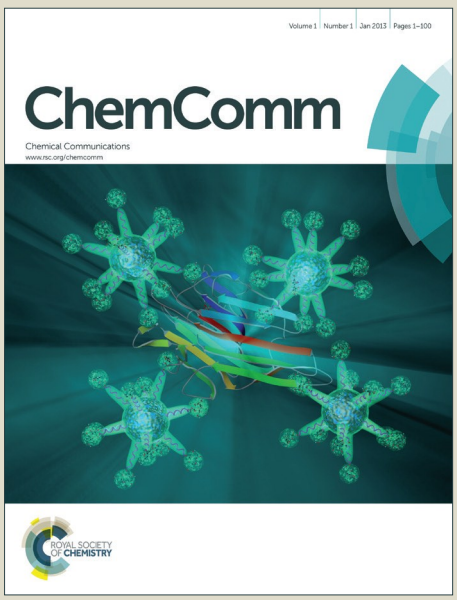
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

ChemComm

COMMUNICATION

Flexible DNA junction assisted efficient construction of stable gene nanoparticles for gene delivery†

Received 00th September 2015,
Accepted 00th September 2015

DOI: 10.1039/c0xx00000x

www.rsc.org/chemcomm

Jianbing Liu, Yanyan Li, Dejun Ma, Di Ouyang and Zhen Xi*

Flexible DNA junction was designed to assist the construction of stable gene nanoparticles with multiple target gene copies, which can be used as the efficient gene cargo with successful expression in eukaryotic cells for gene delivery.

Gene therapy is a promising strategy to the treatment of many gene related diseases.[1] Safe and efficient gene delivery vector is always the first key step for the development of gene medicine. Till now, the gene delivery vectors mainly include two categories, viral vectors and non-viral vectors.[2] On the safe side, much attention has been paid to the construction of various non-viral vectors, such as lipoplexes and polyplexes.[3] And, the gene cargo involved in these non-viral vectors is almost the traditional plasmid. As we know, the plasmid is easy to construct and amplify through the classic protocol. While, the numerous unmethylated CpG motifs and the obstinate bacterial remnants such as lipopolysaccharides (LPS) of the plasmid can induce adverse immune response.[4] To address this, numerous studies have been performed to the construction of newly generated gene cargos, such as the PCR amplified gene expression cassette.[5] To these small linear gene expression cassettes, unnecessary DNA sequences and the inherent obstacles of the plasmid are avoided. However, the poor stability and low packaging efficiency of the linear gene cargos are the main challenges to the further gene delivery research.[6] We therefore hypothesize that crosslinked linear gene cargos with the condensed DNA structure would be easier for encapsulation by the well-developed non-viral nanocarriers[7] and with enhanced stability.

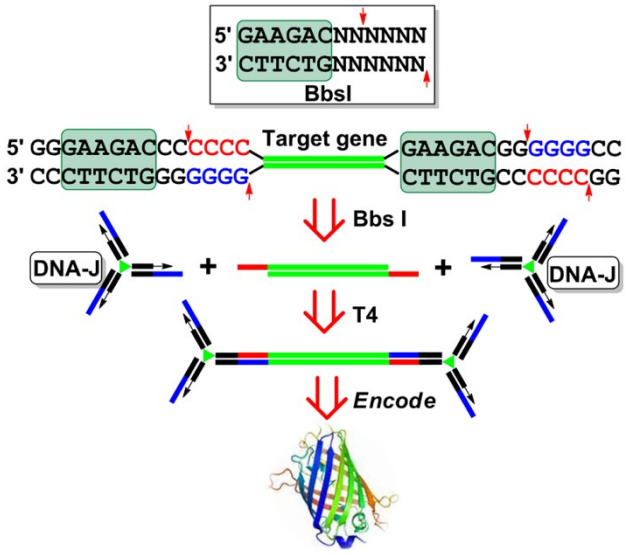
Meanwhile, much effort has been devoted to getting these condensed DNA structures. Among these construction methods, DNA Holliday junction is the main strategy to realize the purpose. The self-assembly of these DNA Holliday junctions almost depends on the terminal DNA sequences design, such as the sticky ends and i-motif structures.[8] While, the vertexes of the traditional DNA Holliday junctions are almost composed of the DNA duplexes, which would be too rigid to crosslink the long gene expression cassettes for condensed DNA structures.[9] Fortunately, branched molecules assisted flexible DNA junctions (DNA-J) have been developed, which would be a great substitute for the traditional DNA Holliday junctions.[10] Before the long gene expression cassette is involved in the crosslinking process with these flexible DNA-J, the suitable DNA restriction endonucleases should be employed to produce the sticky ends of the gene sequences for self-assembly. Importantly, *in vivo* delivery of these crosslinked gene products and their biological effects in cell are worth carefully exploring.

