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Efficient construction of a stable linear gene based on a TNA loop modified primer pair for gene delivery†

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A terminal-closed linear gene with strong exonuclease resistance and serum stability was successfully constructed by polymerase chain reaction (PCR) with an α-L-threose nucleic acid (TNA) loop modified primer pair, which can be used as an efficient gene expression system in eukaryotic cells for gene delivery.

Gene therapy is a promising strategy for the treatment of various human diseases at the genetic level through the regulation of target gene expression.[1] Tailored genetic manipulation technologies have been developed for gene therapy, such as gene delivery (target gene and mRNA), gene silencing (antisense and small interference RNA), and gene editing (sgRNA/Cas9).[2] Construction of a safe and efficient delivery system for these nucleic acid drugs is the first key step for the development of gene therapy in clinical application. Till now, the developed gene vectors are broadly classified as viral and non-viral types.[3] The viral gene vectors with efficient cellular uptake have been approved for clinical treatments, such as glybera for lipoprotein lipase deficiency (LPLD) and strimvelis for adenosine deaminase-deficient severe combined immune deficiency (ADA-SCID).[4] Considering potential immunogenicity and insertional mutagenesis, the wide application of viral gene vectors is limited.[5] Much attention has been drawn to the fabrication of various non-viral vectors.[6] The traditional circular plasmid is the most representative non-viral vector in the research of gene delivery. The obstinate bacterial remnants such as lipopolysaccharides (LPS) and numerous unmethylated CpG motifs in the backbone of the plasmid can induce an adverse immune response.[7] To address this issue, polymerase chain reaction (PCR) amplified linear gene expression cassettes including only the necessary transcription unit (promoter and target gene) are attracting much interest.[8] However, the poor stability of the linear gene expression cassettes is the main challenge for their further application in gene delivery. Much effort has been devoted to improving the stability of this linear nucleic acid, such as the construction of a dumbbell-shaped nucleic acid.[9] This kind of terminal-closed linear gene is conventionally fabricated step by step through PCR amplification, restriction endonuclease digestion, and T4 ligation processes, which are complex and time-consuming. We, therefore, hypothesize that PCR amplification with the unnatural nucleic acid loop modified DNA primer pair may result in the facile and efficient construction of a stable terminal-closed linear gene in large scale for gene delivery.

Xeno-nucleic acids (XNAs) are synthetic nucleic acid analogues with unnatural sugar backbones.[10] Because of their high stability towards the nuclease, XNAs have been widely employed in various biomedical applications, such as bio-imaging, diagnosis, and therapeutics.[11] In particular, α-L-threose nucleic acid (TNA), first synthesized by Eschenmoser's group twenty years ago, demonstrates several unique properties for biological application, such as excellent biocompatibility, strong enzymatic resistance, and efficient cellular uptake.[12] TNA also possesses the ability to form a stable antiparallel Watson–Crick base pairing with itself and natural nucleic acids (DNA and RNA), which can be tailored to fabricate unnatural nucleic acid loop modified linear genes. To the best of our knowledge, the construction of stable TNA loop modified linear gene expression cassettes for gene delivery has not been previously reported.

Herein, we describe a facile and universal strategy to construct a terminal-closed linear gene with two TNA loops for efficient gene expression (Scheme 1). A TNA loop modified primer pair is rationally designed and employed to amplify the linear gene through PCR. After T4 ligation, the terminal-closed linear gene with strong exonuclease resistance and serum stability is

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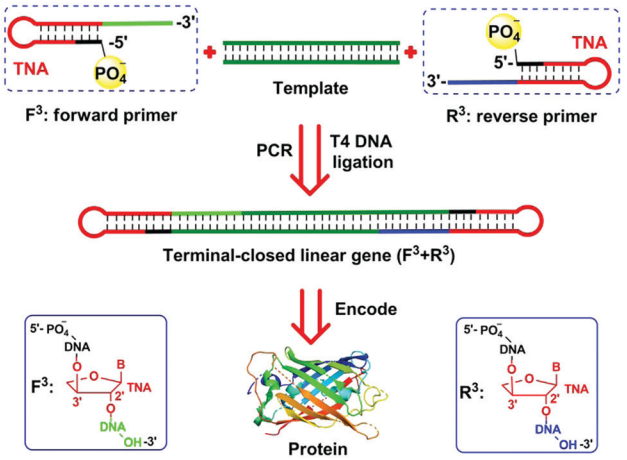

Scheme 1 Schematic representation of the construction of the terminal-closed linear gene through PCR amplification with a TNA loop modified primer pair for gene expression.

