Electronic Supplementary Material (ESI) for Materials Advances.

Supporting information for

Promoting neural differentiation of embryonic stem cells by using thermosensitive nanocomposite

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1. Result and Disscusion

1.1. Synthesis of polymer

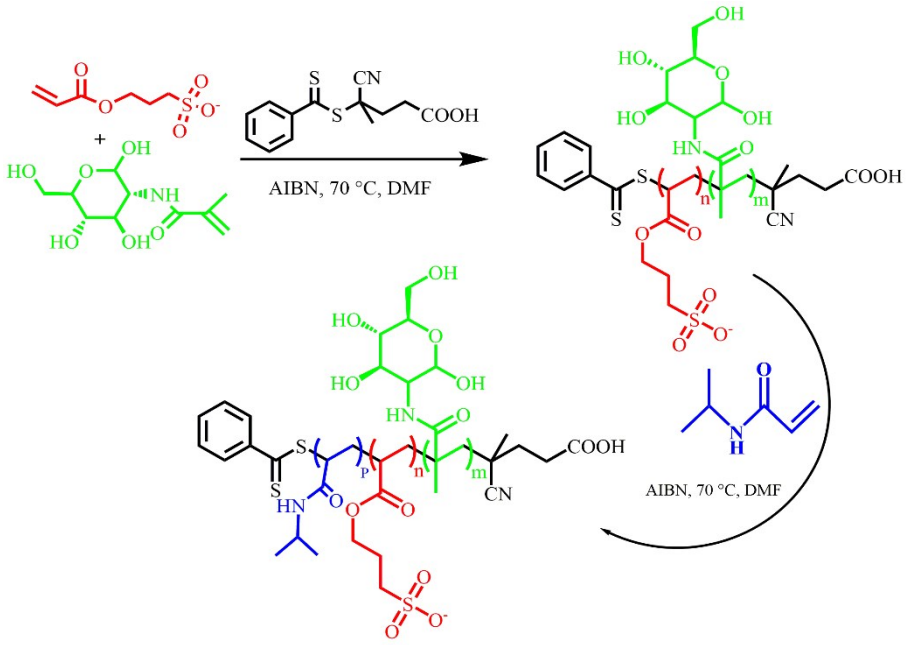

Fig. S1 Synthesis of p(MAG-co-SPA) and pNIPAAm-b-p(MAG-co-SPA) via reversible addition-fragmentation chain transfer (RAFT) polymerization

1.2. ^{1}H NMR spectrum of monomer and polymer

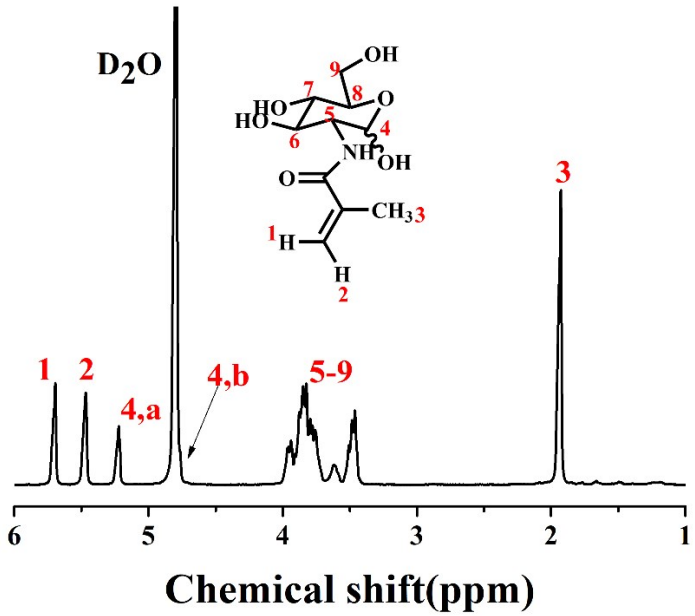

Fig. S2 ^{1}H NMR spectrum of MAG. ^{1}H NMR (400 MHz, D_2O) 5.71 (s, 1H), 5.48 (s, 1H), 5.24 (s, 1H), 4.04 –3.39 (m, 6H), 1.95 (s, 3H).

