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6-*O*-Sulfated Chitosan Promoting the Neural Differentiation of Mouse Embryonic Stem Cells

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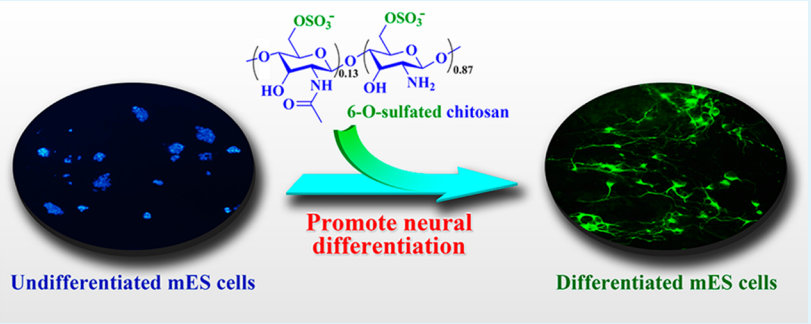
Supporting Information



ABSTRACT: Embryonic stem cells (ESCs) can be induced to differentiate into nerve cells, endowing them with potential applications in the treatment of neurological diseases and neural repair. In this work, we report for the first time that sulfated chitosan can promote the neural differentiation of ESCs. As a type of sulfated glycosaminoglycan analog, sulfated chitosan with well-defined sulfation sites and a controlled degree of sulfation (DS) were prepared through simple procedures and the influence of sulfated glycosaminoglycan on neural differentiation of ESCs was investigated. Compared with other sulfation sites, 6-*O*-sulfated chitosan showed the most optimal effects. By monitoring the expression level of neural differentiation markers using immunofluorescence staining and PCR, it was found that neural differentiation was better enhanced by increasing the DS of 6-*O*-sulfated chitosan. However, increasing the DS by introducing another sulfation site in addition to the 6-*O* site to chitosan did not promote neural differentiation as much as 6-*O*-sulfated chitosan, indicating that compared with DS, the sulfation site is more important. Additionally, the optimal concentration and incubation time of 6-*O*-sulfated chitosan were investigated. Together, our results indicate that the sulfate site and the molecular structure in a sulfated polysaccharide are very important for inducing the differentiation of ESCs. Our findings may help to highlight the role of sulfated polysaccharide in inducing the neural differentiation of ESCs.

KEYWORDS: *sulfated chitosan, neural differentiation, heparin, embryonic stem cells*

1. INTRODUCTION

Embryonic stem cells (ESCs) are pluripotent cells having the potential to differentiate into nearly all cell types in the body; additionally, they are able to self-propagate. Generally, ESCs can be induced by specific growth factors or other bioactive molecules to differentiate into defined cells or tissues in vitro,[1−3] allowing for their application in the fields of genetic screening, drug development, organ transplantation and cell therapy. The use of nerve cells derived from ESCs in vitro has brought new hope for the treatment of neurological diseases, such as depression, Parkinson's and Alzheimer's disease, even though there still remain challenging issues in the medical field.[4,5] Therefore, it is of significant interest to investigate how to guide the differentiation of ESCs into neural cells. There are many methods to induce ESCs into neural progenitor cells and nerve cells.[6,7] The supplementation with exogenous induction factors in the culture medium is one common strategy. The induction factors are mainly growth factor molecules, such as nerve growth factor (NGF), epidermal growth factor (EGF) and fibroblast growth factor (FGF).[6,8,9] However, high costs and short half-lives of these protein drugs strongly limit their entry into mass marketed commercialized products. In recent years, it was discovered that heparin and heparan sulfate (HS) also can promote the neural differentiation of ESCs.[10−13] This suggests that sulfated polysaccharides can affect the directional differentiation of stem cells.

The molecular structure of the sulfated polysaccharide, especially the sulfate groups and carbohydrate units, determines its bioactivity. Heparin and HS are sulfated glycosaminoglycans with structures consisting of alternating *N*-acetylglucosamines

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(GlcNAc) and glucuronic acids (GlcA). Sulfate groups and the overall saccharine structure are key factors that affect the bioactivities of heparin and HS. It was reported that heparin and HS with sulfate groups have the ability to promote the neural differentiation of mouse embryonic stem cells (mESCs),[10] but lose the ability after the removal of sulfate groups.[11] The K5 polysaccharide has a carbohydrate backbone similar to heparin and HS, but without sulfate groups, and could not promote the neural differentiation of ESCs.[12] This result indicated that the sulfate groups in sulfated glycosaminoglycans play a very important role in promoting the neural differentiation of ESCs. Furthermore, several common sulfated glycosaminoglycans (e.g., chondroitin sulfate and dermatan sulfate) have been shown to promote neural differentiation in vivo, albeit at a lower intensity than that of heparin and HS. Because the polysaccharide backbones of chondroitin sulfate and dermatan sulfate do not contain glucosamines and *N*-acetylglucosamines as heparin and HS do, it is likely that the glucosamine and *N*-acetylglucosamine in sulfated polysaccharides are essential for promoting the neural differentiation of ESCs.