In this work, through rationally designed flexible DNA junctions and a particular DNA restriction endonuclease Bbs I, we succeed in constructing the crosslinked gene cargos with nanoparticle-like morphology efficiently. Importantly, these gene nanoparticles show excellent target protein expression in living cells. This is the first application of crosslinked gene cargos assisted by the flexible DNA junctions for the gene delivery research in living mammalian cells.

As shown in Scheme 1, we chose a particular DNA restriction endonuclease Bbs I to work as the efficient biological scissor. Only the sequence [5'-GAAGAC] is essential for the recognition process. The following 2nd and 6nd random bases are the cleavage sites to produce the [CCCC] overhangs in the both 5' terminals. Bbs I was employed for the consideration including: 1) we can design the DNA sequences among the cleavage sites artificially; 2) without the traditional palindromic sequences, the self-crosslinking problem of the digested gene products can be avoided; 3) more stable GC base pairs can be chose to favour the following crosslinking process. In this way, the rationally designed total gene sequences with two GC pairs in both ends to make sure the digestion process of Bbs I well showed in Table S1, ESI†. The final reconstructed target gene CMV-EGFP (1667 bp) was obtained through the one-step normal PCR amplification from the pEGFP-N1 with the common primer pair (sequences showed in Table S2, Fig. S1, ESI†).

Department of Chemical Biology, State Key Laboratory of Elemento-Organic Chemistry, National Engineering Research Center of Pesticide (Tianjin), Collaborative Innovation Center of Chemical Science and Engineering (Tianjin), Nankai University, Tianjin 300071, China. E-mail: zhenxi@nankai.edu.cn;
Tel: (+ 86)22-2350-4782

† Electronic Supplementary Information (ESI) available: Experimental details of the DNA sequence information, synthetical method of DNA juction, PCR process and additional figures for analysis. See DOI: 10.1039/c0xx00000x



Scheme 1 Schematic illustration of the construction of poly linear gene cargos assisted by the flexible DNA junctions (DNA-J) and the particular DNA restriction endonuclease Bbs I for gene delivery research.

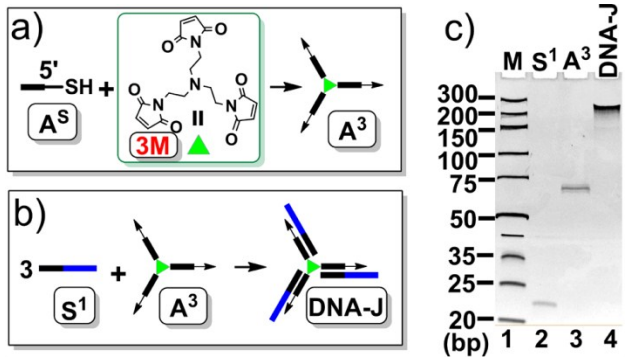


Fig. 1 Synthesis of the flexible branched DNA junction (DNA-J). (a) Synthetic strategy for the branched DNA structure A^3 from the 5' thiol modified oligos A^S. (b) Construction method for the flexible DNA junction through the annealing process between S^1 and A^3. (c) 15% native PAGE analysis of the linear DNA S^1, branched DNA structure A^3 and the constructed flexible DNA junction (DNA-J). M: DNA marker.