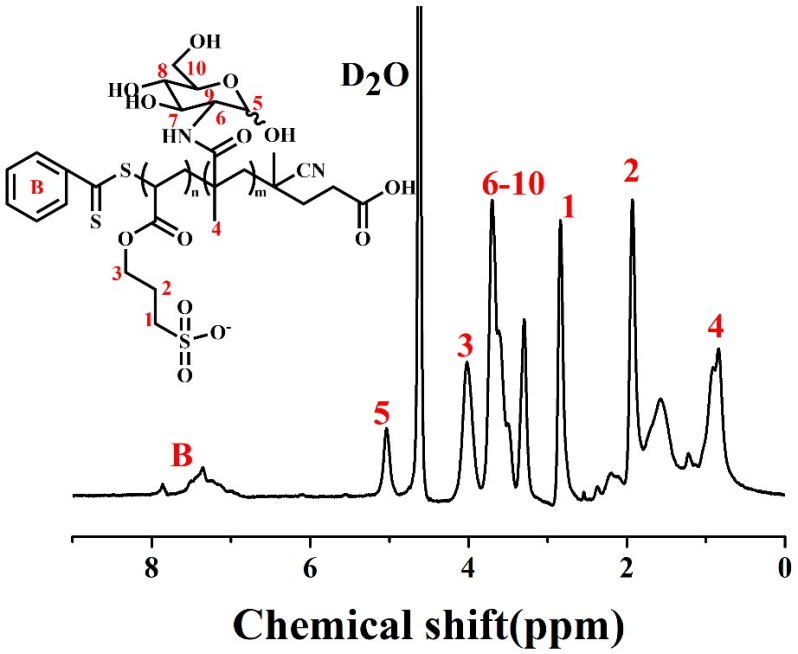

Fig. S3 ^{1}H NMR spectrum of p(MAG-co-SPA). ^{1}H NMR (400 MHz, D_2O) 5.21 (s, 1H), 4.19 (s, 2H), 3.68 (d, J = 159.8 Hz, 6H), 2.88 (s, 2H), 1.98 (s, 2H), 0.96 (d, J = 30.0 Hz, 3H).

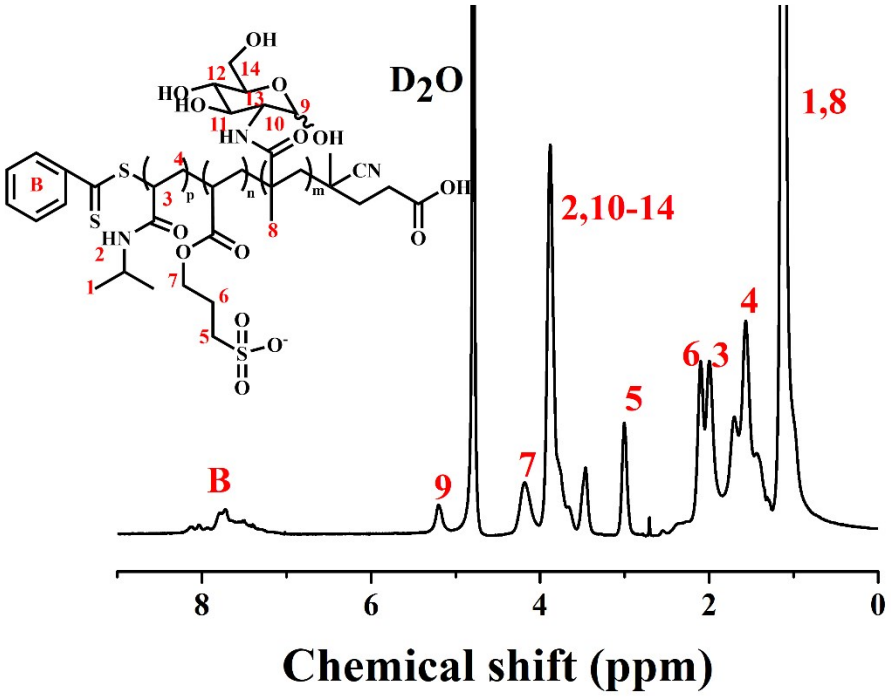

Fig. S4 ^{1}H NMR spectrum of pNIPAAm-b-p(MAG-co-SPA). ^{1}H NMR (400 MHz, D_2O) 5.21 (s, 1H), 4.19 (s, 2H), 3.68 (d, J = 159.8 Hz, 7H), 3.02 (s, 2H), 2.11 (s, 2H), 1.98 (s, 1H), 1.52 (s, 2H), 1.06 (d, J = 30.0 Hz, 6H).

1.3. The feed ratio and molecular weight of the polymer

Table S1 Details in synthesizing copolymers by RAFT polymerization

Copolymer	Monomer	CTA	I	[M]:[CTA]:[I]
pMS	MAG, SPA	CPADB	AIBN	75:2:1
pN1MS	NIPAAm	pMS	AIBN	100:2:1
pN2MS	NIPAAm	pMS	AIBN	200:2:1
pN3MS	NIPAAm	pMS	AIBN	400:2:1
pN4MS	NIPAAm	pMS	AIBN	600:2:1
pN1	NIPAAm	CPADB	AIBN	97:2:1
pN2	NIPAAm	CPADB	AIBN	150:2:1
pN3	NIPAAm	CPADB	AIBN	256:2:1
pN4	NIPAAm	CPADB	AIBN	362:2:1

