**Supporting Information**

# Novel Multi-Drug Delivery Hydrogel Using Scar-Homing Liposomes Improves Spinal Cord Injury Repair

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## SUPPLEMENTAL METHODS

### Fourier transform infrared spectroscopy (FTIR) assay of the hydrogels

FTIR was used to analyze the structural changes of HP, CAQK-LIP@HP and CAQK-LIP-GFs/DTX@HP following the protocols by FTIR 8400 spectrophotometer (Shimadu, Japan) using the potassium bromide disk. Each spectrum was acquired in transmittance mode with a resolution of 2 $cm^{-1}$ and spectral range of 4000–400 $cm^{-1}$. The analysis was finally focused on the range of 1700–700 $cm^{-1}$ as the most informative in the IR spectra of HP for these assays.

### *In Vitro* Release Profiles of aFGF/BDNF/DTX from CAQK-LIP-GFs/DTX and CAQK-LIP-GFs/DTX@HP.

The releases of aFGF, BDNF and DTX were measured according to the literature [30, 31]. In brief, 1 ml aliquots of the aFGF, CAQK-LIP-GFs/DTX, and CAQK-LIP-GFs/DTX@HP (containing 5 μg aFGF, 5 μg BDNF and 5 mg DTX) were placed in dialysis bags (MW cutoff of 25 KDa), and the bags were immersed in 10 ml PBS (the release medium for aFGF and BDNF) or PBS containing 0.5% (v/v) Tween-80 (PBST, the release medium for DTX) under shaking at 100 rpm/min at 37 °C. At specific time points, aFGF, DTX, and BNDF were collected in the supernatant, and the supernatant was replaced with the same volume of fresh solution. The amounts of released GFs (aFGF and BDNF) were quantified with an aFGF and BDNF enzyme-linked immunosorbent assay kit (ELISA, Westtang System, Shanghai, China),

and the amount of released DTX was detected by high-performance liquid chromatography (HPLC).

**Primary Cortical Neurons Cultures**

Primary cortical neurons were extracted from the embryos of pregnant Sprague-Dawley (SD) rats (E18). In brief, the cerebral cortex was separated and cut into approximately 1-mm pieces in precooled Hank's buffer (Gibco-Invitrogen). Subsequently, the tissues were digested with 0.125% trypsin-EDTA (Solarbio, Beijing, China) for 25 mins at 37 °C. After trypsinization, the solution was filtered using a 100-μm cell strainer (BD Falcon) and then was centrifuged at 1200 rpm for 5 min. The cell pellet was resuspended in complete Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, Gibco-Invitrogen) medium and incubated in 5% $CO_2$ at 37 °C. After 4 h, the cells were refreshed and cultured in neurobasal medium (Gibco-Invitrogen) with 0.5 mM L-Glutamine and 2% B27 (GlutaMAX™ Supplement, Gibico).

**Biocompatibility of CAQK-LIP@HP and CAQK-LIP-DTX@HP**

The cytotoxicity of CAQK-LIP@HP hydrogel was assessed in neurons by using the Cell Counting Kit-8 kit (CCK-8, Dojindo Laboratories Inc., Kumamoto, Japan) and LDH-kit (Beyotime Institute of Biotechnology, Shanghai, China). The neurons were seeded into 48-well transwell plates with a seeding density of $2 \times 10^4$ cells per well and incubated for 48 h in the complete neurobasal medium at 37 °C. CAQK-LIP@HP (from 1 μL/mL to 50