After getting the reconstructed target gene CMV-EGFP successfully, we next aimed to construct the flexible DNA junctions (DNA-J). As shown in the Fig. 1a, the flexible branched DNA structure A^3 was firstly synthetized through the thiol-Michael addition reaction to the crosslinking molecule 3M by A^S (20 nt DNA oligo, superscript 's' stands for the 5' thiol modification with the flexible spacer of six carbons) efficiently (sequences showed in Table S3, synthetic process see Fig. S2, mass spectra of A^3 was consistent with the calculated values, ESI†).[11] Then, we designed a corresponding complementary DNA sequence S^1 (24 nt DNA oligo, with 5' phosphate modification for the following T_4 ligation process) to anneal with the flexible branched DNA structure A^3 in a ratio of 3:1 (Fig. 1b, sequences showed in Table S3, ESI†). Till now, the flexible DNA junction with three [GGGG] overhangs (sequences in blue colour of Fig. 1b) has been constructed successfully. Such obtained DNA structures were also analyzed by the 15% native PAGE to ensure their synthetic purity (Fig. 1c). Depending on their branched structures, we found that the newly generated flexible DNA junctions exhibited much slower mobility than their corresponding DNA marker.

Encouraged by these results, we next investigated the conditions for the crosslinking process between the flexible branched DNA junctions and the above-mentioned reconstructed target gene CMV-EGFP. As shown in the Fig. 2a, DNA restriction endonuclease Bbs I was responsible for the digestion of the target gene CMV-EGFP to result in two [CCCC] overhangs in the both 5' ends (Fig. 2b, line 3). After the purification process, the flexible branched DNA junction with three [GGGG] overhangs was introduced into the crosslinking system with a 2/3 ratio to the digested target gene CMV-EGFP for the sufficient self-assembly of the whole sticky ends. In order to ensure the thermodynamic stability of the crosslinked gene cargos, T_4 ligase was employed in the crosslinking process (Fig. S3, ESI†). Obviously, we found that the T_4 ligated products were with very low mobility and mainly persisted in the sample loading zone during the agarose gel electrophoresis analysis (Fig. 2b, line 4). This phenomenon may illustrate that multiple target gene copies have been crosslinked successfully through the self-assembly process with the efficient recognition of stable GC base pairs.

To these crosslinked gene cargos, we also evaluated their enzyme compatibility. During the sequences design, we intentionally inserted two common DNA restriction endonuclease sites Ase I and Afl II into the both ends of the reconstructed target gene CMV-EGFP. Line 5 of Fig. 2b showed that the crosslinked gene cargos can also be normally recognized and subsequently digested by the Ase I and Afl II to release the linear gene fragment (Fig. S4, ESI†).

Next, atomic force microscopy (AFM) was used to observe the morphology of the crosslinked gene cargos. Interestingly, we found that these crosslinked linear gene cargos were with clear nanoparticle-like morphology (Fig. 2c). The mean diameter of these condensed gene nanoparticles fitted well with the hydrodynamic diameter obtained through the dynamic light scattering (DLS) detection (Fig. S5, ESI†).

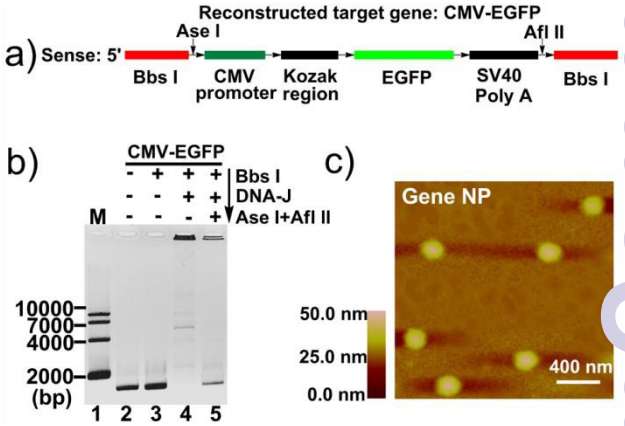


Fig. 2 Flexible DNA junction assisted construction of the target gene nanoparticles. (a) Sequence design of the reconstructed target gene CMV-EGFP. (b) 1% agarose gel electrophoresis analysis for the PCR product CMV-EGFP (line 2), Bbs I digestion product (line 3), T_4 ligated product (line 4) and the digested fragment of ligated products by Ase I+Afl II (line 5), respectively. (c) AFM analysis of the crosslinked linear gene cargos, about 210-250 nm in diameter. Scale bar: 400 nm.

