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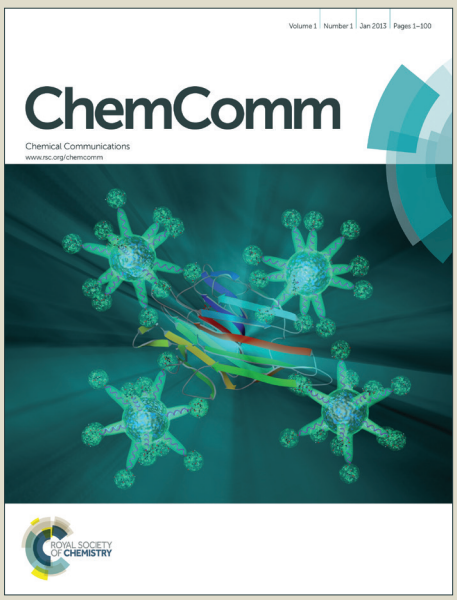

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Efficient construction of stable gene nanoparticles through polymerase chain reaction with flexible branched primers for gene delivery†

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Cite this: DOI: 10.1039/xoxxoooooox

Received ooth March 2015,
Accepted ooth March 2015

DOI: 10.1039/xoxxoooooox

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Flexible branched primers were designed to construct stable gene nanoparticles with multiple target gene copies through polymerase chain reaction, which can be used as efficient transcription template in eukaryotic cells for gene delivery.

Gene therapy has become more and more important in the development of biomedicines due to the potential to cure many gene related diseases in recent years. How to efficiently transfer target gene into specific tissues is the first key step for gene therapy. To address this, much attention has been paid to develop various gene delivery vehicles (*e.g.*, lipoplexes, polyplexes) and encapsulated gene cargoes.[1] The traditional gene cargo of non-viral vectors for gene therapy is plasmid. However, numerous unmethylated CpG motifs in the plasmid backbone can induce several immunotoxic effects to the transfected mammalian cells.[2] And the obstinate bacterial remnants such as lipopolysaccharides (LPS) of the plasmid can also cause adverse immune response.[3] Because of these obstacles, numerous research have been performed to construct new gene cargos, such as minicircle DNA,[4] minimalistic, immunologically defined gene expression vector (MIDGE),[5] Micro-Linear vector (MiLV)[6] and PCR amplified DNA fragment.[7] These work all depend on the reduction of the unnecessary DNA sequences of plasmid to reduce the total number of unmethylated CpG motifs and avoid the complicated purification process for plasmid. However, these uncompacted small vectors are more difficult to be packaged by the corresponding vehicles than that of supercoiled plasmid.[8] Meanwhile, normally one copy of the gene expression cassette is involved in single vector. We therefore hypothesize that crosslinked small vectors with multiple target gene copies would result in a condensed DNA structure (gene cargo) to assist the encapsulation process for gene delivery. Fortunately, various non-viral nanocarriers for plasmid have been developed with high gene transfection efficiency,[9] which could be also suitable for the condensed gene cargo.

On the other hand, much effort has been devoted to get these crosslinked DNA structures, such as origami,[10] holliday junction[11] and crosslinking molecule[12] assisted DNA self assembly. While, when the long gene expression cassette is involved, these DNA self-assembly processes become much more complex and time-consuming. Fortunately, polymerase chain reaction (PCR) is a very mature technology to produce multifold long linear DNA products in a short time. When branched primers are employed in PCR process, various crosslinked DNA structures with target gene can be efficiently constructed.[13] So far, *in vivo* delivery of these chemically branch-PCR products and their biological effects in cell have not been explored.

In this work, through PCR with flexible branched primers, we succeed in constructing the crosslinked DNA with nanoparticle-like morphology efficiently. When target gene expression cassette is involved in the template, the gene cargos can be efficiently produced in large scale. These condensed gene nanoparticles with multiple gene copies show efficient protein expression in living cells. This work presents the first application of branch-PCR gene cargos to the study of gene delivery in living cells.