Chitosan is a linear polysaccharide whose structure consists of a backbone of GlcA and GlcNAc. It is prepared by the deacetylation of the natural chitin polymer. The production is cost-effective and accessible, and the molecule can be chemically modified to obtain the desired molecular structure. It has been reported that after sulfation, chitosan acquires biological activities similar to heparin and HS.[14−16] Recently, sulfated chitosan has been widely used as a substitute for heparin or HS in biomedical applications, such as in anticoagulant drugs, antiviral drugs, promoting osteoblast differentiation, and in the specific binding of proteins.[16−18] The regulatory mechanism of sulfated chitosan is similar to heparin, which affects the activity of proteins and cells by strongly interacting with specific cells and bioactive substances in vivo.[14,19] Therefore, it is likely that sulfated chitosan may promote the neural differentiation of ESCs in a manner similar to heparin/HS.

It has been reported that the position of sulfate groups in the molecular structure is an important factor that affects the bioactivities of heparin and HS;[20,21] the removal of the 2- or 6-sulfate groups removes the ability for heparin and HS to induce ESCs to differentiate directly into neural cells.[12] In addition to sulfation sites, the degree of sulfation (DS) of a sulfated polysaccharide also affects the neural differentiation of ESCs. On the basis of this information, we prepared sulfated chitosan with defined sulfation sites by introducing sulfate groups to specific sites in glucosamine residues of chitosan and sulfated chitosan with different DS to investigate the molecular effects of different sulfation sites and DS on the neural differentiation of ESCs. Neural differentiation of ESCs was studied by measuring the expression levels of βIII-tubulin, a neuron-specific marker.

2. MATERIALS AND METHODS

Reagents. *Chemical Reagents.* Chitosan oligopolysaccharide with an average molecular weight of approximately 5000 Da and a degree of deacetylation of 0.87 was purchased from Jinan Haidebei Marine Bioengineering Co., Ltd. Na_2CO_3, SO_3·pyridine, H_2SO_4, chlorosulfonic acid ($HClSO_3$), dichloroacetic acid, formamide and *N*,*N*-dimethylformamide (DMF) were purchased from Shanghai Chemical Reagent Co. DMF was purified according to standard methods before use. Deionized water, purified to a minimum resistivity of 18 MΩ·cm by a Millipore water purification system, was used in all experiments.

Biological Reagents. GlutaMAX, nonessential amino acids (NEAA) and fetal bovine serum (FBS) were purchased from Gibco. Leukemia inhibitory factor (LIF) was obtained from Chemicon. High glucose Dulbecco's modified Eagle's medium (DMEM) was purchased from Hyclone.

Preparation and Characterization of Sulfated Chitosan. According to the previously published procedures, we prepared different types of sulfated chitosan with different sulfated sites.[22−25] Furthermore, we altered the degree of sulfation of sulfated chitosans by mixing sulfuric acid with chlorosulfonic acid in different proportions. Detailed methods are described in the Supporting Information (Figure S1).

Cell Culture and Differentiation. MESCs (R1/E, Stem Cell Bank, Chinese Academy of Sciences) were maintained on mouse embryonic fibroblasts (mEFs) inactivated with 10 μg/mL mitomycin C (Solarbio) in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 0.1 mM 2-mercaptoethanol, 2.0 mM GlutaMAX, 0.1 mM NEAA and 1000 U/mL LIF. Medium was changed daily, and cells were passaged every second day at a subculture ratio of 1:4.

Before the differentiation experiments, undifferentiated mESCs were detached from the feeder layer with 0.25% trypsin, centrifuged and then preplated on tissue culture flask (Nunc) for 2 h to deplete the mEFs from the culture. Then the mESCs cells were differentiated in differentiation medium (i.e., DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 0.1 mM 2-mercaptoethanol, 2.0 mM GlutaMAX and 0.1 mM NEAA) or neural differentiation medium (i.e., differentiation medium supplemented with 1.0 μM retinoic acid) on gelatin-coated tissue culture flasks at 3×10^5 cells per flask or gelatin-coated 48-well tissue culture plates at 8000 cells per well. Sterilized sulfated chitosan with different sulfation site and DS were added to the media at desired concentration. Medium was replaced every 2 days. All cells were maintained in a 95% air−5% CO_2 humidified incubator at 37 °C.

