Supporting Information for

Cascaded bio-responsive delivery of eNOS gene and ZNF_{580} gene to collaboratively treat hindlimb ischemia via pro-angiogenesis and anti-inflammation

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MATERIALS AND METHODS

Materials

Nε-Carbobenzoxy-L-lysine (noted as Lys-Cbz), triphosgene and N-hexylamine were purchased from Aladdin Bio-Chem Technology Co., LTD (Shanghai, China). 33.3 wt.% HBr/HAc, trifluoroacetic acid and deuterated water (for ^{1}H NMR characterization) were obtained from Beijing Hwrkchemical Company Limited. 3,3'-Dithiobispropanoic acid bis(N-hydroxysucciniMde ester) (noted as DSP), anhydrous solvents and epsilon-polylysine (ε-PLL), collagenase IV, N-Succinimidyl 3-(2-Pyridyldithio) propionate (noted as SPDP) were purchased from HEOWNS Biochem Technologies, LLC (Tianjin, China). Citraconic anhydride and Succinic anhydride were obtained from J&K Scientific Ltd (Beijing, China). Arg-Glu-Asp-Val-Gly-Gly- Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Gly-Cys (REDVGGYGRKKRRQRRRGC, REDV-GG-TAT-GC) and MMP-2-responsive peptide (Gly-Pro-Leu-Gly-Leu-Ala-Gly-Cys, GPLGLAGC) (abbreviated as MMPSP) were purchased from GL Biochem. (Shanghai) Ltd. ZNF_{580} gene in plasmid ($pZNF_{580}$), eNOS gene in plasmid (peNOS) and Cy5 labeled oligonucleotide (Cy5-oligonucleotide) were acquired from Sangon Biotech Co., Ltd. (Shanghai, China). Human umbilical vein endothelial cells (HUVECs) were obtained from the Cell Bank of Typical Culture Collection of Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), 0.01 M phosphate buffers (PBS, pH=7.2-7.4), LysoTracker Green and Hoechst 33342 dyes were purchased from Invitrogen Biotechnology Co., Ltd (Carlsbad, CA). NO assay kit and ELISA kits for TNF-α, IL-6, IL-8 and MCP-1 were purchased from Jian-Cheng Biotechnology Co., Ltd. (Nanjing, China). The polyclonal antibody rabbit anti-ZNF_{580} and anti-CD31 were bought from Abcam Ltd. (Shanghai, China). Male Sprague-Dawley (SD) rats (4-5 weeks old, 15-20 g) were purchased from Chinese

Academy of Medical Science & Peking Union Medical College Institute of Biomedical Engineering.

Characterization of the gene complexes

Transmission electron microscopy (TEM) was performed on a HT7700 TEM (HITACHI, Japan) transmission microscope operated at the accelerating voltage of 100 kV.

Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to measure the plasmid DNA (pDNA) condensing ability of PLL-DSP and dual-layer gene complexes. Briefly, PLL-DSP/pDNA at various w/w ratios were prepared by mixing different amounts of PLL-DSP solution and pDNA. The mixed solutions were incubated for 30 min at the room temperature (RT) to make the $pZNF_{580}$ fully loaded and thereupon electrophoresed on 0.8% (w/v) agarose gel at 120 V for 25 min, then the location of pDNA was recorded by a UV illuminator. In order to observe the PLL-DSP/pDNA complexes behaviors under reductive environments, DTT was added to the complexes solution and incubated for 1 h. After incubation DTT was added to break the disulfide binds between PLL and then the samples were electrophoresed as described above. To exam whether adding the anionic outer layers will destroy the loading of PLL-DSP to pDNA, the gene complexes with outer layers of different kinds and masses were formed and electrophoresed as described above.

In Vitro Cytocompatibility

The cytotoxicity of gene carriers is the chief factor of practical application. For The cytocompatibility measurement of ds-CPs, as-CPs, es-CPs and is-CPs, HUVECs were seeded on 96-

well plates at a density of 4×10^3 cells/well. After 24 h incubation, replace the culture medium of HUVECs with serum-free medium and added various gene complexes at different concentrations (20 μg/mL-120 μg/mL). Followed by 4 h incubation, MTT assay was performed to quantify the viability of HUVECs. The HUVECs culture wells were replaced with new DMEM containing MTT (500 μg/mL) and cells were cultured for another 4 h. Then, the medium was discarded carefully and 150 μL DMSO was added to dissolve the formed formazan crystal. The absorbance of each well was measured at 490 nm using a multi-function microplate reader.

In Vitro Cellular Uptake

The uptake capacity of LPS (1μg/mL) activated HUVECs transfected with different gene complexes was evaluated by the cellular uptake and mean fluorescence intensity (MFI) using a flow cytometry. The ds-CPs, as-CPs, es-CPs and is-CPs with Cy5-oligonucleotides were respectively transfected HUVECs that activated by 1μg/mL LPS for 4 h before transfection. HUVECs were incubated for 4 h and washed three times by PBS (pH = 7.4) to remove the Cy5-oligonucleotides attached to HUVECs surface. Afterwards, HUVECs were trypsinized and collected in PBS for the following measurements via a flow cytometry (Beckman MoFlo XDP, USA).

Confocal Laser Scanning Microscopy (CLSM)

CLSM was used to observe the distribution of Cy5-oligonucleotide to verify how the acid-responsive and enzyme-responsive layer work. HUVECs were seeded in confocal dishes and cultured for 24 h. The HUVECs were activated by 1μg/mL LPS for 4 h and then transfected in the same method as above. After 24 h of transfection, the HUVECs were dyed by LysoTracker Green and Hoechst 33342.

LysoTracker Green was added to stain the endo/lysosome at 75 nM for 30 min and subsequently Hoechst 33342 was used to stain the nucleus for 20 min. Then, wash HUVECs three times with PBS (pH=7.4) to remove extracellular dyes. Before observing by CLSM (Olympus FV1000, Japan), the wavelengths of 649, 504 and 350 nm were set up for Cy5, LysoTracker Green and Hoechst 33342.

Measurement of Transfection Efficiency

LPS (1μg/mL) activated HUVECs treated with $pZNF_{580}$ carrying the enhanced green fluorescent protein (EGFP) gene for 24 h. The transfection efficiency of different carriers was determined by observing the green fluorescence expression of HUVECs via an inverted fluorescence microscope.

