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DNA display for drug discovery†

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A novel DNA display strategy, based on a new puromycin modifier, has been developed. The 5′-end puromycin-tethered oligonucleotide was synthesized to hybridize with mRNA, so that it could attack the nascent polypeptides during *in vitro* translation. The DNA–peptide fusion molecule can tolerate more harsh and stringent selection conditions, therefore, this DNA display may become a useful tool for *in vitro* display technologies for the selection of peptide drug candidates.

A major issue in pharmaceutical science is the identification of small organic molecules capable of specific binding to target proteins of interest. Conventional technologies for drug discovery are based on screening individual molecules against enzymatic activity of the target protein or by displacement of labeled ligands. High-throughput screening allows a researcher to quickly conduct millions of chemical, genetic or pharmacological tests.[1] However, the success of screening depends on the amount and structural diversity of the libraries of compounds. The use of "virtual screening" based on the analysis of structural information may reduce the number of compounds that need to be evaluated.[2,3] Alternatively, a fragment-based approach may be used for the identification of multidentate ligands with suitable binding affinities and pharmacological properties.[4–6] However, the display of ligands such as peptides and proteins on the surface of bacteriophage (Phage display) has revolutionized the field of ligand isolation from a large library.[7,8] Billions of polypeptides, physically linked to their encoding DNA and target proteins can be immobilized on a solid support. The enrichment of ligands from these libraries is achieved by the co-selection of target-binding peptides. The transfection efficiency is one of the main limitations of phage display and other *in vivo* display technologies such as plasmid display,[9] which leads the library diversity to approximately 10^9 independent molecules. Ribosome display is an *in vitro* system, which has the potential to screen peptide libraries of three to six orders of magnitude more than the *in vivo* display.[10] Despite, the ribosome–mRNA–peptide complex is extremely labile in cell-free conditions and it is difficult to preserve its integrity during the selection process. The complete *in vitro* selection strategy relies on the covalent linkage between mRNA and peptides, known as mRNA display or *in vitro* virus, and was proposed by Szostak and Nemoto independently in 1997.[11,12] Puromycin, which has a similar structure to the aminoacyl end of tRNA, was tethered on the 3′-end of mRNA to capture the nascent peptides after translation by the ribosome. However, the use of RNA as the library-encoding nucleic acid is less convenient compared to the use of DNA due to its susceptibility towards RNase degradation, which limits the application of mRNA display in drug discovery. Recently, covalent display technologies have been developed to allow direct binding between peptides and encoding DNA sequences without the help of any enzymes.[13,14] Therefore, a branched DNA primer tethered with puromycin can be applied to accept the nascent peptide during *in vitro* translation, and the code of the linked peptide can be obtained by reverse transcription of this primer on an mRNA template. The branched DNA primer with the puromycin modifier can only be synthesized in a professional laboratory, and is not available on a commercial basis for ordinary use.

Herein, we report a novel DNA display strategy based on a new puromycin modifier, that can be attached to the 5′-end of a DNA primer (Fig. 1). As this primer is complimentary to the 3′-end sequence of mRNA, it can hybridize with mRNA to form a duplex tail. During the *in vitro* translation of mRNA, when the ribosome reaches the duplex domain, puromycin can enter the ribosomal A-site and form a stable amide linkage with the nascent polypeptide. Afterwards, the 3′-end of the DNA primer can be re-transcribed to obtain the DNA–peptide complex (Fig. 1). Such a system has the following features: (1) the displayed polypeptides and the nucleic acids encoding them are covalently linked. (2) Unlike RNA, which is subjected to RNases, the more robust and stable DNA is employed to encode the peptide. Therefore, the DNA–peptide fusion molecules can tolerate more harsh and