Immunofluorescence Assay. Differentiated cells were washed three times in phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 10 min. The cells were permeabilized with 0.1% Triton X-100 for 5 min after washing with PBS and then blocked with 3% bovine serum albumin (BSA) in PBS for 30 min. Cells were then incubated with the primary antibody in 1% BSA/PBS overnight at 4 °C. After the cells were washed with PBS for 3 times, the corresponding secondary antibody was added and the culture was incubated at room temperature for 1 h, followed by PBS washes and a subsequent 5 min of incubation with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) stain. We used anti-βIII-tubulin antibody (Cell Signaling) as the primary antibody; the secondary antibody was fluorescein isothiocyanate (FITC)-labeled goat antirabbit (Invitrogen). Immunofluorescence images were obtained using an Olympus IX 71 microscope with a mercury lamp.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Quantitative Real-Time Polymerase Chain Reaction (qPCR). Total RNA was extracted from differentiated cells by using the Total RNA extraction kit (Tiangen Biotech Co., Ltd.) according to the manufacturer's instructions. The samples were stored at −20 °C until they were processed for quantitative real-time polymerase chain reaction (qPCR) analysis. The total RNA was reverse transcribed for first-strand cDNA synthesis (RevertAid First Strand cDNA Synthesis Kit, Thermo Scientific) using oligo (dT) as a reverse transcription primer.

The qPCR was performed on a StepOnePlus real-time PCR system using SYBR Green master mix with a ROX reference dye (ABI). Reactions contained each primer at 200 nM in 20 μL total volume. Conditions were as follows: 95 °C for 20 s, followed by 50 cycles of 95 °C for 15 s, and 60 °C for 30 s. Gene expression levels were calculated using the comparative cycle time (ΔΔCT) according to the manufacturer's protocol; β-actin was used as the housekeeping gene. The primers used for qPCR are described in Table S3 (see the Supporting Information).

For RT-PCR, the pluripotency gene Nanog and Oct-4 were selected to determine the pluripotency of ESCs. RT-PCR amplification was performed using a standard procedure with Taq DNA polymerase

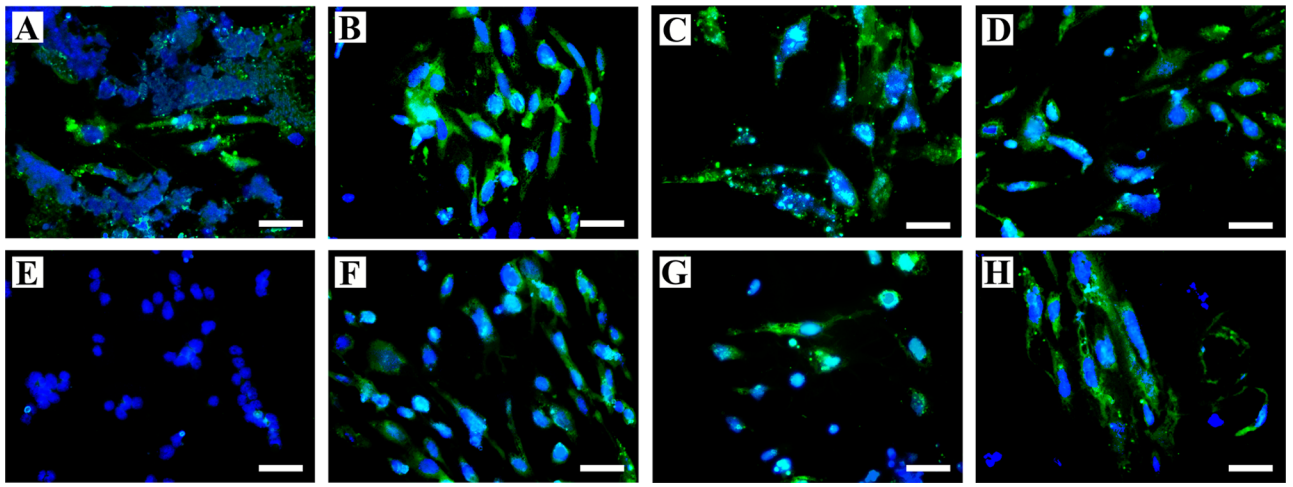

Figure 1. Effects of sulfated chitosan on expression of neural marker protein βIII-tubulin in cells cultured in differentiation medium (A–D) without $NaClO_3$ and (E–H) with $NaClO_3$. (A, E) ESCs cultured in medium without heparin or sulfated chitosan. ESCs cultured in medium supplemented with (B, F) heparin, (C, G) 2S and (D, H) 6S. βIII-tubulin was detected with anti-βIII-tubulin antibody and FITC-labeled antimouse IgG (green), and cell nuclei were stained with DAPI (blue). Scale bar = 50 μm.

(Thermo Scientific). Conditions were as follows: 95 °C for 30 s, followed by 50 cycles of 95 °C for 15 s, 65 °C for 45 s and 72 °C for 45 s. A final extension of 10 min at 72 °C was also performed. Following RT-PCR, samples were run on 1.2% agarose gels labeled with ethidium bromide solution. The gels were then imaged using an EC3 imaging system (UVP, LLC). The primers used for RT-PCR are described in Table S4 (see the Supporting Information).