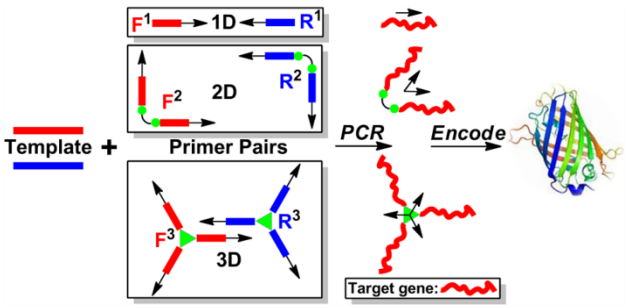

Fig. 1 PCR of the target gene with three groups of primer pairs F^1 and R^1, F^2 and R^2, F^3 and R^3 for the construction of these poly linear vectors encoding the target protein.

Three groups of primer pairs are designed (sequences showed in Table S1, ESI†), such as F^1 and R^1, F^2 and R^2, F^3 and R^3 (F: forward primer, R: reverse primer, superscript stands for the number of extending direction of PCR) corresponding to the 1D, 2D and 3D extending respectively (Fig. 1). F^1 and R^1 is the normal primer pair without any terminal modification of the DNA oligos. F^2 and R^2, F^3 and R^3 were constructed through thiol-Michael addition reaction to 2M or 3M by F^S and R^S (superscript 's' stands for the 5' thiol modification with the flexible spacer of six carbons) (Fig. 2a, Fig. S1, ESI†).[14] Such obtained three groups of primer pairs were analyzed by the 15% denatured PAGE to ensure their synthetic purity (Fig. 2b). F^3 and R^3 migrated slower than the 75 nt DNA marker, depending on their branched structures. At the same time, the mass spectra of F^3 and R^3 were consistent with the calculated values (see ESI†).

After getting these primer groups successfully, we subsequently studied the conditions for the special PCR system. Firstly, we chose a short 50 bp template as a test run (sequences showed in Table S1, ESI†). Following the traditional PCR operation by replacing the normal primer pair with the branched primer pairs (Fig. S2, ESI†), we got the PCR products as the main band result in the 2% agarose gel analysis (Fig. 2c). Interestingly, we also found that the PCR product based on tri-branched primer pair F^3 and R^3 exhibited a clean nanoparticle morphology in the AFM detection (Fig. 2d).

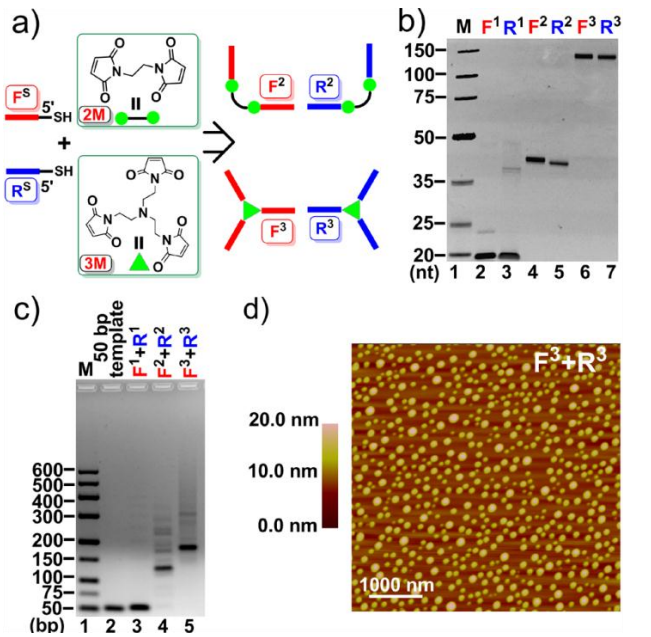

Fig. 2 Synthesis of branched primers and branch-PCR with 50 bp template. (a) Synthetic strategy for the primer pairs F^2 and R^2, F^3 and R^3 from the 5' thiol modified oligos F^S and R^S. (b) 15% denatured PAGE (7 M urea) analysis of the three groups of primer pairs. (c) 2% agarose gel electrophoresis analysis for the PCR products of the 50 bp template with the above three groups of primer pairs. M: DNA marker. (d) AFM analysis of the PCR product F^3+R^3 based on primer pair F^3 and R^3, about 80-130 nm in diameter. Scale bar: 1000 nm.

