The regulatory role of sulfated polysaccharides facilitating rhBMP-2-induced osteogenesis

Han Chen,[a, b] Yuanman Yu,[a, b] Chenmin Wang,[a, b] Jing Wang* [a, b] and Changsheng Liu* [b, c]

a.The State Key Laboratory of Bioreactor Engineering,East China University of Science and Technology, Shanghai, 200237 People's Republic of China Email: biomatwj@163.com, liucs@ecust.edu.cn; Tel: +86-21-64251308

b.Engineering Research Center for Biomedical Materials of Ministry of Education, East China University of Science and Technology, Shanghai, 200237, People's Republic of China

c. Key Laboratory for Ultrafine Materials of Ministry of Education East China University of Science and Technology, Shanghai, 200237, People's Republic of China

1. Materials and methods

1.1 Characterization of materials immobilized surface

The Energy Dispersive Spectrometer (EDS) can analyze the type and content of elements by detecting the X-ray photon characteristic energy of different elements on the surface of the sample. In this experiment, the content of N, O, S elements on the surface of Au, Au-SAM-26 SCS, Au-SAM-DSS, and Au-SAM-PSS were analyzed by EDS.

In this experiment, the wettability of the four groups was judged by measuring the contact angles of the surfaces of Au-SAM, Au-SAM-26 SCS, Au-SAM-DSS and Au-SAM-PSS. Generally, the greater the contact angle, the weaker the wettability of the surface of the material. When measuring, put the chips on the leveling contact angle analyzer; take 4 points of each chip, drop a drop of deionized water, respectively. Take a photo after 30 s, and calculate the static contact angle of the sample by two-point method. Take the average of 4 points as the final results.

Atomic force microscopy (AFM) can be utilized to detect the surface structure and properties of the nano-areas in the sample. We fixed the sample on the sample stage with the view of 2 μm × 2 μm and scanned it with a tapping mode, then calculated the arithmetic mean roughness Ra and root mean square roughness Rq of the material surface in the field of view.

1.2 Cell Toxicity

C2C12 cultured on a 96-well plate with a seeding density of 6000/well. After the cell completely adhered to the bottom of culture bottle (12 h later), the medium were replaced by 200 μL/well fresh ones containing different concentration of materials with the cells incubated in pure culture medium as the control. 1 and 3days later, 20 mL/well thiazolyl blue tetrazolium bromide (MTT) were added to the samples and incubated it in a 5% CO_2 atmosphere at 37°C for 4 h. Then the mixture were replaced by 100 μL/well dimethyl sulfoxide (DMSO) to dissolve

the Formazan in the well. 10 min later, the optical density (OD) of the samples were quantified at the wavelength of 492 nm by using a microplate reader (SPECTRAmax 384, Molecular Devices, USA). The cell toxicity were the percentage ratio of OD sample / OD control.

1.3 Alkaline phosphatase (ALP) activity and concentration screening

C2C12 cells were seeded in 96-well plate at a density of 1×10^4 cell/mL. After 12 h incubation, the growth medium were refreshed with 200 μL/well maintenance medium (DMEM containing 2% fetal calf serum) beside 0.8 μg/mL rhBMP-2 and gradient concentrations of materials (0, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8 μg/mL) with only 0.8 μg/mL rhBMP-2 medium set as the positive control group. The bone formation ability was evaluated by alkaline phosphatase (ALP) assay. After 3days incubation, samples were washed with PBS twice and added 70 μL/well 1% Nonidet P-40 (NP-40) solution and incubated at 37°C for 1.5 h to obtain cell lysate. Pipetted 20 μL of the cell lysate into a new plate, add 200 μL/well of BCA reagent and incubate at 37°C for 30 min. Perform colorimetric comparison at 562 nm on a microplate reader. Drew a standard curve, the fetal bovine serum protein content is the abscissa and the absorbance value is the ordinate. The protein content of the sample was determined from the standard curve based on the absorbance of the sample. Pipetted 50 μL of the cell lysate into another plate, added 100 μL/well of p-nitrophenylphosphate (Sigma, USA) and incubate at 37°C for 30 min. Optical density (OD) of the samples were measured at 405 nm by the microplate reader. The ALP activity were calculated by the percentage ratio of ALP OD value / BCA OD value.

