Electronic supplementary information

Stiffness of cationized gelatin nanoparticles is a key factor determining RNAi efficiency in myeloid leukemia cells

Weiqi Zhang[1,2,#], Bo Han[1,#], Xinning Lai[1], Chen Xiao[3], Shilin Xu[1], Xianghui Meng[1], Zifu Li[3,4,5], Jie Meng[1], Tao Wen[1], Xiangliang Yang[3,4,5], Jian Liu[1*], Haiyan Xu[1*]

[1]Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100005, P. R. China
[2]State Key Laboratory of Medical Molecular Biology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100005, P. R. China
[3]Department of Nanomedicine and Biopharmaceuticals, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, 430074, China
[4] National Engineering Research Center for Nanomedicine, Huazhong University of Science and Technology, Wuhan, 430074, China
[5] Hubei Key Laboratory of Bioinorganic Chemistry and Materia Medica, Huazhong University of Science and Technology, Wuhan, 430074, China

[#] These authors contributed equally to this work.

[*] Corresponding authors: H Xu, xuhy@pumc.edu.cn; J Liu, liujian@ibms.pumc.edu.cn

Material and methods

Materials

Gelatin from porcine skin (type A, bloom 300), 1,2-Ethanediamine (hydrochloride) and glutaraldehyde were purchased from VETEC (St. Louis, Missouri, USA), Aladdin (Shanghai, China) and Macklin (Shanghai, China), respectively. CXCR4 siRNA, BCR-ABL siRNA and NC siRNA-FAM were synthesized by Gene pharma (Shanghai, China). Sequences are shown as follow: Noncoding (NC)-siRNA-FAM: sense sequence 5'-UUC UCC GAA CGU GUC ACG UTT-3', antisense 5'-ACG UGA CAC GUU CGG AGA ATT-3'; BCR-ABL siRNA: 5'-GCA GAG UUC AAA AGC CCU UdTdT-3', 5'-AAG GGC UUU UGA ACU CUG CdTdT-3'. PCR primers were synthesized by Sango (Shanghai, China). Fetal Bovine Serum (FBS) was purchased from Thermo Fisher Scientific (Massachusetts, USA). Penicillin-streptomycin antibiotic and RPMI 1640 medium were obtained from Hyclone (Los Angeles, USA).

Preparation of cationized gelatin

Eight gram of gelatin and 49.5879 g 1,2-Ethanediamine (hydrochloride) were dissolved in 200 mL PB solution (pH 5.0), respectively. After mixing these two solutions, the pH was adjusted to 5.0 by adding NaOH. Further, 4.28g EDC was added into the solution to catalyze the gelatin to react with ethylenediamine, which convert the carboxyl groups to the amino groups. The reaction mixture was agitated at 40 °C for 18 h, and then dialyzed against ddH_2O for 72 h at room temperature. After the dialysis, cationized gelatin was freeze-dried.

Preparation of cationized gelatin nanoparticles (CGNP)

The 3.5 %, 3 %, 2.5 % and 2 % cationic gelatin solutions were stirred at 40 °C for 2h to allow full dissolution. After filtering, the pH of the solution was adjusted to 2.5 using HCI. Then 3.6-folds of acetone (v/v) was added dropwisely at a speed of 1mL/min with continuous stirring. Glutaraldehyde (25%) was utilized to crosslink the cationized gelatin for 24 h at 40 °C. The mass ratio of cationic gelatin (Gel) to Glutaraldehyde (GA) was controlled at 40:1, 30:1, 20:1, 10:1, 5:1, respectively. Next, the mixture was centrifuged at 14,000 rpm for 12 min. The sediment was resuspended in 0.1M glycine solution for 12 h

to terminate the reaction. Finally, redundant glutaraldehyde was removed. Four CGNPs of similar size but with synthesis condition of 3.5% Gel and Gel:GA=5:1; 3% Gel and Gel:GA =10:1; 2.5% Gel and Gel:GA=20:1; 2% Gel and Gel:GA=30:1 were named as CGNP-1, CGNP-2, CGNP-3 and CGNP-4, respectively.

