Sustained release of hydrogen sulfide from anisotropic ferrofluid hydrogel for the repair of spinal cord injury

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ABSTRACT

Spinal cord injury (SCI) results in massive neuronal death, axonal disruption, and cascading inflammatory response, which causes further damage to impaired neurons. The survived neurons with damaged function fail to form effective neuronal circuits. It is mainly caused by the neuroinflammatory microenvironment at injury sites and regenerated axons without guidance. To address this challenge, a ferrofluid hydrogel (FFH) was prepared in a magnetic field to acquire anisotropy. Moreover, Fe3S4 can release hydrogen sulfide (H2S) with anti-inflammatory effects under acidic conditions. Regarding in vitro experiments, 0.01g/ml Fe3S4 FFH significantly reduced the inflammatory factors produced by LPS-induced BV2 cells. Oriented and longer axons of the induced neural stem cells loaded on anisotropic FFH were observed. In vivo experiments showed that FFH reduced the activated microglia/macrophage and the expression of pro-inflammatory factors in SCI rats through the NF-κB pathway. Moreover, it significantly promoted directional axonal regrowth and functional recovery after SCI. Given the critical role of inhibition of neuroinflammation and directional axonal growth, anisotropic Fe3S4 FFH is a promising alternative for the treatment of SCI.

1. Introduction

Spinal cord injury (SCI) is an extremely devastating traumatic injury with massive neuronal death and axonal disruption [1,2]. A key factor limiting the effectiveness of SCI treatment is regenerated axons without guidance [3,4]. Recently, several anisotropic hydrogels with aligned particles formed a directional microtube, in which the axonal growth can be guided. This contributes to the proliferation and differentiation of neural stem cells (NSCs) and also increases functional connections [5]. There are many approaches to achieving directional alignment of particles, such as electrospinning, 3D printing, and electricity [6-9]. However, their application is limited due to disadvantages, such as poor mechanical properties, low biocompatibility, complex fabrication process, and additional ionization. Recent advances in magnetism made magnetic anisotropy with benefits including a homogeneous, contact-free approach and ease of operation [10–12].

After the primary injury, subsequent inflammation further creates a harsh environment for survived neurons and impedes axonal regeneration in the injured site [13,14]. Current strategies, such as drugs, surgery, and hyperbaric oxygen, were used to provide a favorable condition for axonal regeneration in SCI repair [15,16]. But the effort is not satisfying. Therefore, better-designed protective strategies are highly urgent matters. Readily deliverable biological gases can preserve nerve cells by regulating oxidative, inflammatory, and apoptosis processes. Multimodal homeostatic and highly accessible biological gases may represent a promising pharmaceutical approach to treating SCI [17]. But...
the continual stalling of strategies due to limitations of administration methods, pungent smell, and instability keep therapeutics from clinical application. A slow-release and direct-acting gas at the injury site may be a natural candidate. Ferric tetrasulfide (Fe₄S₄), commonly used in ferrofluids, can release hydrogen sulfide (H₂S) in weak acidic conditions. As paramagnetic, ferrofluid is formed by the homogeneous dispersion of magnetic substances in a liquid. Its particles can be arranged in a magnetic field and maintained stably over time [18]. Anisotropic hydrogel with linear channels can promote linear axon extension [19]. H₂S can directly scavenge reactive oxygen species (ROS) and upregulate c-glutamylcysteine synthetase, increasing the transport of cysteine, which is the major source of glutathione. H₂S also reduces the inflammatory response and promotes the proliferation and differentiation of NSCs [20]. Despite its high solubility and rapid clearance, H₂S has acted as a neuroprotective agent in multiple neurological disorders.

Although many biomaterials that guide axonal regeneration or reduce inflammation have been studied for SCI, few reports have combined anisotropy and anti-inflammatory effects. Based on the previous findings, our study aimed to address SCI repair limitations by developing a dual-function hydrogel to orderly guide axonal regeneration and inhibit harsh inflammation. Fe₄S₄ ferrofluid hydrogel (FFH) was prepared with Fe₄S₄, carboxymethyl chitosan, and gold. During synthesis, the particles inside the FFH can be oriented and stabilized in a magnetic field. Axons can be guided under this physical signal. The release of H₂S was slow and sustained, providing a beneficial effect to the inflammatory microenvironment. Then, its effect on the differentiation of induced neural stem cells (iNSC) and the treatment of SCI was explored.

