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Materials Advances

ARTICLE

Promoting neural differentiation of embryonic stem cells by using thermosensitive nanocomposite

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Nerve cells differentiated from embryonic stem cells (ESCs) play an important role in the treatment of neurodegenerative diseases. Heparin has great potential for inducing ESCs to differentiate into nerve cells, but due to the shortcomings of heparin itself, its effect on inducing differentiation of ESCs is limited. In this study, thermosensitive copolymer pNIPAAm-b-p(MAG-co-SPA) (pNMS) was synthesized with N-isopropylacrylamide (NIPAAm) as monomer and heparin mimic p(MAG-co-SPA) (pMS) as macromolecular chain transfer agent. Considering the biocompatibility of AuNPs, the nanocomposites were prepared, and their effects on inducing differentiation of ESCs were studied in detail. Compared with heparin, nanocomposites have a stronger effect on the differentiation of ESCs, especially AuNPs-pNMS, which can better promote the expression of β3-tubulin and stimulate the synthesis of proteins required for neuronal maturation. The results show that AuNPs-pNMS has a stronger ability to induce differentiation than AuNPs-pMS due to the presence of the pNIPAAm segment. The synergistic effect of pNIPAAm and pMS in AuNPs-pNMS greatly increases the efficiency of nanocomposite binding to FGFR, further enhancing the effect of the neural differentiation of ESCs. The effect of AuNPs-pNMS in promoting the differentiation of ESCs is also related to the relative molecular weight of pNMS. Under the premise of controlling the relative molecular weight of pMS to be 8900 Da, when the molecular weight of pNMS is 28100 Da, AuNPs-pNMS has the strongest effect of promoting neural differentiation of ESCs. This thermosensitive nanocomposite provides a new strategy for promoting the neural differentiation of ESCs.

1. Introduction

Embryonic stem cells (ESCs) are totipotent cells with long-term self-reproduction ability and the potential to differentiate into almost all cell types in the body.1,2,3 In recent years, neurodegenerative diseases have threatened the safety and quality of life of human beings and have attracted social attention. These diseases are considered to be mainly caused by nerve cell damage or dysfunction. In the treatment of these diseases, nerve cell-based replacement therapy or transplantation has become a promising strategy to repair damaged nerve cells and restore their dysfunction.4,5 Under normal circumstances, ESCs can be induced to differentiate into specific cells or tissues through some biological, chemical, and physical treatments6,7,8 when they are cultured in vitro. Its application provides convenience for the treatment of neurodegenerative diseases.

Scheme 1. Schematic diagram of AuNPs-pNMS promoting neural differentiation of ESCs.
There are many ways to induce stem cells to differentiate into nerve cells, such as adding exogenous growth factors,10, 11 biologically active molecules,12 chemical inducers,13 electric and magnetic fields,14 etc. But exogenous growth factors, including nerve growth factor (NGF),15 and fibroblast growth factor (FGF),16. 17 are expensive, and its half-life is short, so their practical applications are greatly restricted. In addition, although chemical inducers can promote the differentiation of ESCs into nerve cells,18 their toxicity can also cause damage to cells, and affect cell self-proliferation and even lead to apoptosis. Physical stimulation, such as electrical stimulation or magnetic field stimulation, may cause some undesirable differentiation directions. In recent years, studies have found that glycosaminoglycans (GAGs), especially heparin and its mimics, can promote the neural differentiation of ESCs.19-21

GAGs is a kind of natural polysaccharide polymer with complex structure and versatile application.22, 23 Most GAGs exist as proteoglycans on the cell membrane. Proteoglycans on the cell membrane have not great effect in promoting the neural differentiation of ESCs, and may cause ESCs to differentiate into different germ layers. Previous studies have shown that heparin has a prominent role in promoting the differentiation of stem cells. A large number of sulfonic acid groups in its structure can bind to receptors on the stem cell membrane to promote differentiation.24-27 However, heparin can bind to different biologically active factors, thereby affecting the differentiation direction of ESCs. Therefore, heparin mimic containing sulfonic acid groups is the better choice to promote ESCs differentiation.12, 19, 21 Wang et al. proposed a unit reorganization strategy to synthesize a heparin-mimicking polymer by using independent units with functional groups similar to natural heparin.19 Lei et al. used the unit reorganization strategy to synthesize heparin mimics containing different sulfonic acid units. And the heparin mimic forms a heparin mimic-FGFR-FGFR ternary complex which on the cell membrane to promote the neural differentiation of ESCs.28 However, because it cannot precisely control the sequence of structural units in molecular weight, and the structural sequence of heparin has an important relationship with promoting the neural differentiation of ESCs, this strategy still has some shortcomings. According to reports, heparin mimic can bind to FGFR and FGF through the sulfonic acid group on the chain, which promotes the formation of a stable ternary complex of heparin mimic-FGFR-FGFR on the cell membranes. It can make FGFR form dimers and activate the formation of signal pathways that promote neural differentiation of ESCs.20 In such way, improving the binding ability of heparin mimic and FGFR is more conducive to promoting the neural differentiation of ESCs.