Real-time Polymerase Chain Reaction (RT-PCR)

The LPS activated HUVECs were transfected by ds/$pZNF_{580}$, ds/peNOS and ds/$pZNF_{580}$/peNOS for 24 h and the HUVECs treated with PBS were used as a control group. Total RNA of HUVECs was extracted by Trizol reagent, and then the total RNA was reverse transcribed into cDNA. Afterwards, the RT-PCR was performed with a SYBR Green in an 7500 PCR system. The specific primers of GAPDH, ZNF_{580} and eNOS (TNF-α, IL-6, IL-8) were designed as follows: The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The forward and reverse primer sequences of GAPDH were 5'-AGGTGAAGGTCGGAGTCAAC-3', 5'-CGCTCCTGGAAGATGGTGAT-3', respectively. ZNF_{580} forward 5'-AAAAAGCTTGTGGAGGCGCACGTGCTG-3', and ZNF_{580} reverse 5'-AAAAAGATCTTGCCCGGAGTGCGCCCGTG-3'. The forward and reverse primers for the eNOS gene were 5'-GCTGCGCCAGGCTCTCACCTTC-3' and 5'-GGCTGCAGCCCTTTGCTCTCAA-3',

respectively. The results were analyzed using StepOne software v2.1.

Western Blot Assay

HUVECs were seeded on a 6-well plate and pre-processed by LPS (1μg/mL), transfected with ds/pZNF$_{580}$, ds/peNOS and ds/pZNF$_{580}$/peNOS for 24 h. Then, the HUVECs were washed by PBS (pH=7.4) and lysed by RIPA lysis buffer for 30 min on ice. The total protein was quantified by Coomassie Brilliant Blue G-250 method and separated by 12% SDS-PAGE. Then, the separated protein was transferred onto polyvinylidene difuoride (PVDF) membranes. Three primary antibodies cross-reacted respectively with β-Actin, ZNF$_{580}$ and eNOS overnight at 4℃. Then the membranes were incubated with secondary antibody and developed by an enhanced hemiluminescence (ECL) kit.

CCK-8 Proliferation Assay

The growth of LPS (1μg/mL) activated HUVECs was evaluated by CCK-8 assay. HUVECs were seeded in 96-well plate and cultured for 24 h with complete DMEM. After starving 12 h with serum-free medium and pre-processing by 1μg/mL LPS, the HUVECs were treated with ds/pZNF$_{580}$, ds/peNOS and ds/pZNF$_{580}$/peNOS at a concentration of 3μg/mL of gene. The cells treated with PBS were used as a negative control. The old medium was replaced with fresh medium of CCK-8 solution with CCK-8 solution at different fixed times (12 h, 24 h and 48 h) after transfection and incubation continued for 2 h. Then, the absorbance of samples was measured at 450 nm on a microplate reader.

Transwell Migration Assay

Transwell migration assay was performed to assess the migration ability of transfected HUVECs (LPS (1μg/mL) activated). The transfected HUVECs were digested with trypsin after 24 h and were collected to replate in the transwell chambers (aperture 8.0 μm) at 7×10^4 cells per well with serum-free medium. Media containing 10% FBS was added to the lower chambers. After another 6 h incubation, the chambers were washed thoroughly with PBS (pH=7.4) and fixed with 4% formaldehyde fixative for 10 min. Thereafter, the HUVECs passed through the chambers were stained with the Crystal Violet Staining Solution for 5 min and observed by a microscope. The migration ability was evaluated by counting the migrated cells by Image-Pro Plus 6.0.

In Vitro Tube Formation

The angiogenic ability of LPS (1μg/mL) activated HUVECs transfected with ds/pZNF_{580}, ds/peNOS and ds/pZNF_{580}/peNOS was evaluated by tube formation assay in vitro. In brief, the matrigel was melt overnight at 4 ℃ in advance and added in a 96-well plate (60 μL per well). Subsequently, the plate was incubated at 37 ℃ for 1 h. The transfected HUVECs were seeded onto the surface of Matrigel at a density of 4×10^4 cells per well with serum-free medium. After a 6 h incubation, tube formation was obtained by an optical microscope and can be quantified by ImageJ 2.1.

In Vivo Tube Formation

The angiogenesis assay in vivo was performed to further evaluate the angiogenic ability of LPS (1μg/mL) activated HUVECs. The transfected HUVECs were trypsinized in 300 μL serum-free medium (2.4×10^5 cells / mL) and were mixed with 400 μL matrigel. Then, the mixture was injected into the skin of the rats (4-5 weeks old, 15-20 g). After 5 days of feeding, the rats were euthanized

and the matrigel mixtures were removed and fixed with formalin. Paraffin-embedded matrigel was sliced and the sections were used for hematoxylin and eosin (H&E) staining and anti-CD31 immunofluorescence, respectively. Then, the formation of microvessel structure was observed by a fluorescence microscope.