Table S2 GPC results of synthetic copolymers by RAFT polymerization

polymer	M_w (Da)	PDI	Ratio of monomer [NIPAAm]:[MAG]:[SPA]
pMS	8900	1.17	0:1:1.05
pN1MS	13700	1.20	2.2:1:1.05
pN2MS	18500	1.22	4.6:1:1.05
pN3MS	28100	1.25	9.1:1:1.05
pN4MS	37700	1.28	14.2:1:1.05
pN1	4800	1.12	1:0:0
pN2	9500	1.18	1:0:0
pN3	19000	1.23	1:0:0
pN4	28000	1.24	1:0:0

1.4. Primers

Table S3 The primers used in qPCR.

Gene	Forward primer	Reverse primer	Product size (bp)
β-Actin	CCCTAGGCACCAGGGTGTGA	TCCCAGTTGGTAACAATGCCA	128
Oct-4	GGCGTTCTCTTTGGAAAGGT	GGCGTTCTCTTTGGAAAGGT	112
Sox17	GATGCGGGATACGCCAGTG	CCACCACCTCGCCTTTCAC	136
Flk1	CACCTGGCACTCTCCACCTTC	GATTTCATCCCACTACCGAAAG	239
Nestin	CCCTGAAGTCGAGGAGCTG	CTGCTGCACCTCTAAGCGA	166
β3-Tubulin	ACTTTATCTTCGGTCAGAGTG	CTCACGACATCCAGGACTGA	96

1.5. TEM characterization of nanocomposites

Compared with AuNPs, the TEM images of AuNPs-pNMS can clearly show the polymer grafted on the outside of the particles (Fig. S5-7). In Fig. S6, the samples were prepared at 25 °C and the particle sizes of A to D are 23 nm, 31 nm, 40 nm and 50 nm respectively. In Fig. S7, the samples were prepared at 37 °C and the particle sizes of A to D are 20 nm, 27 nm, 33 nm and 40 nm respectively. It can be clearly seen that as the temperature increases, the particle size of AuNPs-pNMS decreases. However, the size was smaller than that measured by DLS. This was mainly due to the process involved in the preparation of sample. In the case of the TEM methods, TEM images depicted the actual size at the dried state of sample, whereas the size measured by the laser light scattering method was a hydrodynamic diameter (hydrated state), and therefore the nanocomposite showed a larger hydrodynamic volume due to solvent effect in the hydrated state.[1]

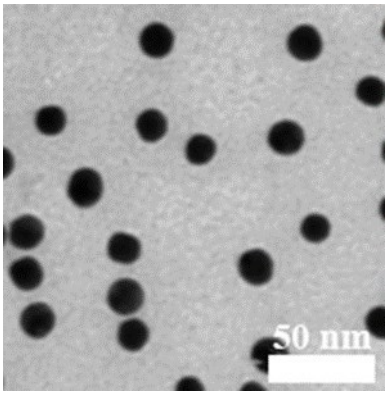

Fig. S5. TEM image of AuNPs

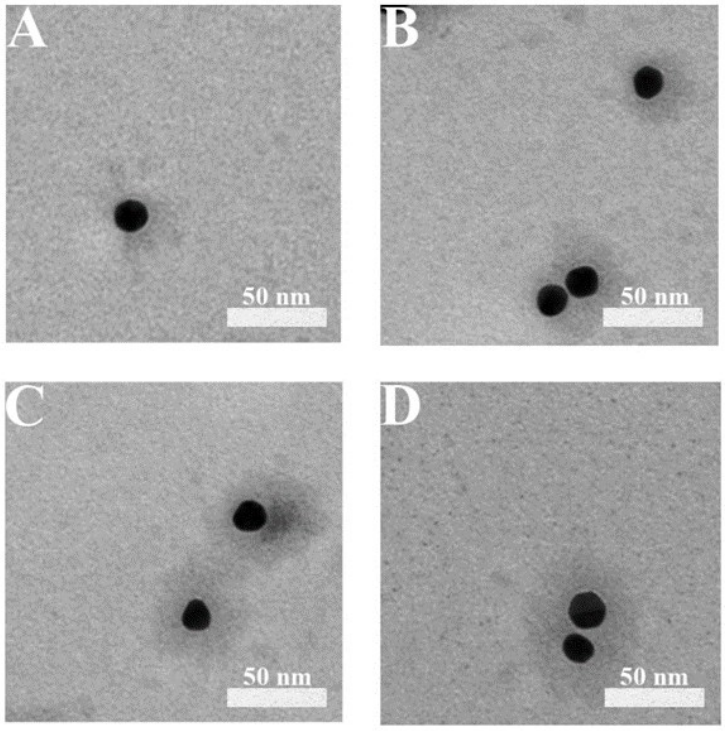

Fig. S6. TEM images of AuNPs-pNMS at 25 °C. (A): AuNPs-pN1MS, (B): AuNPs-pN2MS, (C): AuNPs-pN3MS, (D): AuNPs-pN4MS.