Liu et al. modified a phospholipid group at the end of the polymer and anchored it to the cell membrane, which increased the efficiency of heparin mimic binding to FGFR and improved the effect in promoting differentiation of ESCs. The behavior of heparin mimic in promoting the neural differentiation of ESCs has been studied in depth, and the structural composition of heparin itself has been relatively clear. On this basis, like Liu et al., modifying other functional groups or segments outside of the heparin structure has become the primary choice. In addition to the chemical properties of biological materials that promote the differentiation of ESCs, the physical properties of some biological materials can also assist or induce the differentiation of stem cells. Promoting the differentiation of ESCs through the synergistic effect of the chemical properties and physical properties of biological materials is a promising choice. Seo et al. used temperature-sensitive pNIPAAm-co-Am) nanoparticles to assist the delivery of RA, thereby promoting the neural differentiation of stem cells.29, 30 The two acted synergistically to improve the efficiency of differentiation. It can be seen that the temperature sensitivity of pNIPAAm has certain research significance for the neural differentiation of stem cells. Previous studies have shown that the dynamic presentation of nano-scale ligands can regulate the adsorption and differentiation of stem cells. Kang et al. used nanoswitch controlled by coordination to regulates the mechanosensing and differentiation of stem cells.31 The high specific surface area of AuNPs could enable them to have high carrier capacity for polymer. The good biocompatibility and good dispersion of AuNPs in solution help them well contact with the stem cells surface. So AuNPs were introduced in the design and act attractively as an excellent carrier which can support the well assembly of polymer to promote the neural differentiation of ESCs.32, 33

Responsive polymers are polymers that can respond to external environmental stimuli (such as temperature, light, pH, etc.) to trigger changes in their conformation. When a specific chemical or physical signal changes slightly, responsive polymer can produce a significant response.34 Temperature-sensitive polymers are the most widely used type of stimulus-responsive polymers. For temperature-sensitive polymers with low critical solution temperature (LCST), when the ambient temperature is lower than LCST, the hydrogen bond between the water molecule and the hydrophilic group on the polymer chain causes the polymer chain to form a stable hydration structure. The polymer chains exhibit hydrophilicity and exist in the form of extended coils. When the ambient temperature rises above the LCST, the hydrogen bond is broken and the hydration structure is destroyed. The polymers desolvate and collapse into a dense globular conformation, showing a hydrophobic state.35-37 pNIPAAm (pN) is a typical temperature-sensitive polymer, with LCST around 32 °C. This paper proposed to use NIPAAm and heparin mimic block copolymerization to synthesize temperature-sensitive copolymers pNIPAAm-b-p(MAG-co-SPA) (pNMS). Then AuNPs-pNMS was prepared to promote the neural differentiation of ESCs. Compared to 25 °C, the hydrated diameter of AuNPs-pNMS decreased with the collapse of pNIPAAm fragment at 37 °C. The experimental results showed that AuNPs-pNMS had good temperature sensitivity. The polymer on the surface of AuNPs could specifically bind to FGFR monomer on the cell membrane. In addition, AuNPs as carriers would be enriched on the cell membranes, which was more conducive to the combination of polymer and FGFR monomer. At the same time, when the thermosensitive nanocomposites were placed at 37 °C, the polymer chain collapsed and the hydrated diameter of AuNPs-pNMS decreased, pulling the FGFR monomers, shortening the
distance between the monomers, promoting the formation of FGFR dimers. In addition, FGFR dimer was formed, which activated the signal pathway of neural differentiation of ESCs (Scheme 1). This study provides a new strategy for promoting the neural differentiation of ESCs, which has important biological significance and practical application value.