μL/mL) was added into the transwell inserts and co-incubated for 24h. After that 0 μL of CCK-8 or 250 μL LDH was added into each well. Four hours later, the medium in 48-well transwell plates was transferred to 96-well transwell plates, and the 450 nm (for CCK-8) or 490 nm (for LDH) absorption was measured using a microplate spectrophotometer (Spectramax 190, Molecular Devices Corporation, Sunnyvale, CA). The Biocompatibility of CAQK-LIP@HP and CAQK-LIP-DTX@HP was also determined by performing Annexin V-fluorescein isothiocyanate (Annexin V-FITC)/Propidium iodide (PI) staining and calcium fluorescein-AM/PI double staining assay using SH-SY5Y cells (ScienCell, Carlsbad, CA, USA). Cells were seeded on 6-well transwell plate at $2 \times 10^5$ cells/well and incubated for 24 h to adhere. Then CAQK-LIP@HP or CAQK-LIP-DTX@HP was added into the transwell inserts for 24h incubation. After that, cells were collected and stained using PI/Annexin V-FITC kit (Invitrogen, Carlsbad, CA, USA); the fluorescence intensity was analyzed using a flow cytometer (BD, Biosciences). For AM/PI staining, cells were gently washed twice with PBS, 2 μM of calcein AM and 15 μg·M-1 PI were added to the cells, and culture plates were incubated at 37°C for 30 min. Finally, the dye solution was removed, and the samples were washed with PBS three times. A fluorescence microscope (Nikon) was used to assess the slides. All experiments were performed in triplicate. To evaluate the biocompatibility of the CAQK-LIP@HP or CAQK-LIP-DTX@HP complex, spinal cord injury model was created and 100 μl hydrogel was orthotopically injected and 100 μl saline solution was injected as a control. After 6h, 1, 3 and 7 days, the spinal cords were

collected and the inflammation was detected by quantitative real-time PCR (qRT-PCR) as described previously. White blood cells were measured in blood samples, which were collected 3 days after injury.

**Western Blot.**

For the in vivo protein analysis, spinal cord segment (0.5 cm in length) at the contusion epicenter was dissected and rapidly stored at −80 °C for western blot assays. For protein extraction, the tissue was homogenized in modified radioimmune precipitation assay (RIPA) lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Ethylenediaminetetraacetic acid (EDTA), 1% Triton-X100, 0.5% sodium dexoycholate, 1 mM phenylmethanesulfonylfluoride (PMSF) and 10 μg/mL leupeptin). In vitro, cells were lysed in RIPA buffer (25 mM Tris-HCl, 150 mM NaCl, 1 % Nonidet P-40, 0.5 % sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS)) containing with 1 mM PMSF and 500g/mL DNase I. The extracts above were quantified with bicinchoninic acid (BCA) reagents (Solarbio, Beijing, China) and 40 μg proteins were loaded and separated using 10% sodium do-decyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Next, the separated proteins were transferred onto polyvinylidenedifluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA), followed by 5 % non-fat milk (Bio-Rad) block for 1h. Membranes were incubated overnight at 4 °C with the following primary antibodies: Nestin (1:200, Abcam), Growth associated protein 43 (GAP-43) (1:500, Abcam), Laminin (1:1000, Abcam), Neurocan (1:200, Abcam), Neuron-glial antigen 2

(NG2) (1:200, Abcam), myelin basic protein (MBP) (1:1000, Cell Signaling Technologies), Ace-tubulin (1:2000, Cell Signaling Technologies), Tau (1:1000, Abcam), Dynein (1:500, Sigma-Aldrich) and GAPDH (1:5000, Santa Cruz Biotechnology); later the membranes were immersed for 1h in corresponding horseradish peroxidase-conjugated secondary antibodies. The blots were visualized by Chemi DocXRS + Imaging System (Bio-Rad, Hercules, CA, USA); the grey density was analyzed by the Image Lab software. All experiments were repeated three times.

**Magnetic Resonance Imaging (MRI)**

Spinal MRI was performed to evaluate the damaged and inflammatory area 7 days after SCI. All MRI experiments were performed on a 3 T/10 cm horizontal bore magnet (GE Signa HDxT 3.0T, America) using a spin-echo T2-weighted MRI sequence (TR =1500 ms, TE=92 ms, FOV=9.0 mm, Slice thickness=1.5 mm, Spacing=0 mm).