Statistical Analysis. Relative expression levels were expressed as the mean ± standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA). Comparisons between groups were considered statistically significant for $P < 0.05$.

3. RESULTS AND DISSCUSSION

Effect of Sulfated Chitosans with Different Sulfated Sites on Neural Differentiation of ESCs. To examine the effect of sulfated chitosans with different sulfation sites on the neural differentiation of ESCs, we prepared 2-*N*-sulfated chitosan (2S) and 6-*O*-sulfated chitosan (6S) according to previously reported synthetic methods.[22,23,25] In the Fourier transform infrared (FTIR) spectrum of 6S (Supporting Information, Figure S2), new peaks appeared at approximately 1226 cm^{-1} (ν_{as} O═S═O) and 810 cm^{-1} (ν_{as} C—O—S) compared with the FTIR spectrum of chitosan, indicating that chitosan was successfully sulfated at the 6-*O* position. For 2S, new peaks appeared at approximately 1226 cm^{-1} (ν_{as} O═S═O) but not at 810 cm^{-1} (ν_{as} C—O—S), suggesting that chitosan was sulfated only at the 2-*N* position. We further analyzed their ^{13}C NMR spectra (Supporting Information, Table S1); results showed that two types of sulfated chitosans with different sulfated positions, a 6-*O*-sulfated chitosan and a 2-*N*-sulfated chitosan, were successfully obtained. In addition, elemental analyses were performed to obtain elemental compositions (Supporting Information, Table S2); the results indicated that the DS for 2S and 6S were similar at 0.51 and 0.46, respectively.

Cells were cultured in medium supplemented with 1 μg/mL of 2S, 6S or heparin, respectively, and neural differentiation was measured by the immunofluorescence staining of βIII-tubulin. As shown in Figure 1, after 14 days, βIII-tubulin was strongly expressed in the cells cultured in the differentiation medium supplemented with sulfated chitosan or heparin. This result is consistent with that form quantification analysis (Figure S4A in the Supporting Information), indicating that sulfated chitosan can markedly promote the neural differentiation of ESCs, similar to the function of heparin. However, the βIII-tubulin was also measured in medium without sulfated chitosan or heparin. This may be due to autocrine heparin and HS, which can promote neural differentiation under natural growth conditions of stem cells. To eliminate the influence of endogenous heparin, $NaClO_3$ was added to the differentiation medium. It has been reported that the addition of $NaClO_3$ can specifically inhibit the bioactivity of ATP-sulfurylase, an important enzyme that affects the sulfation of glycosaminoglycan, and thus can prevent the sulfation of heparin.[26] Furthermore, desulfated heparin cannot induce differentiation of ESCs including neural differentiation,[11,12,27,28] which was confirmed by our results (see the Supporting Information, Figure S5).

Therefore, in our subsequent experiments, 20 mM $NaClO_3$ was added to the differentiation medium containing heparin, 2S or 6S to eliminate the effect of autocrine heparin and HS on neural differentiation. After 14 days of culture, there was no significant expression of βIII-tubulin in the control cells, indicating that these cells could not differentiate into neural cells (Figure 1E). However, ESCs cultured in medium supplemented with exogenous heparin significantly expressed βIII-tubulin, indicating that these heparin-treated ESCs underwent neural differentiation. These differentiation conditions were similar to published results, suggesting that exogenous heparin can compensate for the lack of $NaClO_3$-inhibited autocrine heparin and HS and promote the neural differentiation of ESCs (Figure 1F).[29] Cells grown in medium supplemented with 2S or especially 6S also significantly expressed βIII-tubulin (Figure 1G,H). It was discovered that after culturing in medium containing 6S, most ESCs rearranged to a shuttle shape similar to the shapes of axons and dendrites of nerve cells (Supporting Information, Figure S3). These results indicated that sulfated chitosan has the ability to promote the neural differentiation of ESCs even in the absence of autocrine heparin or HS. Between these two types of sulfated chitosans, 6S is more effective than 2S at inducing ESCs to differentiate into cells with neural characteristics, as evidenced by the expression of neural markers and altered cell