2. Experimental

2.1 Materials

2,2-Azobis-(isobutyronitrile) (AIBN) and D-Glucosamine hydrochloride were purchased from TCL. 3-sulfopropyl acrylate potassium salt (SPA), N-methacryloyl chloride, 4-Cyano-4-(phenylcarbonothioylthio) pentanoic acids, sodium citrate dihydrate and paraformaldehyde were purchased from Sigma-Aldrich. N-isopropylacrylamide (NIPAAm) was bought from Energy Chemical. Hydrogen tetrachloroaurate hydrate (HAuCl₄•4H₂O) and fluorescein omethacrylate (FluMA) were obtained from Sinopharm Chemical Reagent Co. (Shanghai, China) and Alfa Aesar Chemical Co., Ltd. (China), respectively. Dulbecco’s modified Eagle’s medium (DMEM, high glucose), Roswell Park Memorial Institute (RPMI-1640) medium, 0.25% (1×) Trypsin, and 10× phosphate buffer solution (PBS) were purchased from Gibco. Leukemia inhibitory factor (LIF), bovine serum albumin (BSA), and β-mercaptoethanol were bought from Merck Millipore, Solarbio, and Beijing Dingguo Biotechnology Co., Ltd., respectively. Heparin (MW≈ 3.5−8.0 kDa), N,N-dimethylformamide (DMF) and methyl alcohol were purchased from Shanghai Chemical Reagent Co. All aqueous solutions were prepared in purified water with a resistivity of 18.2 MQ.cm (deionized water, DIW) from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2 Synthesis of Citrate-Protected AuNPs

The citrate-protected AuNPs were obtained as previous reported. Briefly, all glass containers were soaked overnight with aqua regia solution (HNO₃/HCl = 1:3, v/v) and washed with double-distilled water before using. A mixture of double-distilled water (100 mL) and HAuCl₄ (12 mM, 516 μL) were fed into a 250 mL round-bottom flask equipped with a condenser. When the solution in the flask was boiling, sodium citrate (10% w/v, 4.4 mL) was added with vigorous stirring. Then the color changed from gray to red. Keep the solution boiling for another 15 min and then cool it down to room temperature.

2.3 Synthesis of copolymers

2-Methacrylamido glucopyranose (MAG), the monomer, was synthesized as reported previously. The heparin mimic, pMS, was synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization as previously reported. Potassium 3-sulfonyl acrylate (SPA) and MAG were used as monomers ([SPA]:[MAG]=1:1). 4-Cyano-4-(phenylcarbonothioylthio) pentanoic acid (CPADB) and AIBN were served as chain transfer agent (CTA) and initiator (I), respectively. Monomers, CTA, and initiator ( [M]:[CTA]:[I]=75:2:1) were dissolved in 5 mL of mixed solvent (DMF:DIW = 1:1, v/v), which was bubbled with nitrogen for 30 min to remove oxygen in the container before transferring into a glovebox for further reaction at 70 °C for 24 h (Fig. S1). The products were obtained via lyophilization after dialysis.

A series of thermosensitive heparin mimic, pNMS, were synthesized with N-isopropylacrylamide as monomer and pMS as macromolecular chain transfer agent according to the above RAFT polymerization method. At the same time, according to the molecular weight of thermosensitive heparin mimic, pH of corresponding molecular weight is prepared (see Table S1). Information about the number-average molecular weights (Mn) and polymer dispersity index (PDI) values of all copolymers were acquired by gel-permeation chromatography (GPC) experiments as shown in Table S2.

2.4 Preparation of nanocomposites

The copolymers (0.5 g) were dissolved in 1 mL of double-distilled water in a glass round-bottom flask. Then, sufficient ethanalamine (0.2 mL) was added dropwise into the solution under stirring for 3 h to achieve thiol end groups of the copolymers. The products were dialyzed against water for 48 h and then lyophilized. The achieved mercaptolated polymers were analyzed by UV-vis spectroscopy. AuNPs were centrifuged at 4 °C and 12,000 rpm (5810 R, Ependorff) for 15 min to remove the supernatant. Mercaptolated polymers were dissolved in ultrapure water at a concentration of 0.5 mg/mL, and was used to resuspend the AuNPs. The mixture was incubated at 25 °C for 24 h to ensure the assembly of polymers on AuNPs. After the reaction, the supernatant was removed by centrifugation at 4 °C and 12000 rpm.