**Biotinylated Dextran-amine (BDA) Anterograde Tracer**

Two weeks post-SCI, rats were anesthetized and the procedures were performed as methods described in these publications 28-29. In brief, after placing the animal in a stereotaxic apparatus, the skin was incised in the midline to expose the skull. Approximately 500 nl of a mixture of dextran amine conjugated with Texas Red (10% BDA; MW 10,000; Invitrogen) was injected through a glass micropipette (diameter -50 mm) at 8 positions on the left hemisphere, approximately spanning the rostrocaudal

extent of the hindlimb sensorimotor cortex. BDA delivery took 5 min at each site. The micropipette tip remained in place for 20s before withdrawal. After two weeks, the spinal cord tissues were collected and handled with color rendering.

**Transmission Electron Microscopy (TEM)**

Spinal cord tissue samples were fixed in 2.5% (w/v) glutaraldehyde solution overnight. Following post-fixation in 2% (v/v) osmium tetroxide, the tissues were blocked with 2% (v/v) uranyl acetate and dehydrated in a series of acetone washes followed by Araldite embedding. Semi-thin section and toluidine blue staining were carried out to observe the location. Finally, ultra-thin sections of at least six blocks per sample were cut and observed using a TEM.

**Detection of the individual and synergistic effects of aFGF, BDNF and DTX on multiple trials in vitro.**

To emphasize the new drug combination and compared with our previous types of heparin-poloxamel hydrogel drug delivery systems, multiple trials in vitro were added. To test the individual and synergistic effects of aFGF, BDNF and DTX on the axonal extension, wound healing of neurons was performed. Primary cortical neurons were plated into poly-L-lysine-coated 12-well plates, half the medium was replenished every 2-3 days, a scratch was made across the center of each well with a plastic pipet tip (ART10, Thermofisher) on DIV10. After scratching, the medium was supplemented with

3.34 μg/ml CSPGs and 20 μl hydrogels (CAQK-LIP @HP-aFGF, CAQK-LIP-BDNF@HP, CAQK-LIP-DTX@HP, CAQK-LIP-GFs/DTX@HP) (containing 5 μg/mL aFGF/BDNF and/or 50 μg/mL DTX) were added into the transwell inserts (pore size 0.4 mm) and co-incubated for 48h. After that (DIV12), cells were fixed with paraformaldehyde (4%) and stained with Tuj-1 (1:1000, Abcam). To detect the individual and synergistic effects of the drugs on the migration of fibroblasts, wound healing of Human Brain Vascular Adventitial Fibroblasts (HBVAFs, Sciencell, Carlsbad, CA, USA) was used. HBVAFs were plated into poly-L-lysine-coated 12-well plates and cultured in the complete Fibroblast Medium (FM, Sciencell) which was changed every two days. When the culture reaches above than 90% confluency, a scratch was made across the center of each well, then 20 μl hydrogels (CAQK-LIP @HP-aFGF, CAQK-LIP-BDNF@HP, CAQK-LIP-DTX@HP, CAQK-LIP-GFs/DTX@HP) (containing 5 μg/mL aFGF/BDNF and/or 50 μg/mL DTX) were added into the transwell inserts and co-incubated for 72h. After that, the cells were fixed with paraformaldehyde (4%) and stained with F-actin (1:2000, Abcam).

**Comparisons between multiple experimental groups**

In vivo experiments, we set six groups firstly as SCI, CAQK-LIP-GFs@HP, CAQK-LIP-DTX@HP, CAQK-LIP-GFs/DTX@HP, LIP-GFs/DTX@HP and CAQK-LIP-GFs/DTX to measure the functional effects of the different groups. Results from multiple comprehensive evaluations systems including motor function, histology

and magnetic resonance imaging (MRI) (Figure 3), however, showed that there was no significant improvement in rats treated with CAQK-LIP-GFs/DTX compared with SCI rats. We speculated that drugs without HP can easily flow away so that their efficacy cannot be exerted. Thus, in the further experiments which were performed to confirm the roles and mechanism of GFs, DTX, CAQK, and their combined effects (Figure 6, Figure 7B-F, etc.), we established the model only from these five groups: SCI, CAQK-LIP-GFs@HP, CAQK-LIP-DTX@HP, CAQK-LIP-GFs/DTX@HP, and LIP-GFs/DTX@HP group.