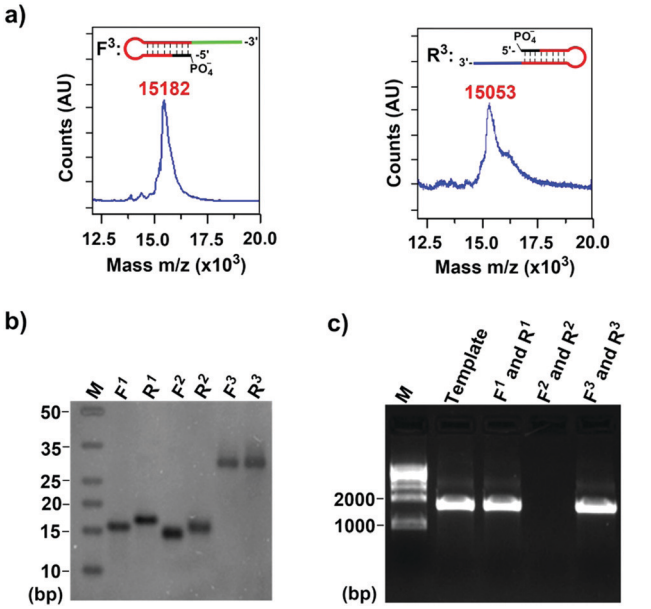

Fig. 1 Synthesis of the terminal-closed linear gene. (a) MALDI-TOF-MS analysis of F^3 and R^3. (b) 8% native polyacrylamide gel electrophoresis analysis of primer pairs, including F^1 and R^1 (DNA_{20}), F^2 and R^2 (TNA_{20}), and F^3 and R^3 (3′-DNA_{20}-TNA_{26}-DNA_4-P-5′). The gel was stained by Stains-All (M: DNA marker). (c) 1% Agarose gel electrophoresis analysis of PCR products (F^1 and R^1, F^2 and R^2, and F^3 and R^3) of the linear CMV-EGFP template based on the primer pairs (F^1 and R^1, F^2 and R^2, and F^3 and R^3), respectively. The gel was stained using EB (M: DNA marker).

successfully fabricated with a high yield. The stable gene expression cassette elicits an efficient and long-term gene expression in eukaryotic cells. Our report is the first example of the use of a TNA loop modified primer pair to amplify the target gene as the efficient gene expression system.

We initially synthesized the four 2′-phosphoramidites based on Chaput's reports (TNA-A, TNA-T, TNA-G, and TNA-C monomers, Scheme S1, ESI†) for the solid-phase synthesis of TNA.[13] These TNA monomers were characterized using ^1H, ^{13}C, ^{31}P NMR and ESI-MS analyses (shown in ESI†). Then, we designed three groups of primer pairs including F^1 and R^1 (DNA_{20}, the subscript represents the base number of the nucleic acid sequences), F^2 and R^2 (TNA_{20}), and F^3 and R^3 (3′-DNA_{20}-TNA_{26}-DNA_4-P-5′) for PCR analysis (detailed nucleic acid sequences are shown in the ESI†). The common DNA primer pair (F^1 and R^1) was commercially available to amplify the naked linear gene (F^1 + R^1). The TNA primer pair (F^2 and R^2) was employed as a control to evaluate the tolerability of DNA polymerase (La-Taq) towards the TNA primer. The TNA loop modified primer pair (F^3 and R^3) includes four tailored functional components to facilitate the construction of the terminal-closed linear gene (Scheme 1). In the 3′ to 5′ direction of F^3 and R^3, a common DNA primer with the same sequences as that of F^1 and R^1 for PCR (green and blue), a TNA loop for terminal closing and PCR stopping with the same sequences as that of eukaryotic tRNA D-loop (red), four DNA bases [GCGC] for efficient base pairing with the stem region of TNA loop (black), and a phosphate group for T4 ligation (yellow) were all covalently linked step by step in one pot for solid-phase synthesis (detailed synthetic method is shown in the ESI†).

After the desalination process using a SEP-PAK classic C18 column, we further employed 15% denatured polyacrylamide gel electrophoresis (PAGE) to purify the primer pairs obtained by solid-phase synthesis. The purified primer pairs (F^2, R^2, F^3 and R^3) were subsequently characterized using MALDI-TOF mass spectrometry (Fig. 1a and Fig. S1, ESI†). We found that the observed values of molecular weights are consistent with the calculated values. We subsequently analyzed these three groups of primer pairs using 8% native PAGE, as shown in Fig. 1b. In order to clearly visualize the TNA involved primers, we employed an efficient nucleic acid dye (Stains-All) for gel staining. With the same nucleic acid sequences, the TNA primer pair (F^2 and R^2) showed slightly faster mobility than the DNA primer pair (F^1 and R^1) as a whole, which may be attributed to the one less methylene group on the TNA molecular backbone compared with DNA. We also observed the clear bands of the TNA loop modified primer pair (F^3 and R^3) in the gel image (Fig. 1b). These results of MALDI-TOF MS and gel characterization indicated that the TNA loop modified primer pair (F^3 and R^3) was successfully synthesized.