2.5 Cell culture and Differentiation

Mouse ESCs were seeded in a 96-well plate with the cell number of 6 × 10³ for each well, which was coated with gelatin in ESCs medium overnight as mentioned above. After the treatment of the nanocomposites, the culture medium was replaced with fresh medium every other day. Cell viability was tested by a Cell Count Kit-8 (CCK-8) at days 1, 3 and 5, respectively. For L929 cells, the number of cells was 3 × 10⁵ for each well. The treatment and CCK-8 test were similar with mouse ESCs. Primary generation of ESCs was purchased from Shanghai Institutes for Biological Science, CAS. Cells were cultured in a T25 culture flask on the feeder layer with humidified atmosphere in a 37 °C incubator (Ependorf Galaxy 170 R) with 5% (v/v) CO₂. Feeder layers were mitomycin C-inactivated mouse embryonic fibroblasts (MEF). ESCs with the number of 2 × 10⁵ were seeded in each well of a 6-well plate that was coated with gelatin in ESCs medium overnight as mentioned above. Before treatment of the nanocomposites, the cells were rinsed with PBS. Then, fresh medium suspended nanocomposites and differentiation medium (DMEM high glucose medium containing 10% FBS, 1% penicillin/streptomycin, 100 U/mL Plasmocin prophlactic, 0.1 mM NEAA, and 0.1 mM β-mercaptoethanol) were added into the cell culture. The differentiation medium was changed every second day.
2.6 Reverse Transcription–Polymerase Chain Reaction (RT-PCR) and Real-Time PCR.

Total RNAs were extracted from ESCs incubated in a sample-added differentiation medium for 14 d using a Total RNA Kit (Tiangen) following the manufacturer’s instruction. RNAs was reverse transcribed into cDNA using a REVERTAID 1ST CDNA SYNTH KIT (Fermentas). The real-time PCR was carried on a Step One Plus real-time PCR system and marked by a Fast SYBR Green Master Mix with a ROX reference dye (ABI). The cDNAs for target genes, Oct-4, Sox17, Flk1, Nestin, and β3-tubulin, were detected with β-Actin as the reference gene (Table S3). Real-time PCR was performed with an initial denaturation of 95 °C for 20 s, and followed by 50 cycles of 30 s denaturation at 95 °C, 45 s annealing at 60 °C, and 45 s elongation at 72 °C. The level of expression of target genes was calculated by 2−ΔΔC_T algorithm. All the samples were normalized to heparin-supplemented medium, which was set to 1.

2.7 Immunofluorescence Assay.

Cells were fixed with 4% paraformaldehyde solution for 15 min, and washed 3 times with PBS. Then, the cells were blocked with 3% BSA (in PBS) for 30 min, and incubated with 1% BSA (in PBS) containing the primary antibody against β3-tubulin at 4 °C overnight. The primary antibody was removed and the cells were rinsed 3 times with PBS. The secondary antibody, Goat anti-Rabbit IgG H&L, diluted in 1% BSA, was added to the cells and incubated for 40 min at room temperature (RT). After rinsing, Fluorescence intensity of the cells was detected by Microplate spectrophotometer.