Characterization of CGNP

CGNP-1 to CGNP-4 were diluted to 100 μg/mL. Dynamic light scattering analysis (DLS) was employed to measure the diameter and zeta potential (Nano ZS, Malvern, Worcestershire, UK). To evaluate the size of CGNP in presence of FBS, CGNP was incubated in 10% FBS, a serum concentration equal to cell culture condition, for 4 h before the DLS measurements. The size and morphology were observed by scanning electron microscopy (SEM) (SU-8010, Hitachi, Japan) and Atomic Force Microscope (AFM). AFM images and force-distance curves were performed on a Multi-Mode 8 AFM (Bruker, Santa Barbara, USA) with clean coverslips in water. All measurements were carried out under the same ScanAsyst-Fluid probe. The AFM, with 10 μm piezoelectric scanner, was set to ScanAsys-Fluid mode to obtain images. Based on the images, the force-distance curves of selected nanoparticles were obtained subsequently and 8-50 shooting target measurements were performed. The Young's modulus of samples was calculated according to the Hertz model:

$$F = \frac{4}{3}\frac{E}{(1-\nu^2)}\sqrt{R}\delta^{3/2}$$

where F is force from force curve, E is Young's modulus, ν is Poisson's ratio (sample dependent, with a value of 0.3 in this study), R is the radius of the indenter tip (with a value of 20 nm in this study), δ is the indentation depth. The original force curves were analyzed using NanoScope Analysis software (version 1.5).

Gel electrophoresis

Appropriate CGNPs were mixed with 0.5 μg of siRNA to obtain a mass ratio of 10:1, 15:1, 20:1, 25:1, and 30:1. The mixture was then incubated at room temperature for 30 min. The complex was mixed with 2 μL of loading buffer and the total volume was adjusted to 20 μL. The 10 μL mixture was pipetted into the loading wells of 1% agarose gel and

electrophoresed (150 V, 20min) in 1×TAE running buffer. The gel was then visualized with a UV transilluminator (Binda, Japan). To probe the siRNA integrity, 3 μL of NC siRNA-FAM or its' complexes with different CGNPs (mass ratio of 30:1, 0.3 μg siRNA) was incubated with 7 μL of FBS or RNAase-free water and incubated at 37 °C for 4 h, then the siRNA samples were electrophoresed in 1% agarose gel at 150 V for 15 min. FBS only was included as control. The FAM fluorescence in the gel was imaged using a Tanon 4800Multi imaging system (Tanon Science & Technology, Shanghai, China).

Cell culture

Human chronic myeloid leukemia K562 cells was purchased from the Cell Resource Center of Chinese Academy of Medical Sciences & Peking Union Medical College (Beijing, China). Cells were cultured in RPMI 1640 medium (HyClone) containing 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells in dishes and well plates were maintained at 37 °C in a humidified incubator with 5% CO_2.

CCK8 cytotoxicity test

CCK8 (Dojindo Molecular Technologies, Kumamoto, Japan) assay kit was used to evaluate the cell viability of CGNPs. Before adding CCK8, K562 cells were incubated with OPTI-MEM containing CGNP-1 to CGNP4 in 96 well plates. CGNPs in the culture medium were varied from 0 to 20 μg/mL. After 4 hours incubation, cells were washed and cultured in fresh medium for another 20 hours. Then the cells were and incubated with CCK8 working solution for 2 h (1640 RPMI basic medium: CCK8=10:1). The absorbance at 450 nm of the supernatant was detected using a 96-well plate reader (BioTek Synergy 4，BioTek Instruments, USA).

Flow cytometry (FACS) analysis

Each type of CGNP was mixed with NC siRNA-FAM as prepared above to obtain a Gel:siRNA ratio of 30. The K562 cells were incubated with CGNPs and siRNA-FAM complex in OPTI-MEM medium for 4 h (5, 10, 15 μg/mL CGNP). Then cells were washed and collected by centrifugation at 1200 rpm for 5 min and resuspended in PBS. Half of the collected cells were treated with 5 μL of trypan blue (100 μg/mL) for 4 min to quench the

siRNA-FAM fluorescence out of the cells. After sifting all cells, the fluorescence was measured by flow cytometry analysis (BD Accuri C6, USA) in FL1 Channel.