1.4 Effect of materials on the stability of rhBMP-2

In order to investigate the maintaining effect of three materials on the osteogensis effect of rhBMP-2, 0.8μg/mL of the three materials were mixed with 0.8 μg/mL rhBMP-2, the group contained 0.8μg/mL rhBMP-2 was used as the control group. Then they were pre-incubated for 0 days, 1 day and 3 days in 37 °C culture medium, and evaluated the 3 days' ALP activity of them. The procedure was the same as 2.5.3.

1.5 Effect of chitosan and dextran on rhBMP-2 induced ALP activity

Unsulfated chitosan and dextran were used to investigate their effect on rhBMP-2 induced osteogenic differentiation. The group contained only 0.8 μg/mL rhBMP-2 was set as the control group. The groups contained 0.8 μg/mL chitosan/dextran mixed with 0.8 μg/mL rhBMP-2 were set as experimental groups. The 3 days' and 7 days' ALP activity were measured with the same procedure as 2.5.3.

1.6 Effect of chitosan and dextran on rhBMP-2 induced ectopic osteogenesis

C57BL/6 male mice were used to assess the effects of chitosan and dextran with rhBMP-2 on ectopic bone formation (n = 2). The procedure was same as 2.6.

2. Results

2.1 Characterization of QCM chip surface

The surface elements of Au-SAM, Au-SAM-26 SCS, Au-SAM-DSS, Au-SAM-PSS were analyzed by energy dispersive spectroscopy (EDS) and compared with the surface of Au (Fig. S1). We could found the S element on the SAM-Au surface in Table S1, indicated that the SAM reagent was successfully grafted on the Au surface; The S content of Au-SAM-26 SCS, Au-SAM-DSS and Au-SAM-PSS surface were 7.4%, 4.3%, and 3.5% higher than that of Au-SAM surface, respectively, because of all three materials contained $-SO_3$, which indicated that the three materials were grafted into the surface of SAM-Au successfully.

As the 2D and 3D image of AFM (Fig. S2A-B) and the roughness analysis (Table S2) indicated, the surface of the chips were both evenly flat before and after grafted the SAM reagent and 26 SCS, DSS and PSS. The Ra and Rq of Au-SAM surface were both decreased slightly, which were 0.2 nm and 0.16 nm lower than that of Au surface, respectively. The Ra and Rq were increased slightly after the three materials grafted on the Au-SAM surfaces, but they were still lower than that of Au surface. This may be due to the small size of the SAM reagent molecule, which were filled into the Au surface and lead to the decreasing of roughness of the chips, while the molecules of three materials were larger, so the surface roughness of the chips were slightly increased.

As depicted in Fig. S2C, the surface of Au chip itself was relatively hydrophobic, and the contact angle was about 89.2°. The hydrophilicity was remarkably improved by the SAM reagent modification. After combined with the three sulfated polymers, the surface of the Au was formed into a bi-layer structure, and the polar end of the outer layer had a strong affinity to water, increased the degree of surface wetted by water. The difference of the contact angle in each group was not too much and both between 74 and 78 degrees, since the three sulfonates had hydrophilic sodium sulfonate group. Among them, Au-SAM-PSS had the smallest contact angle of about 74.8°. PSS was often used as a dispersant. The hydrophobicity of the main chain and the hydrophilicity of the sodium sulfonate group facilitated the regular arrangement of PSS on the surface of the substrate. The above experimental results showed that the three materials successfully grafted on the surface and improved the hydrophilicity of the surface, which facilitated further rhBMP-2 modification.

