Supplementary information

Materials and methods

Chemicals and siRNAs

Purified HIPCO® single-walled carbon nanotubes (CNTs) were purchased from NanoIntegris (Unidym). PL-PEG2000-Amine (cat. no. DSPE-020PA) was purchased from the NOF Corporation. Sulfo-LC-SPDP was purchased from Pierce (cat. no. 21650). FITC-labeled NC-FITC-siRNA (5'-thiol-: UGCGCUACGAUCGACGAUG3'-FITC) was purchased from BIONEER, and siCAS3 (5'--thiol UUGAUGGAUAGUGUUUCUAUU-3') was purchased from GenePharma.

Functionalization of SWNTs

One milligram of SWNTs, 5 mg of PL-PEG2000-Amine, and 5 mL of water were mixed together with continuous stirring. The reaction was allowed to continue for 60 min at 20 °C. We replaced the water once every 20 min in a water bath to prevent overheating. We centrifuged the SWNT suspension for 6 h at 24,000 × g at 20 °C and collected the supernatant solution. Then, the absorption spectrum of the SWNT solution was obtained using UV-VIS-NIR spectrometry. The final SWNT concentrations ranged from 40 to 70 mg/L. The SWNT solution was stored at 4 °C. We added 1 mL of prepared SWNT solution into a 4 mL Amicon centrifugal filter device with a molecular weight cutoff (MWCO) of 100 kDa. We added 3 mL of water and centrifuged for 10 min at 4000 × g at 20 °C. We filled the filter device with water to 4 mL. In order to completely remove excess PL-PEG in the SWNT solution, we washed the solution 5–6 times by repeating the centrifuge/water addition steps. After the final washing step, we measured the concentration of the SWNT solution using UV-VIS-NIR spectrometry with a weight extinction coefficient of 0.0465/mg·cm at 808 nm. We adjusted the concentration of the functionalized SWNTs (F-CNTs) to ~50 mg/L by adding the required amount of water.

siRNA conjugation to SWNTs through cleavable disulfide bonding

We mixed 500 µL F-CNT and 0.5 mg Sulfo-LC-SPDP in 50 µL 10 × PBS and incubated them at 20 °C for 2 h. A 10 mM DTT solution was prepared by dissolving 1.54 mg DTT in 1 mL water. Then, 15 µL of 100 µM siRNA (FITC-siRNAnc, siRNAcas3, or siRNAnc) was mixed with 1.5 µL of DTT solution. The reaction was carried out at 20 °C for 1.5 h. An Amicon centrifugal filter (MWCO = 100 kDa) was used to remove excess Sulfo-LC-SPDP from the SWNT solution. We added 3–4 mL of DNase/RNase-free water, washed the solution 5–6 times, and centrifuged it at 10,000 × g for 6–8 min at a time. Then, the DTT treated with siRNA was purified using a NAP-5 column according to the manufacturer's protocol. The siRNA in the column was eluted with 500 µL of 1 x PBS without DNase/RNase. The activated SWNT was resuspended with 500 µL of purified siRNA solution. The binding was allowed to proceed at 4 °C for 24 h. The final SWNT and siRNA concentrations were ~40 mg and ~2.5 µM, respectively.

Cardiomyocyte isolation and cell culture

Primary cardiomyocytes were isolated from one-day-old Sprague-Dawley rats and cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA, USA) containing

15% FBS (Invitrogen, Carlsbad, CA, USA) at 37 °C and 5% CO2. The culture medium was changed every day. All animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Chinese Academy of Military Medical Science (Beijing, China). All experimental protocols were approved by the Committee on the Ethics of Animal Experiments of the Chinese Academy of Military Medical Science.

Cytotoxicity assay

A Live/Dead Viability/Cytotoxicity Kit (Molecular Probes, Invitrogen, Grand Island, NY, USA) was used to evaluate the percentage of live cells. Neonatal rat ventricular myocytes (NRVMs) grown on the substrates for 1 and 3 d were incubated for 15 min with 2 μM calcein-AM and 4 μM EthD-1 in phosphate-buffered saline (PBS). Live and dead cells were counted in 10 randomly selected fields for each group under a fluorescence microscope (Nikon AZ-100 multipurpose microscope, Tokyo, Japan) through a 10× objective lens. The images were analyzed using the ImageJ software.

Agarose gel electrophoresis

The stability of the bond between siRNA and F-CNT was analyzed using agarose gel electrophoresis. Bare siRNA (200 nM) was dissolved with RNase-free water in the siRNA group. F-CNT and siRNA were directly mixed in the F-CNT group (10 mg/L, 200 nM). Different concentrations of F-CNT (1.25 mg/L, 2.5 mg/L, 5 mg/L, and 10 mg/L) and siRNA (200 nM) were connected through a cleavable disulfide bond according to the method described above. From each group, a 6 μL solution was loaded onto 1% agarose gel containing ethidium bromide (0.5 mg/mL) in 1X Tris-acetate-EDTA (TAE) buffer. The gel was run for 10 min at 90 V and visualized under UV light using the Binta 2020D imaging system (Binta, Beijing, China).