2. Methods

2.1. Synthesis of Fe₄S₄ ferrofluid hydrogel

0.2 g Fe₄S₄ particles were dispersed uniformly in 2 ml deionized water. 10 ml of deionized water and 0.15 g of highly-purified carboxymethyl chitosan (Damao, China) were added to the magnetic stirrer, followed by high-speed stirring with the chloroaucic acid solution (Aladdin, China). Then the mixture was transferred to a water bath for 3 h and then cooled to room temperature. The Fe₄S₄ dispersion was added to the above mixture and maintained with high-speed mechanical stirring for 2 h. Subsequently, it was transferred to an ultrasonic dispersion apparatus with high frequency shaking for 30 min to obtain Fe₄S₄ ferrofluid. 0.06 g of aldehyde-based chitosan, 0.45 g of carboxymethyl chitosan, and 2 mg of carbon-dotted copper were mixed in 10 ml of deionized water and mechanically stirred until a viscous gel was obtained. Subsequently, 1 g of Fe₄S₄ ferrofluid was added and stirred continuously until a hydrogel was obtained. Meanwhile, an appropriate amount of Lactide (Meilunbio, China) was added and stirred until homogenous. The hydrogel was placed in a magnetic field, in which the particles were rearranged during the synthesis to form aligned structures.

2.2. Characterisation of hydrogel swelling properties

The equilibrium swelling rate (Ws) of the hydrogels in 37 °C PBS solution for different times was determined. The weight of Fe₄S₄ ferrofluid hydrogel before swelling was recorded as W₀. The weights of the hydrogel at each specific time after soaking in PBS solution were recorded as Wt. The swelling ratio was recorded as Ws=(Wt-W₀)/ W₀.*100%

The remaining percentage of the hydrogel in PBS at different times was used to evaluate the degradability of hydrogel. The weight of the dried Fe₄S₄ FFH was recorded as W₀. The Fe₄S₄ FFH was immersed in 10 ml of PBS solution, which was changed daily. After drying at different times, the weight was recorded as Wx. The remaining rate was recorded as Wx/W₀*100%

2.3. Characterisation of hydrogel anisotropy

The hydrogel was laid flat on a slide with a thickness of 1 mm and the fine structure was observed under a light microscope. Then the optical scattering pattern was observed by a red laser (wavelength 650 nm) that directed towards hydrogel at a vertical angel.

2.4. Concentration of H₂S released by Fe₄S₄ FFH

The concentrations of H₂S released in vitro were determined by the methylene blue standard curve. Firstly, Na₂S standard solutions (concentrations of 5, 10, 20, 40, 60, 80, and 100 μM) were prepared with sodium sulfide (Na₂S) and distilled water. 1 ml of each concentration of Na₂S standard solution was taken, and repeated three times. The reaction solution was fully reacted with methylene blue reagent for 30 min at room temperature. The absorption spectrum was detected by a UV spectrophotometer. Then a standard curve was plotted as a control. Then 1 g of Fe₄S₄ FFH and 10 ml of deionized water were mixed, 1 ml of each sample was taken at different times, and the reaction with methylene blue reagent was also carried out at room temperature for 30 min. The maximum absorbance at 670 nm was detected. The concentration of H₂S was calculated according to the previous standard curve.

2.5. Culture and induction of iNSC

In this experiment, human iPSC-derived iNSC were used to explore the effect of Fe₄S₄ FFH. iPSC was obtained from the Key Laboratory of Stem Cell and Tissue Engineering, Ministry of Education. A reported induction protocol for the spinal cord derived iNSC was used [21–23]. The iPSCs were cultured with mTeSR medium (Stemcell, Canada) to proliferate in the form of colonies. ReLeSR (Stemcell, Canada) was used every 5 days at a 1:3 ratio for passaging. When 70% of cells were contacted, a neural induction medium (NIM) was added. Accutase (Gibco, USA) was used to passage every 3 days at a ratio of 1:3. After 10 days, when the cells were induced into iNSC, NIM was replaced by a neural maintenance medium (NMM). iNSC differentiation was induced in vitro with a neural differentiation medium (NDM). Neurospheres were identified by immunofluorescence. iPSCs were labeled with Nanog and Oct4, iNSC with Nestin and Pax6, neurons with TUJ1, astrocytes with GFAP, and oligodendrocytes with MBP.