For in vitro experimentation, first we aimed to evaluate the effects of the DTX and GFs without HP on neurons growth in vitro, and confirm the mechanisms of the drugs, so we initially chose Con, CAQK-LIP-GFs, CAQK-LIP-DTX, CAQK-LIP-GFs/DTX, and CAQK-LIP-GFs/DTX@HP group to investigate the roles of GFs, DTX, and their combined effects by staining Ace-tubulin/Tyr-tubulin (Figure 7H-J). However, by comparing the effects of CAQK-LIP-GFs/DTX and CAQK-LIP-GFs/DTX@HP, we found that HP also improved the functions of the drugs in vitro, which further demonstrated the positive role of HP. Therefore, in the following experiments, we chose these four sample groups: Con, CAQK-LIP-GFs@HP, CAQK-LIP-DTX@HP, and CAQK-LIP-GFs/DTX@HP group, and further investigated the functions and mechanisms of GFs, DTX, and their combined effects.

To emphasize the new drug combination and compared with our previous types of heparin-poloxamel hydrogel drug delivery systems, we added some in vitro experiments

with six groups (Con, CAQK-LIP @HP-aFGF, CAQK-LIP-BDNF@HP, CAQK-LIP-DTX@HP, CAQK-LIP-GFs/DTX@HP) under the comments of the reviews (Figure S4).

## SUPPLEMENTAL FIGURE LEGENDS

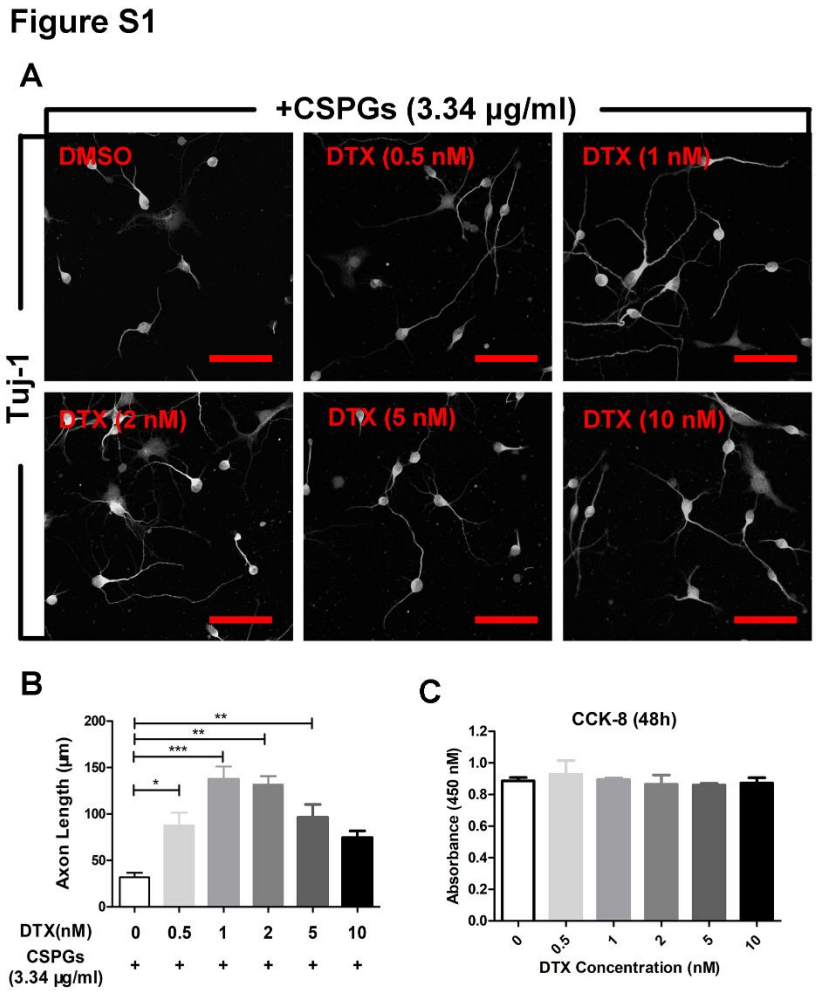
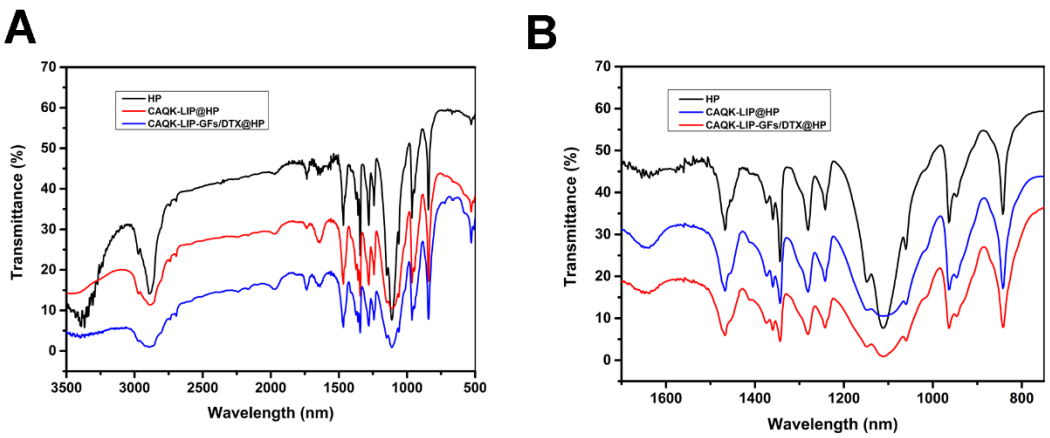