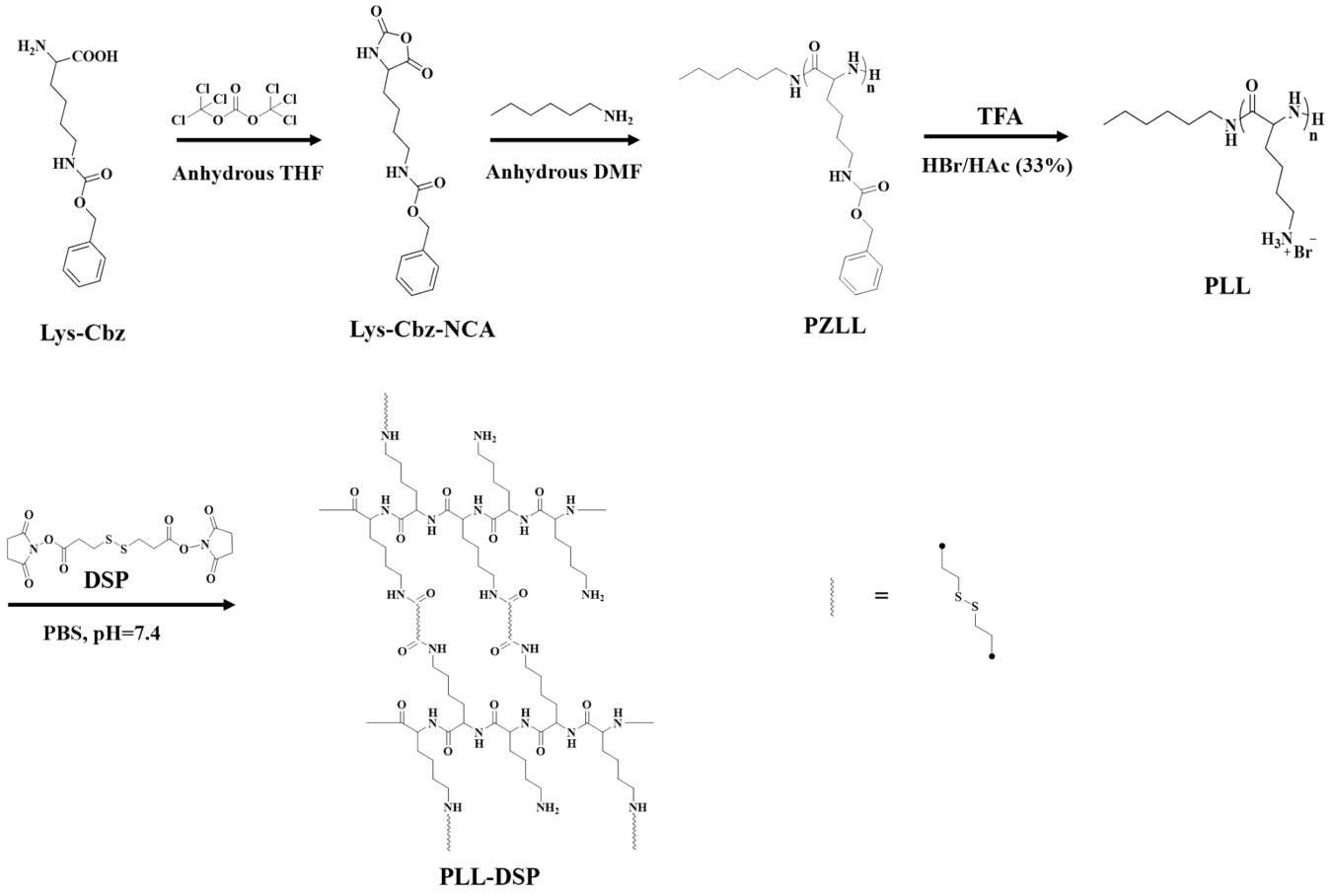


Fig. S1. Synthesis pathway of PLL-DSP.

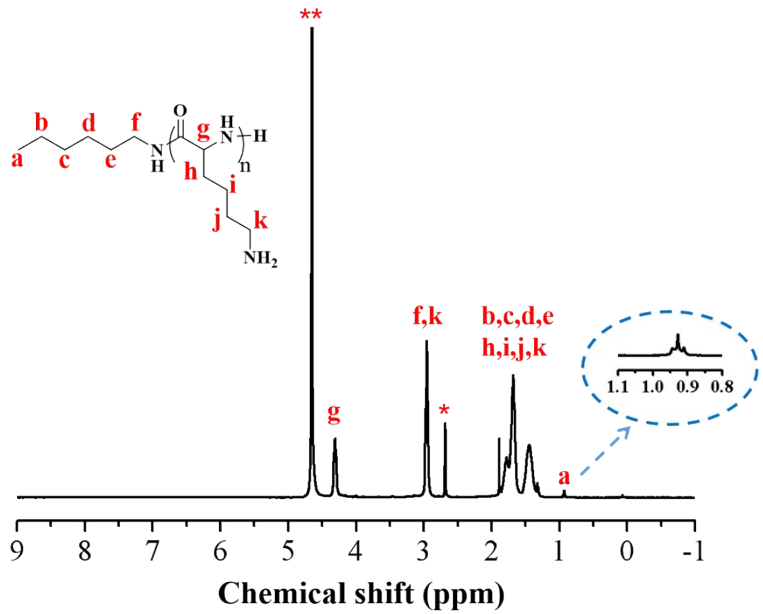


Fig. S2. [1]H NMR spectra of PLL using deuterated water as solvent (**).

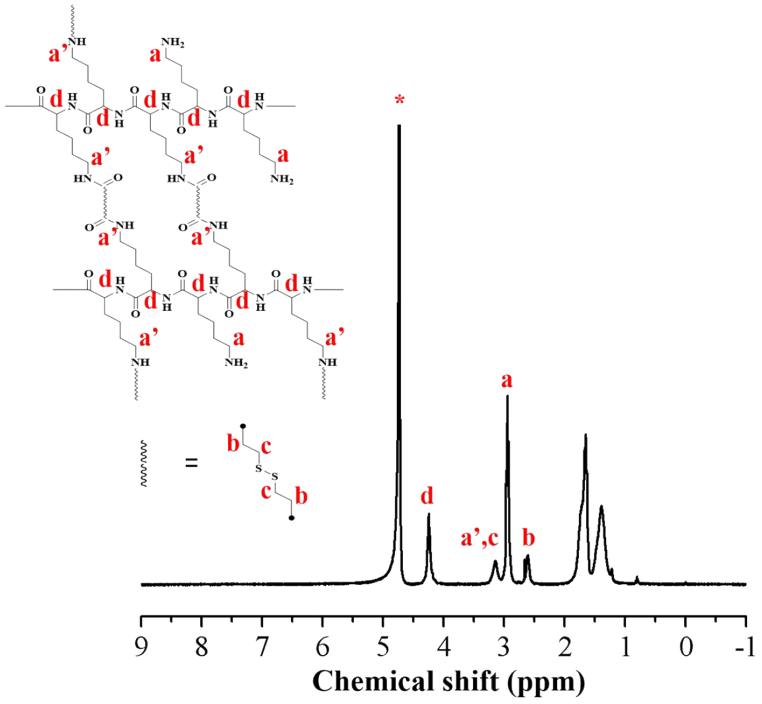


Fig. S3. [1]H NMR spectra of PLL-DSP using deuterated water as solvent (*).