2.6. Live/dead staining and CCK-8

iNSC in neurosphere form was cultured on the Fe₄S₄ FFH. On the 4th day, Calcein-AM/PI (Dojindo, Japan) was added to iNSC for 15 min. A confocal reflection microscope (Leica, Germany) was used to capture images. The CCK-8 (Dojindo, Japan) solution was added to culture plates at a ratio of 1:10 to detect cell proliferation on day 1, 4, 7. After incubation for 2 h, 100 μl of the supernatant mixed solution was transferred into 96-well plates and measured with an enzyme-labelling instrument (SpectraMax M5, USA) at the 450 nm wavelength.

2.7. BV2 culture and stimulation

BV2 cells were cultured with DMEM high glucose(Gibco, USA) supplemented with 10% fetal bovine serum (FBS)(Gibco, USA) and 1% penicillin-streptomycin(PS)(Gibco, USA)at 37 °C in 5% CO₂. For inflammatory stimulation, BV2 cells were reseeded on hydrogels with different Fe₄S₄ ferrofluid concentrations in the serum-free medium for 12 h to avoid excessive activation. 100ng/ml LPS (Gibco, USA) was added to the medium for 24 h and BV2 cells were collected for RT-PCR analysis.
2.8. Directional axon extension analysis

PC12 cells were cultured with DMEM low glucose (Gibco, USA) supplemented with 10% FBS and 1% PS at 37 °C in 5% CO₂. PC12 cells were reseeded on CCH, FFH(−), and FFH(+) hydrogels for 12h and switched to the differentiation medium (DMEM low glucose supplemented with 1% FBS and 1% PS) for 7 days for axonal outgrowth. iNSC were also reseeded on CCH, FFH(−), and FFH(+) hydrogels for 12h and switched to NDM for 7 days. Immunofluorescence staining was performed at the end of the experiment. The length and angle of axons were calculated using Image J.

3. Ethics statement

All animal procedures were approved by the Animal Ethics Committee of the South China Agricultural University (2021d066) and performed according to the protocols approved by the Centre of Laboratory Animals.

3.1. Complete transection of spinal cord and grouping

Adult female SD rats (6–8 weeks old, 200–220g) were supplied by the Guangdong Medical Laboratory Animal Center. All rats were divided into four groups: SCI group without treatment after injury, CCH group implanted carboxymethyl chitosan after injury, Fe₃S₄ FFH without anisotropy after injury, FFH(+) group implanted Fe₃S₄ FFH with anisotropy after injury. After anesthesia, a laminectomy at the T10 level was carried out to expose the spinal cord. The dura was incised longitudinally, then 3 mm of the spinal cord was removed, and different hydrogels were placed into the defective area. The dura was kept and sutured, and the dorsal incision was closed (Fig. S1). Manual emicition was given twice daily after the operation until their automatic micturition function recovered. Gentamicin(4000U/Kg/day) was given for 3 days after surgery to prevent additional infections.

3.2. Behavioral tests

Basso–Beattie–Bresnahan (BBB) test was performed to assess the hindlimb function of rats weekly after SCI. Rats moved freely in an open field for 3min to assess the joint move range of the hindlimbs, the capacity of weight-bearing, and the resting state of the feet. Inclined grid climbing test was also performed to assess the accuracy of foot placement and coordination.

3.3. Electrophysiological analysis

The evoked potentials of rats under anesthesia were recorded by the BL-420s biological signal acquisition system (TECHMAN, China). The lamina rostral to the injury was removed as previously described. The stimulating electrode was placed on the spinal cord rostral to the injury. The recording electrode was connected to the left sciatic nerve. The ground electrode was clamped on the tail. Both the stimulating and receiving electrode was connected to the BL-420S. The waveform was recorded to analyze the amplitude and latency. Parameters setting: single square wave stimulation, 10mv, 50Hz.

3.4. Immunofluorescence

Rats were transcardially perfused sequentially with 0.9% saline and 4% paraformaldehyde after being deeply anesthetized. Spinal cord sections were washed with 1 PBS solution, then incubated with 1% normal goat serum (Beyotime, China) and 0.3% Triton X-100 (Bio-Froxx, Germany) in PBS for 1 h at room temperature. The primary antibodies (details in Supplementary Table 1) were diluted in antibody-dilution buffer (New Cell &Molecular, China), then added and incubated overnight at 4 °C. The next day, Alexa Fluor-coupled secondary antibodies were diluted and incubated for 1 h. DAPI (Servicebio, China) was then incubated for 15 min.

3.5. RT-PCR

mRNA from cells and spinal cord tissue was extracted using an RNA extraction kit (Sigma, Germany) and then converted into cDNA by a reverse transcription kit (Takara, Japan). Quantitative PCR was performed using the SYBR Green PCR Master Mix and QuantStudio 5 detection system (Thermofisher, USA). Actin-β was used as the housekeeping gene for normalization. Each sample was measured three times for each gene. The primers used were shown in Supplementary Table 2.