In order to study the property of the obtained DNA nanoparticles, we next evaluated their response to the biological enzymes such as restriction endonuclease and T4 ligase. During the sequences design, we have employed a restriction endonuclease site AvaI in the middle of the 50 bp template (Fig. S3a, ESI†). The branch-PCR product F^3+R^3 could be cleaved and subsequently re-ligated by the restriction endonuclease AvaI and T4 ligase, respectively (Fig. S3, ESI†).

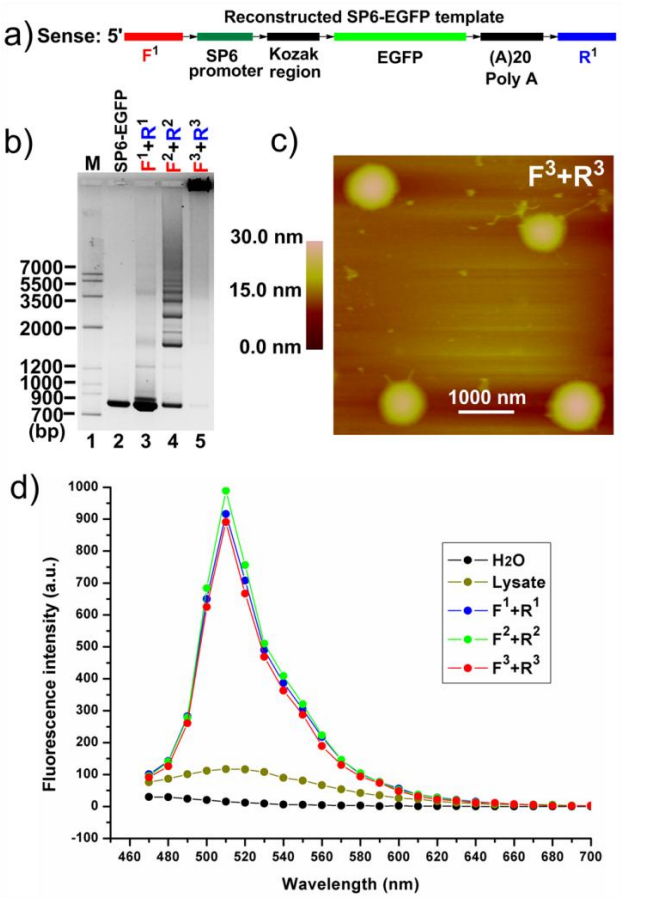

Fig. 3 Branch-PCR with reconstructed SP6-EGFP template for cell-free expression. (a) Sequence design of the reconstructed SP6-EGFP template. (b) 1% agarose gel electrophoresis analysis for the PCR products of the reconstructed SP6-EGFP template with three groups of primer pairs. (c) AFM analysis of the PCR product F^3+R^3 based on primer pair F3 and R3, about 850-950 nm in diameter. Scale bar: 1000 nm. (d) The EGFP fluorescence detection of the cell-free system with three kinds of PCR products.

Encouraged by these results, we next investigated much longer reconstructed SP6-EGFP template (809 bp) (Fig. 3a, sequences showed in Table S2, ESI†) to check this new PCR method. The sequences of the primer pair F^1 and R^1 were the same as before, and between the primer F^1 and EGFP gene, SP6 promoter and Kozak region have been inserted. Following the EGFP, 20 nt poly A sequence was added to make sure the stability of the transcripted mRNA.

The reconstructed SP6-EGFP template was obtained through one-step normal PCR amplification from the pEGFP-N1 with the common primer pair (sequences showed in Table S2, ESI†). Then, the branch-PCR over this template was carried out similarly to the 50 bp template. The newly obtained branch-PCR products showed remarkably slower mobility than its SP6-EGFP template (809 bp) (Fig. 3b). Interestingly, such obtained

branch-PCR product F^3+R^3 based on tri-branched primer pair F^3 and R^3 showed a clean larger nanoparticle morphology in the AFM detection (Fig. 3c).