3. Results and Discussion

3.1 Preparation and Characterization of Nanocomposites

The random copolymer, pMS, was synthesized by RAFT polymerization using potassium 3-sulfonyl acrylate (SPA) and MAG as monomers. The achieved pMS was served as the macromolecular chain transfer agent for the synthesis of the block copolymer, pNMS. The exact structure of each copolymer can be further proved by the 1H NMR spectrum of the purified copolymers (Fig. S2-4), from which the characteristic peaks of MAG, sulfonated unit and NIPAAm are clearly visible. Their structural characteristics were detected by FTIR spectroscopy (Fig. 1). Since they had similar structures from the monomers of MAG and SPA, some characteristic peaks of these functional groups correspondingly present in pMS and pNMS were in the same wavenumbers. Both copolymers displayed obviously a peak at around 3300 cm⁻¹ that attributes to the N−H/O−H bonds of MAG, and two peaks at 1665 cm⁻¹ and 1530 cm⁻¹, which could be assigned to the amide I band (mainly due to the C=O stretching, vibration) and the amide II band (a combination of the N−H bending vibration and C−N stretching vibration) of MAG, respectively. Both copolymers displayed obviously a peak at around 3300 cm⁻¹ that attributes to the N−H/O−H bonds of MAG, and two peaks at 1665 cm⁻¹ and 1530 cm⁻¹, which could be assigned to the amide I band (mainly due to the C=O stretching, vibration) and the amide II band (a combination of the N−H bending vibration and C−N stretching vibration) of MAG, respectively. The absorption peaks around 1200 cm⁻¹ and 1050 cm⁻¹ in the two copolymers corresponded to the SO stretches from SPA. While in comparison to pMS, pNMS displayed three specific peaks at 1465 cm⁻¹, 1385 cm⁻¹ with 1% BSA (in PBS) containing the primary antibody against FGFR1 at 4 °C overnight. The primary antibody was removed and the cells were rinsed 3 times with PBS. The secondary antibody, Goat anti-Rabbit IgG H&L, diluted in 1% BSA, was added to the cells and incubated for 40 min at room temperature (RT). After rinsing, Fluorescence intensity of the cells was detected by Microplate spectrophotometer.

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and 1368 cm\(^{-1}\), respectively, attributing to the (CH\(_3\))\(_2\)CH-bending vibration. The FT-IR results indicated that both pMS and pNMS had MAG and SPA in their structures, and NIPAM was only in the block copolymer pNMS.

The terminal groups of the polymer chain underwent sulfhydrylization by ethanolamine. According to the ultraviolet-visible light absorption spectra before and after the sulfhydrylization of the polymer, the disappearance of the characteristic absorption peak at 304 nm for the dithioester in the copolymers could be observed after the sulfhydrylization (Fig. 2A). At low temperatures, polymer coils are extended and steric hindrance is relatively small. The achieved thiol end groups on the polymers offered a convenient way to graft the polymers on AuNPs because Au-S is easy to form and very stable (Scheme 2).

Since the conjugation of polymers would significantly increase the particle size of AuNPs, the hydrated diameters of these nanoparticles were investigated by dynamic light scattering (DLS). The results showed that the citrate-protected AuNPs had an average diameter of about 16 nm, which was identical with the other reports. Compared with the unmodified AuNPs, the modification of polymers on AuNPs could obviously increase the hydrated diameters. As shown in Fig. 2B, there was an increase in the hydrated diameter of around 6 nm in AuNPs-pMS, attributing to the polymer grafted on the surface of AuNPs. While the difference was more significant between AuNPs and AuNPs-pNMS, which could be due to the longer polymer chain in pNMS than pMS. Considering that the specific SERS peak at 520 nm from AuNPs would always change after the conjugation of polymers, it was investigated by ultraviolet-visible spectroscopy. The spectra showed that AuNPs’ absorption peaks at 520 nm and several red-shifted absorption peaks at 524 nm, 522 nm and 524 nm, respectively for AuNPs-pMS, AuNPs-pN and AuNPs-pNMS, corresponding to the polymer modifications (Fig. 2C). Meanwhile, the modification of polymers could also affect the surface potentials of the nanocomposites because of the negative charges on the sulfated groups in the side chains of the polymers (Fig. 2D). These results proved that the polymers

![Fig. 2 Characterization of polymers and nanocomposites. (A) UV-Vis absorption spectra of polymer before and after sulfhydrylization. (B) The hydrated diameters of nanoparticles. (C) Visible light absorption spectra of AuNPs and nanocomposites. (D) The Zeta potential of AuNPs and nanocomposites. Data are presented as the mean ± SD (n = 3), *, p < 0.05; **, p < 0.01; ***, p < 0.001 by t-test (AuNPs was the control group for analysis of significant differences).](image)

![Scheme 2 Schematic diagram of assembly of SH-end group polymer and AuNPs. (A) assembly of pMS and AuNPs, (B) assembly of pNMS and AuNPs, (C) assembly of pN and AuNPs.](image)
could be well grafted to AuNPs through Au-S bonds, forming different nanocomposites.

