

**Electronic supplementary information for:**

**Promoting neural differentiation of embryonic stem cells using  $\beta$ -cyclodextrin sulfonate**

Zhonglin Lyu,<sup>‡</sup> Xiujuan Shi,<sup>‡</sup> Jiehua Lei, Yuqi Yuan, Lin Yuan, Qian Yu,\* and Hong Chen\*

State and Local Joint Engineering Laboratory for Novel Functional Polymeric Materials, College of Chemistry, Chemical Engineering and Materials Science, Soochow University, Suzhou 215123, P. R. China.

E-mail: yuqian@suda.edu.cn, chenh@suda.edu.cn

<sup>‡</sup> These authors contributed equally to this work.

## **1 Materials and Instruments**

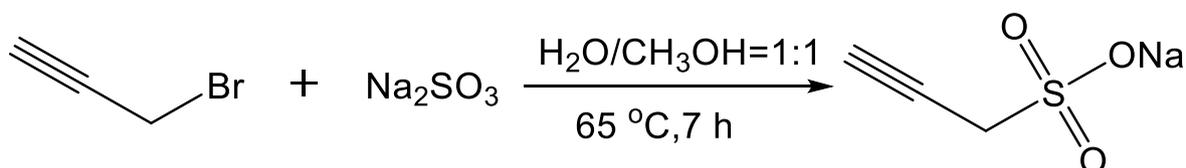
$\beta$ -cyclodextrin ( $\beta$ -CD, 97%, Aladdin) was recrystallized twice from water and dried in a vacuum oven at 100 °C for two days prior to use. Triphenyl phosphine ( $\text{Ph}_3\text{P}$ , 98.5%, Sigma-Aldrich), *N*-bromosuccinimide (NBS, 99%, Sigma-Aldrich) were used as received. Bathophenanthrolinedisulphonic acid disodium salt (BPS) and sodium hydride (60 % dispersion in mineral oil) were from Sigma-Aldrich. Propargyl bromide (80% in Toluene, ca. 9.2 mol/L) were received from TCI Co.  $\beta$ -CD-( $\text{N}_3$ )<sub>7</sub> was synthesised as in our previous report.<sup>1</sup> Bovine serum albumin (BSA), paraformaldehyde, gelatin, dexamethasone, Triton X-100, DAPI (40,6-diamidino-2-phenylindole), fluorescein isothiocyanate (FITC)-labelled phalloidin were from Sigma-Aldrich. MTT (3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2H-tetrazolium bromide, 98%) was from Amresco. PCR primers were from Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). All other solvents, which were of analytical reagent grade, were from Sinopharm Chemical Reagent Co. (Shanghai, China). All aqueous solutions were prepared in 18.2 MU cm purified water from a Milli-Q water

purification system (Millipore, Bedford, MA, USA).

$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded on Bruker DPX-400 and DPX-400 spectrometers using deuterated solvents obtained from Aldrich. FTIR spectra were recorded on a Bruker VECTOR-22 FTIR spectrometer using a Golden Gate diamond attenuated total reflection cell. MALDI-ToF data was collected using a Bruker Ultraflex II MALDI ToF Instrument which has a mass range up to 500 kDa and analysed using FlexAnalysis software. The MALDI contains a 337 nm wavelength  $\text{N}_2$  (pulse energy of 100  $\mu\text{J}$ ) at a maximum repetition rate 50 Hz. The system is kept inside a vacuum at  $5 \times 10^{-6}$  Torr to avoid contamination of the sample reading. A matrix was used (CHCA and DHB) to protect the product from being destroyed from direct laser beam and to facilitate vaporisation and ionisation.

## 2 Synthesis and characterization of compound and polymer

### 2.1 Synthesis and characterization of per-6-substituted $\beta$ -CD sulfonate (CD-S) via CuAAC



**Scheme S1.** Synthetic route to sodium propargyl sulfonate ( $\text{C}_3\text{H}_3\text{SO}_3\text{Na}$ ).

Sodium propargyl sulfonate was prepared according to the previous report of Y. Zhao.<sup>2</sup> The  $^1\text{H}$  NMR spectra were consistent with the literature.  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ , 298 K, 400 MHz):  $\delta$  (ppm) = 3.82 (d, 2H,  $J = 2.4$  Hz), 2.69 (t, 1H,  $J = 2.7$  Hz).