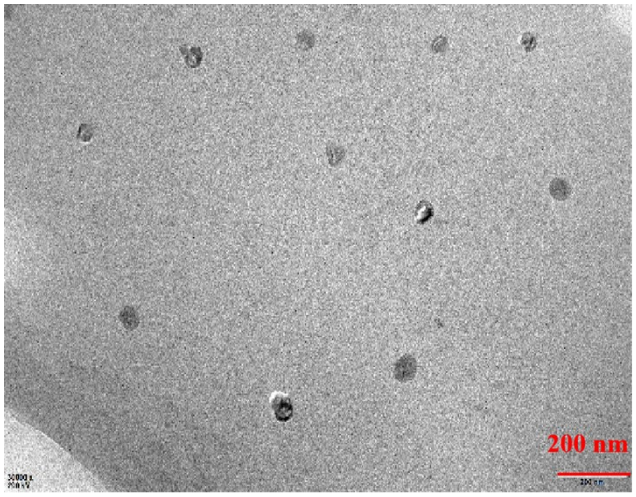


Fig. S4. Morphology of PLL-DSP in aqueous solution detected by TEM.

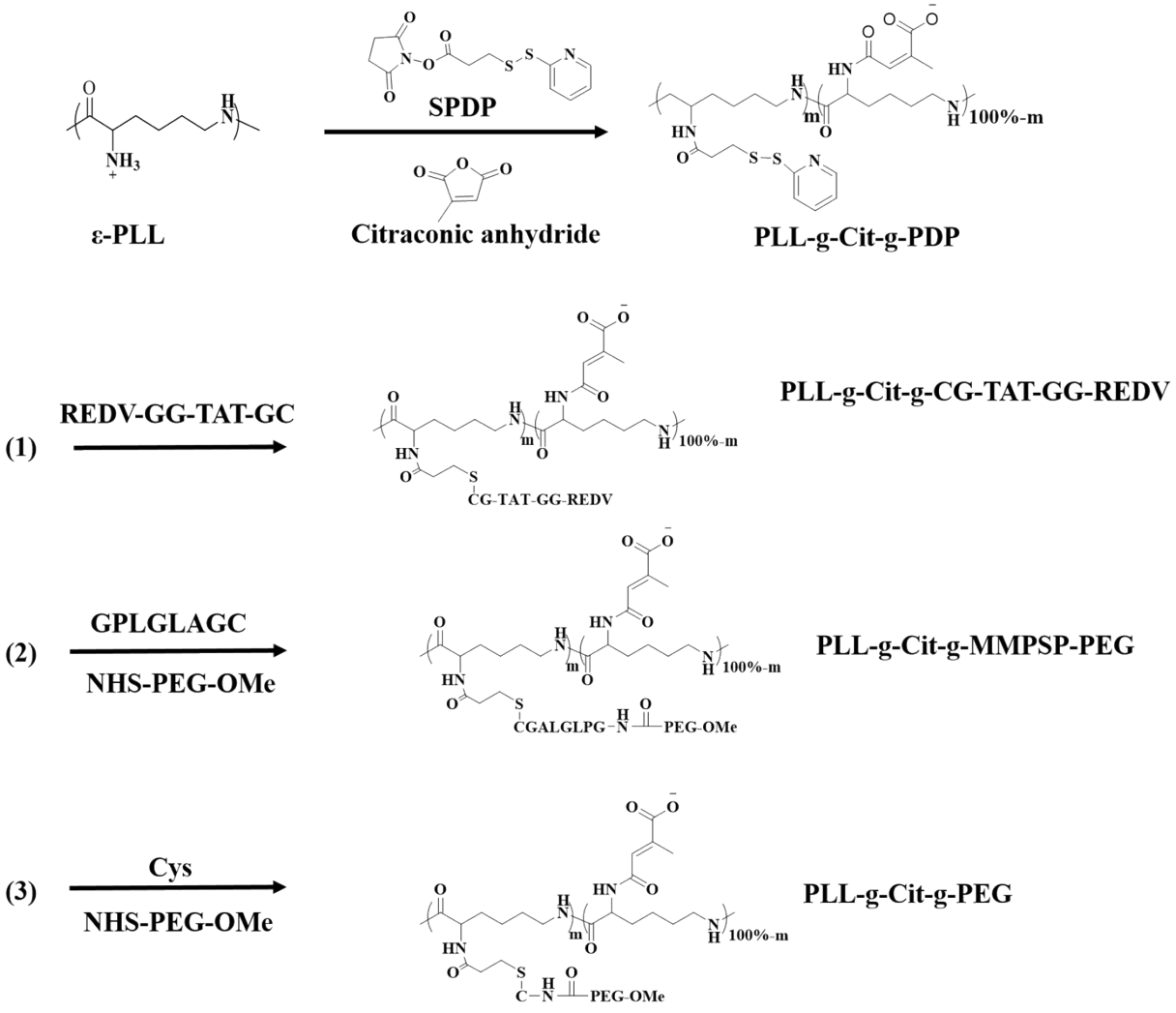


Fig. S5. Synthesis pathway of PLL-g-Cit-g-PDP, PLL-g-Cit-g-CG-TAT-GG-REDV, PLL-g-Cit-g-

MMPSP-PEG and PLL-g-Cit-g-PEG.

