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Supporting information

Agmatine-grafted bioreducible poly(L-lysine) for gene delivery with low cytotoxicity and high efficiency

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1. Experiments Section

1.1 Materials

L-lysine monohydrogen chloride (L-lysine HCl) and N-boc-butylenediamine (BDA) were purchased from TCI, Tokyo Chemicals Industry Japan. Cystaminebisacrylamide (CBA), agmatine sulfate salt, trifluoroacetic acid (TFA) and dithiothreitol (DTT) were purchased from Sigma-Aldrich, Beijing, China. Fresh human whole blood was obtained legally from a healthy human volunteer. Human umbilical vein endothelial cells (HUVECs) were obtained from the Cell Bank of Typical Culture Collection of Chinese Academy of Sciences (Shanghai, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide (MTT) and cell counting kit-8 (CCK-8) were purchased from Solarbio Co., Ltd (Shanghai, China). The pEGFP-ZNF580 plasmid (pZNF580) was purchased from Abcam (HK) Ltd (Hong Kong, China). A Cy5-labeled oligonucleotide (Cy5-oligonucleotide) was purchased from Sangon Biotech (Shanghai) Co. Ltd (Shanghai, China). LysoTracker Green DND-26 and Hoechst 33342 dyes were purchased from Shanghai Invitrogen Biotechnology Co., Ltd (Shanghai, China). Chlorpromazine and Amiloride hydrochloride were purchased from Sigma-Aldrich (St. Louis, USA). Filipin III was purchased from Cayman Chemical (Michigan, USA). Transwell chambers and Matrigel (Cat. Nos. 356234) were obtained from Corning Incorporated (New York, USA). All other chemicals and solvents were used from Sigma-Aldrich.

1.2 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to investigate the DNA condensation ability of NPs. Briefly, the gene complexes with various w/w ratios ranging from 1/1 to 20/1 were prepared and incubated for 30 min at room temperature. The mixed solution was loaded into the agarose gel (0.8%) containing 5 μL of GoldView™ in 1 × TAE buffer at 100 V for 30 min. The pDNA retardation was recorded using a UV illuminator Gel Documentation Systems (Bio-Rad, Hercules, CA) at 254 nm. To investigate the cleavage of disulfide bonds, polyplexes were incubated in 5 mM DTT for 1 h at 37 ℃ and then electrophoresed as described above.

A DNase I protection assay was performed to investigate the ability of SSL-AG to protect pZNF580 from DNase I degradation. pZNF580 complexes at weight ratio 5/1 containing 1 μg pZNF580 were prepared and then electrophoresed as described above.

1.3 Biological assays

The hemolysis experiments were conducted in accordance with ISO 10993-4:2009. In vitro cell assays were carried out in the HUVECs cells. HUVECs cells were cultured in high glucose DMEM supplemented with FBS (10%) in a humidified incubator at 37 ℃ with 5% CO_2 atmosphere, until 70-90% confluence was achieved.

1.3.1 Hemolytic test

1 mL of human blood was freshly obtained from a healthy human donor in Hospital of the Armed Police. A total of 200 μl of diluted human blood was added to 800 μL of nanoparticles solution, and the mixture was incubated at 37 ℃ for 2 h. 200 μL of diluted human blood incubated with 800 μL of PBS or water was used as the negative or positive control, respectively. Red blood cell aggregation was detected through phase contrast microscope (DMI400B, Leica, Germany) at 100 × magnification. The absorbance of liquid at a wavelength of 543 nm was recorded on a Synergy HT Multi-Mode Microplate Reader (BioTek, USA). The percent of hemolysis was calculated by the following formula.

$$R = \frac{C - C_0}{C_1 - C_0} \times 100\%$$

where R is the hemolysis rate (%), C is the absorbance of the sample (%), C_0 is the absorbance of the negative control (%), and C_1 is the absorbance of the positive control (%).