*Heptakis*{6-(4-sulfonylmethyl-1H-[1,2,3]triazol-1-yl)-6-deoxy}- $\beta$ -cyclodextrin (**5**, CDS-C) was prepared according to the procedure reported by H. Le *et al*<sup>3</sup> with a little change. Detailed procedures

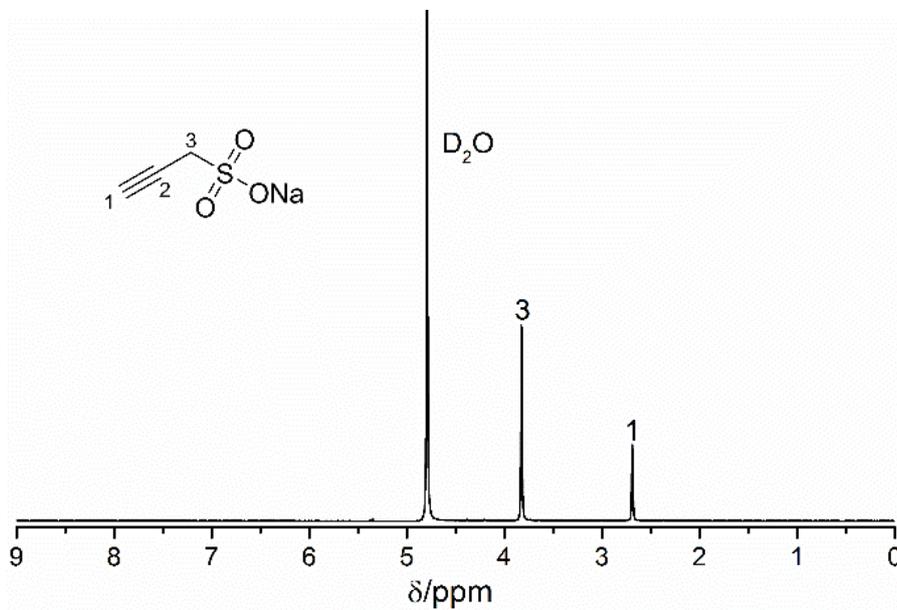
are shown below.

6-Azido-6-deoxy- $\beta$ -CD (100 mg, 0.0763 mmol), sodium propargyl sulfonate (108 mg, 0.763 mmol, Supplementary data 1.4), TEA (107  $\mu$ L, 0.8 mmol), CuSO<sub>4</sub>·5H<sub>2</sub>O/BPS (134  $\mu$ L of 100 mM stock in water, 1:2 molar ratio), and sodium ascorbate (107  $\mu$ L of 500 mM stock in water) were mixed with 24 mL of THF/0.1M phosphate buffer (PB, pH = 7)/EtOH (5:5:2). The mixture was stirred at 80 °C for 1 day. The reaction mixture was evaporated to remove solvent. The solid was dissolved in water (20 mL) and the insoluble impurities were filtered off. The filtrate was shaken with CupriSorb™ resin for overnight. The supernatant was evaporated and precipitated with cooled methanol (20 mL). The solid was dissolved in 1 mL water and acetone (20 mL) was added. The precipitate was dissolved in 3 mL of water and stirred overnight. The mixture was centrifuged at 12,000 rpm for 30 min. The supernatant was desalted on a Sephadex G-25 column and lyophilized using a Freeze dryer (Ilshin Europe B.V) to give a white powder (165 mg, yield 93.7 %).

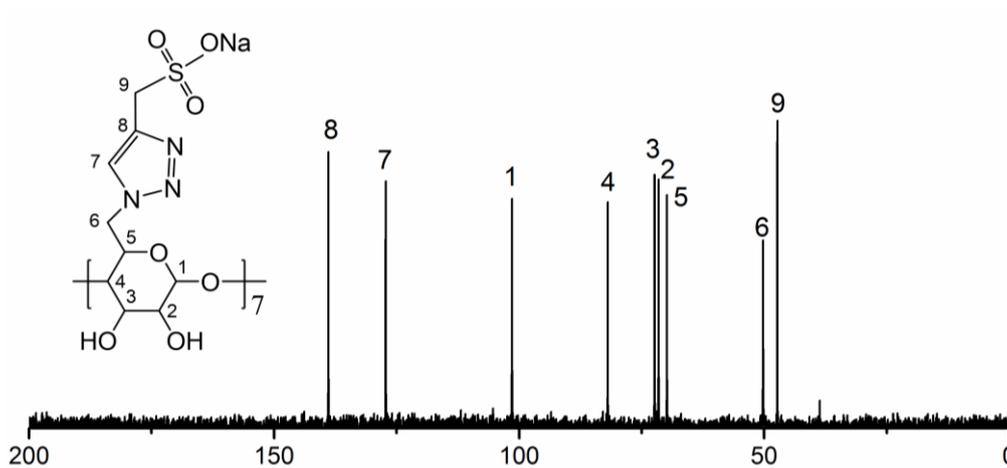
<sup>1</sup>H NMR (D<sub>2</sub>O, 298 K, 400 MHz):  $\delta$  (ppm) = 8.02 (s, 7H, H7), 5.16 (d, 7H, J = 2.8 Hz, H1), 4.59 (app d, 7H, J = 13.6 Hz, H6), 4.39 (dd, 7H, J = 14.8, 5.2 Hz, H6), 4.32 (br, 7H, H5), 4.13 (app q, 14H, J = 14.8 Hz, H9), 3.99 (app t, 7H, J = 9.2 Hz, H3), 3.57 (dd, 7H, J = 10.4, 3.2 Hz, H2), 3.33 (app t, 7H, J = 9.2 Hz, H4). <sup>13</sup>C NMR (D<sub>2</sub>O, 298 K, 400 MHz):  $\delta$  (ppm) = 138.91 (C8), 127.23 (C7), 101.46 (C1), 81.92 (C4), 72.35 (C3), 71.54 (C2), 69.83 (C5), 50.22 (C6), 47.31 (C9). FTIR  $\nu$  (cm<sup>-1</sup>): 3441 (OH); 2928 (CH); 1195 (SO<sub>2</sub>) cm<sup>-1</sup>. MALDI-ToF-MS (DHB, negative) *m/z*: C<sub>63</sub>H<sub>84</sub>N<sub>21</sub>Na<sub>7</sub>O<sub>49</sub>S<sub>7</sub>, [M-4Na+3H]<sup>-</sup>, calculated 2214.30, found 2211.5. [M-3Na+2H]<sup>-</sup>, calculated 2236.28, found 2233.4. [M-2Na+H]<sup>-</sup>, calculated 2258.26, found 2256.2. [M-Na]<sup>-</sup>, calculated 2280.24, found 2280.5.