These branch-PCR nanoparticles were then translated into the target EGFP protein through SP6 high-yield wheat germ protein expression system in a cell-free manner. In the 25 μL reaction system, 15 μL cell-free lysate and 3.0 μg (6 pmol) of each PCR product (10 μL) have been applied, then these mixtures were incubated at 25 °C for 48 hours. After that, we took 10 μL of the each sample to detect the fluorescence intensity of EGFP directly. Based on the similar fluorescence intensity at about 510 nm (characteristic peak of EGFP), we could find that these PCR products with primer pairs F^1 and R^1, F^2 and R^2, F^3 and R^3 can all be employed to work as the template for the translation in the SP6 cell-free expression system efficiently (Fig. 3d). The residual SP6-EGFP template made negligible contribution to the fluorescence (Fig. S4, ESI†).

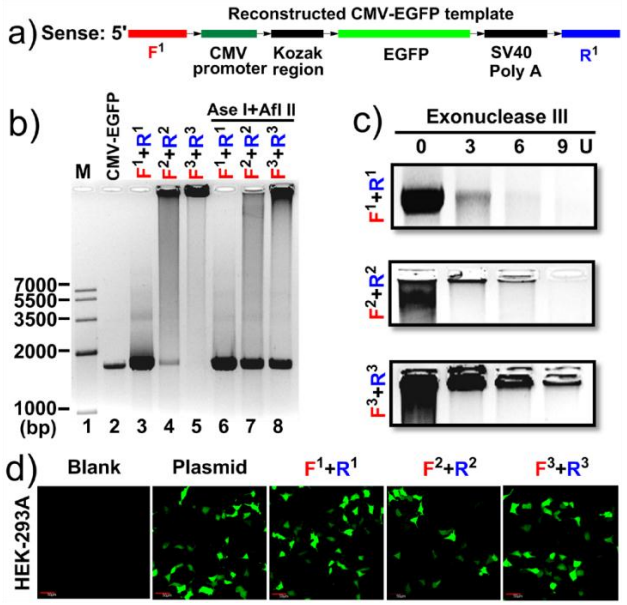

Fig. 4 Branch-PCR with reconstructed CMV-EGFP template for translation in eukaryotic cells. (a) Sequence design of the reconstructed CMV-EGFP template. (b) 1% agarose gel electrophoresis analysis for the PCR products of the reconstructed CMV-EGFP template with three kinds of primer pairs and the digestion results with restriction endonucleases Ase I and Afl II. (c) 1% agarose gel electrophoresis analysis for the resistance of the three kinds of PCR products to Exonuclease III digestion. (d) Confocal microscopy images of the expression of EGFP in eukaryotic cells HEK-293A transfected with 250 ng pEGFP-N1 plasmid and three kinds of PCR products respectively. Scale bar: 50 μm.

As we know, compared with the normal linear DNA, the crosslinked DNA nanoparticles are easier to be encapsulated for delivery through the endocytosis.[15] In order to evaluate the expression ability of our poly linear vector in the eukaryotic cells, we next constructed the CMV-EGFP template (1679 bp) (Fig. 4a, sequences showed in Table S3, ESI†). It should be noted that the SV40 poly A section was also involved in the template to stabilize the transcripted mRNA in the cellular environment. Similarly, the common primer pair (sequences showed in Table S3, ESI†) was applied to amplify the reconstructed CMV-EGFP template from the pEGFP-N1. Two restriction endonuclease sites Ase I and Afl II (Fig. S5, ESI†) at each end of the template were inserted to test the enzyme compatibility of these special PCR products. The PCR product F^3+R^3 based on tri-branched primer pair F^3 and R^3 was much more resistant to the digestion by these restriction enzymes (Fig. 4b). The exonuclease III digestion assay also showed that the branch-PCR nanoparticle F^3+R^3 (Fig. S6, ESI†) remained almost 50% even after 1 hour treatment by 6 U of exonuclease III (Fig. 4c, Table S4, ESI†).

Next, we investigated whether these DNA nanoparticles can be translated into the target EGFP protein in the eukaryotic cells. 250 ng control plasmid pEGFP-N1 and three kinds of purified branch-PCR products 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 EGFP fluorescence of the experiment groups indicated that these DNA nanoparticles can efficiently work as the template for gene expression in the cellular environment (Fig. 4d). Similarly, the residual DNA template made negligible contribution to the fluorescence (Fig. S7, ESI†).