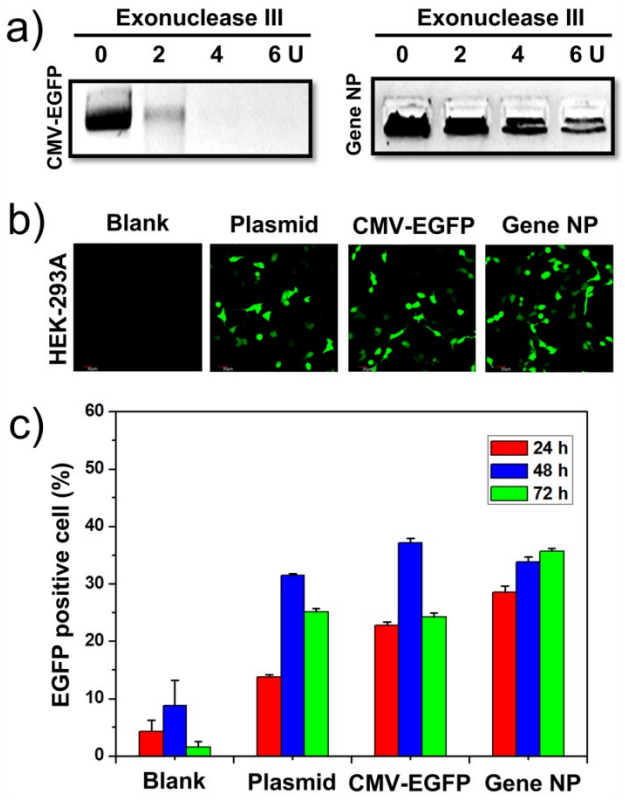


Fig. 3 Evaluation of the stability and translational ability in the HEK-293A cells for the condensed gene nanoparticles. (a) 1% agarose gel electrophoresis analysis for the resistance of the DNA products (the linear PCR product CMV-EGFP and condensed gene nanoparticles) to the digestion of Exonuclease III. (b) Confocal microscopy images of the expression of EGFP in HEK-293A transfected with 250 ng pEGFP-N1 plasmid, linear PCR product CMV-EGFP, and condensed gene nanoparticles for 24 h, respectively. Scale bar: 50 μm. (c) Flow cytometry analysis of the expression of EGFP in HEK-293A transfected with 250 ng pEGFP-N1 plasmid, linear PCR product CMV-EGFP, and condensed gene nanoparticles for 24, 48 and 72 h, respectively.

Inspired by the above results, we further investigated the stability of these condensed gene nanoparticles through the classic exonuclease III digestion assay. The linear reconstructed target gene CMV-EGFP was almost completely digested under the treatment by 4 U of exonuclease III during 1 hour (Fig. 3a). While, the crosslinked condensed gene nanoparticles remained almost 50% in the same condition (Fig. 3a, Table S4, ESI†). The efficient crosslinked and protected gene terminals of these condensed gene nanoparticles should be responsible for the much enhanced stability towards the digestion of exonuclease III. This property may facilitate the delivery process of these gene nanoparticles *in vivo*.

With the enhanced stability, we next examined the expression ability of these condensed gene nanoparticles in the eukaryotic cells HEK-293A. To ensure the successful expression process in the eukaryotic cells, the eukaryotic CMV promoter and the SV40 poly A section were both involved in the reconstructed target gene CMV-EGFP (Fig. 2a). 250 ng control plasmid pEGFP-N1, linear PCR product CMV-EGFP and the crosslinked condensed gene nanoparticles were transfected into the HEK-293A by Lipofectamine 2000, respectively. After 4 hours incubation, the medium was replaced by 2 mL fresh DMEM medium supplemented with 10% FBS. 20 hours later, these cell samples were observed through the confocal microscopy directly. The observed intense EGFP fluorescence of the experiment groups indicated that these condensed gene nanoparticles can efficiently work as the template for gene expression in the cellular environment (Fig. 3b). Also, the flow cytometry analysis was employed for the quantitative study of the expression ability of the three kinds of gene cargos. Interestingly, we found that our gene nanoparticles can induce nearly 30% EGFP positive cells even after 72 h (Fig. 3c). The long-lasting effect may be caused by the enhanced stability of the gene nanoparticles as shown above.