3.2 Temperature Responsiveness of Nanocomposites

There were two separated functional fragments in the copolymer pNMS. The fragment of pMS was a heparin mimic, responsible for the binding of FGF and FGFR and promoting neural differentiation.42 While the fragment of pN was a polymer of NIPAAm, which was always applied as the temperature-sensitive polymer.43 The hydrated diameters of the AuNPs-pNMS was carefully investigated respectively at 25 °C and 37 °C by DLS. As expected, the hydrated diameter of AuNPs-pNMS was almost 56 nm at 25 °C, while it decreased to as small as 40 nm at 37 °C (Fig. 3A). However, the hydrated diameter of AuNPs-pMS at 25 °C and 37 °C had no significant difference. This specific regulation of the hydrated diameter of AuNPs-pNMS at different temperatures could be attributed to the fragment of pN in the polymer chain. Although pN was close to the surface of AuNPs and covered by pMS fragment, its excellent thermosensitivity could still lead to the hydrated diameter change of the nanocomposites. Meanwhile, the difference of hydrated diameters of the nanocomposites at 25 °C and 37 °C had no obvious effect on their surface potentials (Fig. 3B). These results indicated that AuNPs-pNMS had a bigger hydrated diameter at 25 °C, but a smaller one at 37 °C, and the alteration of temperature didn’t change neither the dispersion nor the surface potential of the nanocomposites. Considering that the hydrated diameter change would result in a change of the distances between the polymer chains located on AuNPs, the treatment of nanocomposites on the ESCs was mainly performed at 25 °C for 2 h and changed to 37 °C for cell culture and differentiation.

3.3 The effect of nanocomposites on Cell viability

The nanocomposites were produced from AuNPs, pN, pMS and pNMS. It has been proved that AuNPs are the biocompatible nanomaterials widely applied in clinical detection, imaging and therapy.44, 45 And both pN and pMS showed little toxic effect on cell growth, which offers them the advantages in the studies of drug delivery, protein adsorption and cell differentiation.28, 46 In this study, the effects of the nanocomposites were investigated mainly on the growth and neural differentiation of ESCs. Compared with normal somatic cells, ESCs have the potential abilities in the unlimited proliferation and pluripotent differentiation. However, they are more sensitive to the environment. Any improper condition can affect the growth of ESCs. Our results showed that all the nanocomposites of AuNPs-pMS, AuNPs-pN and AuNPs-pNMS had the expected hydrate
performance on the growth of ESCs (Fig. 4A). During the treatments within 5 days, there was just a small decrease, no more than 5%, observed on the nanocomposites treated cells. And from the statistic analysis, such differences were not significant (p > 0.05), which suggests that no obvious cytotoxic effect happens to the growth of ESCs after the treatment of nanocomposites. Similar phenomena also occurred on the treated L929 cells (Fig. 4B), which is the fibroblast, using as a cell model in evaluating the cytotoxic effect of the biomaterials. These results indicated that AuNPs-pNMS had little cytotoxic effect on cell proliferation due to their desirable biocompatibility, and could be then applied for neural differentiation.

3.4 The Effect of Nanocomposites on the Pluripotency of ESCs

ESCs are the undifferentiated cells that have self-renewal and pluripotent abilities. They were used to differentiate into all types of functional cells for clinical studies. During the differentiation, there are many genes changing their expression level. Among them, Oct-4, which is recognized as the specific markers for the pluripotency of ESCs, always shows a decreased expression along with cell differentiation. The function of Oct-4 is to maintain the pluripotency of ESCs through its regulation in promoting the gene expression for pluripotency and preventing the gene expression for cell differentiation. Accordingly, a lower expression level of Oct-4 exists in the differentiated cells. Since the level of gene expression can be precisely detected by real-time PCR, the pluripotent state of ESCs was determined from Oct-4 expression in the cells after the treatment of different nanocomposites. As shown in Fig. 5A, it was obvious that the levels of Oct-4 expression in the cells treated with AuNPs-pMS, and AuNPs-pNMS had a great decrease at around 65% compared with the negative control. While AuNPs-treated ESCs maintained almost 65% expression of Oct-4, which indicated that the decrease of Oct-4 expression by the treatment of nanocomposites might not come from AuNPs, but from the grafted copolymers. The suppression of Oct-4 expression by AuNPs-pMS had already been observed, which was due to the similarity of pMS with natural heparin. Meanwhile, the suppression of Oct-4 expression by AuNPs-pN also had been observed. Considering that pMS and pN were served as one of the fragment in the block copolymer pNMS, the decrease of Oct-4 expression level induced by AuNPs-pNMS was understandable. As a result of the suppression of Oct-4 expression, some cell differentiation events might have been initiated on ESCs.