To evaluate the PCR amplification ability of these obtained primer pairs, we constructed a linear CMV-EGFP gene template (1679 bp) as a proof of concept (detailed gene sequences are presented in the ESI†). As shown in the 1% agarose gel electrophoresis analysis (Fig. 1c), the naked linear DNA template (CMV-EGFP) can be efficiently amplified using the common DNA primer pair (F^1 and R^1). However, no PCR product was observed in the PCR system with the TNA primer pair (F^2 and R^2). This negative result indicated that the TNA sequences cannot be recognized and subsequently elongated using La-Taq DNA polymerase. Because of this PCR blocking phenomenon induced by TNA, we rationally organized the TNA loop in the 5′ terminal of the common DNA primer for terminal closing and PCR stopping. As shown in Fig. 1c, we observed an obvious target PCR product with the TNA loop modified primer pair (F^3 and R^3) in the agarose gel image. This result demonstrated that the terminal TNA loop

modifications of DNA primers will not affect the PCR process to amplify the target gene.

After obtaining the terminal TNA loop modified EGFP gene, the terminal-closed linear EGFP gene (F^3 + R^3) was subsequently fabricated using T4 DNA ligation at 25 °C for 5.0 h. This ligation process was facilitated by the efficient complementary base pairing between the 5′ terminal DNA [GCGC] and the stem region of the TNA loop involved in the primer pair (F^3 and R^3). A 1% denatured agarose gel electrophoresis analysis and exonuclease III (a kind of 3′ to 5′ exonuclease) digestion assay were performed to evaluate the efficiency of terminal-closed ligation. After T4 ligation, the PCR product based on the primer pair F^3 and R^3 showed obviously slower mobility than the non-ligated control group (Fig. S2a, ESI†). The enhanced resistance to exonuclease III digestion also confirmed the successful construction of the terminal-closed linear gene (F^3 + R^3) (Fig. S2b, ESI†). After purification using a gel extraction kit, the terminal-closed linear gene (F^3 + R^3) can be directly used in the following evaluations.

We next investigated the exonuclease resistance and serum stability of the terminal-closed linear gene (F^3 + R^3). The constructed linear EGFP gene was incubated with different units of lambda exonuclease (a kind of 5′ to 3′ exonuclease) at 37 °C for 1.5 h. As shown in Fig. 2a, the naked linear EGFP gene (F^1 + R^1) was almost completely digested after the treatment of lambda exonuclease in a relatively low concentration (5.0 U). In contrast, the terminal-closed linear EGFP gene (F^3 + R^3) demonstrated a strong exonuclease resistance even at a higher concentration of lambda exonuclease (10.0 U). The efficient terminal-closing by the stable TNA loop is responsible for the greatly enhanced exonuclease resistance. Meanwhile, we incubated the linear EGFP gene with 10% FBS in DMEM at 37 °C for different time periods to evaluate the serum stability. After incubation for 4 h, most of the naked linear EGFP gene (F^1 + R^1) disappeared and an evident smear band of digested gene was detected (Fig. 2b).

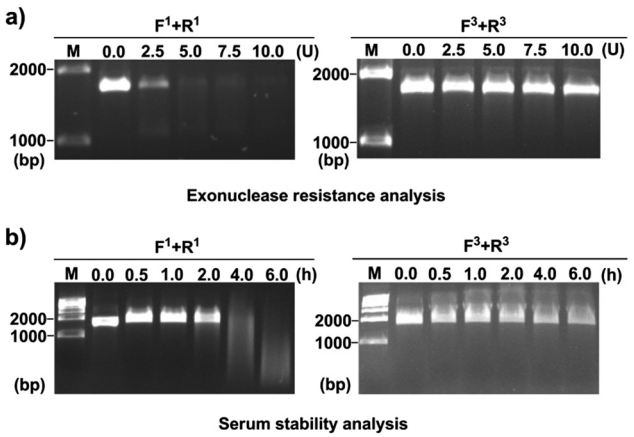

Fig. 2 Evaluation of exonuclease resistance and serum stability. (a) 1% Agarose gel electrophoresis analysis for the exonuclease resistance of the naked linear EGFP gene (F^1 + R^1) and terminal-closed linear EGFP gene (F^3 + R^3). (b) 1% Agarose gel electrophoresis analysis for the serum stability of the naked linear EGFP gene (F^1 + R^1) and terminal-closed linear EGFP gene (F^3 + R^3) (M: DNA marker).