1.3.2 Cell viability assay

The cytotoxicity of PLL, SSL-BDA, SSL-AG and their gene complexes for HUVECs were evaluated by MTT assay. PEI25kDa and Lipofectamine™ 3000 was used as control groups. Briefly, HUVECs cells (1×10^4 cells per well) were seeded in 96-well plates and incubated for 24 h. Then the freshly prepared polymers and their complexes with concentrations of 10, 20, 40, 60, 80 and 100 μg/mL were added into the medium. After additional incubation for 48 h, 20 μL of stock solution of MTT (5 mg/mL in PBS) was added to each well. After 4 h of incubation, the medium was changed with 150 μL DMSO to dissolve the formazan crystals formed by live cells. The absorbance of the solution was measured at 490 nm using a microplate reader (Bio-Rad, IMARKTM). The cell viability was expressed as the percentage of viability relative to control group (non-transfected HUVECs cells).

1.3.3 Cellular uptake and transmembrane mechanism

The cellular uptake and mean fuorescence intensity (MFI) in HUVECs were quantitatively evaluated by a flow cytometry. Cells were seeded into 6-well plates (3×10^5 cells/well) and transfected with different Cy5-oligonucleotide complexes (1 μg Cy5-oligonucleotide for each well, w/w ratio = 5/1). After 4 h incubation, cells were washed three times with 0.01 M PBS (pH = 7.4) and trypsinized with 0.25% trypsin. Then, the cells were centrifuged, collected and re-suspended in 300 μL PBS (pH = 7.4), followed by analyzing with a flow cytometer (Beckman MoFlo XDP, USA).

To further investigate the transmembrane mechanism of SSL-BDA/Cy5-oligonucleotide and SSL-AG/Cy5-oligonucleotide complexes, HUVECs were pretreated with different endocytosis inhibitors for 1 h before transfection. Chlorpromazine (CPZ, 30 μM), Amiloride hydrochloride (Amil, 50 μM) and Filipin Ⅲ (Filip, 5 μg/mL) were used to inhibit clathrin-mediated endocytosis, macropinocytosis and caveolae-mediated endocytosis, respectively. After 4 h incubation with Cy5-oligonucleotide complexes, the cellular uptake was analyzed as described above.

1.3.4 Intracellular distribution study

Confocal laser scanning microscopy (CLSM) was performed to investigate the intracellular distribution of SSL-AG/Cy5-oligonucleotide complexes after internalization. The cells were seeded at 1×10^5 cells per well into a confocal dish and cultured for 4 and 24 h. After transfection, the cells were washed twice with PBS (pH = 7.4) to remove residual complexes. Subsequently, the cells were treated with LysoTracker Green dye (0.5 mM) to mark endosomes and Hoechst 33342 (2 μg/mL) to mark nuclei. The intracellular localization of complexes was observed by a confocal scanning laser microscope (CSLM; Olympus FV1000, Japan) at 649 nm, 504 nm and 350 nm excitation wavelengths for Cy5 (red), LysoTracker (green) and Hoechst 33342

(blue), respectively. The co-localization ratio (CLR) was calculated by Image-Pro Plus 6.0 software according to the following equation.

$$Co - localization\,ratio = \frac{Number\ of\ yellow\ or\ pink\ pixels}{Number\ of\ yellow, red\ and\ pink\ pixels} \times 100\%$$

1.3.5 In vitro transfection

The transfection efficiency of SSL-AG/pZNF580 complexes was evaluated by HUVECs according to previous studies. In brief, HUVECs cells were seeded in a 24-well plate at a density of 1×10^4 cell per well and cultured for 24 hours until 70–80% confluence. Before transfection, cells were starved using the serum-free medium for 12 hours. Gene complexes (w/w ratio = 5) (4 μg pZNF580 per well) were added into the wells. After 4 hours, the medium was replaced by a fresh growth medium (10% FBS DMEM). The green fluorescence protein (GFP) in cells was observed and imaged after 24 h of incubation under an inverted fluorescence microscope (ECLIPSE Ti-U, Nikon, Japan).