<sup>1</sup>H NMR showed the successful synthesis of sodium propargyl sulfonate (**Fig. S1**). <sup>13</sup>C NMR revealed the appearance of the carbon on triazole and CH<sub>2</sub> linked to sulfonyl group (**Fig. S2**). FTIR

revealed the complete disappearance of azide peak at around  $2110\text{ cm}^{-1}$  and the appearance of sulfonyl peak at around  $1195\text{ cm}^{-1}$  (**Fig. S3**). The results of MALDI-ToF mass spectra showed the peak from per-6-sulfonated  $\beta$ -CD (**Fig. S4**).



**Fig. S1**  $^1\text{H}$  NMR spectrum of sodium propargyl sulfonate.



**Fig. S2**  $^{13}\text{C}$  NMR spectrum of CD-S

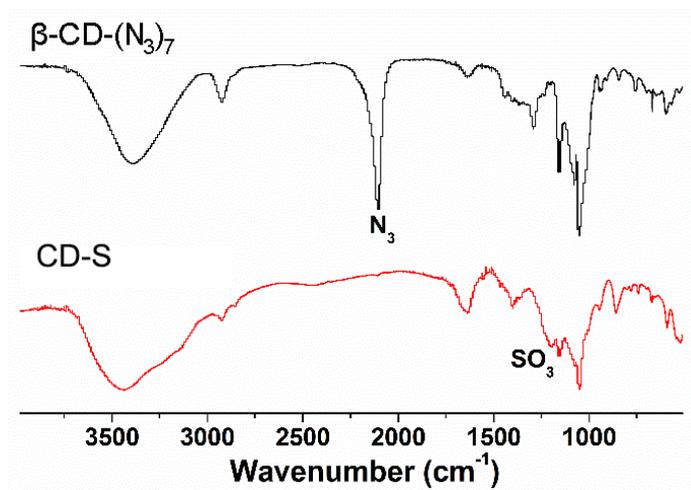


Fig. S3 FTIR spectra of  $\beta$ -CD-(N<sub>3</sub>)<sub>7</sub> and CD-S.

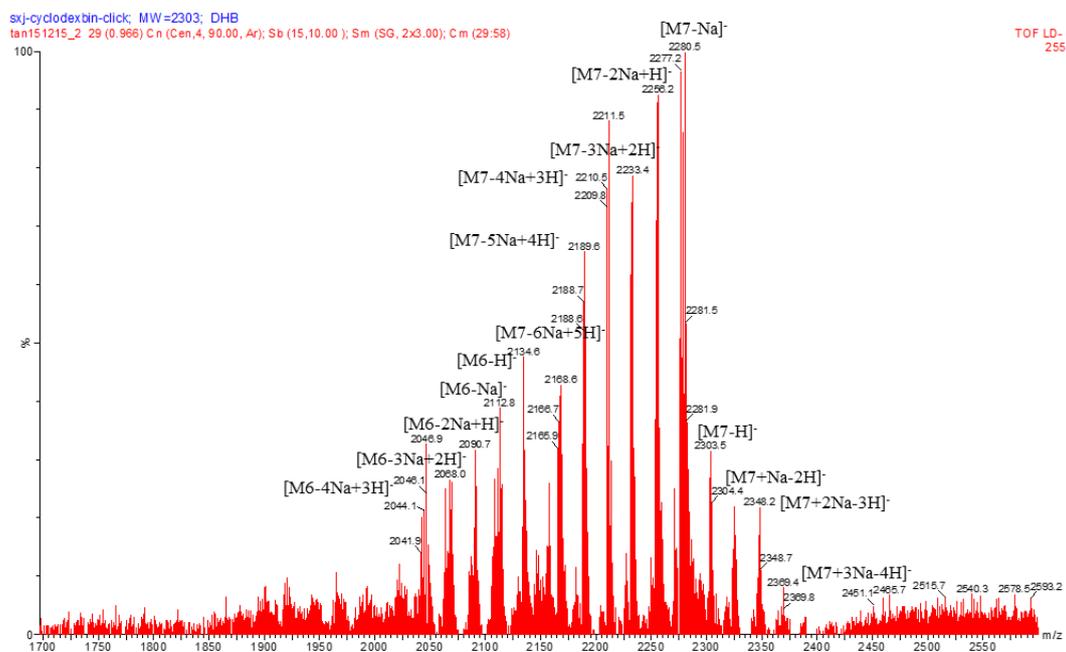


Fig. S4 MALDI-ToF spectrum of CD-S.