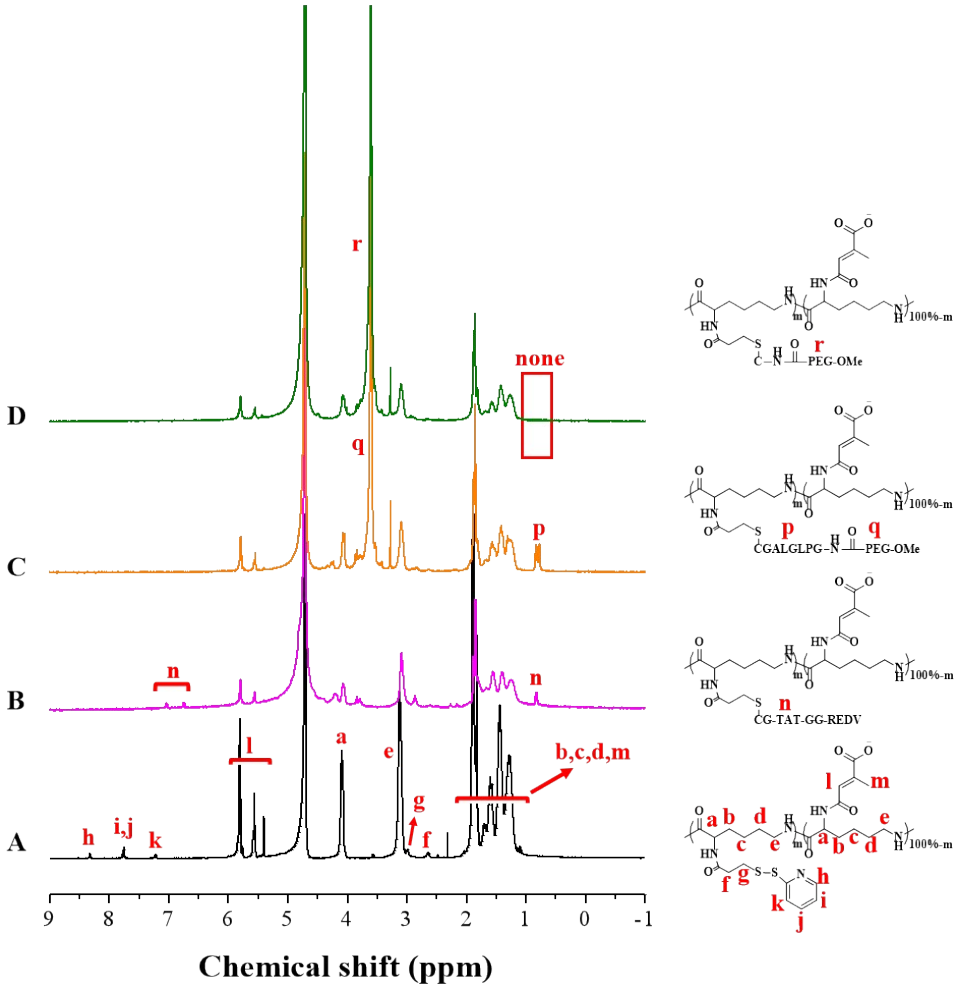


Fig. S6. ^{1}H NMR spectra of PLL-g-Cit-g-PDP, PLL-g-Cit-g-CG-TAT-GG-REDV, PLL-g-Cit-g-MMPSP-PEG and PLL-g-Cit-g-PEG using deuterated water as solvent.

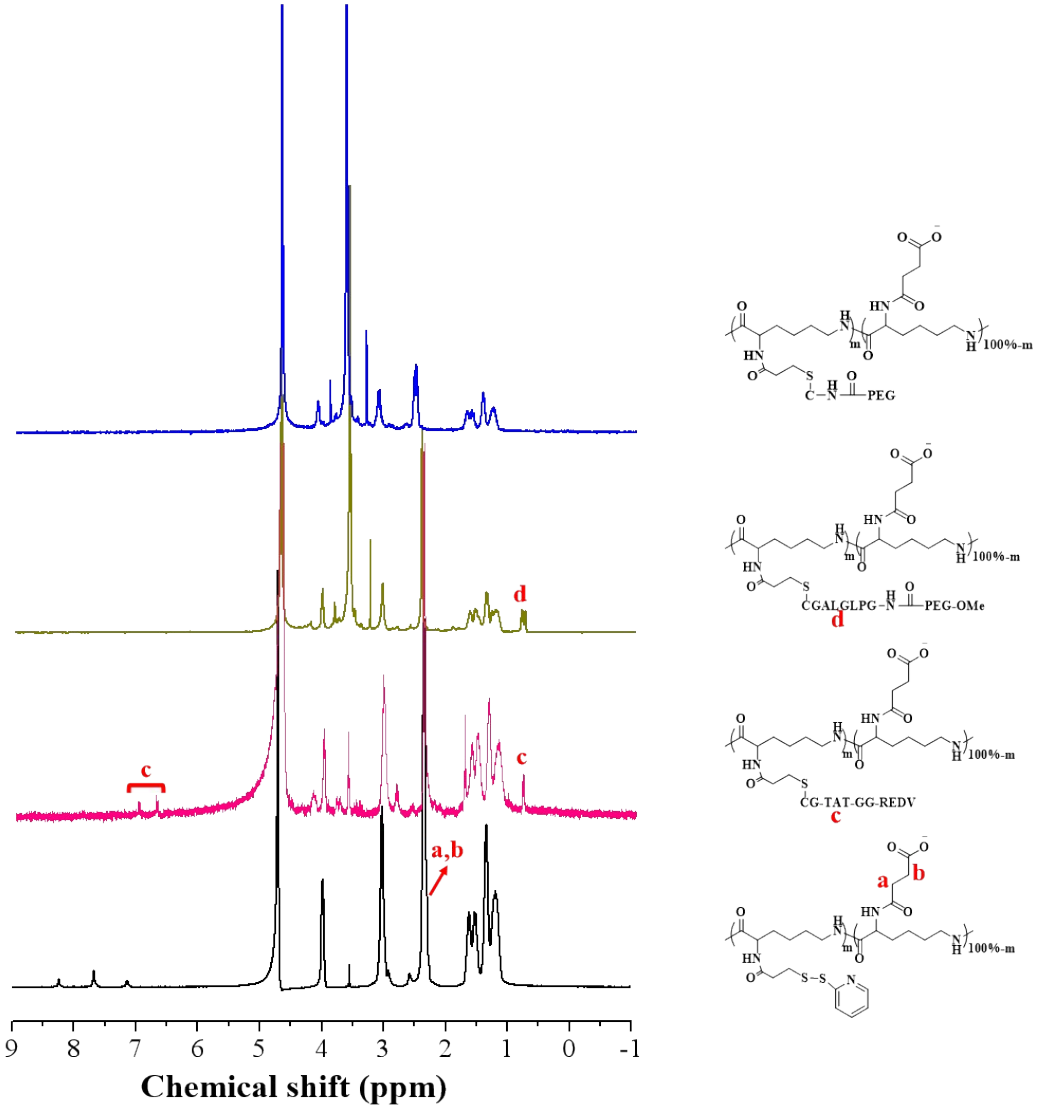
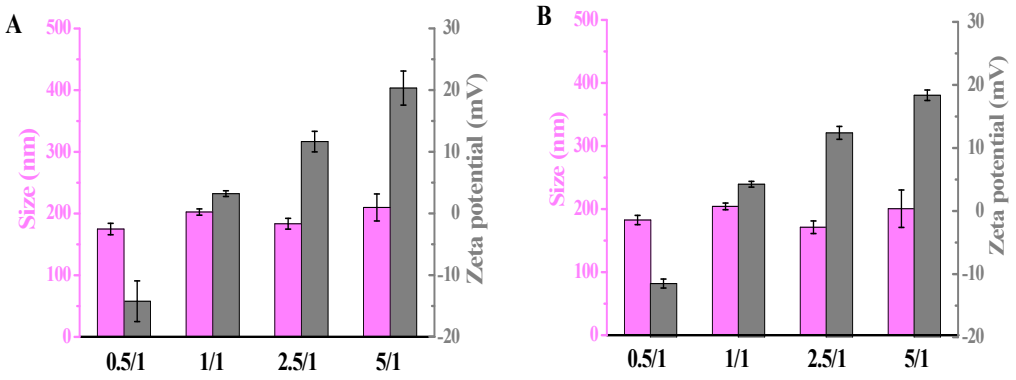