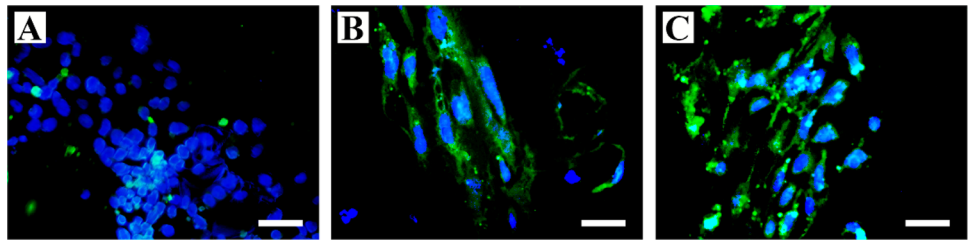

Figure 2. Effect of different DS of 6-*O*-sulfated chitosan on neural differentiation of ESCs. βIII-tubulin was detected with anti-βIII-tubulin antibody and FITC-labeled antimouse IgG (green), and cell nuclei were stained with DAPI (blue). ESCs were cultured for 14 days in medium supplemented with (A) chitosan (DS = 0), (B) 6S (DS = 0.46) and (C) 6S-H (DS = 0.81). Scale bar =50 μm.

morphology. Thus, sulfate chitosans can promote the neural differentiation of ESCs, with functions similar to those of exogenous heparin.

Effect of 6-*O*-Sulfated Chitosans with Different DS on Neural Differentiation of ESCs. Pickford et al. found that when 80% of the sulfate groups were removed by chemical methods, neural differentiation by heparin significantly decreased.[12] On the basis of this evidence, we synthesized two types of 6-*O*-sulfated chitosans, 6S with less DS (0.46) and 6S-H with higher DS (0.81), to explore the effect of DS of 6-*O*-sulfated chitosans on ESC neural differentiation. Nonsulfated chitosan, 6S or 6S-H was added to the $NaClO_3$-supplemented differentiation medium of cell cultures for 14 days. Afterward, cells were stained for βIII-tubulin. The immunofluorescence images showed that in cells grown in medium containing nonsulfated chitosan, there was no significant expression of βIII-tubulin and no neural-like morphology was observed, indicating that nonsulfated chitosan had no effect on promoting neural differentiation (Figure 2A). However, ESCs cultured in medium supplemented with 6S or 6S-H both showed noticeable neural differentiation. 6S-H-treated cultures showed markedly higher levels of neural differentiation, as evidenced by the higher concentration of βIII-tubulin and morphological outgrowths similar to that of axons and dendrites of nerve cells (Figure 2B,C). These results suggest that chitosan sulfation enables the induction of neural differentiation. Moreover, higher DS sulfated chitosan have a greater ability to promote ESC neural differentiation.

Neural marker genes Nestin and βIII-tubulin, which are expressed in the early and late stages of neural differentiation, respectively, were examined by qPCR. Almost no Nestin or βIII-tubulin expression was observed in ESCs cultured in medium with nonsulfated chitosan (Figure 3). However, both βIII-tubulin and Nestin genes were expressed in the 6S-treated and 6S-H-treated cells. The relative expression level of βIII-tubulin in 6S-H-treated cells was approximately 11 times greater than the level in 6S-treated cells, and the relative expression level of Nestin was 0.2 times of that of 6S-treated cells. This suggests that more cells can be induced to differentiate into mature nerve cells when they are treated with sulfated chitosans with higher DS. Altogether, these results showed that without sulfation, chitosan was not capable of inducing neural differentiation at the early and late stages. Sulfated chitosans with 0.46 and 0.81 DS can significantly enhance the expression of both early and late stage neural marker genes. The 6-*O*-sulfated chitosan with higher DS can better support the formation of mature nerve cells, as evidenced by the greater expression of neural marker genes and in the more altered cell morphology, compared with the 6-*O*-sulfated chitosan with lower DS.

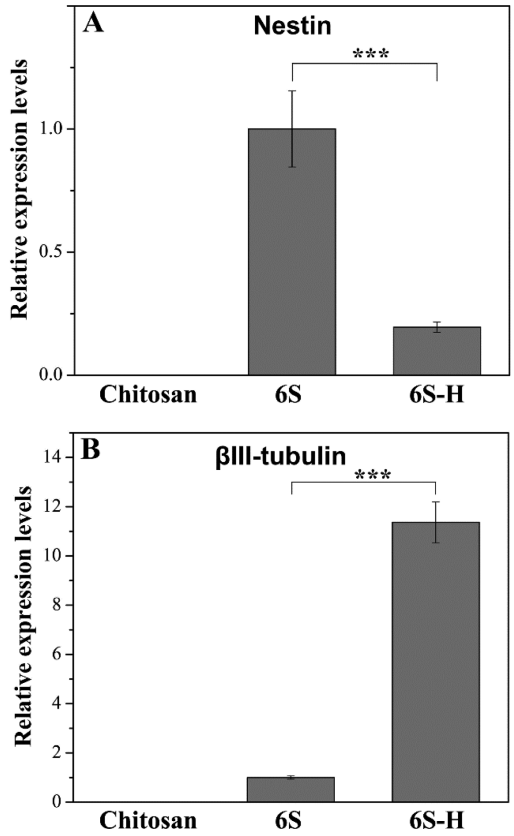

Figure 3. Effect of different DS of 6-*O*-sulfated chitosan on neural differentiation of ESCs. The ESCs were cultured in medium supplemented with chitosan, 6S or 6S-H for 14 days followed by qPCR analysis for (A) Nestin gene and (B) βIII-tubulin gene expression levels. The DS of chitosan, 6S, and 6S-H were 0, 0.46 and 0.81, respectively. ***$P < 0.001$ ($n = 3$).