## 2.2 Synthesis of poly(sodium 4-vinylbenzenesulfonate) (pSS)

pSS was synthesized using previously reported method *via* reversible addition-fragmentation chain transfer (RAFT) polymerization.<sup>4</sup> Aqueous gel permeation chromatography (GPC) was conducted on a waters system comprised of a Waters 1515 HPLC pump and a Waters 2414 refractive

index detector with a PL aquagel-OH MIXED-M column. The mobile phase consisted 70% 0.2 M NaNO<sub>3</sub> and 0.1 M NaH<sub>2</sub>PO<sub>4</sub> (adjusted to pH 7) and 30% methanol cosolvent at a flow rate of 1.0 mL/min. Calibration was with near-monodisperse PEO standards.

The results from <sup>1</sup>H NMR and FTIR showed the success of the synthesis. The characteristic peaks of SS are clearly visible from <sup>1</sup>H NMR (Fig.5, ESI) and the peaks at 1176, 1120 and 1036 cm<sup>-1</sup> from FTIR correspond to the SO stretches (Fig.6, ESI). Gel permeation chromatography (GPC) traces suggested that pSS was unimodal with narrow polydispersity index of 1.17. The molecular weight of pSS used in the present study was about 8.6 kDa (M<sub>n</sub>, from GPC).

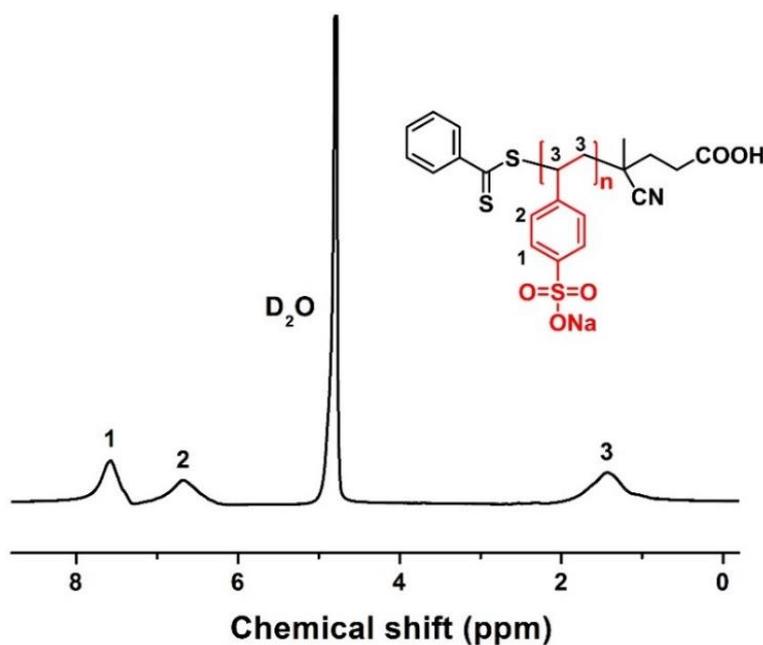
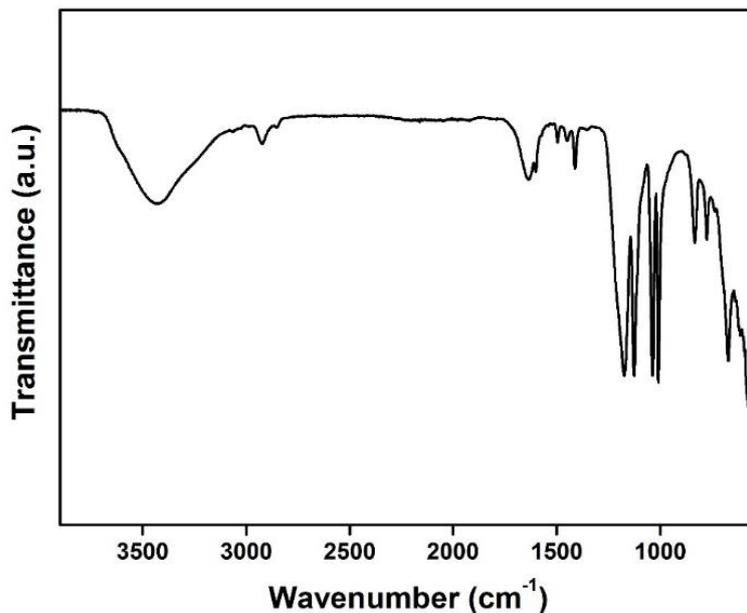


Fig. S5 <sup>1</sup>H NMR spectrum of pSS.