In summary, we have developed a facile and efficient strategy to construct the condensed gene nanoparticles through the flexible DNA junctions assisted self-assembly process. These condensed gene nanoparticles show several advantages for gene delivery. Firstly, these condensed gene cargos can be easily produced through the self-assembly process with the efficient recognition of stable GC base pairs. Secondly, these condensed gene cargos with multiple gene copies have well-defined nanoparticle-like morphology. Thirdly, these gene nanoparticles show much enhanced stability towards the digestion of exonuclease III. Finally, our gene nanoparticles can efficiently act as the poly linear vectors for *in vivo* target protein expression in eukaryotic cells. This construction strategy for gene nanoparticles can be potentially general to other target genes for gene delivery.

This work was supported by the MOST of China (2012AA022501) and NSFC (21332004).

Notes and references

1 (a) I. M. Verma and N. Somia, *Nature*, 1997, **389**, 239; (b) S. O. Han, R. I. Mahato, Y. K. Sung and S. W. Kim, *Mol. Ther.*, 2000, **2**, 302; (c) C. E. Walsh, *Gene Ther.*, 2003, **10**, 999; (d) I. A. McNeish, S. J. Ben and N. R. Lemoine, *Gene Ther.*, 2004, **11**, 497; (e) G. D. Schmidt Wolf and I. G. Schmidt-Wolf, *Trends Mol. Med.*, 2003, **9**, 67; (f) A. Kotterman and D. V. Schaffer, *Nat. Rev. Genet.*, 2014, **15**, 445; (g) C. de Mendoza, P. Barreiro, L. Benitez and V. Soriano, *Expert. Opin. Biol. Th.*, 2015, **15**, 319; (h) J. L. Gori, P. D. Hsu, M. L. Maeder, Shen, G. G. Welstead and D. Bumcrot, *Hum. Gene Ther.*, 2015, 2 443.

2 (a) S. Li and L. Huang, *Gene Ther.*, 2000, **7**, 31; (b) T. Niidome a L. Huang, *Gene Ther.*, 2002, **9**, 1647; (c) C. E. Thomas, A. Ehrhar and M. A. Kay, *Nat. Rev. Genet.*, 2003, **4**, 346; (d) S. D. Li and Huang, *Gene Ther.*, 2006, **13**, 1313; (e) H. Yin, R. L. Kanasty, A. Eltoukhy, A. J. Vegas, J. R. Dorkin and D. G. Anderson, *Nat. Re Genet.*, 2014, **15**, 541; (f) R. G. Crystal, *Hum. Gene Ther.*, 2014, **25**, 3; (g) C. H. Sum, S. Wettig and R. A. Slavcev, *Curr. Gene. Ther.*, 2014, **14**, 309; (h) V. Gaspar, D. de Melo-Diogo, E. Costa, Moreira, J. Queiroz, C. Pichon, I. Correia and F. Sousa, *Expert. Opi Biol. Th.*, 2015, **15**, 353.

3 (a) Y. Liu, L. Wenning, M. Lynch and T. M. Reineke, *J. Am. Che Soc.*, 2004, **126**, 7422; (b) C. A. H. Prata, Y. Zhao, P. Barthelemy, Y Li, D. Luo, T. J. McIntosh, S. J. Lee and M. W. Grinstaff, *J. A Chem. Soc.*, 2004, **126**, 12196; (c) T. Ooya, H. S. Choi, A. Yamashit N. Yui, Y. Sugaya, A. Kano, A. Maruyama, H. Akita, R. Ito Kogure and H. Harashima, *J. Am. Chem. Soc.*, 2006, **128**, 3852; (d) S.