Effect of Sulfated Chitosan with Double Sulfation Sites on Neural Differentiation of ESCs. Because the DS of 6S-H is a relatively high 0.81, it is difficult to further increase the DS of a single-site-sulfated chitosan. To further study the effect of DS on neural differentiation, we attempted to increase the overall DS of sulfated chitosan by adding another sulfation site to the 6-*O*-sulfated chitosan.

Therefore, 2-*N*,6-*O*-sulfated chitosan (26S) and 3,6-*O*-sulfated chitosan (36S) with double sulfation sites were prepared based on the 6-*O*-sulfated chitosan. Elemental analyses showed that the DS values of the 26S and 36S were 1.39 and 1.32, respectively, which were higher than the DS of 6S-H. After ESCs were cultured in the differentiation medium supplemented with 26S or 36S for 14 days, the results from immunofluorescence staining of βIII-tubulin (Figures 4 and

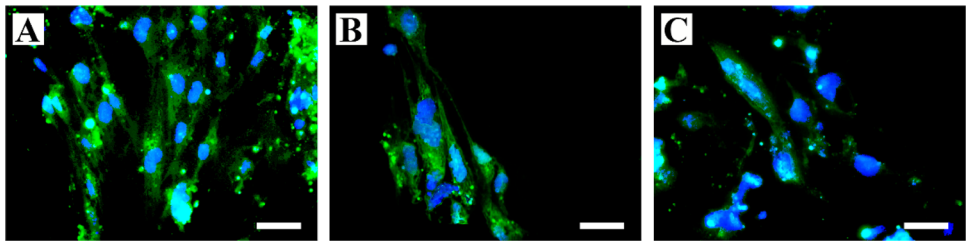

Figure 4. Effect of double-site-sulfated chitosans on neural differentiation of ESCs. ESCs in 6S-H group (A) were cultured for 14 days in medium supplemented with 6-*O*-sulfated chitosan. The other groups and were supplemented with (B) 2-*N*,6-*O*-sulfated chitosan (26S) and (C) 3,6-*O*-sulfated chitosan (36S). βIII-tubulin was stained with anti-βIII-tubulin antibody and FITC-labeled antimouse IgG (green), and cell nuclei were stained with DAPI (blue). Scale bar = 50 μm.

S4B, Supporting Information) showed that the 26S can promote neural differentiation with effects similar to that of 6S-H (i.e., it can promote the expression of βIII-tubulin and the formation of a dendrite-like morphology of nerve cells).

In contrast, although the DS of 36S is similar to that of 26S, the expression level of βIII-tubulin and the synapse number in ESCs treated by 36S were significantly lower than those in cells treated with 26S or 6S-H.

Our results suggest that increasing the DS of sulfated chitosans by introducing a second sulfation site does not further enhance neural differentiation. Moreover, the introduction of some sulfation sites, such as the 2-*N* site, decreased the ability of sulfated chitosans to induce ESC neural differentiation. These results may be caused by the spatial configurations of sulfate groups affecting the interaction between sulfated chitosan and ESCs. The presence of a sulfate group at the 6-*O* position may be enough for the activation of the neural differentiation signaling pathway; additional sulfate groups may have negligible or even negative effects. Another likely reason why the introduction of the 3-*O* sulfate site had no improvement on neural differentiation is that the 3-*O* sulfate group is rarely observed in heparin and HS.[30] Additionally, it has been reported that 3-*O*-sulfated heparin has no effect on neural differentiation.[12] Collectively, we can conclude that, compared with the DS of sulfated chitosan, the sulfated position is more important for promoting the neural differentiation of ESCs. However, further investigation is required to determine the exact mode of action.

Influence of the Culture Time of 6-*O*-Sulfated Chitosan on Neural Differentiation of ESCs. In an attempt to understand the entire 6-*O*-sulfated chitosan-induced differentiation process of wild-type ESCs to mature nerve cells, the expression levels of βIII-tubulin in ESCs was measured at specific culture periods. The results showed that βIII-tubulin was significantly expressed in ESCs after 10 days. When the culture time was extended to 18 days, the βIII-tubulin expression continued to increase. The βIII-tubulin expression at day 14 increased by 60% compared with that at day 10, and at day 18, a 4-fold increase in the expression of βIII-tubulin was observed. There was no significant difference in the βIII-tubulin expression between day 22 and day 18, suggesting that the βIII-tubulin expression had reached a stable maximum level (Figure 5). Our results showed that the induction effects of 6-*O*-sulfated chitosan on the differentiation of ESCs into mature nerve cells increased over culture time; the maximum level of βIII-tubulin expression was reached after 18 days culture.