In vitro transfection in cardiomyocytes and confocal microscopy

NRVMs were seeded and grown on 10 mm x 10 mm coverslips in 24-well plates (1 x 106 cells/well). One hundred microliters of RNase-free water was added to the control group, 100 μL of NC-FITC-siRNA (with a final siRNA concentration of 200 nM) was added to the naked siRNA group, and the F-CNT-siRNA group was transfected with NC-FITC-siRNA (with a final siRNA concentration of 200 nM) connected with F-CNT by a cleavable disulfide bond. The cells were washed twice with cold PBS buffer solution after 6 h of incubation with 4% formaldehyde for 30 min at 20 °C. After washing three times with cold PBS, the solution was then permeabilized with 0.3% Triton X-100 in PBS for 30 min and then blocked with 2% BSA and 0.05% sodium azide in PBS for 30 min at 37 °C. Next, NRVMs were incubated with rabbit polyclonal anti-connexin 43 (AbCam, 1:2000) antibodies overnight at 4 °C. After that, cells were washed with PBS three times and then incubated with Cy3 Conjugated Goat Anti-Rabbit IgG (Invitrogen, 1:200) for 2 h at 20 °C. Finally, the cells were stained with Hoechst 33258 4-6-Diamidino-2-phenylindole (DAPI) in PBS (1:200, Sigma) and analyzed under a confocal microscope with Volocity Demo 6.1.1 (Zeiss).

Gene expression

NRVMs were cultured in Dulbecco's Modified Eagle Medium (DMEM; Cellgro) with 15% fetal bovine serum (FBS; Hyclone) in 6-well plates until reaching 80% confluency. One hundred microliters of RNase-free water was added to control group, naked siRNA-Caspase3 (with a final siRNA concentration of 200 nM) was added to the siCas3 group, and the F-CNT-siNC group was transfected with siRNA-NC connected with F-CNT by a cleavable disulfide bond (the final siRNA concentration was 200 nM). The F-CNT-siCas3 group was transfected with siRNA-Caspase3 connected with F-CNT by a cleavable disulfide bond (the final concentration of siRNA was 200nM). The transfection was conducted for 24 h. Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's protocol and stored at -20 °C prior to use. cDNA reverse-transcribed from total RNA by the Takara kit was amplified by Power SYBR Green (Invitrogen) in an Applied Biosystems StepOne Plus real-time PCR system with the following pairs of oligonucleotides: *Capase3*: CAATGGTACCGATGTCGATG (forward primer) and GACCCGTCCCTTGAATTTCT (reverse primer). *GADPH*: CGGAGTCAACGGATTTGGTCGTAT (forward primer) and AGCCTTCTCCATGGTGGTGAAGAC (reverse primer). Gene expression levels were normalized relative to that of the housekeeping gene *GADPH*.

Protein isolation and western blot analysis

NRVMs were prepared and transfected as described above. Laemmli Sample Buffer (Bio-Rad) was used to lyse NRVMs on the third day after transfection. The extracted proteins were measured using a BCATM Protein Assay Kit (Thermo Scientific). Equal quantities of proteins were electrophoresed using SDS-PAGE. Separated proteins were transferred to a PVDF membrane (Millipore, Corporation, MA, USA), blocked with 5% skim milk for 1 h at 20 °C, and incubated with primary antibody overnight at 4 °C. Anti-Caspase3 was purchased from Cell Signaling Technology (Beverly, MA, USA). The membranes were incubated for 1 h with appropriate HRP-conjugated secondary antibodies (Invitrogen) after washing, and labeled proteins were visualized using the ECL chemiluminescence reagent. The expression of GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was detected as the loading control. The intensities of bands were analyzed using ImageJ software 1.8.0.

Animal studies

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the Chinese Academy of Military Medical Science (Beijing, China). A randomized and double-blinded study consisting of four groups was conducted using a rat acute myocardial infarction (AMI) injury model. Male Sprague-Dawley (SD) rats (250 ± 25 g) were anesthetized with sodium pentobarbital (30 mg/kg), and permanent left anterior descending artery ligation (6–0, Prolene, Ethicon) was performed. Immediately after coronary artery ligation, 100 μL PBS, 100 μL naked siRNA-Caspase3 (200nM siRNA), 100 μL siRNA-NC (200nM siRNA) connected with F-CNT by a cleavable disulfide bond, or 100 μL siRNA-Caspase3 (200 nM siRNA) connected with F-CNT by a cleavable disulfide bond were administered by intramyocardial injection to three regions of the border zone of the infarcted area. After injection, the chests were closed, and the rats were allowed to recover under a heating light. Seven days after surgery, the rats were anesthetized, and echocardiography (Sequoia 512, Siemens, Germany) was conducted to evaluate cardiac function. Data from the left

ventricular shortening fraction (LVFS) and left ventricular ejection fraction (LVEF) were noted according to the previous report.1 The animals were sacrificed after the echocardiography evaluation, and the hearts were removed and cut into two transverse slices from the middle of the infarcted area. The heart samples were frozen in O.C.T. and cut into 4 mm frozen sections. The sections were stained with Masson's Trichrome; afterwards, the infarct size and left ventricular wall thickness were determined under a microscope.