1.3.6 Quantitative real-time PCR and Western blot assay.

The gene expression efficiency of HUVECs was evaluated at mRNA and protein levels by RT-PCR and Western blot assays.

Total RNA was extracted from transfected cells using TRIzol reagent and then reverse-transcribed into cDNA using the HiFiScript cDNA Synthesis Kit. The resulting cDNAs as templates were quantified via Ultra SYBR Mixture (low ROX) on 7500 Real Time PCR system according to the manufacturer's instructions. GAPDH was used as the reference gene for normalization of target genes. The forward and reverse primer sequences were 5'-GAGGTTACTGCCTTACCCTGG-3' and 5'-ACCCAGTTCCGACTGGTTC-3' for ZNF580, 5'-AGGTGAAGGTCGGAGTCAAC-3' and 5'-CGCTCCTGGAAGATGGTGAT-3' for GAPDH, respectively.

Total protein was extracted from transfected cells using RIPA lysis buffer containing 1% volume of PMSF and quantified by BCA protein assay kit. Before measuring, the protein was denatured by adding 6×SDS. All samples were separated by SDS-polyacrylamide gel electrophoresis and subjected to western blot. Membranes were incubated with rabbit anti-ZNF580 polyclonal antibody in TBST overnight and horseradish peroxidase conjugated anti-rabbit secondary antibody for 1 h. Membranes were scanned with the Tanon-5200 automatic chemiluminescence image analysis system and analyzed by Image J software. All results are expressed as the means ± SD of three independent experiments.

1.3.7 Cell proliferation assay

A CCK-8 assay was performed to evaluate the proliferation of transfected HUVECs. After transfection for 12, 24, 48 and 72 h, cell culture medium was removed and 100 μL of free serum medium containing 10% Cell Counting Kit-8 assay (CCK-8) was added to each well of a 96-well plate. After incubation for another 4 h, the absorbance was measured at a wavelength of 450 nm.

1.3.8 Cell migration assay

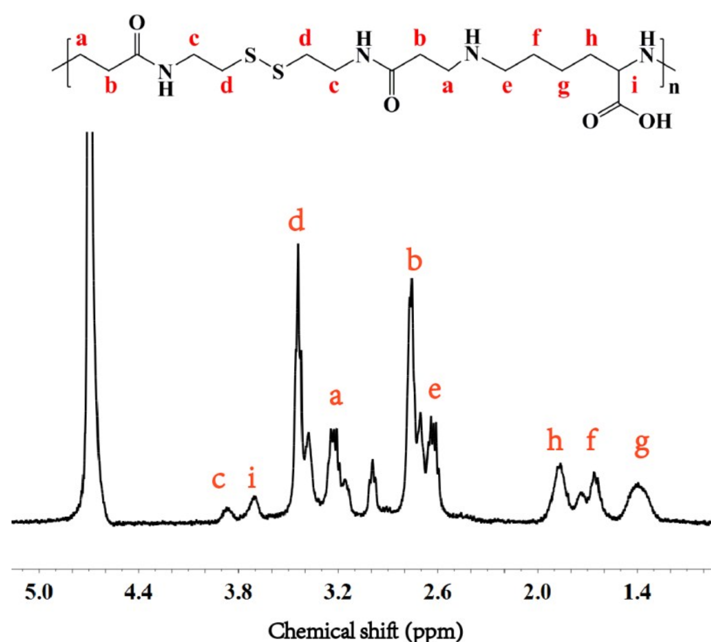
To evaluate the migration capability of the transfected HUVECs by different complexes, the wound healing assay and transwell migration assay were performed. For wound healing assay, the transfected cells were grown on 6-well dish plates to a 100% confluent monolayer and then scratched to form a “wound” using sterile 200 μL pipet tips. Cellular debris was removed by washing with D-hanks buffer (pH = 7.4). The images were recorded at 10 h and 20 h after the scratch by using an inverted microscope; the migration area was calculated using Image J 2.1 based on the images after 20 h. The wounded area was calculated by the following formula.

$$Relative\ migration\ area\ (\%) = \frac{wounded\ area\ -\ nonrecovered\ area}{wounded\ area} \times 100\%$$

For transwell migration assay, the transfected cells were seeded into the upper chambers of inserts with a density of 1×10^5 cells per well. After 6 h of incubation, the migrated cells across the transwell were stained with violet crystal, photographed and counted to calculate the migration rate.