Dose-Dependent Effects of 6-*O*-Sulfated Chitosan on Neural Differentiation of ESCs. Pickford et al. found that 1 to 10 μg/mL heparin can efficiently promote the neural

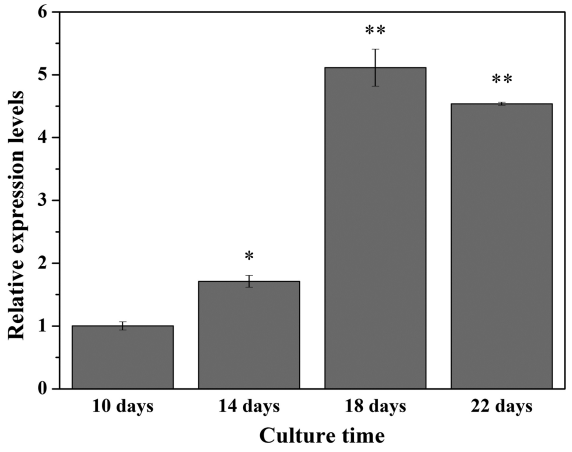

Figure 5. Influence of the culture time of 6-*O*-sulfated chitosan on neural differentiation of ESCs. Levels of βIII-tubulin were assayed by qPCR at days 10, 14, 18 and 22. *$P < 0.05$; **$P < 0.01$ ($n = 3$).

differentiation of ESCs.[12] To obtain the optimal concentration of 6-*O*-sulfated chitosan for inducing neural differentiation, βIII-tubulin expression levels were measured after 14 days of treatment with 1, 2, 4, 6, 8 and 10 μg/mL 6S-H. (Figure 6).

It should be noted that although 1 μg/mL 6S-H promoted βIII-tubulin expression, the number of neuron synapses produced was minimal. For ESCs treated with 2 or 4 μg/mL 6S-H, strong βIII-tubulin expression was observed. Additionally, large numbers of neuron synapses were produced, leading to the development of mutual contacts between neuron synapses (similar to the behavior of mature neurons). When the concentration of the 6-*O*-sulfated chitosan increased to 6–10 μg/mL, the expression levels of βIII-tubulin decreased, but remained higher than that of ESCs treated with 1 μg/mL 6S-H. These results demonstrated that there exists an optimal concentration of approximately 2 μg/mL 6S-H for inducing ESC neural differentiation, which is lower than the optimal concentration for heparin, i.e., 6 μg/mL.[12] Therefore, 6-*O*-sulfated chitosan showed an efficient enhancement of ESC neural differentiation under low concentrations. It should be noted that higher concentrations resulted in a significant reduction in neural differentiation. It is likely that 6S in medium can inhibit differentiation by binding growth factors related to neural differentiation and may sequester the factors away from the cell surface, disrupting essential 6S-growth factor binding complexes.[31]

Therefore, 6-*O*-chitosan can increase βIII-tubulin expression and also promote the production of neuron synapses and their mutual contacts. In addition, 6-*O*-sulfated chitosan showed no cell toxicity (see the Supporting Information, Figure S7). So it