3.6. Western-Blot

Spinal cords of the injury site including one segment caudal and rostral were collected in RIPA lysis buffer and phosphatase inhibitor buffer (Beyotime, China). After centrifuging at 12,000g for 35min, the supernatant was collected. The protein concentration was quantified using a BCA protein analysis kit (Thermo Fisher, USA), SDS-PAGE protein loading buffer (5X) was added to the samples and heated for 5min at 100 °C. Equal amounts of protein(20 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel in electrophoresis and then transferred to polyvinylidene fluoride (PVDF) membranes. After being blocked in 5% BSA at room temperature for 1 h, the PVDF membranes were incubated with the primary antibodies overnight at 4 °C. After washing in 1X Tris-buffered saline (TBS) with 1% Tween 20 the next day, the PVDF membranes were incubated with the secondary antibodies for 1 h at room temperature. An enhanced chemiluminescence kit (Thermo Fisher, USA) was used to detect the bands.

3.7. Hemolysis analysis

Blood samples were collected and co-incubated with FFH for 4 h, followed by centrifugation at 12000g for 5 min at 4 °C. PBS and Triton-100X were used as the blank and positive group, respectively. The absorbance was measured, and the hemolysis percentage was calculated using the following Equation:

\[
\text{Hemolysis}(%)= \frac{\text{Sample absorbance} – \text{Negative control}}{\text{Positive absorbance} – \text{Negative control}} \times 100%
\]

3.8. Statistics

To statistically analyze the collected data, SPSS 22.0 statistical analysis software and GraphPad Prism software were applied. And multiple groups were compared using analysis of one-way ANOVA and Student’s t-test. P < 0.05 was considered a statistically significant difference.

4. Results

4.1. Fe₃S₄ ferrofluid hydrogel with paramagnetic properties and proper degradability

Fe₃S₄ ferrofluid hydrogel (FFH) was synthesized according to Fig. 1. When a magnet was held close to the hydrogel, the surface of which was attracted to bulge upward (Fig. 2A, B), indicating that the hydrogel was paramagnetic. The orientation of Fe₃S₄ FFH particles was unified in the magnetic field to achieve anisotropy. The structures of the intermediate and final products were observed under a scanning electron microscope (SEM). Fe₃S₄ particles were agglomerated and of different sizes, with uneven distribution and irregular shapes (Fig. 2C). As the intermediate product, particles in Fe₃S₄ ferrofluid (FF) were connected to rod-like polymer crystals, leading to a tightly connected rivet-like structure on the surface (yellow box) (Fig. 2D). Fe₃S₄ FFH had a porous structure (red
FFH (Fig. 2E), through which water and nutrients were transported to cells. After swelling in phosphate-buffered saline (PBS) solution and achieving equilibrium after 100 h, both Fe₃S₄ FFH and Carboxymethyl chitosan-based hydrogel (CCH) increased in volume (Fig. S2A). Fe₃S₄ FFH and CCH could absorb water more than 10 and 18 times their weight, respectively (Fig. 2F). The hydrogels should be biodegradable after serving as an extracellular matrix without hindering tissue regeneration. The weight remaining of Fe₃S₄ FFH and CCH could absorb water more than 10 and 18 times their weight, respectively (Fig. 2F).

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**4.2. Slow release of H2S and anisotropy in FFH**

According to Methods 4, the concentrations of H₂S released from Fe₃S₄ FF and Fe₃S₄ FFH at different times were calculated. In Fe₃S₄ FF, H₂S release increased rapidly and maintained a concentration of more than 30 μM after 50 h (unstable). However, H₂S released from Fe₃S₄ FFH was stable at 10 μM after equilibrium (Fig. 2H). Using different concentrations of the direct donor Na₂S, CCK-8 experiments showed that 10μM-1mM H₂S promoted the cell proliferation of iNSC (Fig. S3). Fe₃S₄ FFHs with or without anisotropy were classified into the FFH (+) and FFH (−) groups, respectively. When stretching on the universal testing machine (Fig. S2H), anisotropy made the maximum strain of Fe₃S₄ FFH decrease slightly, but the maximum stress increased to more than 30 KPa (Fig. 2I). This means that the mechanical property of the hydrogel was enhanced in the anisotropic direction. Subsequently, a microscope was used to examine the microstructures of hydrogels. The particles in the FFH (−) were arranged irregularly, whereas those in the FFH (+) were almost in the same direction (Fig. 2J and K). In addition, the anisotropy had unique scattering patterns in light experiments. When the hydrogel of FFH (−) was irradiated with a laser, the pattern formed through the hydrogel was circular. It was due to the randomly scattered light in all directions. In contrast, when FFH (+) was irradiated, the scattering was enhanced along the orientation of the particles, resulting in a clear shuttle-shaped pattern (white dotted lines in Fig. 2L, M). The patterns in the white box were formed on the glass slide by laser irradiation.