Fig. S7. ^{1}H NMR spectra of PLL-g-Suc-g-PDP, PLL-g-Suc-g-CG-TAT-GG-REDV, PLL-g-Suc-g-MMPSP-PEG and PLL-g-Suc-g-PEG using deuterated water as solvent (**).



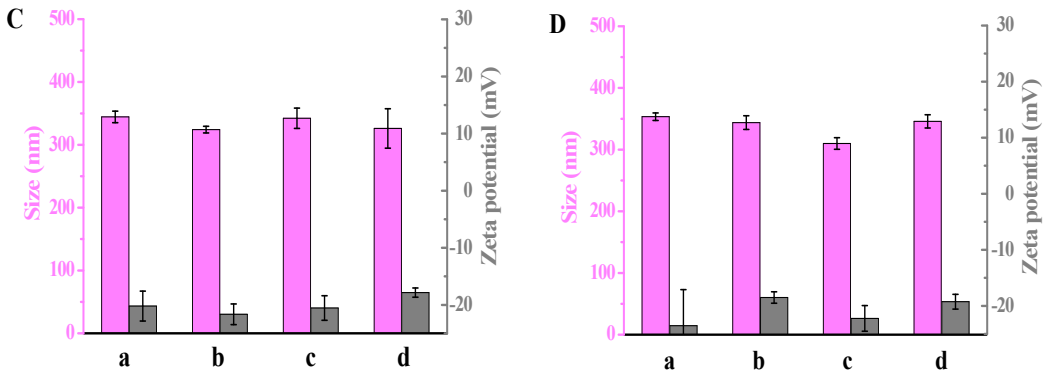


Fig. S8. The size and zeta potential of binary gene complexes loaded (A) ZNF_{580}, (B) eNOS, and ternary gene complexes loaded (C) ZNF_{580}, (D) eNOS with the w/w/w of PLL-DSP/pDNA/outer layers = 2.5/1/5. (a) ds-CPs, (b) as-CPs, (c) es-CPs, (d) is-CPs.

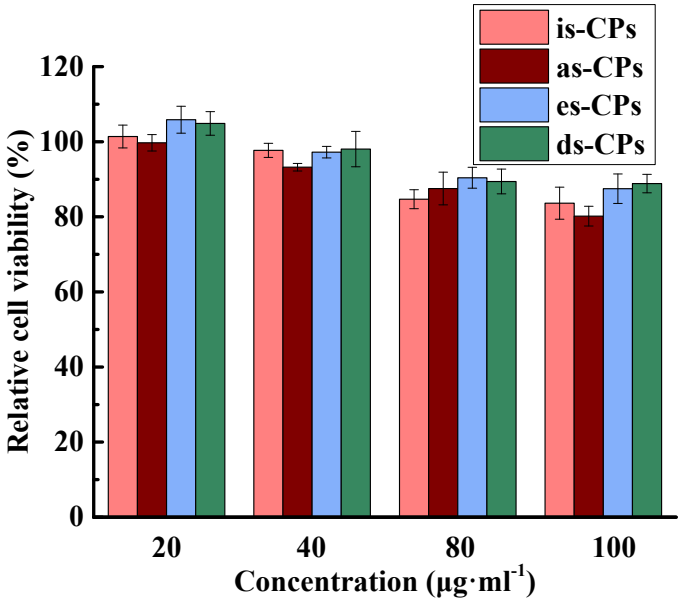


Fig. S9. Relative cell viability of HUVECs treated with different concentrations of nanocomplexes and incubated for 24 h. Data were shown as mean ± SD (n = 3).

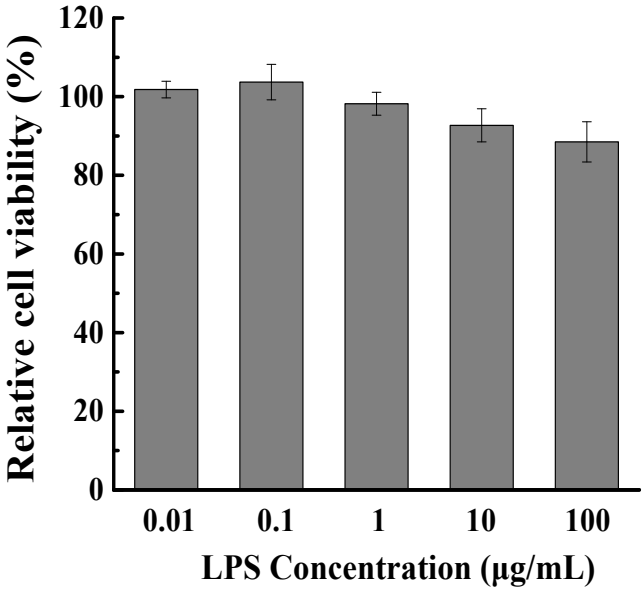


Fig. S10. Relative cell viability of HUVECs treated with different concentrations of LPS for 4 h. Data were shown as mean ± SD (n = 3).