Figure S1 DTX promoted axonal extension. (A) Representative images of neurons stained with Tuj-1 that were treated with different concentration of DTX at DIV5. Scale bar represents 50 μm. (B) Quantification of the mean axonal length (from the junction cell body and axon to the end of the axon) in each group from A. N= 4. (C) Viability of neurons after incubation in different concentrations of DTX for 48 h (CCK-8 assay).

## Figure S2

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203 Figure S2 Characterization of the Complex Hydrogels. (A) FTIR spectra of lyophilized
204 HP, CAQK-LIP@HP and CAQK-LIP-GFs/DTX@HP hydrogels in the range of 4000–
205 400 cm-1. (B) FTIR spectra of lyophilized HP, CAQK-LIP@HP and
206 CAQK-LIP-GFs/DTX@HP hydrogels in the range of 1700–700 cm-1.

**Figure S3**

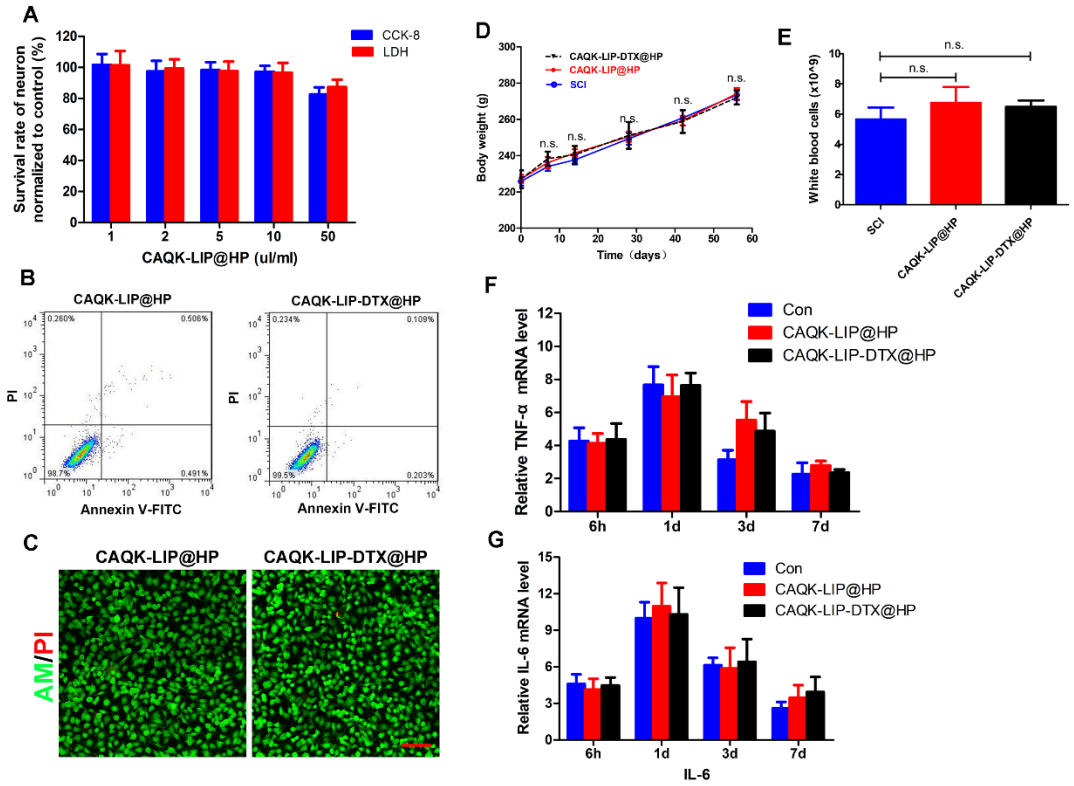


Figure S3 Cytotoxicity and Biocompatibility of the CAQK-LIP-GFs/DTX@HP in vitro and in vivo. (A)The viability of SH-SY5Y cells after incubation with different formulations of CAQK-LIP @HP for 24 h using the CCK-8 and LDH kits. (B-C) The survival rate of SH-SY5Y cells with or without treatment of CAQK-LIP @HP using PI/annexin V-FITC staining and Calcein-AM/PI staining. Scale bar = 10 μm. All experiments were performed in triplicate. (D) Animal body weight at various time-points after spinal cord contusion injury, n = 9. (E) Number of white blood cells in blood samples 3 days after SCI, n = 4. (F-G) Relative mRNA of TNF-α and IL-6 in the injured spinal cord at various time-points after SCI. N = 3. *P < 0.05, **P< 0.01.