In summary, we have developed a facile and efficient strategy to construct the condensed DNA nanoparticles through PCR using flexible branched primers. These condensed PCR products show several advantages for gene delivery. Firstly, these DNA structures can be easily produced by PCR amplification in a short time. Secondly, these condensed PCR products have well-defined nanoparticle-like morphology with multiple gene copies. Thirdly, these gene nanoparticles show much better stability to the digestion of exonuclease III. Finally, these gene nanoparticles can act as poly linear vectors for *in vivo* protein expression in eukaryotic cells. This construction strategy for gene nanoparticles can be potentially general to other target gene for gene therapy.

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

Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental details of the DNA sequences information, organic synthesis, PCR process and additional figures for analysis. See DOI: 10.1039/c000000x/

1 (a) M. A. Mintzer and E. E. Simanek, *Chem. Rev.*, 2009, **109**, 259; (b) R. Srinivas, S. Samanta and A. Chaudhuri, *Chem. Soc. Rev.*, 2009, **38**, 3326; (c) G. D. Schmidt-Wolf and I. G. Schmidt-Wolf, *Trends Mol. Med.*, 2003, **9**, 67; (d) S. Li and L. Huang, *Gene Ther.*, 2000, **7**, 31.

2 (a) B. Goldberg, H. B. Urnovitz and R. B. Stricker, *Immunol. Lett.*, 2000, **73**, 13; (b) A. Reyes-Sandoval and H. C. Ertl, *Mol. Ther.*, 2004, **9**, 249; (c) C. Coban, K. J. Ishii, M. Gursel, D. M. Klinman and N. Kumar, *J. Leukoc. Biol.*, 2005, **78**, 647; (d) A. R. Brooks, R. N. Harkins, P. Wang, H. S. Qian, P. Liu and G. M. Rubanyi, *J. Gene Med.*, 2004, **6**, 395; (e) M. Mitsui, M. Nishikawa, L. Zang, M. Ando,

K. Hattori, Y. Takahashi, Y. Watanabe and Y. Takakura, *J. Gene Med.*, 2009, **11**, 435.

3 (a) D. M. F. Prazeres, G. N. M. Ferreira, G. A. Monteiro, C. L. Cooney and J. M. S. Cabral, *Trends Biotechnol.*, 1999, **17**, 169; (b) I. P. Wicks, M. L. Howell, T. Hancock, H. Kohsaka, T. Olee and D. A. Carson, *Hum. Gene Ther.*, 1995, **6**, 317; (c) C. De Castro, O. Holst, R. Lanzetta, M. Parrilli and A. Molinaro, *Protein Peptide. Lett.*, 2012, **19**, 1040; (d) Y. H. Tan and J. C. Kagan, *Mol. Cell*, 2014, **54**, 212.

4 (a) A. M. Darquet, B. Cameron, P. Wils, D. Scherman and J. Crouzet, *Gene Ther.*, 1997, **4**, 1341; (b) A. M. Darquet, R. Rangara, P. Kreiss, B. Schwartz, S. Naimi, P. Delaere, J. Crouzet and D. Scherman, *Gene Ther.*, 1999, **6**, 209; (c) Z. Y. Chen, C. Y. He and M. A. Kay, *Hum. Gene Ther.*, 2005, **16**, 126; (d) S. Chabot, J. Orio, M. Schmeer, M. Schleef, M. Golzio and J. Teissie, *Gene Ther.*, 2013, **20**, 62.

5 (a) 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; (b) P. L. Mok, S. K. Cheong, C. F. Leong, K. H. Chua and O. Ainoon, *Cytotechnology*, 2012, **64**, 203.

6 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.

7 (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) P. Johansson, T. Lindgren, M. Lundstrom, A. Holmstrom, F. Elgh and G. Bucht, *Vaccine*, 2002, **20**, 3379; (d) K. Hirata, M. Nishikawa, N. Kobayashi, Y. Takahashi and Y. Takakura, *J. Pharm. Sci.*, 2007, **96**, 2251.