1.3.9 In vitro tube formation assay

The formation ability of capillary-like structures was detected in a 96-well plate using growth factor-reduced Matrigel according to the Corning Matrigel Matrix instructions. HUVECs transfected with various gene complexes (4×10^4 cells per well) were plated onto Matrigel (50 μL per well) and cultured for another 6 h. HUVECs treated with pZNF580 were used as a control. The tubule-like structures were observed using an optical microscope and the number was quantified using Image J software.



FigS1. ^{1}H NMR spectra of poly (disulfide-L-lysine).

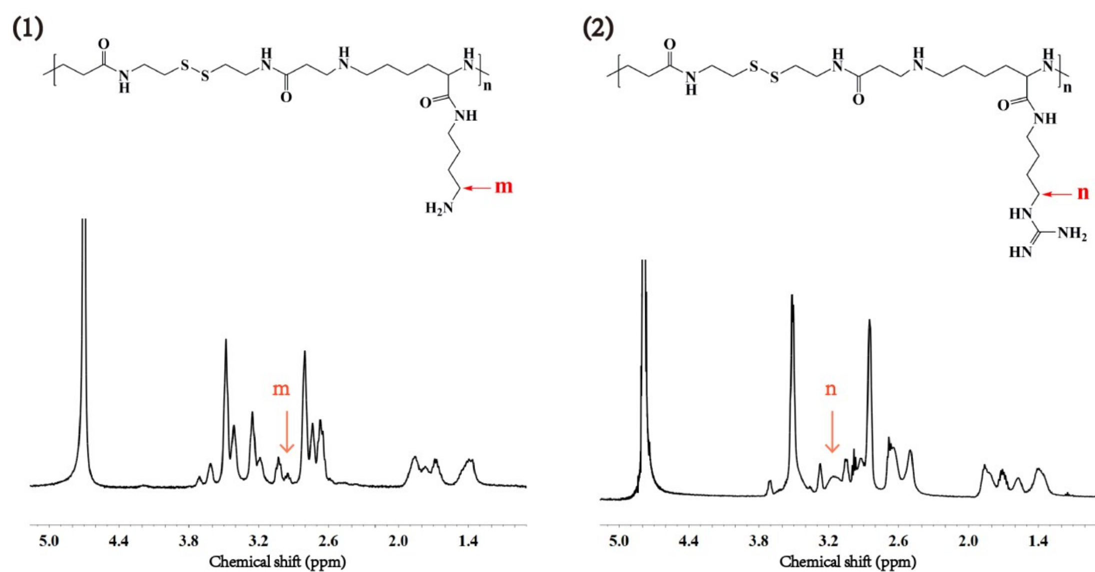


Fig.S2 ¹H NMR spectra of (1) poly(disulfide-L-lysine)-g-butylenediamine (SSL-BDA) and (2) poly (disulfide-L-lysine)-g-agmatine (SSL-AG).