**4.3. The biocompatibility and anti-inflammatory effect of Fe₃S₄ FFH**

The viability of iNSC on 1 g/ml FFHs was less than 80% of the control (Fig. 3A). Therefore, it was excluded from further study. To select the optimal concentration of Fe₃S₄ FF, we further explored the function of anti-inflammation. The results showed that the mRNA of IL-1β, IL-6, iNOS, and TNF-α were significantly elevated after LPS stimulation while decreased in hydrogels with different Fe₃S₄ FF concentrations. The best outcome was observed in the 0.01 g/ml group, indicating the best anti-inflammatory effect (Fig. 3B-E).

Hydrogel with 0.01 g/ml Fe₃S₄ FF was served as the FFH group, and a blank plate as control. After iNSC plantation, live/dead staining was performed on day 4. Live cells were stained green, whereas dead cells were stained red. The results showed that few cells were stained red in both groups (Fig. 3F). The results of CCK-8 suggested that the survival rate of iNSC in the FFH group was more than 80% of the control group on days 1, 4 and 7 (Fig. 3G), indicating that hydrogel with the concentration of 0.01 g/ml FFH had good biocompatibility. Hemocompatibility was an important indicator for evaluating the hemolytic activity of implanted materials in vivo. As shown in Fig. 4, serum extracted from 0.01 g/ml FFH co-incubated whole blood showed slight yellow, close to that of the PBS control group. But the Triton-X100 group was bright red in color. The hemolysis ratio of FFH was 0.3143%. Therefore, the hydrogel with 0.01 g/ml Fe₃S₄ FF was selected for the subsequent experiments.

**4.4. iNSC differentiation and directional axonal extension on FFH**

The immunofluorescence staining showed that most iNSC was differentiated into neurons with axons (Fig. 4A). The length of axons in the CCH group was 26.8 ± 5.34 μm (Fig. 4B), shorter than that of the FFH (−) group (52.14 ± 23.51 μm) and FFH (+) group (47.34 ± 19.35 μm) (P < 0.05). The
growth angle of axons in the FFH (−) group ranged in (±57.27°), which was close to that of the CCH group (±55.40°) (Fig. 4C). Most neuronal axons in the FFH (+) group exhibited almost the same orientation and more concentrated growth angle (±13.99°). Almost the same results were found in PC12 cells (Fig. S6).

4.5. Mobility assessment of SCI rats

The hindlimbs of the rats in 4 groups were completely paralyzed after injury. At week 3, rats in the SCI group failed to move the joints of their hindlimbs, with their legs straight and claws dragging on the ground. In other groups, the claws were able to turn over occasionally by large joints. From week 4, the hindlimb locomotor capacity of rats in the FFH (+) group was close to that of the CCH group (0.23 ± 0.01 mV), which was significantly higher than that of the FFH (−) group (P < 0.01) (Fig. 5A). To evaluate the nerve conduction of injured spinal cord, the amplitude, and latency of evoked potentials were recorded 8 weeks post-injury. The waveforms after single electrical stimulation changes to viscera (Fig. S7).

Inflammation after SCI was mainly mediated by macrophage/microglia. As a marker of activated macrophage/microglia, CD68 immunofluorescence was performed on normal and 7-days injured rats. Only little CD68 staining was found in normal rats, indicating the resting state of macrophage/microglia in the uninjured spinal cord (Fig. 8A). The fluorescence intensity in the SCI group was the strongest among the four groups, indicating extensive macrophage/microglia activation after SCI. Western blotting (WB) also showed the highest CD68 expression in the SCI group, followed by CCH. The FFH (−) and FFH (+) groups had the lowest CD68 expression (Fig. 6C). Fe3S4 FFH did not result in pathological changes to viscera (Fig. S7).

4.6. Anti-inflammatory effect of Fe3S4 FFH after SCI

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inflammation in the early stage of SCI.