8 (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.

9 (a) K. Miyata, N. Nishiyama and K. Kataoka, *Chem. Soc. Rev.*, 2012, **41**, 2562; (b) R. Sheng, F. An, Z. Wang, M. Li and A. Cao, *RSC Adv.*, 2015, **5**, 12338; (c) R. Sheng, T. Luo, Y. Zhu, H. Li and A. Cao, *Macromol. Biosci.*, 2010, **10**, 974; (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) 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.

10 (a) M. Endo and H. Sugiyama, *Chembiochem*, 2009, **10**, 2420; (b) B. Wei, M. Dai and P. Yin, *Nature*, 2012, **485**, 623; (c) Y. Suzuki, M. Endo, Y. Yang and H. Sugiyama, *J. Am. Chem. Soc.*, 2014, **136**, 1714; (d) M. Endo and H. Sugiyama, *Accounts. Chem. Res.*, 2014, **47**, 1645.

11 (a) N. C. Seeman, *Nature*, 2003, **421**, 427; (b) Y. He, T. Ye, M. Su, C. Zhang, A. E. Ribbe, W. Jiang and C. Mao, *Nature*, 2008, **452**, 198; (c) Z. Liu, C. Tian, J. Yu, Y. Li, W. Jiang and C. Mao, *J. Am. Chem. Soc.*, 2015, **137**, 1730; (d) J. Li, C. Zheng, S. Cansiz, C. Wu, J. Xu, C. Cui, Y. Liu, W. Hou, Y. Wang, L. Zhang, I. t. Teng, H.-H. Yang and W. Tan, *J. Am. Chem. Soc.*, 2015, **137**, 1412.

12 (a) M. Scheffler, A. Dorenbeck, S. Jordan, M. Wüstefeld and G. von Kiedrowski, *Angew. Chem., Int. Ed.*, 1999, **38**, 3311; (b) J. Zimmermann, M. P. J. Cebulla, S. Mönninghoff and G. von Kiedrowski, *Angew. Chem., Int. Ed.*, 2008, **47**, 3626; (c) J. K. Lee, Y. H. Jung, J. B. H. Tok and Z. Bao, *ACS Nano*, 2011, **5**, 2067; (d) B. R. Stepp, J. M. Gibbs-Davis, D. L. F. Koh and S. T. Nguyen, *J. Am. Chem. Soc.*, 2008, **130**, 9628; (e) I. Eryazici, I. Yildirim, G. C. Schatz and S. T. Nguyen, *J. Am. Chem. Soc.*, 2012, **134**, 7450; (f) R. V. Thaner, I. Eryazici, O. K. Farha, C. A. Mirkin and S. T. Nguyen, *Chemical Science*, 2014, **5**, 1091.

13 (a) S. Keller, J. Wang, M. Chandra, R. Berger and A. Marx, *J. Am. Chem. Soc.*, 2008, **130**, 13188; (b) W. Chen, A. Bian, A. Agarwal, L. Q. Liu, H. B. Shen, L. B. Wang, C. L. Xu and N. A. Kotov, *Nano Lett.*, 2009, **9**, 2153; (c) M. R. Hartman, D. Y. Yang, T. N. N. Tran, K. Lee, J. S. Kahn, P. Kiatwuthinon, K. G. Yancey, O. Trotsenko, S. Minko and D. Luo, *Angew. Chem., Int. Ed.*, 2013, **52**, 8699; (d) H. Kuang, W. Ma, L. G. Xu, L. B. Wang and C. L. Xu, *Accounts. Chem. Res.*, 2013, **46**, 2341; (e) D. Yang, M. R. Hartman, T. L. Derrien, S. Hamada, D. An, K. G. Yancey, R. Cheng, M. Ma and D. Luo, *Accounts. Chem. Res.*, 2014, **47**, 1902.

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

15 (a) V. Vijayanathan, T. Thomas and T. J. Thomas, *Biochemistry*, 2002, **41**, 14085; (b) A. El-Sayed and H. Harashima, *Mol. Ther.*, 2013, **21**, 1118.

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