Takae, K. Miyata, M. Oba, T. Ishii, N. Nishiyama, K. Itaka, Y. Yamasaki, H. Koyama and K. Kataoka, *J. Am. Chem. Soc.*, 2008, **130**, 6001; (e) R. Srinivas, S. Samanta and A. Chaudhuri, *Chem. Soc. Rev.*, 2009, **38**, 3326; (f) M. Sanjoh, K. Miyata, R. J. Christie, T. Ishii, Y. Maeda, F. Pittella, S. Hiki, N. Nishiyama and K. Kataoka, *Biomacromolecules*, 2012, **13**, 3641; (g) M. Soliman, R. Nasanit, S. R. Abulateefeh, S. Allen, M. C. Davies, S. S. Briggs, L. W. Seymour, J. A. Preece, A. M. Grabowska, S. A. Watson and C. Alexander, *Mol. Pharmaceutics*, 2012, **9**, 1; (h) H. Xu, Z. Li and J. Si, *J. Biomed. Nanotechnol.*, 2014, **10**, 3483; (i) L. Li, Y. Wei and C. Gong, *J. Biomed. Nanotechnol.*, 2015, **11**, 739.

4 (a) I. P. Wicks, M. L. Howell, T. Hancock, H. Kohsaka, T. Olee and D. A. Carson, *Hum. Gene Ther.*, 1995, **6**, 317; (b) D. M. F. Prazeres, G. N. M. Ferreira, G. A. Monteiro, C. L. Cooney and J. M. S. Cabral, *Trends Biotechnol.*, 1999, **17**, 169; (c) B. Goldberg, H. B. Urnovitz and R. B. Stricker, *Immunol. Lett.*, 2000, **73**, 13; (d) N. Bessis, F. J. GarciaCozar and M. C. Boissier, *Gene Ther.*, 2004, **11**, 10; (e) A. R. Brooks, R. N. Harkins, P. Wang, H. S. Qian, P. Liu and G. M. Rubanyi, *J. Gene Med.*, 2004, **6**, 395; (f) A. Reyes-Sandoval and H. C. Ertl, *Mol. Ther.*, 2004, **9**, 249; (g) C. Coban, K. J. Ishii, M. Gursel, D. M. Klinman and N. Kumar, *J. Leukoc. Biol.*, 2005, **78**, 647; (h) M. Mitsui, M. Nishikawa, L. Zang, M. Ando, K. Hattori, Y. Takahashi, Y. Watanabe and Y. Takakura, *J. Gene Med.*, 2009, **11**, 435; (i) C. De Castro, O. Holst, R. Lanzetta, M. Parrilli and A. Molinaro, *Protein Peptide. Lett.*, 2012, **19**, 1040; (j) Y. H. Tan and J. C. Kagan, *Mol. Cell*, 2014, **54**, 212.

5 (a) S. Li, M. Brisson, Y. He and L. Huang, *Gene Ther.*, 1997, **4**, 449; (b) C. R. Hofman, J. P. Dileo, Z. Li, S. Li and L. Huang, *Gene Ther.*, 2001, **8**, 71; (c) F. Schakowski, M. Gorschluter, C. Junghans, M. Schroff, P. Buttgereit, C. Ziske, B. Schottker, S. A. Konig-Merediz, T. Sauerbruch, B. Wittig and I. G. Schmidt-Wolf, *Mol. Ther.*, 2001, **3**, 793; (d) P. Johansson, T. Lindgren, M. Lundstrom, A. Holmstrom, F. Elgh and G. Bucht, *Vaccine*, 2002, **20**, 3379; (e) K. Hirata, M. Nishikawa, N. Kobayashi, Y. Takahashi and Y. Takakura, *J. Pharm. Sci.*, 2007, **96**, 2251; (f) H. S. Wang, Z. J. Chen, G. Zhang, X. L. Ou, X. L. Yang, C. K. Wong, J. P. Giesy, J. Du and S. Y. Chen, *PLOS One*, 2012, **7**, 47159.