**Fig. S6** FTIR spectrum of pSS.

### **3 Cell-related assays**

#### **3.1 Cell culture**

L929 cells from the connective tissue of mouse were maintained in RPMI (Roswell Park Memorial Institute) 1640 medium (Thermo HyClone) with 10% fetal bovine serum (FBS, Gibco), 1% penicillin/streptomycin (Genview). Mouse embryonic stem cells (mESCs, R1/E, Stem Cell Bank, Chinese Academy of Sciences) were cultivated on a feeder layer of mitomycin C-inactivated mouse embryonic fibroblasts (mEFs, ICR MEF, Stem Cell Bank, Chinese Academy of Sciences) in ESC maintenance medium containing DMEM (Dulbecco's Modified Eagle's Medium; Thermo HyClone) supplemented with 10% FBS, 1% penicillin/streptomycin, 0.1 mM non-essential amino acids (Gibco), 0.1 mM  $\beta$ -mercaptoethanol (Amresco) and 1000 U/mL leukemia inhibitory factor (LIF; Chemicon). All cells were cultured at 37 °C in a humidified 5% (v/v) CO<sub>2</sub> incubator (Eppendorf Galaxy 170R) and cell culture medium was changed every 2 days. Before cell proliferation assay and neural

differentiation experiments, mESCs were cultured on gelatin-coated cell culture flask to remove mEFs.

### **3.2 Cell proliferation assay**

Proliferation of L929 cells and mESCs was assessed using an MTT assay. Cells were cultured in a 96-well plate for 12 h to obtain full spreading before adding different molecules into the cell culture medium. After desired incubation time, cells were further incubated in 220  $\mu$ L fresh medium containing 20  $\mu$ L MTT (5 mg/mL) for 4 h. The medium was then replaced with 220  $\mu$ L DMSO to dissolve the formazan crystals generated by living cells and the absorbance of the resulting solution was measured at 490 nm by a microplate reader (Varioskan Flash, Thermo Scientific).

### **3.3 Neural differentiation of ESCs**

For neural differentiation, mESCs at a density of  $2.5 \times 10^5$  cells/cm<sup>2</sup> were grown without feeder cells on gelatin-coated 6-well plate in basic differentiation medium (DMEM containing 10% FBS, 1% penicillin/streptomycin, 0.1 mM non-essential amino acids and 0.1 mM  $\beta$ -mercaptoethanol) or neural differentiation medium (differentiation medium supplemented with 1.0  $\mu$ M RA) supplemented with different molecules. The work from Pickford *et al.* concluded that specific glycosaminoglycans (including heparin) or synthetic mimics can be used as suitable low-cost factors for neural differentiation of ESCs.<sup>5</sup> Inspired by their work, in our previous research, the results proved the conclusion of Pickford *et al.* by showing that specific sulfated chitosan induced neural differentiation of ESCs in a similar way to heparin in basic differentiation medium without any neural inducer instead of neural differentiation medium (N2B27).<sup>6</sup> Considering the synthesized CD-S in the present study is a kind of sulfonated saccharide similar to heparin, its ability to induce neural differentiation was studied using basic differentiation medium as well.<sup>2</sup> Medium was changed every 2 days. ESCs were passaged every 4-5 days at a confluence of 80-90% and 1/3 of the cells were reseeded to a new gelatin-coated 6-

well plate.

### **3.4 Immunofluorescent staining**

Differentiated cells were washed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 10 min at room temperature. After being washed in PBS, cells were permeabilized with 0.1% Triton X-100/PBS for 5 min and blocked with 3% bovine serum albumin (BSA) in PBS for 30 min. Cells were then incubated with the primary antibody (Rabbit anti-mouse anti- $\beta$ 3-tubulin antibody, Cell Signaling) in 1% BSA/PBS overnight at 4°C. The corresponding secondary antibody (FITC-labeled goat anti-rabbit antibody, Invitrogen) was added after washing cells in PBS and the cells were incubated at room temperature for 1 h, followed by a 10 min incubation with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) stain. Immunofluorescent staining images were obtained using an Olympus IX 71 fluorescence microscope. The images were overlapped using Image-Pro Plus 6.0 software (public software from Media Cybernetics, <http://www.mediacy.com/>).