Table S1 Content of elemental atom of the surface of QCM chips in EDS spectra

Substrate	O%	N%	S%
Au	33.3	66.7	-
SAM-Au	39.0	35.2	25.8
26 SCS-SAM-Au	38.8	27.9	33.3

DSS-SAM-Au	34.2	35.7	30.1
PSS-SAM-Au	33.5	37.2	29.3

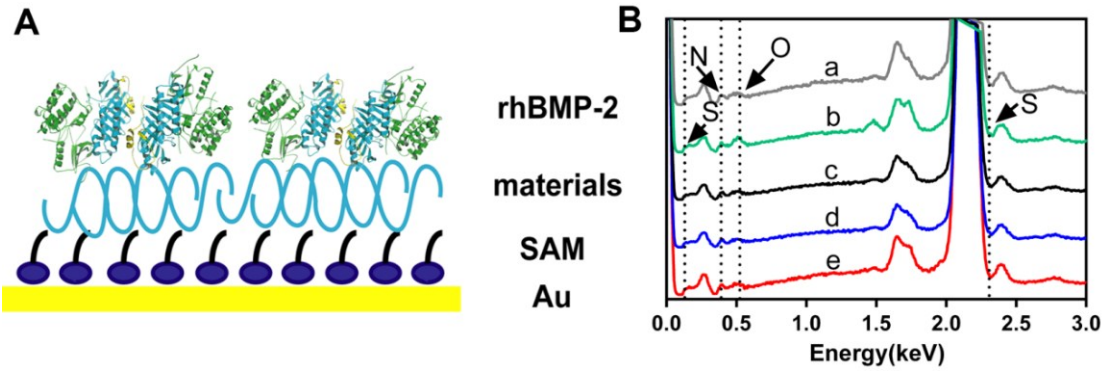

Figure S1. (A) Structure of QCM chip surface; (B) Surface elements (N, O, S) assay of (a) Au (b) Au-SAM (c) Au-SAM-26 SCS (d) Au-SAM-DSS (e) Au-SAM-PSS by EDS.

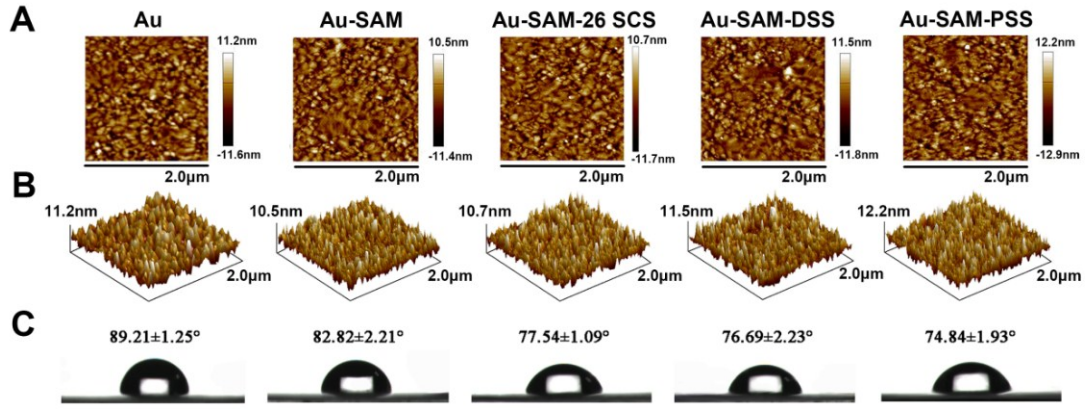

Figure S2. Surface roughness assay by AFM (A.2D and B. 3D) and (C) contact angle.

Table S2 Rq and Ra of different substrate of QCM-D chips.

Substrate	Rq (nm)	Ra (nm)
Au	3.2	2.6
SAM-Au	3.0	2.4
26 SCS-SAM-Au	3.1	2.5
DSS-SAM-Au	3.2	2.5
PSS-SAM-Au	3.2	2.5

2.2 Cell Toxicity

According to the 1 day and 3 days cell toxicity assay (Fig. S3), the 26 SCS showed few effect on cellular activity, while the DSS and PSS showed toxicity to cells when the density beyond 0.8 μg/mL in 3 days assay. According to the OD value to evaluate cytotoxicity refer to

the "American Pharmacopoeia" toxicity grading method, the evaluation criteria are: Level 0, RGR ≥ 100%; level 1, RGR ≥ 80%; level 2, RGR ≥ 50%; level 3, RGR ≥3 0%; level 4, RGR ≥ 0%. When the concentration was less than 0.8 μg/mL, the cell toxicity grade were less than level 1 in the 3 days and 7 days tests.