After incubation for 6 h, a clear band of the terminal-closed linear EGFP gene (F^3 + R^3) could still be observed. After statistical analysis of the bands using Image J (Fig. S3, ESI†), an intuitive increase of serum stability was shown in the group of the terminal-closed linear EGFP gene (F^3 + R^3). These results indicated that the terminal-closed TNA loop modification is a universal strategy to enhance the stability of a target linear gene against the digestion of exonuclease and serum.

Encouraged by the enhanced exonuclease resistance and serum stability, we subsequently evaluated the gene expression potential of this terminal-closed linear EGFP gene (F^3 + R^3) in eukaryotic cells (HEK-293T). The eukaryotic CMV promoter and SV40 poly A section were both involved in the linear DNA template (CMV-EGFP) to ensure successful gene expression in the eukaryotic cells. 0.5 pmol naked linear EGFP gene (F^1 + R^1) and terminal-closed linear EGFP gene (F^3 + R^3) were transfected into the HEK-293T cells using Lipofectamine 2000, respectively. The transfected HEK-293T cells were directly imaged using a fluorescence microscope with an excitation wavelength of 488 nm at different time periods to visualize the expression level of the target EGFP gene. We observed an intense EGFP fluorescence in the group of the terminal-closed linear EGFP gene (F^3 + R^3, Fig. 3a), indicating that the terminal-closed TNA loop modification of the linear gene will not obviously affect the efficiency of gene expression in the cellular environment.

Furthermore, compared with the naked linear EGFP gene (F^1 + R^1), our terminal-closed EGFP expression cassette (F^3 + R^3) elicited a more efficient and long-lasting EGFP gene expression (Fig. 3b). After incubation at 37 °C for 60 h, we employed flow cytometry to quantitatively analyze the level of EGFP expression with the treatments of these constructed gene expression cassettes (Fig. 3c and Fig. S4, ESI†). About a 36% enhancement of the EGFP positive cells was observed upon the treatment of the terminal-closed linear EGFP gene (Fig. S5, ESI†). This efficient and long-lasting gene expression may be attributed to the strong exonuclease resistance and serum stability of the terminal-closed linear EGFP gene with the stable TNA loop modification.

In summary, we have developed a universal and efficient strategy for the construction of a terminal-closed linear gene through PCR with a TNA loop modified primer pair. This terminal-closed linear gene demonstrates several desirable features for gene delivery. First, this terminal-closed linear gene can be easily produced by PCR amplification in a short time without the requirement of restriction endonuclease. Second, this chemically well-defined linear gene shows obviously enhanced exonuclease resistance and serum stability. Finally, our stable terminal-closed linear gene can elicit an efficient and long-lasting gene expression in eukaryotic cells. This construction strategy for a stable gene expression cassette can be potentially generalized to other target genes, which will open a new avenue for the development of gene therapy.

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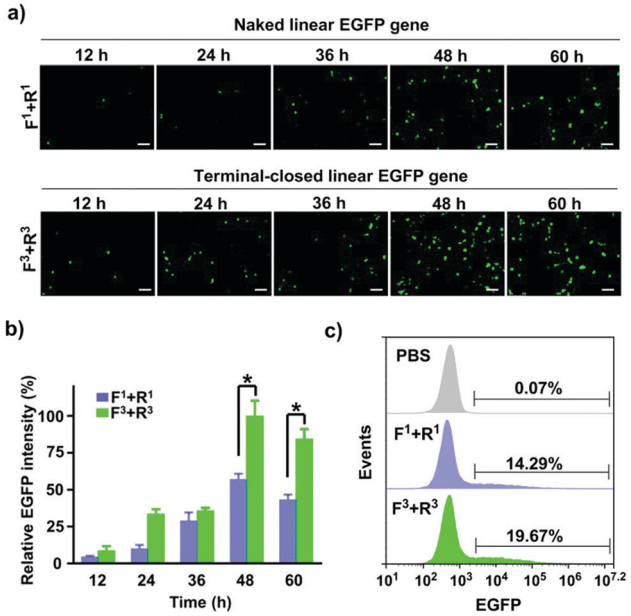

Fig. 3 Gene expression analysis in HEK-293T cells. (a) Fluorescence microscope images of the expression of EGFP in HEK-293T cells transfected with 0.5 pmol naked linear EGFP gene ($F^1 + R^1$) and terminal-closed linear EGFP gene ($F^3 + R^3$) for different time periods. Scale bar: 50 μm. (b) Statistical result of the relative EGFP intensity of HEK-293T cells treated with $F^1 + R^1$ and $F^3 + R^3$ using Image J analysis (*$P < 0.05$). (c) Flow cytometry analysis of the expression of EGFP in HEK-293T cells transfected with PBS, $F^1 + R^1$, and $F^3 + R^3$ for 60 h.

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Conflicts of interest

There are no conflicts to declare.

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