 and 0, 2, 20, 50, 100 or, 500 μg 26SCS (No. 4.2) were implanted into mice to induce ectopic bone formation. After 4 weeks, the implants were retrieved and photos are taken by digital camera (A). Bone wet weight and ash weight were obtained as described in "Materials and methods" (B), $^{*}p < 0.05$ compared with groups without 26SCS. Sections of implants from every group were stained with hematoxylin/eosin (C).

discrepancy. In detail, the experimental dose of heparin supporting the positive effect on BMP-2 was relative low (4 or 5 μg/mL) [6], oppositely, large amount of heparin (up to 100 or 2000 μg/mL) [17,19] resulted in the negative effect on BMP-2. It has been reported that heparan sulfate and heparin inhibit BMP-7 activity in ROS17/2.8 osteosarcoma cells [30]. The discrepancy in BMP subgroup (BMP-2 or BMP-7) and cell line (myoblast, osteoblast-like cell, mesenchymal stem cell and osteosarcoma cell) might also lead to opposite results. In our study, heparin as well as sulfated chitosan at low dose had stimulation on cell differentiation, whereas high dose of sulfated polysaccharides turned to be negative. Some reports proved that low doses and high doses of heparin have biphasic effects on osteoblast-like Saos-2 cells *in vitro* [34]. The matrix deposition and mineralization were inhibited by high dose of heparin (>5 μg/mL), whereas low concentration of heparin (5–500 ng/mL) promoted mineralization. As reported, BMP-2 protein is well expressed in human osteosarcoma [35]. Therefore, we suppose that some sulfated polysaccharides interacting with BMP-2 secreted in osteoblastic-like cells might lead to the above biphasic effects. Our results that exogenous heparin affects the bioactivity of BMP-2 dose-dependently showed some combinations of these studies. Differently, highly sulfated chitosan obtained in this study has much higher density of sulfate group on polysaccharide chains, which might be the possible reason for higher enhancement on the bioactivity of BMP-2 at low dose.

At a relative larger dose, the inhibition of sulfated chitosan to the bioactivity of BMP-2 was not due to its cytotoxicity as demonstrated by cell proliferation assay. BMP-2 signaling pathway would be interfered. During the signal transduction, a class of antagonists,

including Noggin, chordin, gremlin, follistatin, and the DAN/Cerebrus family were found to impair BMP-2 bioactivity and degrade it as a part of negative feedback loop [2]. Noggin has been extensively studied for its ability to bind to BMP-2, -4, and -7 in a manner that inhibits the bioactivity of BMPs [36,37]. Noggin may negatively regulate BMP activity in undifferentiated cells by inhibitory microenvironment to control the rate of cell differentiation [38]. Our results showed that the level of induction of Noggin mRNA by BMP-2 was increased in the presence of 26SCS. It has also been reported that Noggin binds strongly to some sulfated polysaccharides on the surface of cultured cells [39,40]. Hence, we could not exclude the possibility that Noggin might integrate with sulfated chitosan. When treated with 26SCS, higher levels of Noggin mRNA expression might result from the enhanced BMP-2 signal, the inhibition from this antagonist-Noggin was blocked; as a result, the degradation of BMP-2 might be protected. Jiao and Billings [17] postulated that high dose of heparin blocked BMP-2–receptor interactions, Kanzaki et al. [19] suggested that heparin suppressed BMP-2-BMPR binding, this hypothesis was supported by our results from immunofluorescence staining which showed that BMP-2 and its receptor binding were enhanced by low dose 26SCS, whereas the localized BMP-2 on cell surface was reduced by high-dose 26SCS, which means that the signaling pathway was diminished.

Regarding to high risks of treatment with heparin, our study has successfully established a novel agent to substitute the function of heparin to enhance the bioactivity of BMP-2. Therefore, the synthesized 26SCS should be clinically useful to enhance BMP-2 activity at least for local bone regeneration. The C2C12 cells and murine implant model enable us to test for synergistic actions of growth promoting agents on bone formation. From these results, we confirmed that low dose 26SCS, but not high-dose 26SCS, could promote BMP-2 signaling, suggesting that appropriate usage of 26SCS could be exploited to modulate BMP-mediated ectopic bone formation such as dysregulated BMP-2 signaling [41], fibrodysplasia ossificans progressive [42], heterotopic ossification [43]. On the other hand, further delineation of this mechanism and optimal dose in which 26SCS acts synergistically with BMP-2 to enhance bone formation will hasten the therapeutic application of combination therapy.

## 5. Conclusions

In the present study, a series of sulfated chitosans with varied sulfate group, $M_w$ and sulfur content were synthesized to investigate the effects on the bioactivity of BMP-2. Low dose of synthetic sulfated chitosan, especially 26SCS, stimulated not only the osteoblast differentiation induced by BMP-2 *in vitro* but also the ectopic bone formation *in vivo*. It appeared that low dose of 26SCS enhanced the interaction between BMP-2 ligands and receptors, as well as inhibited the function of Noggin, whereas BMP-2 signaling pathway was hampered when high dose of 26SCS was involved. These findings suggest that 26SCS is a more potent enhancer of BMP-2 bioactivity than native heparin and will be promising prospects as a synergistic factor of BMP-2 for local bone regeneration.

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## Appendix

Figures with essential colour discrimination. Certain figures in this article, in particular Figures 5, 7, 8, and 9, may be difficult to interpret in black and white. The full colour images can be found in the online version, at doi:10.1016/j.biomaterials.2008.12.016.

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