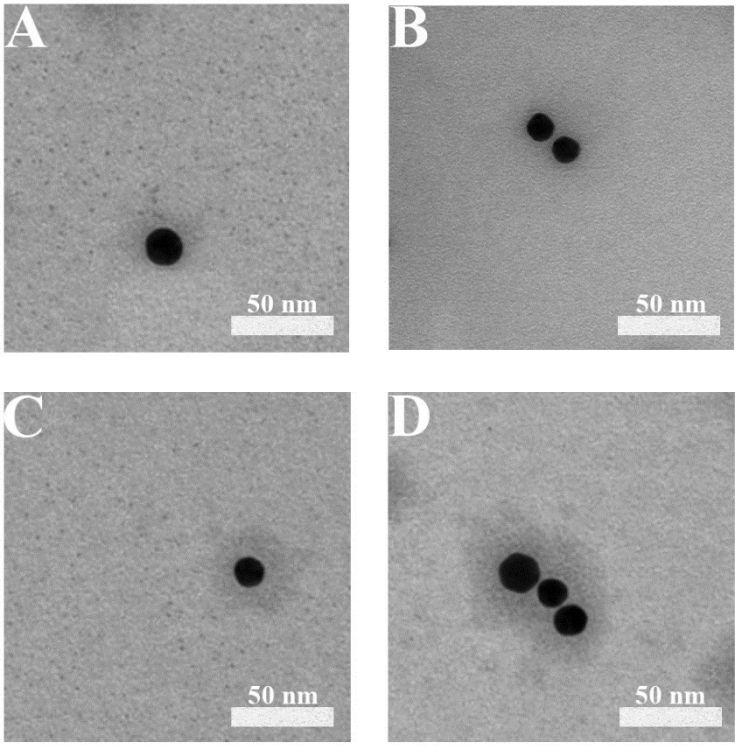

Fig. S7. TEM images of AuNPs-pNMS at 37 °C. (A): AuNPs-pN1MS, (B): AuNPs-pN2MS, (C): AuNPs-pN3MS, (D): AuNPs-pN4MS.

1.6. Immunofluorescence staining

Immunofluorescence staining was performed to detect the protein expression levels of Sox17 and Flk1. In Fig. S8, all sample groups showed the expression of Sox17 protein. However, compared with the negative control group and the heparin group, the expression level of Sox17 protein in the cells treated with the nanocomposite was significantly reduced, especially AuNPs-PNMs, which greatly inhibited the expression of Sox17 protein. This is consistent with the detection results of the relative expression level of SOX17 gene. In Fig. S9, all sample groups also showed the expression of Flk1 protein. And the expression level of Flk1 protein is consistent with the detection results of the relative expression level of Flk1 gene. The nanocomposite greatly inhibits the expression of Flk1 protein, especially AuNPs-pNMS, which has the best effect.