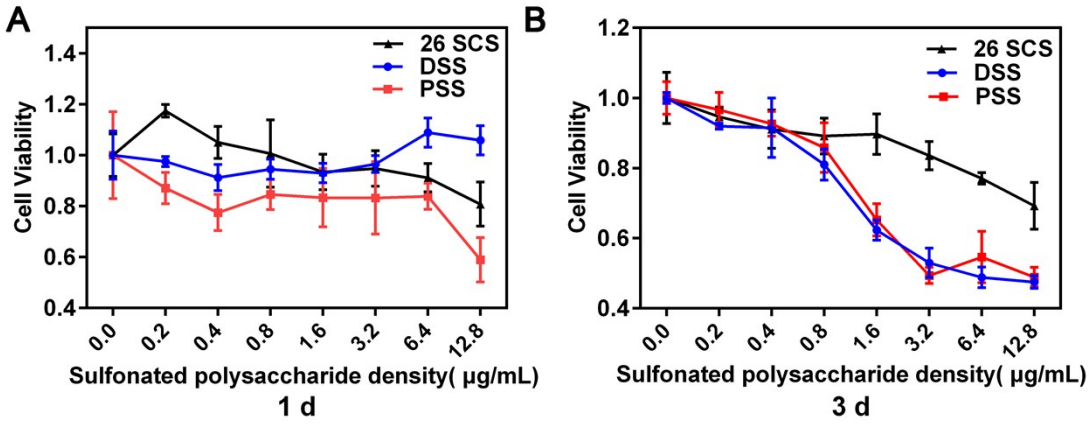

Figure S3. Cell toxicity of materials in (A) 1 day and (B) 3 days assay. C2C12 cells were cultured with graded concentrations of materials. Cell toxicity were measured by using thiazolyl blue tetrazolium bromide. The values represent the mean ± standard deviation (n = 5).

2.3 Cell Differentiation

In our assay, we were tried to find the suitable concentration of sulfated polysaccharides to promote the procedure of differentiation facilitated with 0.8 μg/mL rhBMP-2 and the differentiation between each sulfated polysaccharide. For 26 SCS, there is a significantly uptrend of ALP activity when the concentration increase to 0.4 μg/mL and reached the highest peak at 0.8 μg/mL. While the ALP activity of DSS and PSS were decreased and even became to inhibition in a higher concentration. The results in Fig. S4 showed 26 SCS facilitate with rhBMP-2 has the most potential to promote the differentiation of C2C12 myoblast cells into osteoblastat. The ALP activity of 26 SCS was superior to DSS and PSS in the range of 0–3.2 μg/mL and the values were raised in a dose-dependent way. The highest ALP activity of the group with 0.8 μg/mL 26 SCS and rhBMP-2 was 2.3 times and 1.7 times higher than positive control in 3 days and 7 days, respectively.

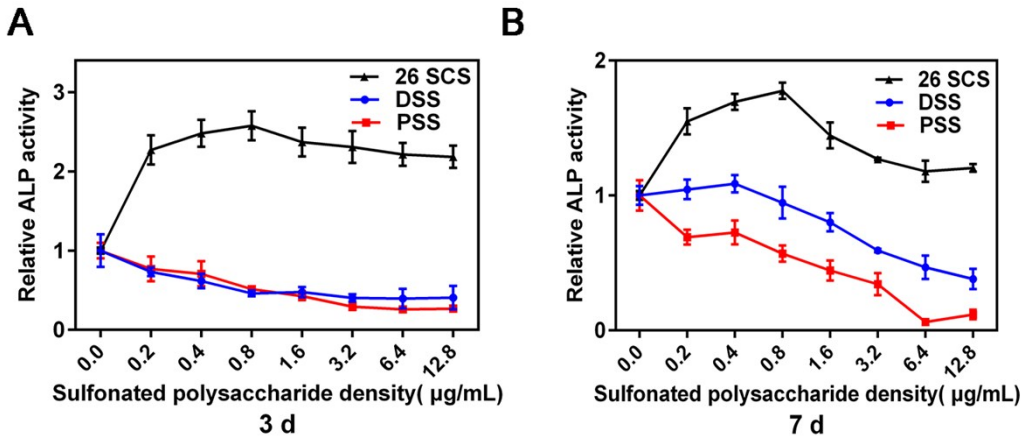

Figure S4. ALP activity of C2C12 cultured with graded concentrations of materials and 0.8 μg/mL rhBMP-2 in (A) 3 days and (B) 7 days. The values represent the mean ± standard deviation (n = 5).

