Supporting Information

Flexible DNA junction assisted efficient construction of

stable gene nanoparticles for gene delivery

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1. General Information

All chemicals and solvents used in organic synthesis were in analytic level. And all solvents were purified or dried according to standard procedures. Millipore water was used to prepare all aqueous solutions. Simple unmodified oligonucleotides were synthesized and purified by Sangon Biotech (Shanghai, China). The 5' thiol-modified C6 S-S DNA sequences were synthesized and purified by BioSune (Shanghai, China). The PCR and enzyme-digested products were purified by the GeneJET PCR Purification Kit from Fermentas (Tianjin, China). Restriction endonuclease and T4 ligase were provided by NEB and Fermentas (Tianjin, China) respectively. HEK-293A cell line was purchased from ATCC (Beijing, China).

Mass spectral analysis was measured on Waters LCT Premier XE (deconvoluted ESI) for modified oligonucleotides. Atomic force microscopic (AFM) images were obtained using a NanoScope IV multimode atomic force microscope (Veeco Instruments, USA) in tapping mode in air at room temperature. HEK-293A EGFP fluorescence was studied by confocal microscope (Olympus FV1000) and flow cytometry analysis (BD LSRFortessa).

2. DNA sequences of the reconstructed target gene and DNA-J

Table S1: DNA sequences of target gene CMV-EGFP.

GGGAAGACCCCCCATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGA Bbs I Ase I

GTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCC ATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCA ATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAG TACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGAC CTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGAT GCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCT CCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATG TCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATAT AAGCAGAGCTGGTTTAGTGAACCGTCAGATCCGCTAGCGCTACCGGACTCAGATCTCGAGCTC AAGCTTCGAATTCTGCAGTCGACGGTACCGCGGGGCCCGGGATCCACCGGTCGCCACCATGGTG AGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTA AACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGAC CTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCT ACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAA(ICCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTAC AAGACCCGCGCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGG ATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCAC AACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCAC AACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGAC GGCCCCGTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCC AACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGC ATGGACGAGCTGTACAAG<mark>TAA</mark>AGCGGCCGCGACTCTAGATCATAATCAGCCATACCACATTTG TAGAGGTTTTACTTGCTTTAAAAAAACCTCCCACACCTCCCCCTGAACCTGAAACATAAAATGA ATGCAATTGTTGTTGTTAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCA

TCACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGGTTTGTCCAAACTCA TCAATGTAT<u>CTTAAG**GAAGACGGGGGGCC-3'**</u>

Afl II Bbs I

Table S2: Target gene CMV-EGFP was amplified from pEGFP-N1 by the following primer pair:

Name	Sequence (5'-3')			
Forward primer	GG<u>GAAGACCCCCC</u>ATTAATAGTAATCAATTACG			
Reverse primer	GG <u>CCCCCGTCTTCCTTAAG</u> ATACATTGATGAGT			

Table S3: DNA sequences of DNA-J.

Name	Sequence
\mathbf{A}^{3}	(5'-3') SCCTCCTCTTCCCTGTCCAAA
S ¹	(3'-5') GGAGGAGAAGGGACAGGTTTGGGG _P

3. PCR process for the reconstructed target gene



Fig. S1 PCR for the reconstructed target gene with 1% agarose gel electrophoresis analysis.

PCR content: 50.0 ng pEGFP-N1 template, 0.25 μ M primer pair, 1 U La-Taq DNA polymerase, 5.0 μ L 5 × PCR reaction buffer and added ddH₂O into 25 μ L PCR system.

PCR program: 95 °C 5 min - (95 °C 30 S - 60 °C 30 S - 72 °C 120 S) \times 30 cycles - 72 °C 5 min - 4 °C 1 h.

4. Synthesis of branched DNA structure A³



Fig. S2 Synthetic method for the branched DNA structure A^3 .

Single DNA with C6-S-S-C6 modification in 5' end (60 µg) (\mathbf{A}^{S-S}) was dissolved in 57 µL ddH₂O. Then 3 µL 100 mM TCEP was added, with a final concentration of 5 mM. The mixture was incubated at 37 °C for next 1 h. After that, the reaction mixture was precipitated twice in 0.3 M NaOAc and 75% ethanol to wash the TCEP residue which may affect the following reaction. Next, the produced C6-SH DNA solid (\mathbf{A}^{S}) was dissolved in 40 µL ddH₂O. Under argon atmosphere, 40 µL Tris-HCl (pH 8.0) was added with a final concentration of 500 mM in 25 °C. Then, **3M** (1/3 eq) in DMF was added carefully in two batches every 30 min. With stirring, the reaction was monitored by denatured PAGE (7 M Urea) until no more products generated, which may take 4 h. Finally, \mathbf{A}^{3} was purified by denatured PAGE (7 M Urea).



5. Restriction endonuclease BbsI digestion and T4 ligation process

Fig. S3 Self-assembly gene products with various DNA junction to CMV-EGFP ratios. (a) Schematic illustration of the construction process. (b) 1% agarose gel electrophoresis analysis for the self-assembly gene products with various DNA junction to CMV-EGFP ratios. (c) 1% agarose gel electrophoresis analysis for the Exonuclease III stability of the various self-assembly gene products. (d) AFM analysis of the various self-assembly gene products. (1/3: 180-270 nm, 2/3: 210-250 nm and 3/3: 180-300 nm in diameter. Scale bar: 400 nm.) (e) Confocal microscopy images of the expression of EGFP in HEK-293A transfected with 250 ng various self-assembly gene products for 24 h, respectively. Scale bar: 100 μ m.

Restriction endonuclease BbsI digestion process:

3 µg purified linear PCR product CMV-EGFP, 10 U BbsI, 2 µL 10 × BbsI reaction buffer, and added ddH₂O into 20 µL system. The mixture was incubated at 37 °C for 5 h, and then purified by the PCR Purification Kit to remove the produced short oligos.