TUNEL staining for apoptosis detection

Apoptosis evaluation was performed by using an in-situ cell death detection kit (Roche), and the number of apoptotic cells was counted under laser scanning confocal microscopy. Five slices were randomly selected from the same specimen, and five visual fields were randomly selected from the same section for counting.

Supplementary figure captions

Figure S1. Characterization of SWNTs. (A) photograph of SWNTs before functionalization. (B) Photograph of SWNTs after functionalization. (C) Transmission electron microscopy (TEM) image of SWNTs functionalized with PL-PEG2000.

Figure S2. Infrared absorption spectra of SWNTs, PEG, and SPDP.

Figure S3. The binding stability of siRNA to SWNTs was determined by agarose gel electrophoresis analysis.

Figure S4. Confocal images of cardiomyocytes. Nuclei were stained blue with DAPI, and cTNT was labeled with green fluorescent FITC (bars = 20 μm).

Supplementary figures

Fig. S1

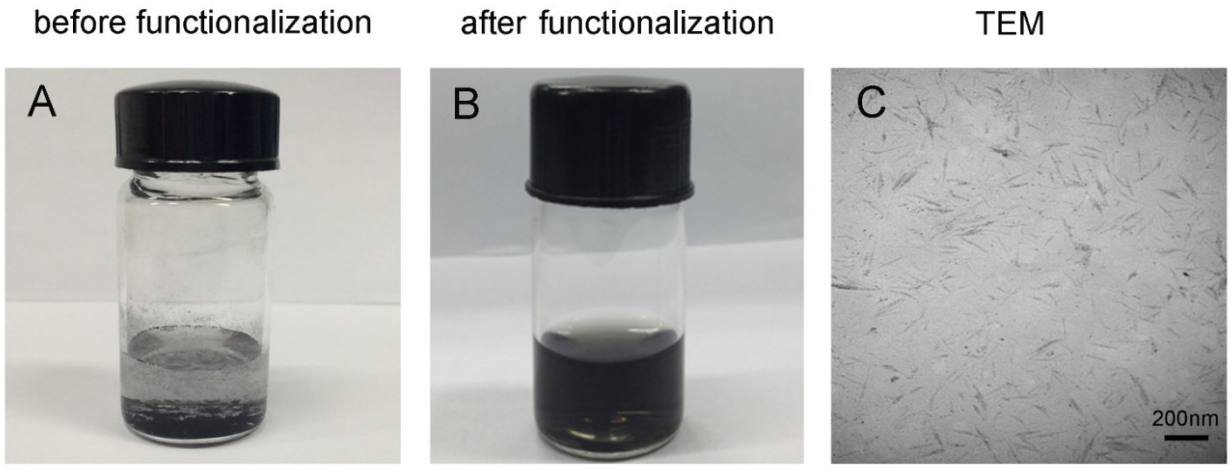

Fig. S2

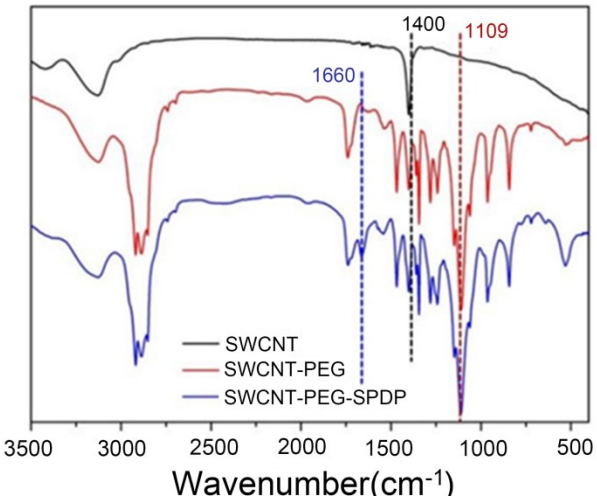

Fig. S3

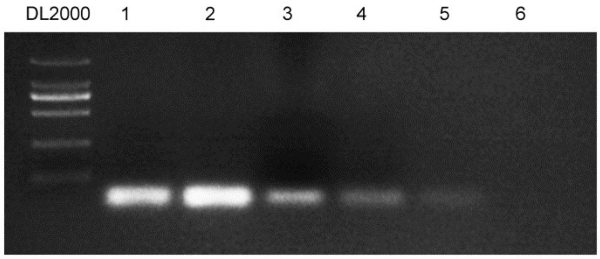

Fig. S4