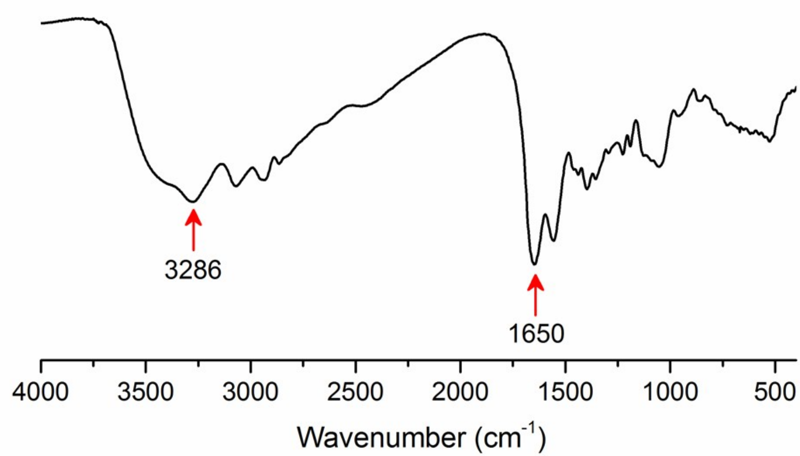


Fig.S3 FTIR spectra of poly (disulfide-L-lysine)-g-agmatine (SSL-AG).

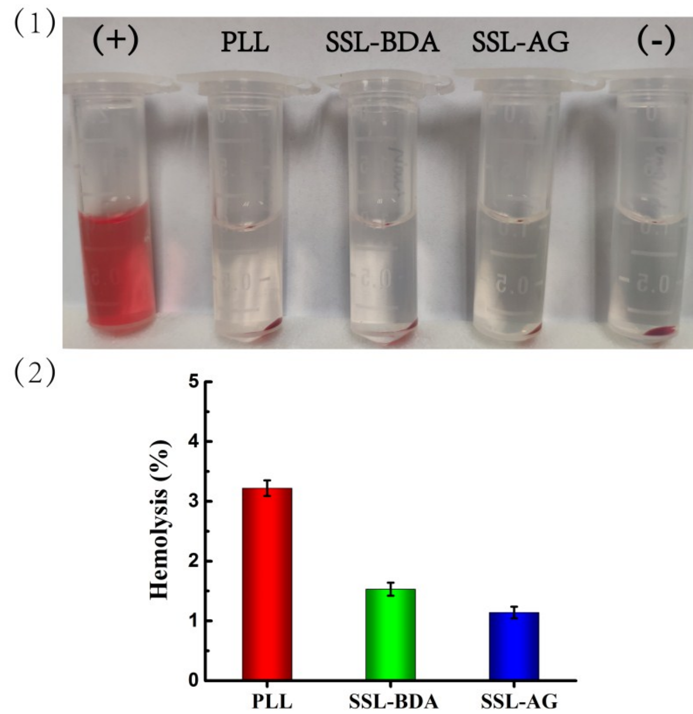


Fig. S4 Visual observation of hemolysis (1) and the hemolysis rates (2) by using double-distilled water (denoted as +) and phosphate buffered saline (PBS, pH = 7.4) (denoted as -) groups as controls. Human whole blood containing 15 mg/mL PLL, SSL-BDA and SSL-AG was separately incubated at 37 ℃ for 120 min and then centrifuged at 3000 rpm for 10 min.