Quantitative real time PCR analysis

The complex of CGNPs and siRNA was prepared as described above (Gel:siRNA ratio of 30). K562 cells were treated with 15 μg/mL CGNP1 to CGNP4 in OPTI-MEM medium for 4 h. Cells were washed with sterile PBS. Complete RPMI 1640 medium was added 20 h. Total RNA was extracted from K562 cells using Trizol reagent (Invitrogen, USA). PrimeScript RT reagent (Takara, Shiga, Japan) was used to synthesize cDNA. Real time-PCR was performed using BioRad CFX96 with SYBR Green Premix Ex Taq (Takara, Shiga, Japan) as indicated. Primer Sequences were used as below:

Human BCR-ABL sense: CAT TCC GCT GAC CAT CAA TAA G

Antisense: GAT GCT ACT GGC CGC TGA AG

Human GAPDH sense: GGT CAC CAG GGC TGC TTT TA

Antisense: GAG GGA TCT CGC TCC TGG A

Western Blot

K562 cells were treated as indicated above. After 4-hours treatment by CGNPs (15 μg/mL) either complexed with BCR-ABL or NC siRNA, the cells were further incubated in fresh medium for 20 hours. Then the total protein was extracted and separated using the SDS-PAGE. Following the transfer of the protein to the nitrocellulose membrane, the sample was detected using the BCR-ABL antibody (CST, #2862) as suggested by the manufacturer. Actin was analyzed using β-Actin (8H10D10) mouse mAb (CST, #3700) as an inner control.

Statistic analysis

The statistic difference was analyzed by two-tailed student's t-test (paired *t*-test). *$P<0.05$ and **$P<0.01$ were regarded as significantly different.

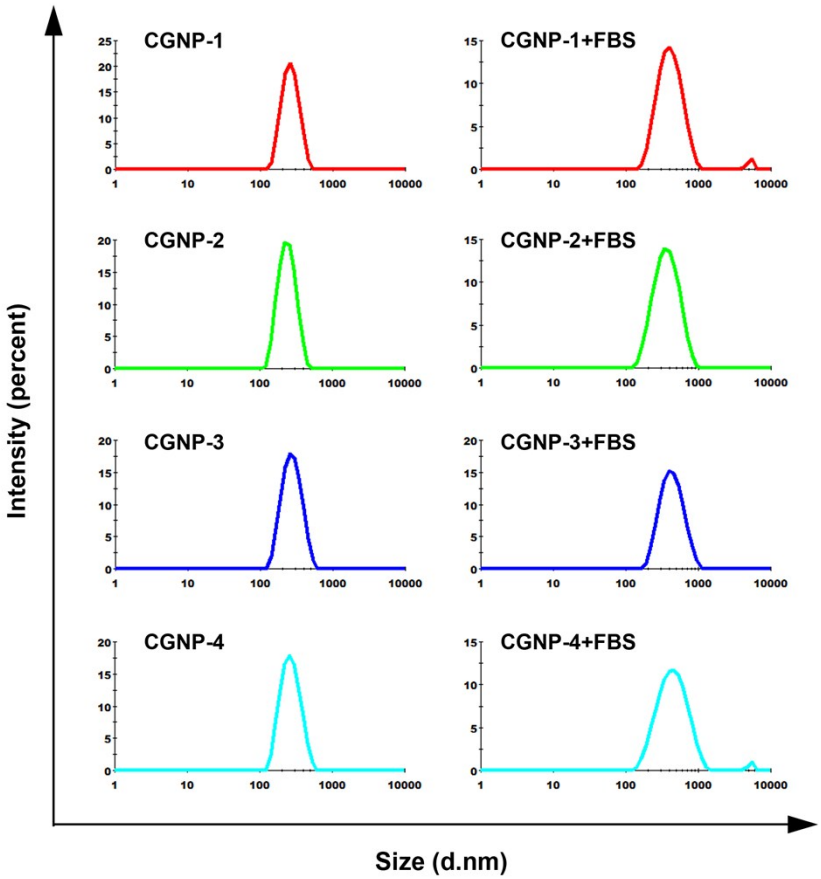

Fig. S1 Representative size distribution graphs of CGNPs incubated with or without fetal bovine serum (FBS) at 37 °C for 4h.