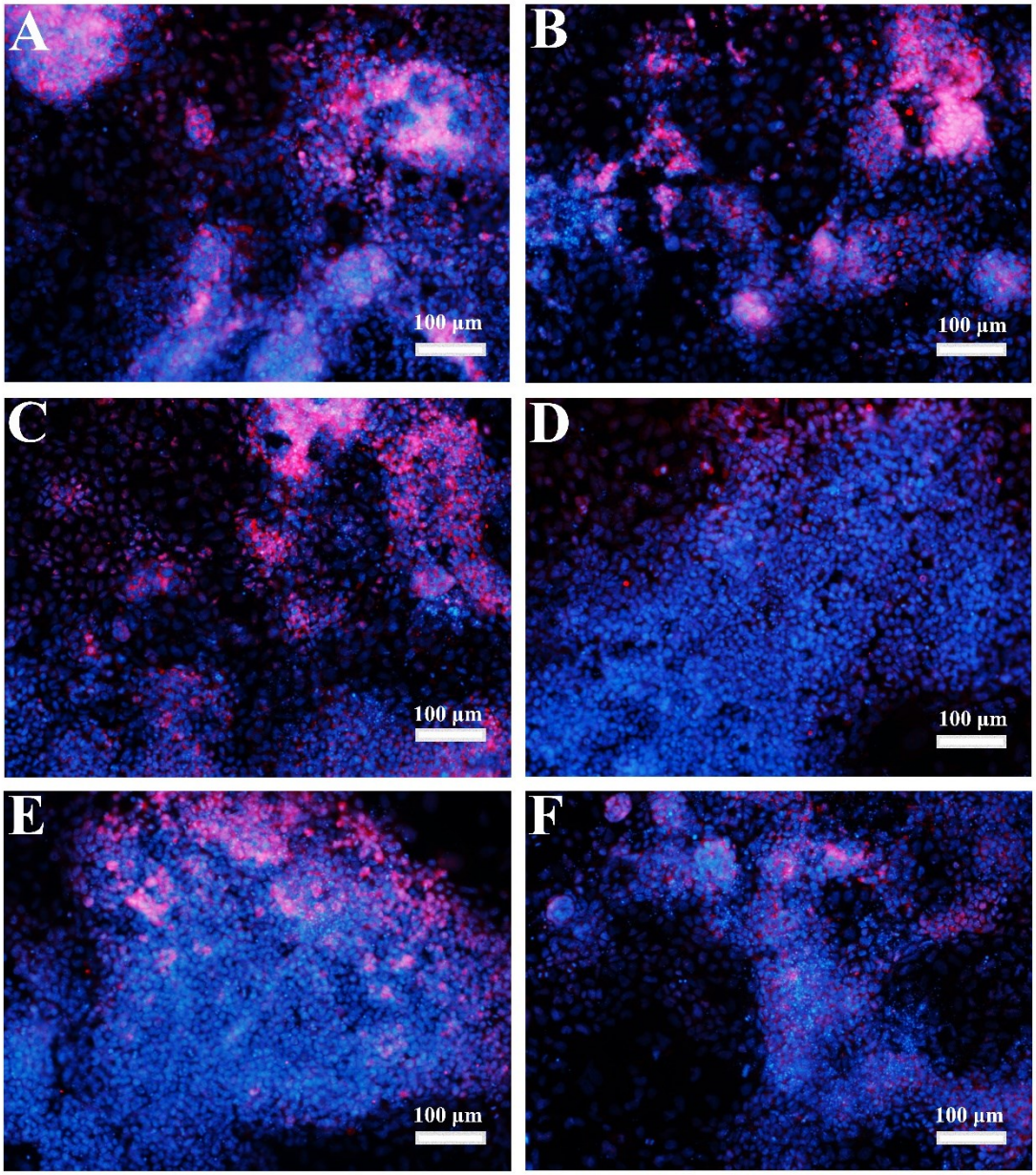

Fig. S8. Immunofluorescence images of ESCs, (A): Blank, (B): Heparin, (C): AuNPs, (D): AuNPs-pMS, (E): AuNPs-pN, (F): AuNPs-pNMS. Scale bar: 100 μm. Sox17 was detected with anti-Sox17 mouse monoclonal antibody and Alexa Fluor 555-conjugated goat anti-mouse IgG (red), and cell nuclei were stained with DAPI (blue).