Generally, there are three major directions initiating the differentiation of ESCs, endoderm, mesoderm and ectoderm. The expression level of the genes, Sox17, Flk1 and Nestin (representing as the markers respectively for endoderm, mesoderm and ectoderm) were then detected on ESCs treated with different nanocomposites. It was shown that the

Fig. 5 Influence of heparin and nanocomposites on the relative expression levels of pluripotency marker (Oct-4), endoderm marker (Sox17), mesoderm marker (Flk1) and ectoderm marker (Nestin) in ESCs. ESCs were treated for 14 days. Data are presented as the mean ± SD (n = 3), * p < 0.05; ** p < 0.01; *** p < 0.001 by t-test (Blank was the negative control group for analysis of significant differences).
expressions of Sox17 and Flk1 were inhibited at a great extent after the treatment of AuNPs-pMS, AuNPs-pN and AuNPs-pNMS for 14 days (Fig. 5B & 5C). AuNPs-pMS and AuNPs-pN could lead to the expression of Sox17 in a level of about 40% of the negative control, while AuNPs-pNMS treatment made another 10% decrease. AuNPs-pMS, AuNPs-pN and AuNPs-pNMS could lead to the expression of Flk1 in a level of about 30% of the negative control, especially AuNPs-pNMS, which had the better inhibition of Flk1 expression. The results suggested that neither endoderm nor mesoderm was the direction of the differentiation for ESCs under the treatment of nanocomposites. However, the expression of ectoderm marker, Nestin, had a totally different appearance. The transcribed Nestin in AuNPs-pMS and AuNPs-pN treated cells had reached to 460% and 480% of the negative control respectively (Fig. 5D). And for AuNPs-pNMS treated cells, the level of Nestin even increased to more than 680% of the negative control. The results clearly demonstrated that the nanocomposites could promote the differentiation of ESCs, and the direction for the differentiation was close to ectoderm.

3.5 The Effect of Nanocomposites on neural Differentiation of ESCs

In previous report, the heparin mimic showed an expected promotion effect on neural differentiation of ESCs, which was developed from the ectoderm. Considering the heparin mimic structures in the copolymers of pMS and pNMS, they would have similar effect on neural differentiation. As shown in Fig. 6, the RT-PCR results showed that nanocomposites could increase the expression of β3-tubulin gene. The transcribed β3-tubulin in AuNPs-pMS and AuNPs-pN treated cells had reached to 35 times and 32 times of the negative control respectively. Holding the two fragments of pN and pMS together, AuNPs-pNMS exhibited a great promotion effect on β3-tubulin expression, which was 91 times of the negative control and 14 times of heparin group.

Correspondingly, as shown in Fig. 7D, there were some cellular structures like axons and dendrites formed in AuNPs-pMS treated cells. Since these structures positively bound with anti-β3-tubulin antibodies and shown in green fluorescence, it suggested that the mature nerve cells could be effectively produced under the treatment. Similar structures were also observed in AuNPs-pNMS treated ESCs, but surprisingly in a high density and a broad area, which meant a much stronger effect of AuNPs-pNMS on promoting neural differentiation (Fig. 7F). The difference between AuNPs-pMS and AuNPs-pNMS in their structures was just a fragment polymer pN served as a linker in AuNPs-pNMS. Therefore, this phenomenon might be possibly caused by the elongation of the polymer chain to reach further FGFR on cell membrane, or the thermosensitivity of pN to regulate the hydrophilicity and length of the polymer chain, or the synergistic effect of the two segments, pN and pMS in AuNPs-pNMS, greatly increases the efficiency of nanocomposite binding to FGFR. The results showed that the temperature-sensitive nanocomposite AuNPs-pNMS had a stronger effect on the differentiation of ESCs and can better promote the expression of β3-tubulin and stimulate the synthesis of proteins required for neuronal maturation.

3.6 The mechanism of Nanocomposites on neural Differentiation

It was found that the optimal molecular weight of the heparin mimic glycopolymers in promoting neural differentiation was around 9×10^3 Da, longer polymer chains could not increase this effect much. Apparently, the elongation of the polymer chain by pN itself would not lead to the higher promotion effect.
of AuNPs-pNMS in neural differentiation, which was also proved in our study by using AuNPs-pMS with different pMS molecular weights.