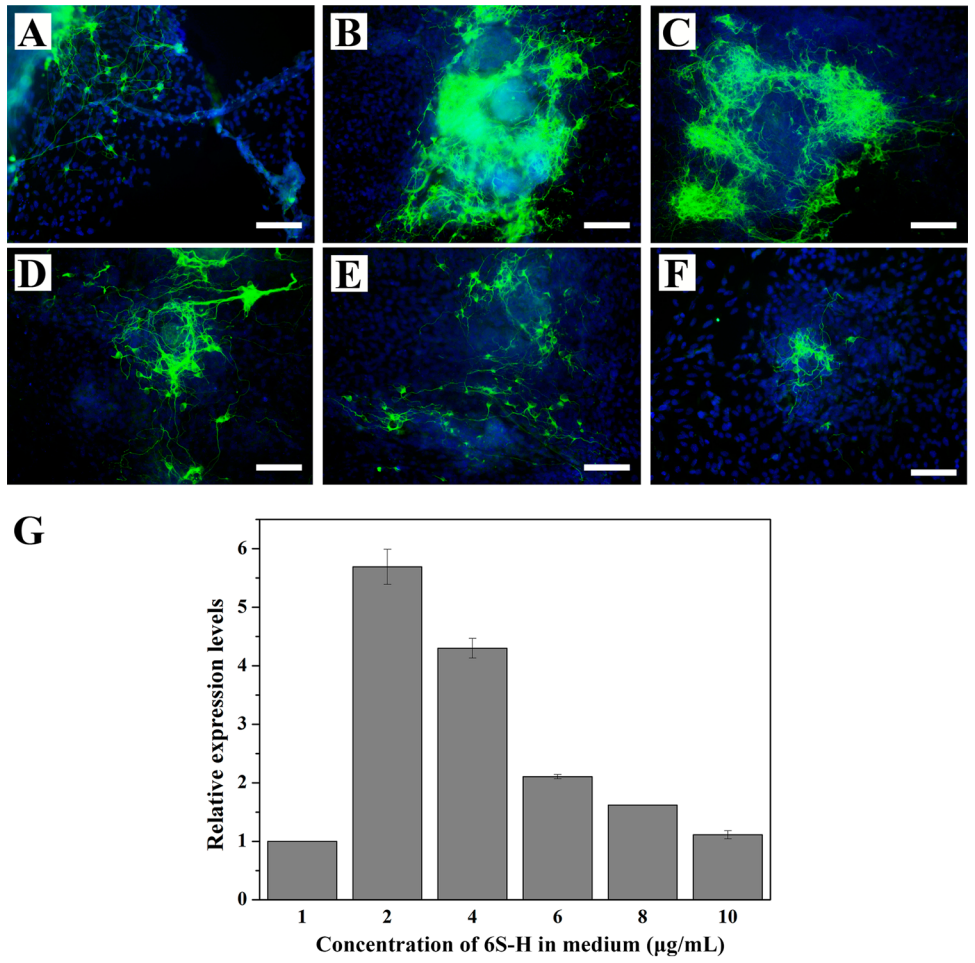

Figure 6. Dose-dependent effects of 6-*O*-sulfated chitosan on neural differentiation of ESCs. The ESCs were treated with 1, 2, 4, 6, 8 and 10 μg/mL (A–F) of 6S-H. Cells were stained for the marker βIII-tubulin. Scale bar = 100 μm. Levels of βIII-tubulin were assayed by qPCR (G).

has the potential to be used as an efficient drug for inducing neural differentiation of ESCs.

4. CONCLUSIONS

In conclusion, sulfated chitosan with different sulfation sites showed different capabilities for inducing the neural differentiation of ESCs. Compared with 2-*N*,6-*O*-sulfated chitosans and 3,6-*O*-sulfated chitosans, 6-*O*-sulfated chitosans most efficiently promoted the neural differentiation of ESCs. In addition, the promotion of neural differentiation correlated with the DS of the sulfated chitosan. 6-*O*-Sulfated chitosan with higher DS better enhanced neural differentiation. However, nonsulfated chitosan was unable to induce neural differentiation. Furthermore, the neural differentiation induced by sulfated chitosan was caused by the synergistic effect of both the sulfation site and DS. Compared with DS, the sulfation site has more influence. Additionally, the induction of neural differentiation was dependent on the concentration of 6-*O*-sulfated chitosan and incubation time. The optimal concentration was approximately 2 μg/mL for the studied concentration range of 1 to 10 μg/mL; the best neural differentiation of ESCs can be achieved after at least 18 days of culture. Considering that sulfated chitosan is derived from chitosan, which is cost-effective, accessible, and easy to be chemically modified, our work shows that sulfated chitosan can be readily used as a candidate for inducing and differentiating ESCs into nerve cells, which is important for the development of neural drugs for nerve repair and regeneration.

■ ASSOCIATED CONTENT

Supporting Information

Scheme and methods for the synthesis, FTIR spectra, ^{13}C NMR spectra and elemental analyses of sulfated chitosan, photomicrographs of mESCs, relative fluorescence intensity of immunofluorescent staining of βIII-tubulin in ESCs, primers used in qPCR and RT-PCR, RT-PCR analysis of the expression of pluripotency genes in mESCs after culture in the media supplemented with $NaClO_3$, the relative expression levels of neural marker gene Nestin in ESCs and cell viability of mESCs. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

2S, 2-*N*-sulfated chitosan
6S, 6-*O*-sulfated chitosan
26S, 2-*N*,6-*O*-sulfated chitosan
36S, 3,6-*O*-sulfated chitosan
BSA, bovine serum albumin
DAPI, 4′,6-diamidino-2-phenylindole
DMEM, high glucose Dulbecco's modified Eagle's medium
DS, degree of sulfation
EGF, epidermal growth factor
ESCs, embryonic stem cells
FBS, fetal bovine serum
FGF, fibroblast growth factor
FITC, fluorescein isothiocyanate
GlcA, glucuronic acids
GlcNAc, N-acetylglucosamines
HS, heparan sulfate
LIF, leukemia inhibitory factor
mEFs, mouse embryonic fibroblasts
mESCs, mouse embryonic stem cells
NEAA, nonessential amino acids
NGF, nerve growth factor
PBS, phosphate buffered saline
q-PCR, quantitative real-time polymerase chain reaction
RT-PCR, reverse transcription polymerase chain reaction

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