The expression of P65 and IκB in the injured tissues was examined by WB to investigate the mechanism underlying the anti-inflammatory effects of Fe₃S₄FFH. There was no significant difference in the expression of P65 and IκB among the four groups. However, the levels of phosphorylated IκB and P65 were lowest in the FFH (−) and FFH (+) groups (Fig. 6G). This trend was consistent with the pro-inflammatory factors described above. Therefore, the anti-inflammatory effect of Fe₃S₄FFH was related to the inhibition of the NF-κB pathway.

4.7. Axonal regeneration in SCI rats by anisotropic Fe₃S₄FFH

Immunofluorescence staining was performed to assess the neural regeneration (Fig. 7A, S8B). GFAP was used to outline the edge of the injury area and NF200 to demonstrate the regenerated neurofilaments (Fig. 7A). The absence of GFAP at the injury sites showed that the spinal cords were completely transected. For NF200 staining, large defects and cavities were observed in the SCI group, with weak neurofilament fluorescence and fewer connections at both ends.

In the CCH group, slightly higher expression of neurofilaments was observed than that in the SCI group. In FFH (−) and FFH (+) groups, the neurofilaments increased significantly with massive neural connections at both ends. However, the NF200 staining of the injured area was stronger in the FFH (+) group, indicating a higher density of neurofilaments. In addition, the magnification image in the FFH (+) group showed a more consistent orientation of neurofilament compared with other groups.

To explore the phenotypes of newly formed neurons, we further performed the 5-HT (descending neurons of cortico-spinal tract) and CGRP staining (ascending sensory neurons) (Fig. S10). Little 5-HT (+) and CGRP (+) staining was observed in the SCI group and CCH group, which may due to the limited neural regeneration. Although lots of CGRP (+) axons were visible in both FFH (−) and FFH (+) group, indicating large numbers of nascent sensory nerves, the 5-HT (+) axons in FFH (−) group were fewer than that in FFH (+) group. It can be inferred that the anisotropy promoted the regeneration of the cortico-spinal tract. However, the detailed mechanisms involved need further study. Protein expressions of TUJ1 (neurons), NF200 (neurofilaments), GAP43 (growth cone), and MBP (myelin sheath) were also used to reflect the neural regeneration at the injury site by Western Blot. It was consistent with the immunofluorescence analysis (Fig. 7C). We also quantified the mRNA of the TUJ1, NF200, and SYNA (synapses) to assess the neural regeneration, in which FFH (+) group was the highest (Fig. 7B). We further analyzed the expression of axon guidance molecules, Semaphorin (Sema3a), Netrin (Netn1), Slit (Slit1), Ephrin B (Efnb1), to determine whether they participate in the orientated growth of axons. There was no difference in Semaphorin, a potent inhibitory cue, between FFH (−) and FFH (+) groups. But both FFH groups were slightly down-regulated compared to the CCH group. This indicated that the released H₂S might be able to inhibit Semaphorin to promote axon regrowth. Netrin, Slit, and Ephrin B showed no difference among each group (Fig. S11). This indicated that the anisotropy of FFH had no significant effect on the expression of axon guidance molecules. The orientated growth of axons was mostly due to the topography of the hydrogel.
To explore the fate of Fe₃S₄ FFH, Prussian iron staining was carried out. The results showed that a large amount of iron was stained in the damaged area at 4 weeks but mostly disappeared at 8 weeks (Fig. 7D). It indicated the degradation of the Fe₃S₄ ferrofluid hydrogel. The images of spinal cords at different times after transplantation also showed that the FFH hydrogel was gradually degraded in the first 6 weeks and disappeared at 8 weeks after transplantation (Fig. S13).

5. Discussion

5.1. The anisotropy of hydrogel

The anisotropic hydrogels can be synthesized by surface patterning, 3D printing, light, current, and magnetism [6–9]. Surface patterning only creates oriented grooves on the surface of the material. 3D printing can arrange all particles in the same orientation, but the fabrication process is complicated. Light-induced anisotropy is mostly applied to photosensitive materials. Electricity allows the internal particles within hydrogels to be oriented in the direction of the current. But additional electrolysis or dissociation may be caused. The magnetic field-induced anisotropic hydrogel is synthesized in a uniform, broad, and contact-free manner. With the recent development in superconducting magnetism, the orientation of internal particles can be done by magnet field conveniently. Once the orientation arrangement is completed during the gelatinization, the particle arrangement becomes stable [24]. Gently handling and placing the hydrogel will not disturb the orientation arrangement.