T4 ligation process:

0.5 pmol purified digested product, 0.5/3, 1/3 and 1.5/3 pmol DNA-J, 10 U T4 ligase, 2 μ L 10 × T4 ligation buffer, and added ddH₂O into 20 μ L system. The mixture was incubated at 16 °C for 18 h. Then the traditional annealing process was needed before the gel analysis.

Exonuclease III digestion:

Ligated products with 6 U Exonuclease III, 2 μ L 10 × Exonuclease III reaction buffer. The mixture was incubated at 37 °C for 1 h, then at 70 °C for 20 min before gel analysis.

Theoretical consideration guided us to make unwasted match to fully use the designed DNA junction is to set DNA junction to CMV-EGFP ratio as 2/3. That made us to start the study with this ratio. Of course, we have further checked DNA junction to CMV-EGFP ratio set as 1/3 and 3/3 by the referee's suggestion.

When the DNA junction to CMV-EGFP ratios were set as 1/3, 2/3 or 3/3, we can find that the ligation gene products were mainly in the gel loading zone with low gel mobility, which is taken as the nanoparticle formation (Fig. S3b).

1) size: We further checked the AFM of these newly formed nanoparticles. As can be seen in Fig. S3d, the nanoparticles of 2/3 ratio was with the narrower size range (1/3: 180-270 nm, 2/3: 210-250 nm and 3/3: 180-300 nm in diameter). These results showed that the sizes of these nanoparticles were affected by the DNA junction to CMV-EGFP ratios, but the newly formed nanoparticles are still centered around 230 nm, except that the mix ratio set as 2/3 gave better nanoparticle size distribution. That may be showing that either excess amount of the material somehow stick to the center particle instead to expand the size distribution. Of course, this needs further study. To our present paper, choosing mix ratio as 2/3 is so far a good choice.

2) stability: Also, the Exonuclease III stability assay suggested that the ligation gene products with different mix ratio yield the product of ratio 2/3 is most resistant to the nuclease among all three different mix ratio (1/3, 2/3, 3/3, Fig. S3c). These results showed that the product of ratio 2/3 of DNA junction to CMV-EGFP has better stability or resistance to nuclease.

3) transfection efficiency: 24h transfection of different mix ratio products yield roughly same strength of EGFP fluorescence (Fig. S3e).

Taking all factors into consideration, especially the stability towards the Exonuclease III, we chose the DNA junction to CMV-EGFP ratio 2/3 with the sufficient self-assembly of the whole sticky ends for the reported experiments.

6. Ase I and Afl II digestion of T4 ligated product



Fig. S4 Ase I and Afl II digestion of the T4 ligated product.

0.5 pmol purified T4 ligated product, 20 U Ase I and Afl II for each, 2 μ L 10 × NEB 2.1 reaction buffer, added ddH₂O into 20 μ L system. The mixture was incubated at 37 °C for 4 h.

7. Diameter analysis for the constructed gene nanoparticles



Fig. S5 Diameter analysis for the constructed gene nanoparticles. (a) AFM: about 210-250 nm in diameter. (b) DLS: about 270-370 nm in diameter. (The obtained diameter of DLS is larger than AFM, which may be caused by the shrinkage of DNA nanoparticles during the drying process in the AFM samples preparation.)

AFM imaging:

0.1 μ M of purified T4 ligated product was diluted into 10 nM by 2 mM Mg(OAc)₂ and 3 μ L of this sample was spotted on a freshly cleaved mica surface. After adsorbing for 5 min, 20 μ L 2 mM Mg(OAc)₂ was used to wash the sample for once. Then the sample was dried at room temperature before imaging.

DLS analysis:

0.1 μ M of purified T4 ligated product was used directly for DLS analysis with a laser wavelength of λ = 678 nm and a scattering angle of 90°.

8. Exonuclease III stability assay

0.5 pmol PCR product CMV-EGFP or condensed gene nanoparticles, 2, 4 and 6 U Exonuclease III, 2 μ L 10 × Exonuclease III reaction buffer, and added ddH₂O into 20 μ L system. The mixture was incubated at 37 °C for 1 h. Then, incubated at 70 °C for 20 min was needed to inactivate the Exonuclease III before gel analysis.

Exonuclease III	2 U	4 U	6 U
CMV-EGFP	22%	1%	0%
Gene NP	76%	55%	41%

Table S4: The statistic results of the residual gene products by the software of ImageJ.

9. Cell culture and transfection

Human embryo kidney cells (HEK-293A) was cultured at 37 °C, 5% CO₂ in DMEM/HIGH GLUCOSE (GIBICO) supplemented with 10% fetal bovine serum (GIBICO), 100 U/mL penicillin, 100 μ g/mL streptomycin, and 4 mM L-glutamine. The cells were maintained in exponential growth, and then seeded in 6-well plates (~30000 cells/well) to reach about 70% confluence for transfection.

The cells were transfected with 250 ng pEGFP-N1 plasmid, linear PCR product CMV-EGFP and condensed gene nanoparticles respectively with Lipofectamine 2000 (Invitrogen, USA) (total transfection volume, 2.0 mL) according to the manufacturer's instructions. Medium was replaced by 2 mL fresh DMEM medium supplemented with 10% FBS 4 hours later. After 20 hours, cells were imaged on a confocal microscope (Olympus FV1000) with 480 nm excitation.

Flow cytometry analysis: 24, 48 and 72 h after the transfection process, the cultured cells were trypsinized for 3 min to obtain the cell suspension. Then, the cells were washed twice by the PBS buffer and filtered through the nylon gauze before the flow cytometry analysis (BD LSRFortessa) with 488 nm excitation.