### **3.5 Real-time quantitative polymerase chain reaction (qPCR)**

Total RNA was extracted from cells using a total RNA extraction kit (Tiangen Biotech Co., Ltd.) according to the manufacturer's instructions. The extracted total RNA samples were then reverse transcribed for first-strand cDNA synthesis (RevertAid First Strand cDNA Synthesis Kit, Thermo Scientific) using oligo (dT) as the reverse transcription primer. qPCR was performed on a StepOnePlus real-time PCR system (Applied Biosystems) using Fast SYBR Green Master Mixed (Applied Biosystems) to quantify the levels of mRNA expression in mESCs. The primer details are summarized as follows:  $\beta$ -actin-f 5'-CCCTAGGCACCAGGGTGTGA-3',  $\beta$ -actin-r 5'-TCCCAGT TGGTAACAATGCCA-3'; Foxa2-f 5'-ACTGGAGCAGCTACTACG-3', Foxa2-r 5'-CCCACATAGGATGACATG-3'; Flk1-f 5'-CACCTGGCACTCTCCACCTTC-3', Flk1-r 5' -

GATTCATCCCACTACCGAAAG-3'; Oct4-f 5'-GGCGTTCTCTTTGGAAAGGT-3', Oct4-r 5'-TCTCATTGTTGTTCGGCTTCCT-3'; Nestin-f 5'-GCAGGGTCTACAGAGTCAG-3', Nestin-r 5'-GCAAGCGAGAGTTCTCAG-3';  $\beta$ III-tubulin-f 5'-ACTTTATCTTCGGTCAGAG TG-3',  $\beta$ III-tubulin-r 5'-CTCACGACATCCAGGACTGA-3'. qPCR amplification was achieved with 50 cycles of 30 s at 95 °C, 45 s at 60 °C, 45 s at 72 °C and a fluorescence measurement.

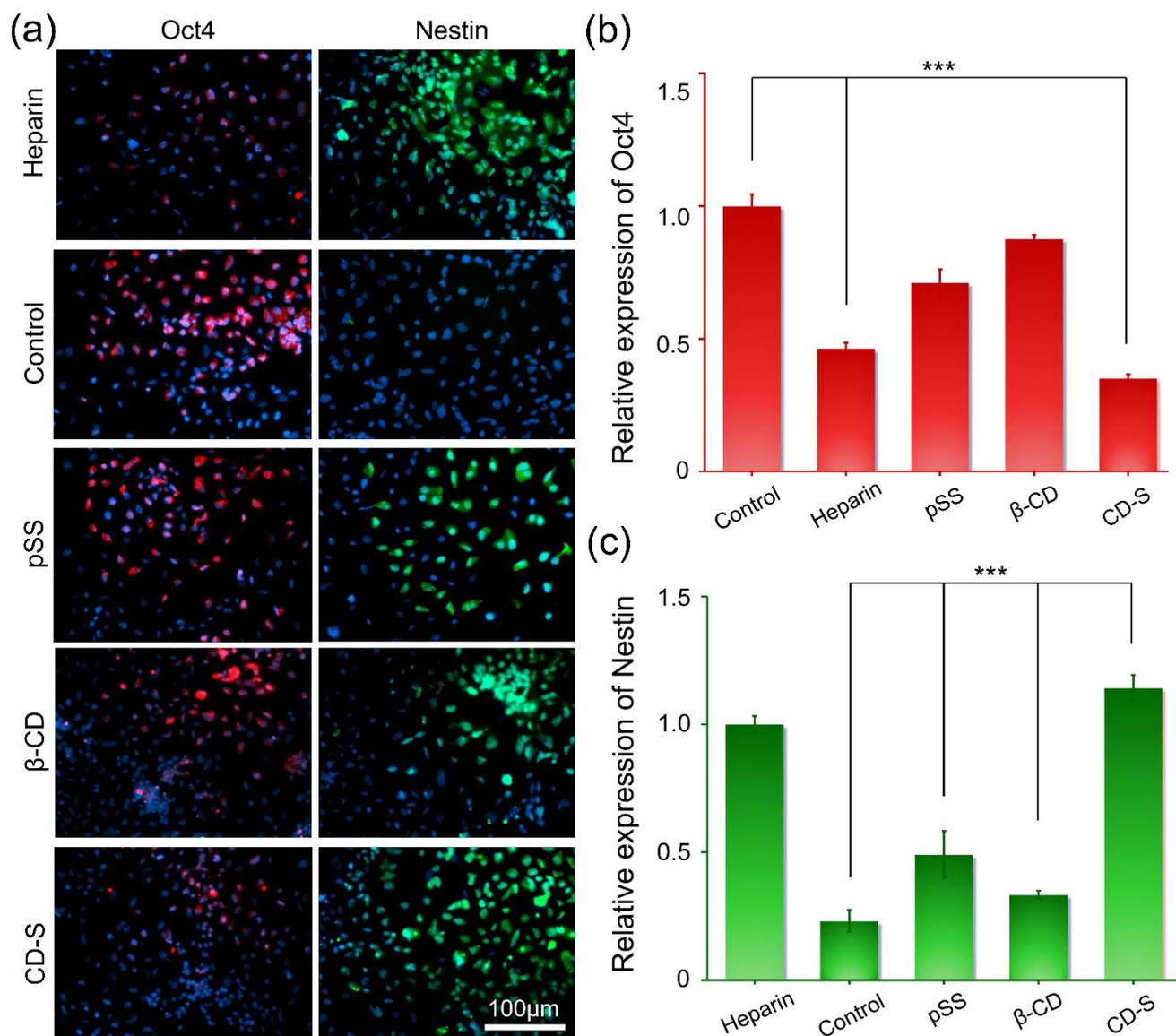
#### **4 Statistical methods**

Every independent experiment was duplicated at least three times and the results were expressed as the mean  $\pm$  standard deviation (SD). *p*-values less than 0.05 were considered significant.