6 (a) J. Y. Cherng, N. M. E. Schuurmans-Nieuwenbroek, W. Jiskoot, H. Talsma, N. J. Zuidam, W. E. Hennink and D. J. A. Crommelin, *J. Controlled Release*, 1999, **60**, 343; (b) P. Chancham and J. A. Hughes, *J. Liposome. Res.*, 2001, **11**, 139; (c) A. von Groll, Y. Levin, M. C. Barbosa and A. P. Ravazzolo, *Biotechnol. Prog.*, 2006, **22**, 1220; (d) M. Muñoz-Úbeda, S. K. Misra, A. L. Barrán-Berdón, C. Aicart-Ramos, M. B. Sierra, J. Biswas, P. Kondaiah, E. Junquera, S. Bhattacharya and E. Aicart, *J. Am. Chem. Soc.*, 2011, **133**, 18014.

7 (a) R. Sheng, T. Luo, Y. Zhu, H. Li and A. Cao, *Macromol. Biosci.*, 2010, **10**, 974; (b) K. Miyata, N. Nishiyama and K. Kataoka, *Chem. Soc. Rev.*, 2012, **41**, 2562; (c) V. Biju, *Chem. Soc. Rev.*, 2014, **43**, 744; (d) Y. He, Y. Nie, G. Cheng, L. Xie, Y. Shen and Z. Gu, *Adv. Mater.*, 2014, **26**, 1534; (e) W. Li, J. Du, K. Zheng, P. Zhang, Q. Hu and Y. Wang, *Chem. Commun.*, 2014, **50**, 1579; (f) R. Sheng, F. An, Z. Wang, M. Li and A. Cao, *RSC Adv.*, 2015, **5**, 12338.

8 (a) N. C. Seeman, *Nature*, 2003, **421**, 427; (b) Y. Li, Y. D. Tseng, S. Y. Kwon, L. d'Espaux, J. S. Bunch, P. L. McEuen and D. Luo, *Nat. Mater.*, 2004, **3**, 38; (c) E. Cheng, Y. Xing, P. Chen, Y. Yang, Y. Sun, D. Zhou, L. Xu, Q. Fan and D. Liu, *Angew. Chem., Int. Ed.*, 2009, **48**, 7660; (d) M. Endo and H. Sugiyama, *Chembiochem*, 2009, **10**, 2420; (e) Y. Xing, E. Cheng, Y. Yang, P. Chen, T. Zhang, Y. Sun, Z. Yang and D. Liu, *Adv. Mater.*, 2011, **23**, 1117; (f) J. Li, C. Zheng, [illegible] Cansiz, C. Wu, J. Xu, C. Cui, Y. Liu, W. Hou, Y. Wang, L. Zhang, [illegible]t. Teng, H.-H. Yang and W. Tan, *J. Am. Chem. Soc.*, 2015, **137**, [illegible] (g) Z. Liu, C. Tian, J. Yu, Y. Li, W. Jiang and C. Mao, *J. Am. Chem. Soc.*, 2015, **137**, 1730.

9 Y. He, T. Ye, M. Su, C. Zhang, A. E. Ribbe, W. Jiang and C. Mao, *Nature*, 2008, **452**, 198.

10 (a) M. Scheffler, A. Dorenbeck, S. Jordan, M. Wüstefeld and G. von Kiedrowski, *Angew. Chem., Int. Ed.*, 1999, **38**, 3311; (b) B. R. Stepp, J. M. Gibbs-Davis, D. L. F. Koh and S. T. Nguyen, *J. Am. Chem. Soc.*, 2008, **130**, 9628; (c) S. Keller, J. Wang, M. Chandra, R. Berger and A. Marx, *J. Am. Chem. Soc.*, 2008, **130**, 13188; (d) U. Feldkamp, B. Saccà and C. M. Niemeyer, *Angew. Chem., Int. Ed.*, 2009, **48**, 5996; (e) R. V. Thaner, I. Eryazici, O. K. Farha, C. A. Mirkin and S. T. Nguyen, *Chem. Sci.*, 2014, **5**, 1091; (e) J. Liu, R. Wang, D. Ma, L. Ouyang and Z. Xi, *Chem. Commun.*, 2015, **51**, 9208.

11 L. Wei, L. Cao and Z. Xi, *Angew. Chem., Int. Ed.*, 2013, **125**, 6629.

Table of contents

Flexible DNA junction was designed to construct stable gene nanoparticles, which can be used as efficient gene cargo to eukaryotic cells.