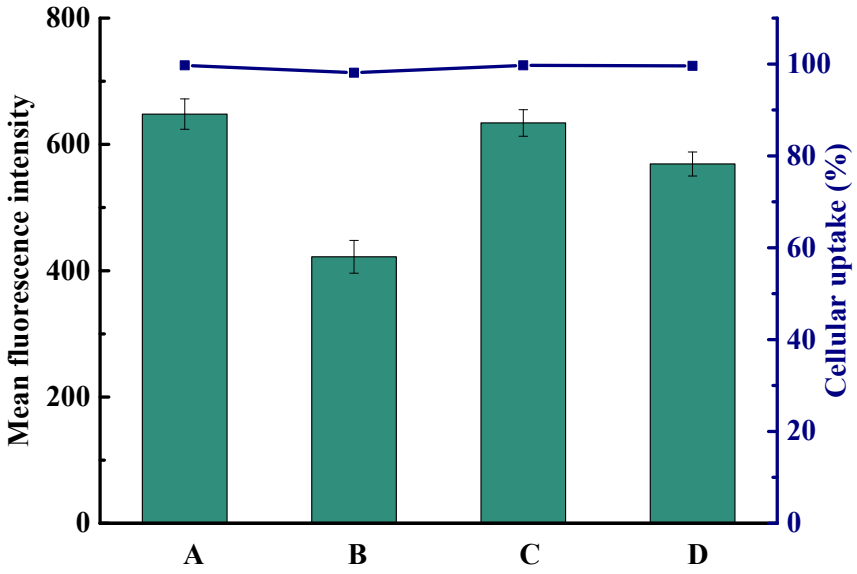


Fig. S11. The flow cytometry to investigate the cellular uptake and mean fluorescence intensity. (A) PLL-DSP/Cy5-oligonucleotide treat HUVECs without LPS activation, (B) ds-CPs treated HUVECs without LPS activation, (C) ds-CPs treated HUVECs with MMP-2 incubation and unactivated by LPS, (D) ds-CPs treated with HUVECs with LPS activation. Data were shown as mean ± SD (n = 3).

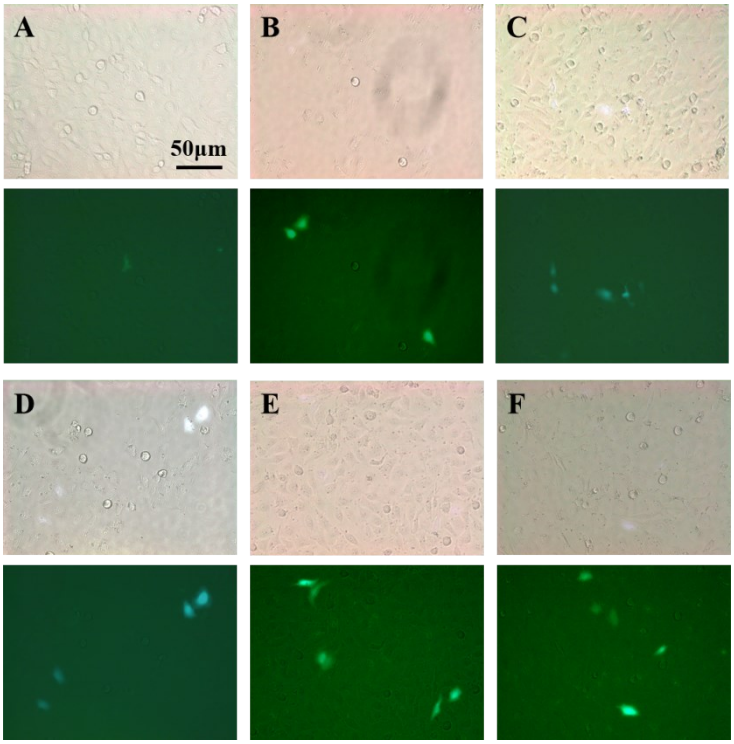


Fig. S12. Fluorescence images of HUVECs transfected for 24 h by (A) $pZNF_{580}$ as a control group, (B) is-CPs, (C) as-CPs, (D) es-CPs, (E) ds-CPs and (F) lipo3000 containing $pZNF_{580}$.

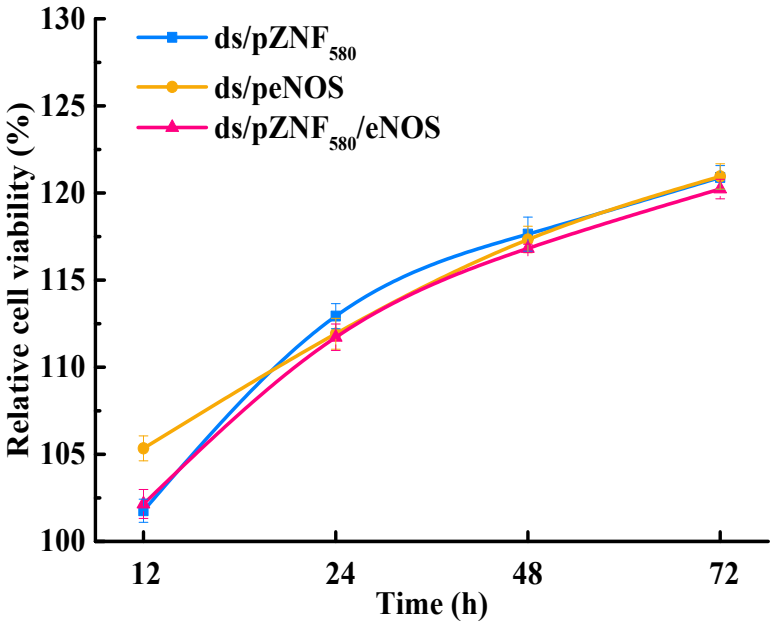


Fig. S13. Relative cell viability of HUVECs treated with ds/$pZNF_{580}$, ds/peNOS, and ds /$pZNF_{580}$/peNOS at different time points. Data were shown as mean ± SD (n = 3).