#### **5 Result of bioassays**

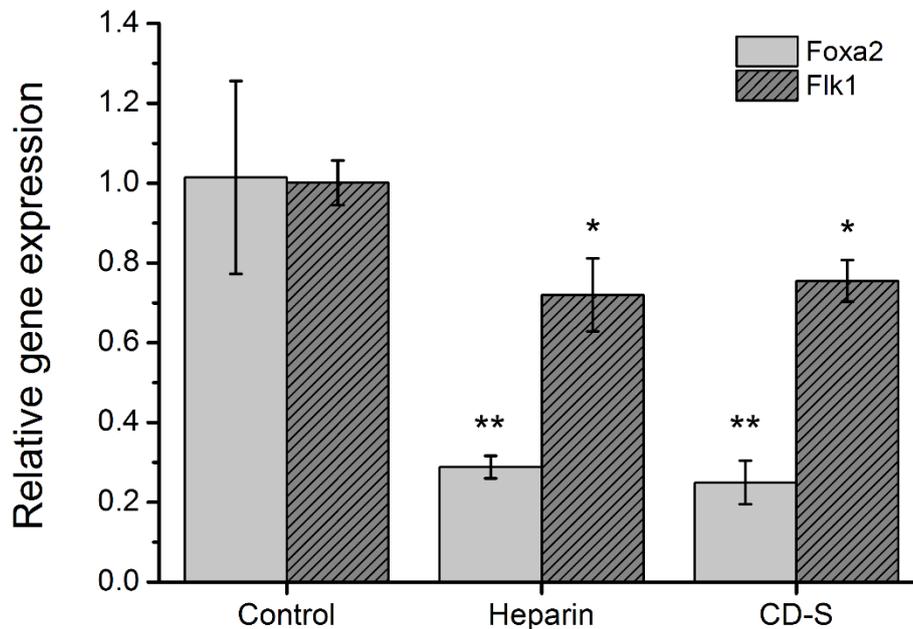
##### **5.1 The expression of Oct4 and Nestin in mESCs cultured in basic differentiation medium**

The expression of Oct4 and Nestin in cells treated with different molecules in basic differentiation medium for 7 days using both immunofluorescence staining and qPCR. The results showed that compared with the control group, the expression of Oct4 in both heparin- and CD-S-treated groups dramatically decreased (Fig. 7a and 7b,  $p < 0.001$ ) while the expression of Nestin in heparin- and CD-S-treated groups increased significantly (Fig. 7a and 7c,  $p < 0.001$ ).



**Fig. S7** The expression of Oct4 and Nestin in mESCs treated with heparin, β-CD, pSS, or CD-S for 7 days in basic differentiation medium. Cells grown in basic differentiation medium were used as control. (a) Immunofluorescence images of mESCs treated with different molecules for 7 days. Red: Oct4, green: nestin, blue: nuclei by DAPI. Scale bar: 100 μm. (b) Oct4 expression of mESCs in different groups were determined by qPCR. Data were normalized with that of control group and presented as mean ± SD (n = 3). \*\*\* $p < 0.001$ . (c) Nestin expression of mESCs in different groups were determined by qPCR. Data were normalized with that of heparin-treated group and presented as mean ± SD (n = 3). \*\*\* $p < 0.001$ .

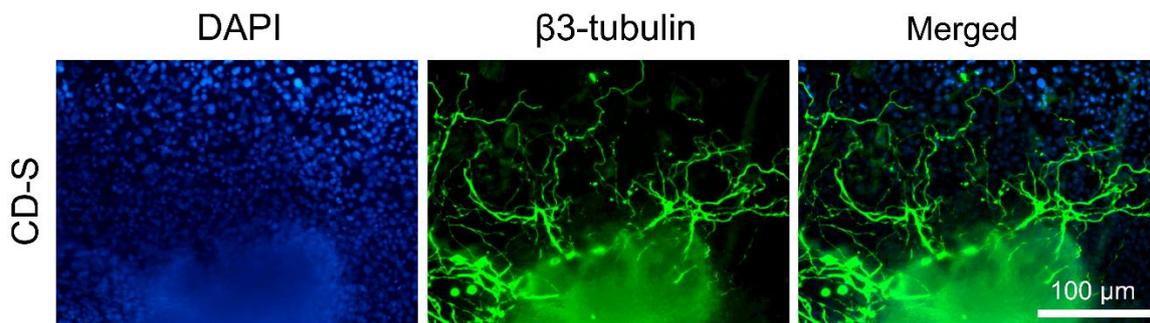
## 5.2 The expression of endodermal and mesodermal marker genes in mESCs cultured in basic differentiation medium



**Fig. S8** The expression of endodermal (Foxa2) and mesodermal (Flk1) marker genes in mESCs treated with heparin or CD-S for 7 days in basic differentiation medium. Cells cultured in basic differentiation medium were used as control. \* $p < 0.05$ , \*\* $p < 0.01$ . Data are the mean  $\pm$  SD ( $n = 3$ ).