5.2. The effect of anisotropy on neuronal regeneration

Without guidance signals, axons regenerated at the lesion site are disordered after SCI, with limited capacity to form a relay [25]. This might be a reason for the limited effectiveness of current therapies. The reinnervation and functional recovery after SCI depend on the longitudinally directed regrowth of injured axons [25,26]. Various anisotropic hydrogels can elicit directed axonal regeneration through the directional arrangement of internal particles, which can increase the effective connections [27,28]. Studies have demonstrated that axonal guidance by physical signals, such as highly anisotropic polymer hydrogels, can induce highly oriented axonal growth and form neuronal networks and functional connectivity [5,29,30]. An anisotropic hydrogel containing rod-shaped microgels also achieved in-situ orientation under a magnetic field. This narrow and long microgel enabled strong axonal guidance to promote axonal growth. Another nanohydrogel with hierarchically anisotropic microstructures provided multiple physical cues to repair SCI. Its aligned microstructure promoted cell migration and orientation, which further stimulated angiogenesis and neuronal extension [31]. It also found that the direction of neuronal growth on the hybrid hydrogel was determined within the first 3 days, followed by enhanced alignment [31]. Long directional axons and interrupted conduction bundles formed a relay with the help of implants. This played an important role in restoring spinal cord conductivity. There might be an angular range that allowed for effective connections between the protrusions of the neurons, and the same orientation of the axons increased this possibility.

5-HT represents descending neurons of the cortico-spinal tract and CGRP represents ascending sensory neurons [32,33]. These two indexes can show anterograde or retrograde axonal regeneration. Better 5-HT fluorescence were observed in the FFH(+) group, indicating that the

Fig. 4. (A) The axonal growth of iNSC on CCH, FFH (−), and FFH (+). (B–C) Quantitative statistics of axon length and angle on the three hydrogels (*P < 0.05, ***P < 0.001, ns means no significance).
anisotropy might be able to facilitate cortico-spinal tract regeneration. We also explored the relationship between topology and axon guidance molecules, the endogenous factors that guide axonal growth by attracting or repelling axons, but discovered no positive results. Probably because axon guidance molecules mainly attract or repel axon migration during the development of the nervous system and will not be affected by topography [34,35].

5.3. Advantages of slowly released H$_2$S

Despite being a protective agent, H$_2$S also has some concerns that hinder its application. A high concentration of H$_2$S would cause brain damage and decrease learning and memory function [36]. Besides, H$_2$S can aggravate antiproliferative and proapoptotic effects during atherosclerosis and exhibit pro-inflammatory effects in pancreatitis, sepsis, and hemorrhagic shock [36]. Strong H$_2$S odor or acute exposure also leads to eye irritations, neurological disorders, skin symptoms cardiovascular abnormalities, and respiratory symptoms [37]. It is generally agreed that the concerning outcomes of hydrogen sulfide are related to the concentration and administration routes [36]. High concentrations of H$_2$S aggravated inflammation while low concentrations alleviated inflammation. Based on the above concerns, the sustained release system was critical for its application.

Commonly used donors of H$_2$S include inorganic salts such as sodium hydrosulfide (NaHS), and sodium sulfide (Na$_2$S). They failed to simulate the biological process of H$_2$S because of their extremely rapid release, which often results in excessive concentration. Although there were H$_2$S compounds with a slow-release property such as GYY4137 and AP39, the short half-life period limits their application [38–40]. Therefore, maintaining an effective concentration of H$_2$S in vivo require additional dosage continuously. We demonstrated the feasibility of Fe$_3$S$_4$FFH to release H$_2$S within an acute therapeutic window (24–72 h). The
releasing of H$_2$S rose rapidly to 10 μM in the first 50 h, followed by an equilibrium. It is consistent with the pathophysiology process of the inflammation after SCI and avoid frequent supplementation [41,42]. With a high clearance rate and consumption in tissues, the concentration of H$_2$S in most tissues was estimated in the low nanomolar range [43]. However, free H$_2$S, acid-labile sulfide, and sulfane-sulfur were interchangeable, and measurement in biological samples can be confounded by side reactivity, leading to highly variable estimates of H$_2$S concentration [44]. Although we failed to provide the concentration of H$_2$S released from FFH in vivo, we demonstrated that the toxic threshold of H$_2$S was 1 mM (Fig. S3), which was 100-fold higher than released from FFH in vitro. We also proved that FFH showed no toxicity to the viscera (Fig. S7), indicating no long-term toxicity. Based on the above reasons, we believed the acute toxidrome of H$_2$S from FFH was minimal. And more accurate measurements are required in future to assess the H$_2$S in vivo.