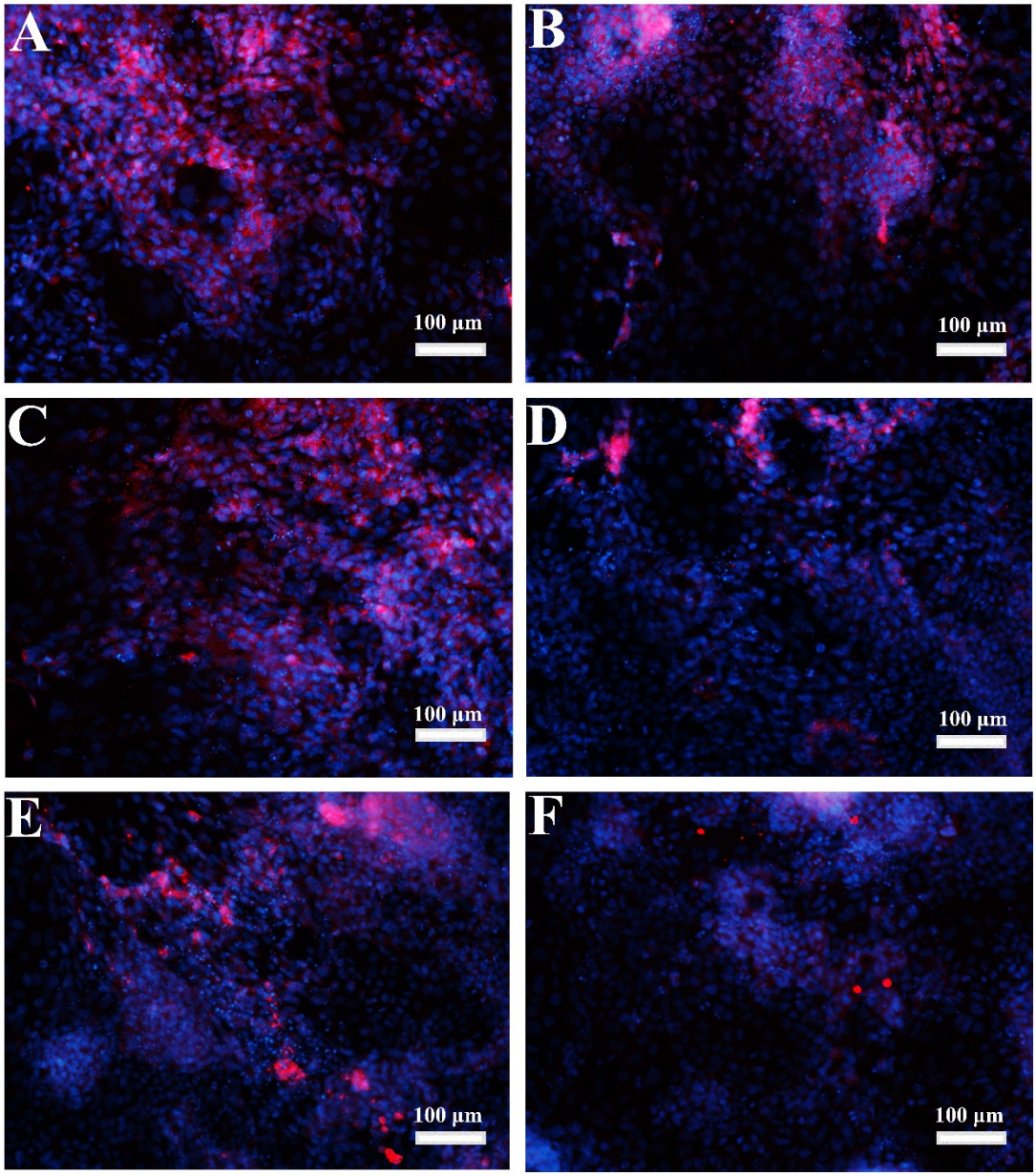

Fig. S9. Immunofluorescence images of ESCs, (A): Blank, (B): Heparin, (C): AuNPs, (D): AuNPs-pMS, (E): AuNPs-pN, (F): AuNPs-pNMS. Scale bar: 100 μm. Flk1 was detected with VEGFR2 polyclonal antibody (rabbit polyclonal) and goat anti-rabbit lgG (H+L), CoraLite594 conjugate (red), and cell nuclei were stained with DAPI (blue).

1.7. Adhesion experiment of nanocomplex to cell

First, the fluorescent labeled thermosensitive heparin mimic (pNMSF) was synthesized

via the reversible addition fragmentation chain-transfer (RAFT) polymerization of NIPAAm, pMS, and FluMA. Briefly, NIPAAm (0.9053 g, 8 mmol), MAG (0.360 g, 0.04 mmol), FluMA (0.032 g, 0.08 mmol), and AIBN (0.0033 g, 0.02 mmol) were dissolved in the solution of DMF and DIW (1:1, v/v). The solution was then degassed by bubbling with nitrogen for 30 min. The polymerization was carried out at 70 °C for 24 h under a nitrogen atmosphere in a glovebox. When the polymerization was complete, the polymerization mixture was dialyzed for 48 h against DIW to remove the excess monomers. The copolymer formed in solution were lyophilized to give a fluffy yellow solid. Then the copolymer was subjected to a sulfhydryl reaction and further fluorescently labeled nanocomposite was prepared. Fluorescence images were taken after the fluorescent labeled nanocomposite acts on the cells for 4 hours. In Fig. S10, the images were taken by a fluorescence microscope. It can be clearly seen that the fluorescent labeled nanocomposites overlap with the cell completely. The adhesion of the fluorescent labeled nanocomposites to a single cell was further observed by confocal microscope. It can be clearly observed that the skeleton of a single cell was perfectly covered with the fluorescent labeled nanocomposites in Fig. S11. Chithrani et. al. determined that after being taken up by cells, AuNPs are wrapped in vesicles and will not be evenly dispersed inside the cells.[2] If there is no nanocomposite attached to the cell membrane surface, the fluorescence image of the nanocomposites will not completely overlap with the cytoskeleton. Therefore, we can determine that the nanocomposite still adheres to the surface of the cell membrane after the material acts on the cell for 4 hours.