2.4 Evaluation of rhBMP-2 stability

The ALP activity of the 26 SCS group was significantly higher than that of the control group when it was not pre-incubated in the medium. While the ALP activity of DSS and PSS groups were lower than that of control group. After 1 day' and 3 days' pre-incubation, the 26 SCS group still has the better ability to promote rhBMP-2 induced osteogensis activity than that of control group, which confirmed that 26 SCS had a better effect maintaining the osteogensis activity of rhBMP-2.

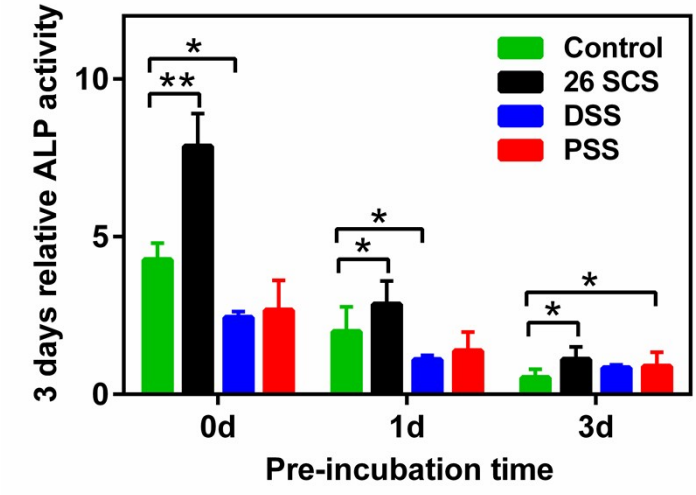

Figure S5. 0.8μg/mL rhBMP-2 and 0.8μg/mL materials were pre-incubated in culture medium for 0 days, 1 day, 3 days, respectively. Then to test the 3 days' ALP activity of them. The values represent the mean ± standard deviation (n = 5). (*$p < 0.05$ and **$p < 0.01$ represent a statistically significant difference compared with the control group).

2.5 Effect of chitosan and dextran on rhBMP-2 induced ALP activity

In the 3 and 7 days' experiments, the ALP activity of the chitosan and dextran groups were basically the same as that of the control group, indicated that the unsulfated chitosan and dextran did not have the promotion effect to rhBMP -2 induced osteogensis differentiation.

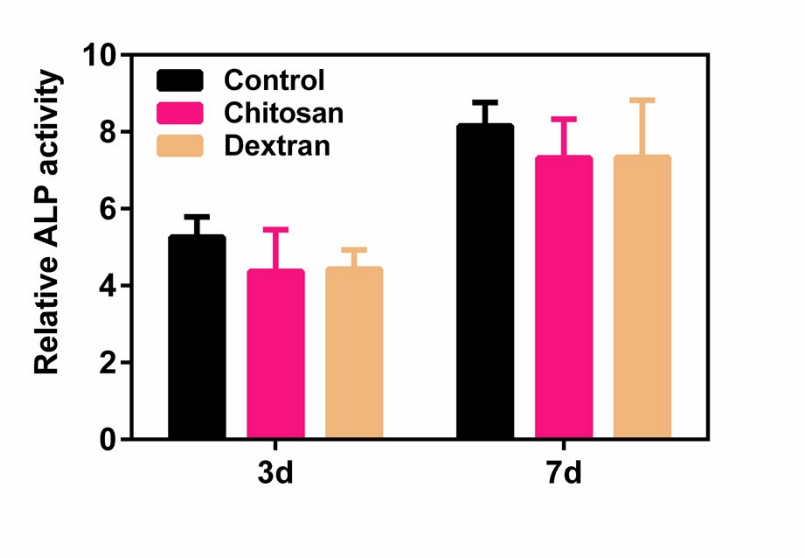

Figure S6. ALP activity of C2C12 cultured with 0.8 μg/mL of materials and 0.8 μg/mL rhBMP-2 in 3 days and 7 days. The values represent the mean ± standard deviation (n = 3).