**Figure S4**

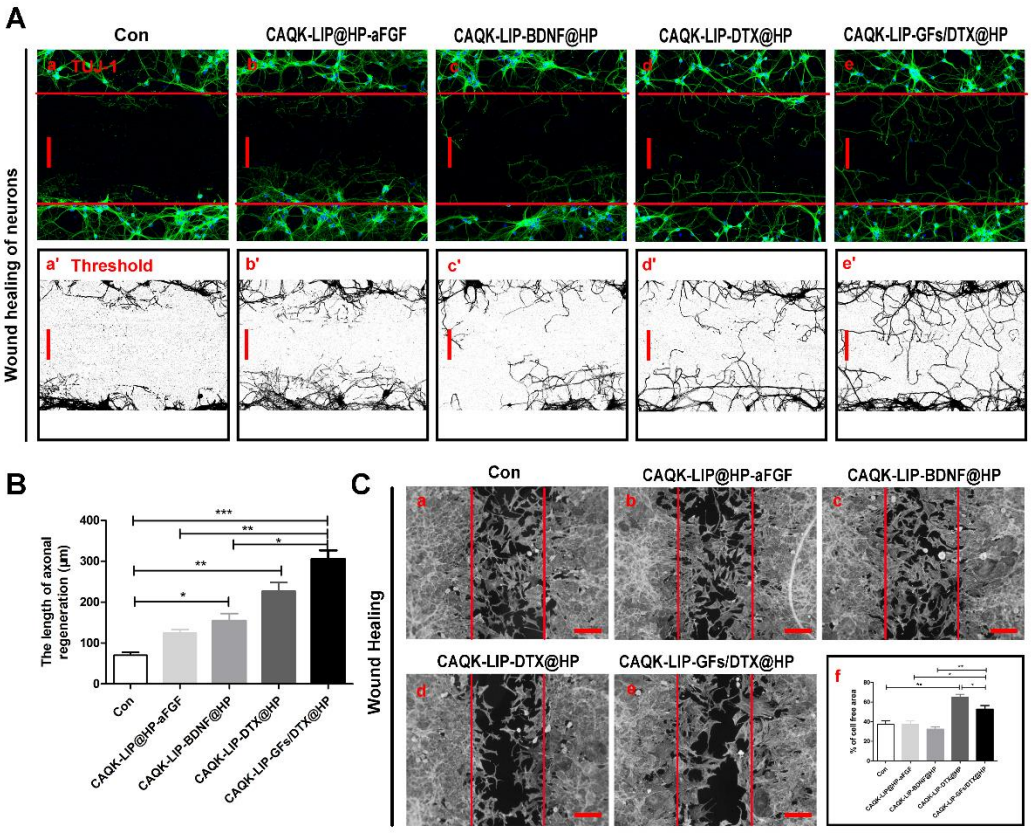


Figure S4 The individual and synergistic effects of aFGF, BDNF, and DTX on multiple trials in vitro. (A) Representative wound healing images of neurons with different treatment and stained with Tuj-1 at DIV12. (B) Quantitative analysis of the length of axonal regeneration. N= 4. (C) Representative wound healing images and quantification data of HBVAFs stained with F-actin at 72h after treatment. Scale bar = 100 μm. *P < 0.05, **P< 0.01.