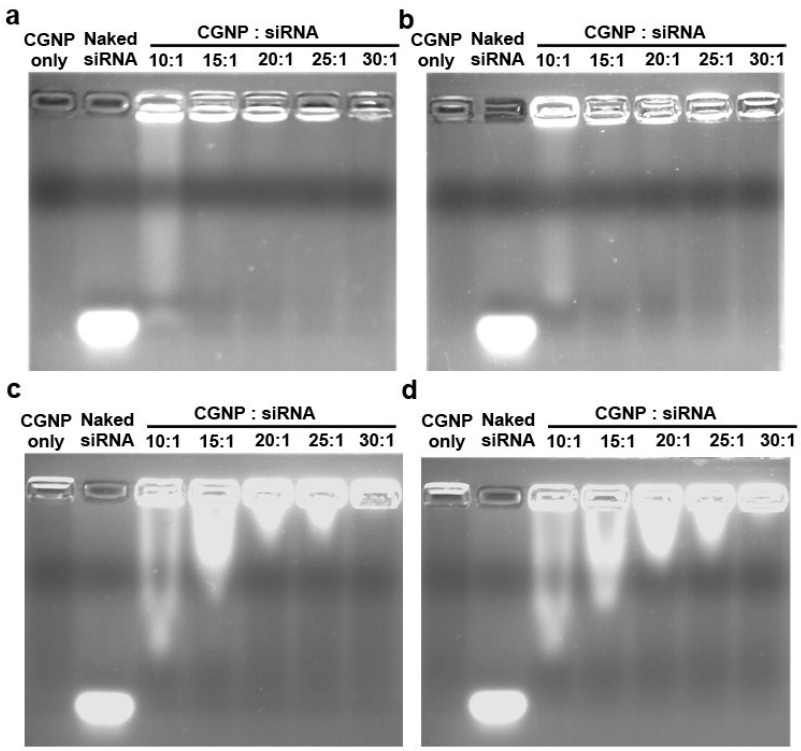

Fig. S2 CGNP's binding with siRNA determined by agarose gel electrophoresis. (a) CGNP-1. (b) CGNP-2. (c) CGNP-3. (d) CGNP-4. CGNP:siRNA represents the weight ratio of CGNP to siRNA.

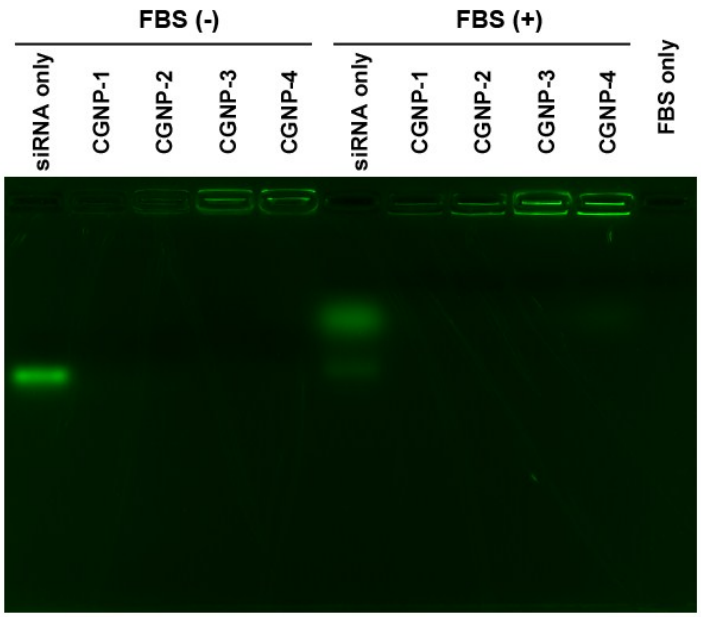

Fig. S3 Agarose gel electrophoresis of siRNA and CGNP-siRNA with or without FBS treatment (37 °C, 4h). The siRNA-FAM was used to visulize its interity by fluorescent imaging.

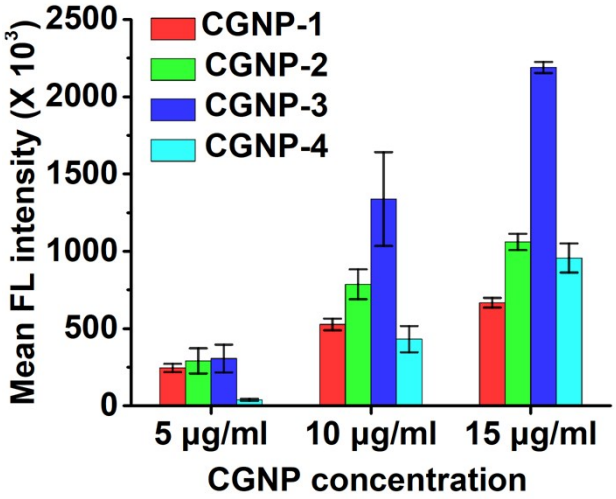

Fig. S4 Mean fluorescent intensity of K562 cells without trypan blue quenching. Cells were treated by CGNPs and siRNA-FAM complex for 4 hours.

Fig. S5 The uncropped western blots images of BCR-ABL and actin.