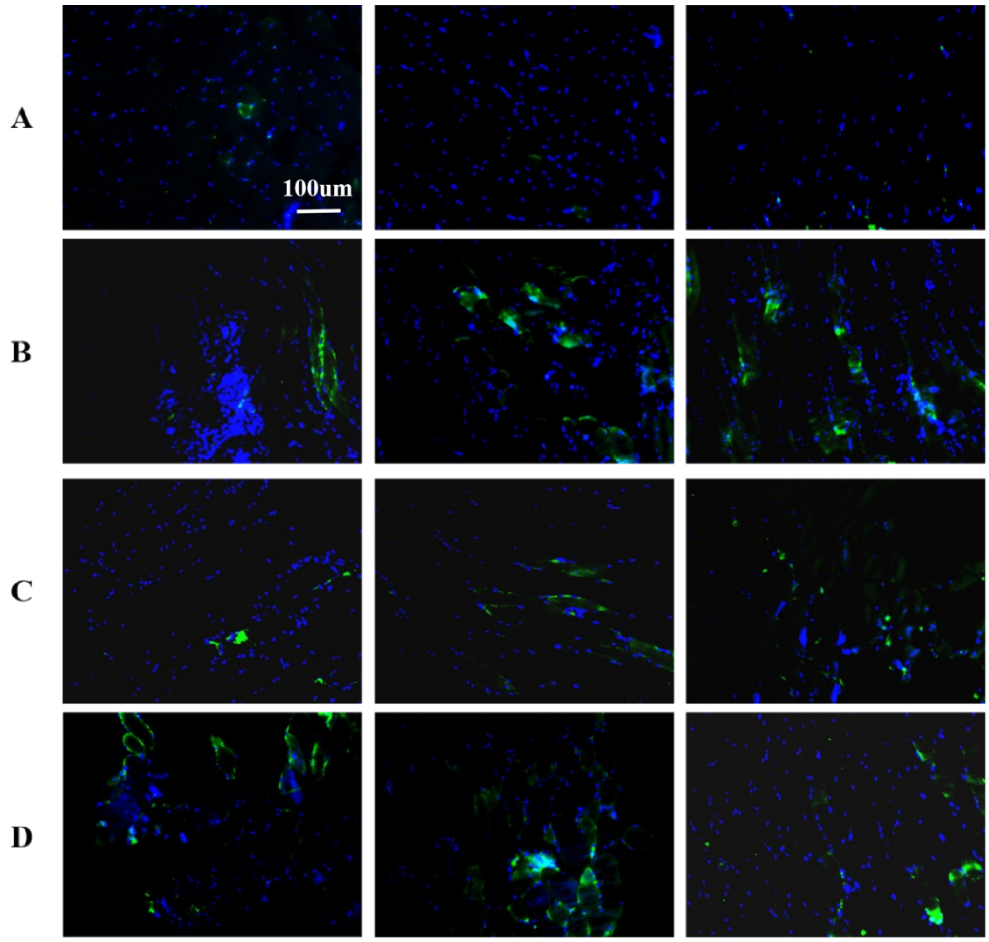


Fig. S14. CD34 staining of endothelial cells in ischemic tissue after the treatment with different genes. (A) PBS as a control group, (B) ds/pZNF$_{580}$, (C) ds/peNOS and (D) ds/pZNF$_{580}$/peNOS. Data were shown as mean ± SD (n = 3).

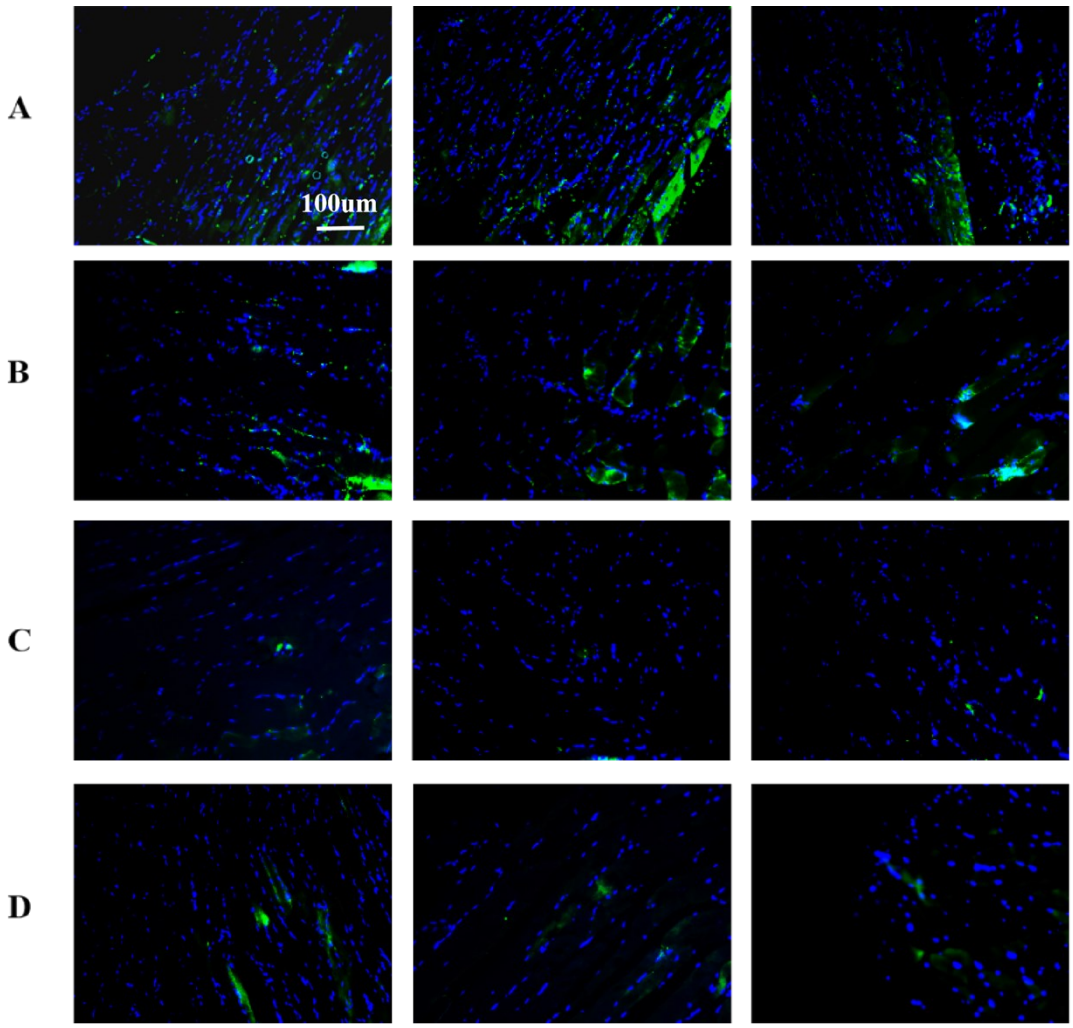


Fig. S15. CD68 staining of inflammatory cells in ischemic tissue after the treatment with different genes. (A) PBS as a control group, (B) ds/pZNF$_{580}$, (C) ds/peNOS and (D) ds/pZNF$_{580}$/peNOS. Data were shown as mean ± SD (n = 3).