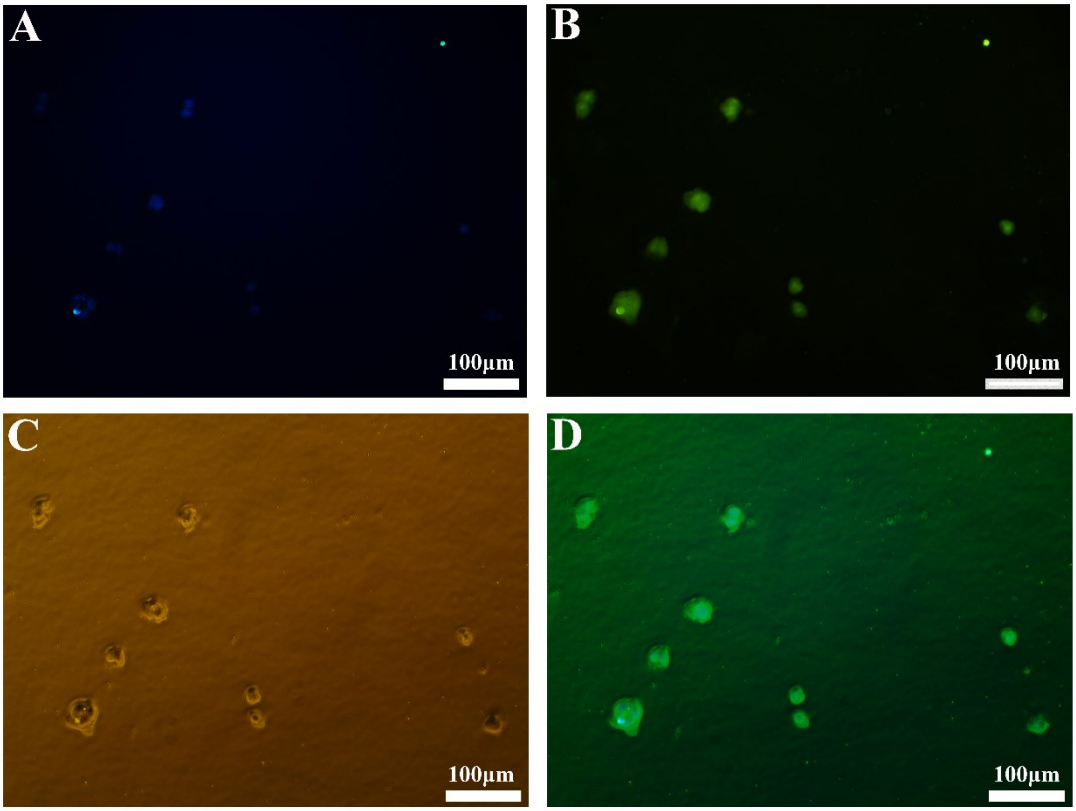

Fig. S10. The adhesion of the nanocomposite after acting on the cells for 4 hours. (A): cell nuclei were stained with DAPI (blue), (B): the fluorescent labeled nanocomposite. (C): ESCs under bright field, (D): Merged image from A-C. Scale bar: 100 μm. The images were taken by a fluorescence microscope.

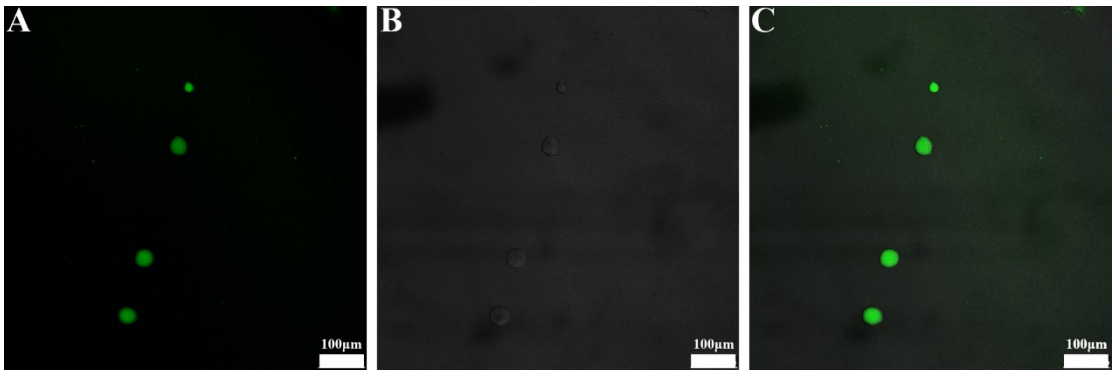

Fig. S11. The adhesion of the nanocomposite after acting on the cells for 4 hours. (A): the fluorescent labeled nanocomposite. (B): ESCs under bright field, (D): Merged image from A and B. Scale bar: 100 μm. The images was taken by a confocal microscope.

1.8. Cell culture with/without serum

We have done the experiments to confirm the neural differentiation effect of nanocomposite on ESCs in the culture medium with/without serum. However, we found that ESCs could not proliferate in the culture medium without serum and would gradually die in the experiment (Fig. S12). On the 3rd day, ESCs in the culture medium with serum proliferated in a large amount, but ESCs in the culture medium without serum did not proliferate and the growth state of ESCs was not very good. On the 5th day, the proliferation of ESCs in the culture medium with serum was very good, however, ESCs in the culture medium without serum had almost all died. Because maintaining the normal proliferation and good growth status of ESCs is the prerequisite for studying the differentiation of ESCs, the experiment to explore the differentiation of ESCs in the culture medium without serum cannot be carried out.