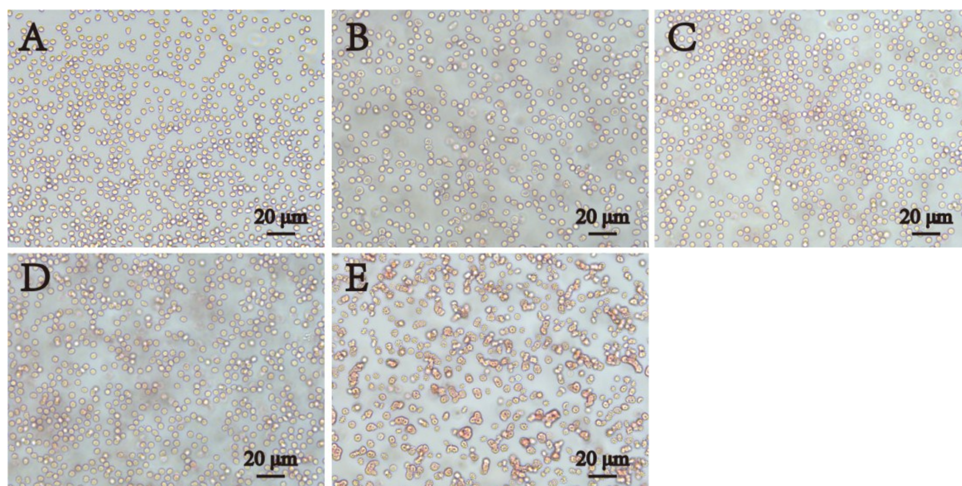


Fig. S5 Microscope images of erythrocytes after incubation with (A) PBS (pH = 7.4), (B) PLL/pZNF580 (w/w ratio = 5/1), (C) SSL-BDA/pZNF580 (w/w ratio = 5/1), (D) SSL-AG/pZNF580 (w/w ratio = 5/1) and (E) PEI25kDa/pZNF580 (w/w ratio = 1/1).

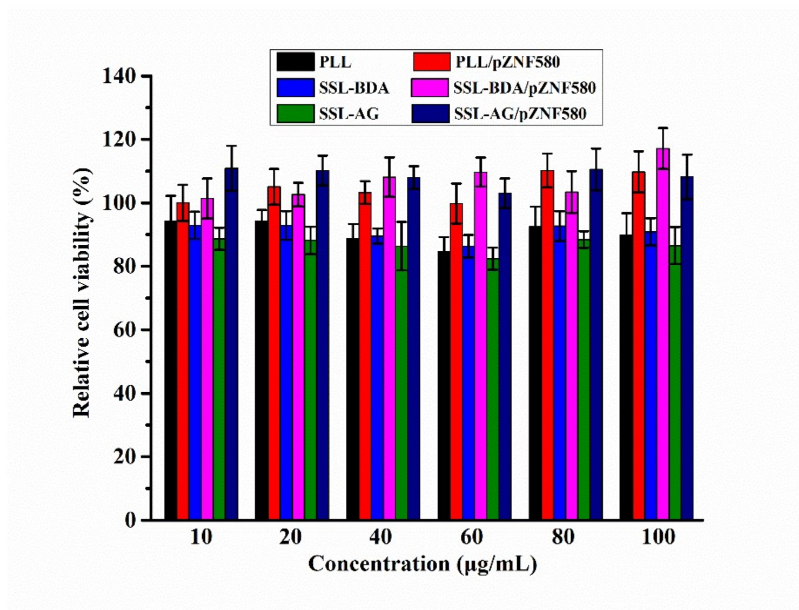


Fig. S6 Relative cell viability of HUVECs at 24 h after treated with PLL, SSL-BDA, SSL-AG and their complexes at w/w ratio of 5/1. The concentration represents the polymer and complex concentration.

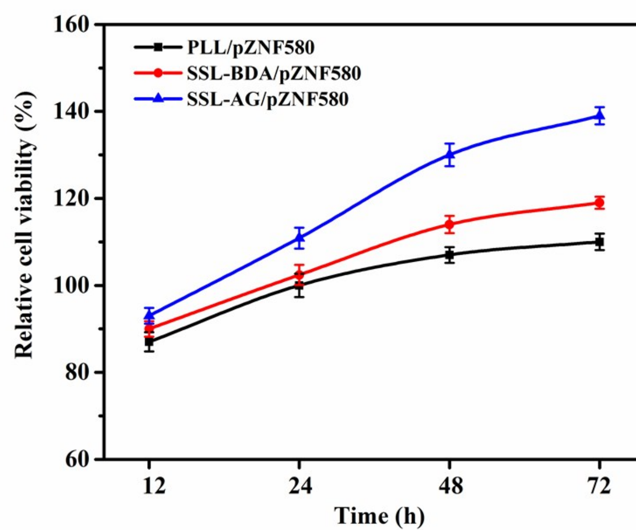


Fig. S7 Relative viability of HUVECs treated with PLL/pZNF580, SSL-BDA/pZNF580, and SSL-AG/pZNF580 at w/w ratio of 5/1 at different time points (mean ± SD, n = 3)