2.6 Effect of chitosan and dextran on rhBMP-2 induced ectopic osteogenesis

Compared to the control group, the wet weight of bone increased slightly in the chitosan group and dextran group, but the ash weight were same as control group (Fig. S7 A-B). There were undegraded gelatin sponges and new bone formed in chitosan group and dextran group in Histological analysis (Fig. S7 C), which were not significantly different from the control group. The above results indicated that the unsulfated chitosan and dextran did not have the promotion effect to rhBMP -2 induced ectopic osteogenesis.

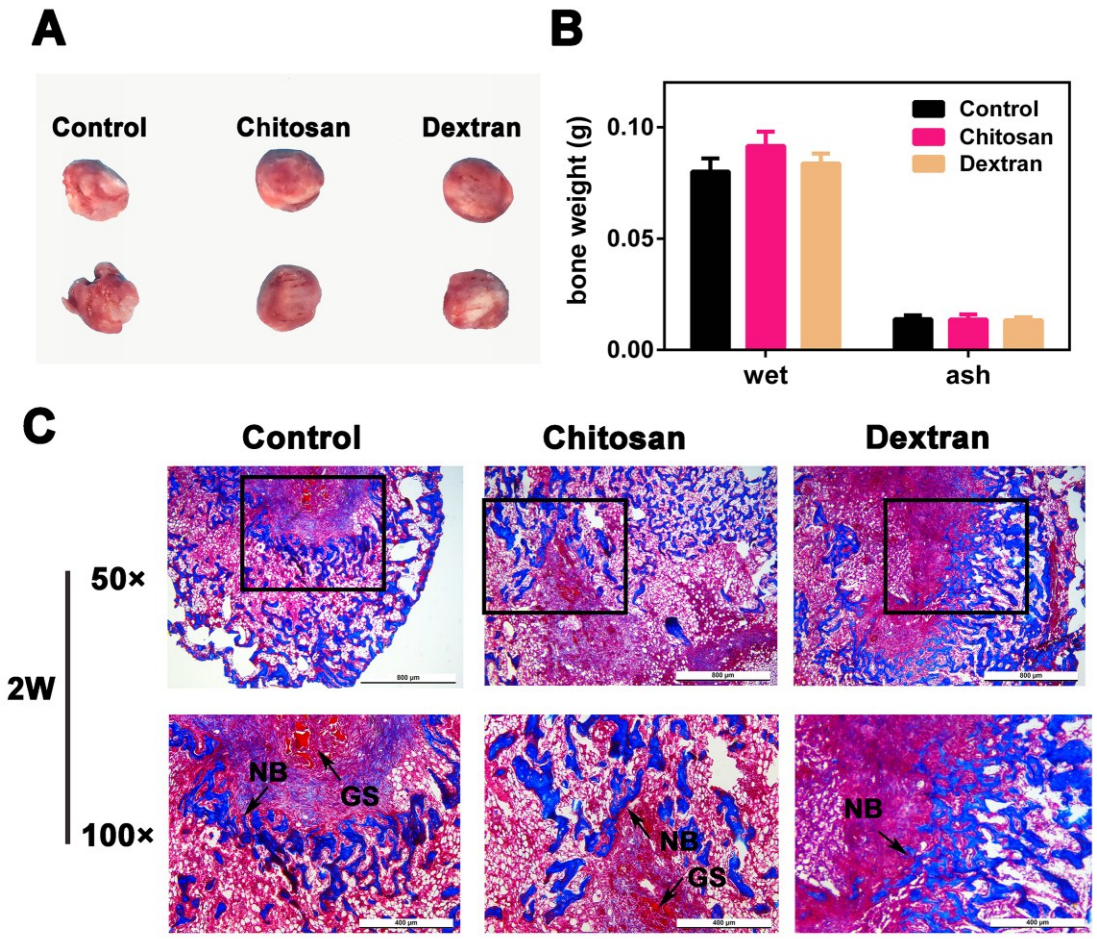

Figure S7. Ectopic bone formation *in vivo*. (A) Morphological evaluation of ectopic bone in 2W assay. (B) Wet weight and ash weight of each group . (C) Histological analysis of ectopic bone. NB: newly formed trabecular bone, GS: non-degraded gelatin sponge.