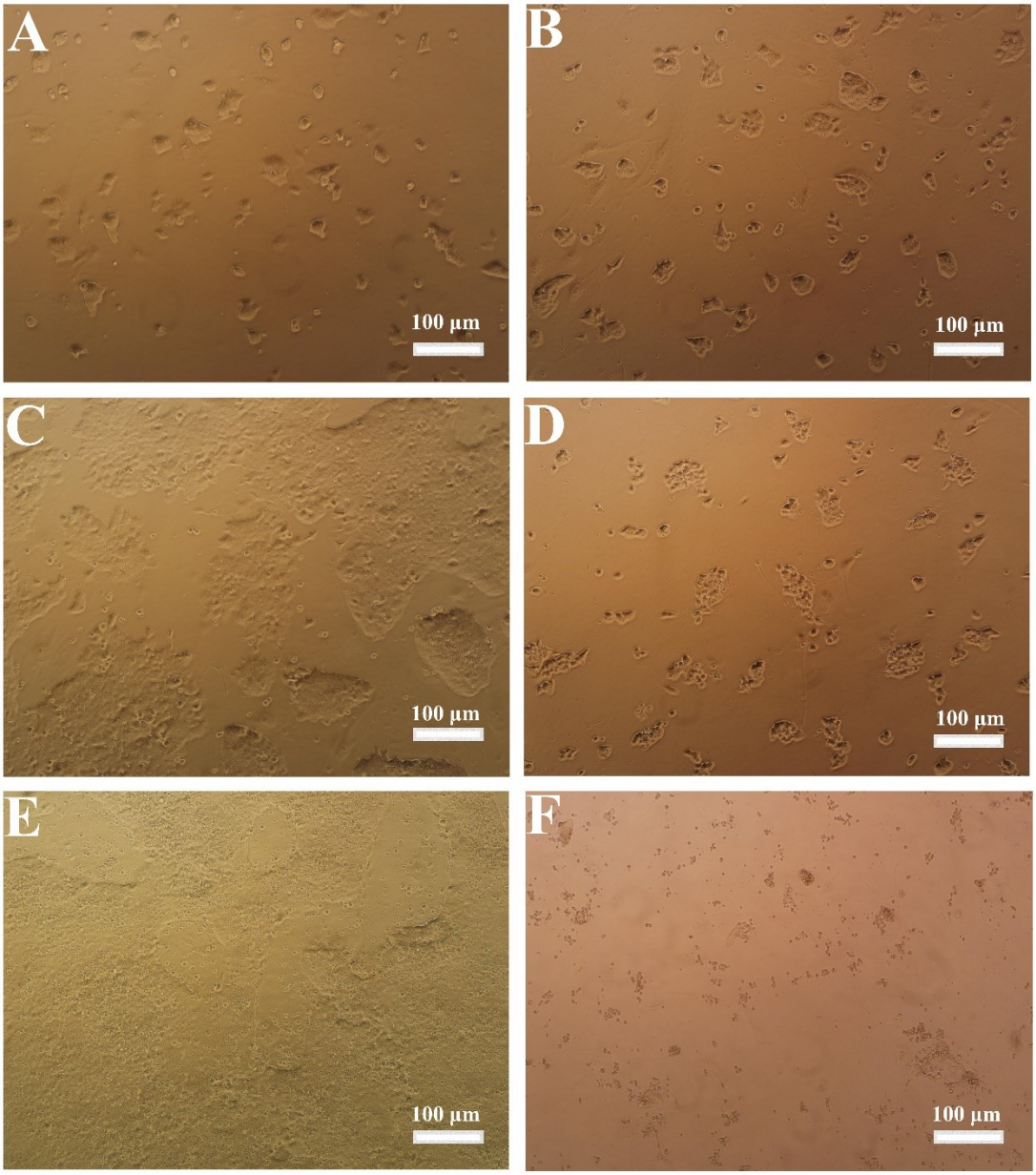

Fig. S12. The proliferation of ESCs. A, C, and E are images of ESCs grown in the culture medium with serum for 1, 3, and 5 days. B, D, and F are images of ESCs grown in the culture medium without serum for 1, 3, and 5 days. Scale bar: 100 μm.

2. Reference

1. W. She, K. Luo, C. Zhang, G. Wang, Y. Geng, L. Li, B. He and Z. Gu, *Biomaterials*, 2013, **34**, 1613-1623.
2. B. D. Chithrani, A. A. Ghazani and W. C. W. Chan, *Nano Lett.*, 2006, **6**, 662-668.