The expression of endodermal (Foxa2) and mesodermal (Flk1) marker genes in mESCs treated with heparin or CD-S for 7 days in basic differentiation medium was studied using qPCR. It was found that similar to heparin-treated mESCs, CD-S-treated cells showed lower expression level of both endodermal and mesodermal marker genes as compared to the control group (**Fig. S8**), indicating the addition of CD-S or heparin favored neural differentiation but suppressed mesodermal and endodermal differentiation in basic differentiation medium.

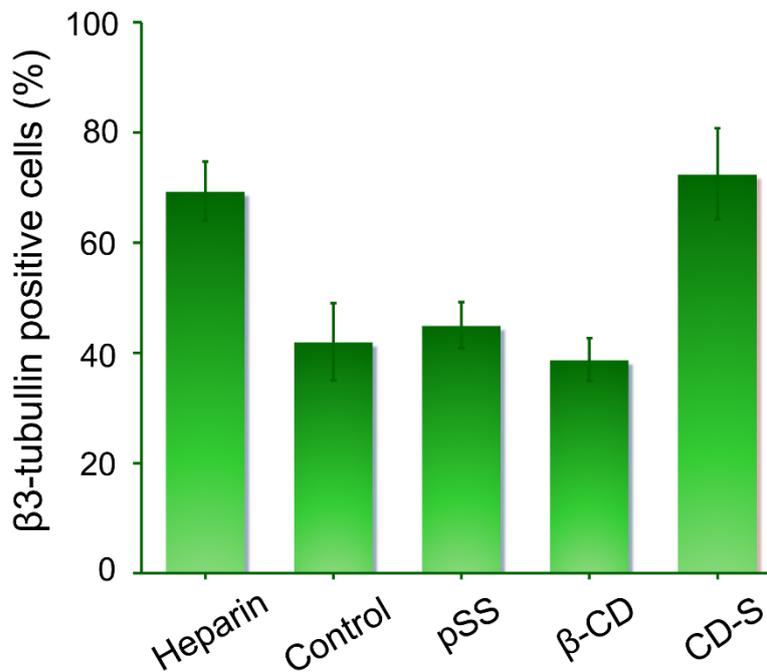
### 5.3 The expression of $\beta 3$ -tubulin in CD-S-treated mESCs in neural differentiation medium



**Fig. S9** Immunofluorescence images of CD-S/RA-treated mESCs (green:  $\beta 3$ -tubulin; blue: DAPI) after culture for 18 days (scale bar: 100  $\mu$ m).

As shown in **Fig. S9**, CD-S/RA-treated ESCs showed neuron-like morphologies with axon outgrowths after 18 days.

### 5.4 The percentage of $\beta 3$ -tubulin positive cells treated with different molecules in neural differentiation medium



**Fig. S10** The percentage of  $\beta 3$ -tubulin positive cells treated with 1  $\mu$ M RA together with heparin, pSS,

$\beta$ -CD, or CD-S for 18 days. Data are presented as mean  $\pm$  SD ( $n = 3$ ).

The amount of  $\beta$ 3-tubullin positive cells in different groups was summarized in **Fig. S10**. The percentage of  $\beta$ 3-tubullin positive cells in CD-S-treated group was 72.5% which is similar to that in heparin-treated group (69.4%) and much higher than that in the control-(42%), pSS-(45%), or  $\beta$ -CD-treated group (38.8%).

## References

1. X. Shi, G. Chen, Y. Wang, L. Yuan, Q. Zhang, D. M. Haddleton and H. Chen, *Langmuir*, 2013, **29**, 14188-14195.
2. E. Ryu and Y. Zhao, *Org. Lett.*, 2005, **7**, 1035-1037.
3. H. T. Le, H. M. Jeon, C. W. Lim and T. W. Kim, *Carbohydr. Res.*, 2014, **391**, 22-28.
4. M. Wang, Z. Lyu, G. Chen, H. Wang, Y. Yuan, K. Ding, Q. Yu, L. Yuan and H. Chen, *Chem. Commun.*, 2015, **51**, 15434-15437.
5. C. E. Pickford, R. J. Holley, G. Rushton, M. P. Stavridis, C. M. Ward and C. L. Merry, *Stem Cells*, 2011, **29**, 629-640.
6. K. Ding, Y. Wang, H. Wang, L. Yuan, M. Tan, X. Shi, Z. Lyu, Y. Liu and H. Chen, *ACS Appl. Mater. Interfaces*, 2014, **6**, 20043-20050.