**Figure S5**

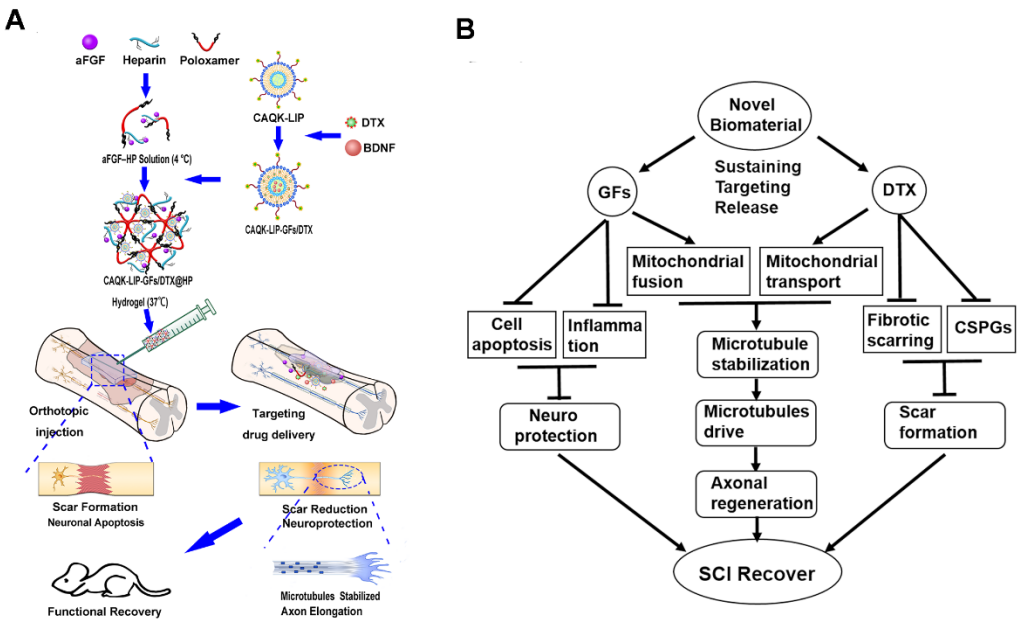


Figure S5 (A) Schematic diagram of injectable hydrogel complex as an in-situ targeting multiple drugs delivery system for the recovery of the spinal cord. (B)Schematic showing CAQK-LIP-GFs/DTX@HP loading multiple drugs has different therapeutic targets, multifunctional and synergistic therapeutic effects in SCI directed to counteract multiple injury mechanisms.