By detecting the fluorescence intensity of FGFR, the efficiency of the combination of nanocomposite and FGFR could be judged. As shown in Fig. 8, the fluorescence intensity of cells treated by AuNPs-pMS, AuNPs-pN and AuNPs-pNMS had a great increase compared with heparin control. Among them, AuNPs-pNMS could promote the fluorescence intensity of the cells as high as 13.7 times of heparin, which was twice of AuNPs-pMS. While AuNPs maintained just 2.1 times of heparin. The result indicated that the increase of the fluorescence intensity mainly not come from AuNPs, but from pNMS grafted onto AuNPs. And it is mainly due to the pN fragment in the structure of AuNPs-pNMS. The effects of these nanocomposites show that the synergistic effect of pN and pMS in AuNPs-pNMS greatly increases the efficiency of nanocomposite binding to FGFR, further enhancing the effect of neural differentiation of ESCs.

4. Conclusion

In summary, the thermosensitive nanocomposites were prepared in this study to promote neural differentiation of ESCs. The copolymer pMS, served as the heparin mimic, is responsible for the specific binding of FGF and FGFR to assemble pMS-FGF-FGFR mimicking complex. The fragment of pN, linking AuNPs and pMS, holds a unique thermosensitive property to extend of pN in their structures. AuNPs-pN1MS, holding the shortest pN, showed a similar promotion effect on neural differentiation with AuNPs-pMS, which had no pN. And to pNMS in the MW range of 13700-28100 Dalton, the higher MW of pN resulted in a stronger promotion effect. The percentage of green fluorescence in AuNPs-pN3MS treated cells was as high as 75% (Fig. 11B), and there were a large number of mature neuronal cells with identical axon and dendritic characteristics (Fig. 10C). However, for the largest one, AuNPs-pN4MS, the promotion effect decreased compared with AuNPs-pN3MS (Fig. 10 & 11). These results indicated that the length of pN in AuNPs-pNMS might play a key role to increase its biological effect. Owing to the thermosensitivity of pN, it could be assumed that AuNPs-pNMS could bind specifically with FGFR monomers on cell membrane at 25 °C, and under the increase temperature, the collapsed polymer chain would pull FGFR monomers together to form the active FGFR dimers, which could initiate the signalling for neural differentiation (Scheme 1). To ensure this promotion effect, a proper length of pN is necessary. If it is too short, there is no enough space for the block copolymers to reach more separated FGFR monomers. While if it is too long, the shrinkage of the polymer still leave excess space and it is difficult to dimerize FGFR. As shown in our study, AuNPs-pN3MS might offer an optimal regulation of the polymer length leading to FGFR dimerization, and then resulting a desirable promotion effect on neural differentiation of ESCs.
the polymer chain at low temperature and collapse at high temperature, which provides a proper space for the dimerization of FGFR. The synergistic effect of pN and pMS in AuNPs-pNMS greatly increases the efficiency of nanocomposite binding to FGFR. While AuNPs can not only carry the functional polymers, but also increase the local amount of heparin mimics on cell membrane. Combined all the above advantages, an excellent promotion effect on neural differentiation is achieved. This study presents a new strategy in raising the dimerization of FGFR on cell membrane and has a great potential for cell differentiation.

![Figure 10](image1.png)

**Fig. 10** Immunofluorescence images of ESCs treated with nanocomposites for 14 day. (A) AuNPs-pN1MS, (B) AuNPs-pN2MS, (C) AuNPs-pN3MS, (D) AuNPs-pN4MS. Scale bar: 100 μm. β3-Tubulin was detected with anti-β3-tubulin antibody (rabbit anti-mouse) and FITC-labeled goat anti-rabbit IgG (green), and cell nuclei were stained with DAPI (blue).

![Figure 11](image2.png)

**Fig. 11** Neural differentiation of ESCs treated with the nanocomposites and heparin for 14 days. (A) Relative expression level of mature neuronal gene marker β3-tubulin. Data are presented as the mean ± SD (n = 3), **, p < 0.01; ***, p < 0.001 by t-test, (AuNPs-pMS was the control group for analysis of significant differences). (B) Proportion of relative fluorescence intensity (RFI) of green and blue in Fig. 7D and Fig. 10. The optical density of images in green and blue tunnels was measured with ImageJ software.
Conflicts of interest

There are no conflicts to declare.

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