5.4. The effect of H$_2$S in the treatment of SCI

H$_2$S was a novel neuro-modulator and neuroprotective agent, with anti-inflammatory, antioxidant, and anti-apoptotic effects in many neurological diseases [44,45]. In the nervous system, H$_2$S reduces inflammation through direct anti-inflammatory effects and indirect effects such as inhibition of the NF-κB pathway, making it a potent immunomodulatory agent [46]. Following SCI, the early phase of inflammation was comprised principally of neutrophils (peaking 1-day post-injury), and macrophages/microglia (peaking 7 days post-injury) [47], and the latter was maintained for several months [48]. In this study, C6D8 was used to label activated microglia/macrophages at the injury site on day 7 after SCI. It was worth noting that, there was no significant differences in C6D8 expression between FFH(−) and FFH(+) groups. This indicated that the anisotropy had no effect on inflammation. The anti-inflammatory effect of H$_2$S in the nervous system was shown in reducing the inflammatory mediators, such as NO, TNF-α, and IL-1β or inhibiting oxidative stress [49,50]. In RT-PCR, Fe$_3$S$_4$FFH reduced the mRNA levels of pro-inflammatory factors such as IL-1β, TNF-α, and iNOS on day 1 and day 7 after SCI. This will help to reduce the damage of inflammation on neurons.

Besides anti-inflammation, H$_2$S was proved to promote axonal regrowth [51] and upregulate the expression of axonal guidance molecules such as NGF and ARTN [52]. NaSH was proved to enhance the proliferation of NSCs through the extracellular signal-regulated kinase (ERK) 1/2 pathway, and regulate the differentiation of NSCs by some factors [53]. In this study, INSC and PC12 loaded on FFH sprouted longer axons than those loaded on CCH. This indicated that H$_2$S can promote axonal extension in vitro. It also increased the CGRP(+) axons and promoted the expression of TUJ1, NF200, and SYN A in vivo. Moreover, Semaphorin, a potent inhibitory axon guidance molecule, was slightly downregulated after ferrofluid hydrogel transplantation. This indicated that the released H$_2$S might be able to inhibit Semaphorin to promote axon regrowth. However, the detailed mechanisms involved need further study.

Fig. 6. (A) CD68 immunofluorescence staining in the spinal cord of four groups at 7 days after injury, (B) Quantitative of CD68 immunofluorescence intensity in four groups, **P < 0.01, ***P < 0.001, ns means no significance, n = 3. (C) CD68 expression in the spinal cord by WB, n = 3. (D-F) The mRNA of IL-1β, iNOS, and TNF-α detected by RT-PCR 7 days post injury, ***P < 0.001, ns means no significance, n = 3. (G) The relationship between the anti-inflammatory effect and the NF-κB pathway was verified by WB, n = 3.
6. Conclusion

We designed an anisotropic Fe$_3$S$_4$ ferrofluid hydrogel with H$_2$S sustained releasing property in this study. The Fe$_3$S$_4$ FFH possesses great biocompatibility and releases H$_2$S at a concentration of 10 μM, displaying excellent anti-inflammatory and neurotrophic effects. Moreover, the preorientation of Fe$_3$S$_4$ particles by magnetic field promotes axonal directional extension in vitro. The in vivo experiments proved that Fe$_3$S$_4$ FFH could attenuate microglia/macrophage activation through the NF-κB pathway. Further, the anisotropy of FFH significantly stimulated directional axonal regeneration and motor function recovery in SCI rats. The immunomodulatory and anisotropic dual effects make Fe$_3$S$_4$ FFH a promising candidate for the treatment of SCI.

CRediT authorship contribution statement

Ruofei Wang: Conceptualization, Methodology, Formal analysis, Writing – original draft. Xiaxiao Wu: Conceptualization, Methodology, Investigation, Data curation, Data curSation. Zhenming Tian: Methodology, Investigation, Validation, Writing. Tian Hu: Methodology, Investigation. Chaoyang Cai: Methodology, Investigation, Resources. Guanping Wu: Methodology, Data curation. Gangbiao Jiang: Conceptualization, Validation, Supervision. Bin Liu: Conceptualization, Formal analysis, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could appear to influence the work reported in this paper.

Acknowledgments

This work was supported by the following grants: the National Key Research and Development Program of China (2017YFA0105400); the National Natural Science Foundation of China (82072455, 81772349); the Guangdong Basic and Applied Basic Research Foundation (2019A1515012181). The graphical abstract was created with BioRender.com.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2022.10.020.

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