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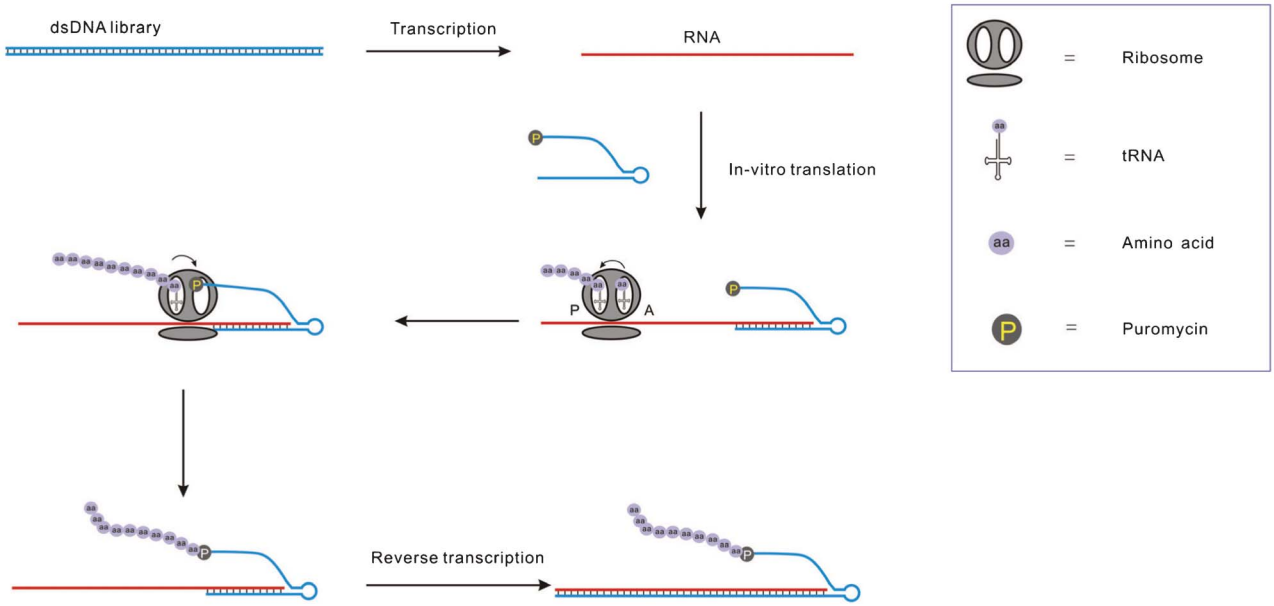
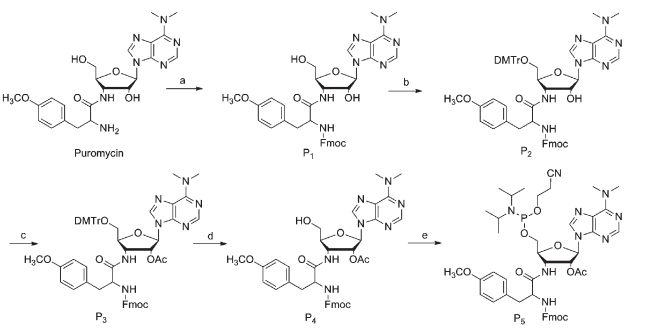


Fig. 1 *In vitro* selection of peptides. First, the puromycin–oligonucleotide is annealed to the DNA near the 3′-end, then the hybridization mixture is added into the *in vitro* translation system. At the end of translation, the ribosome stops when it comes to the junction of single stranded RNA and the duplex DNA region. Here, puromycin enters the A site of the ribosome to capture the nascent peptide. On reverse transcription, the cDNA–peptide fusion is formed and affinity selection is executed. The target cDNA–peptide fusion is amplified by PCR after enrichment and the second round of selection is carried out.

stringent selection conditions. (3) A library with more than 10^{12} DNA–peptide molecules can be constructed for drug discovery.

In this study, first we have synthesized the puromycin phosphoramidite that could be coupled to the 5′-end of oligonucleotides directly by standard DNA synthetic protocols, which is different from the way developed by Szostak and other groups in which puromycin was tethered at the 3′-end of oligonucleotides.[11,15,16] As the puromycin was labeled at the 5′-end, the modified DNA primer could be extended by re-transcription from the 3′-end. To mimic the aminoacyl end of tRNA, puromycin was linked through a phosphate ester bond from the 5′-hydroxyl group.

Therefore, synthetic work was carried out to obtain 5′-phosphoramidite puromycin **P5** (Scheme 1). We initiated our research by protecting the amino group with fluorenylmethoxycarbonyl (Fmoc) to obtain **P1**. As the Fmoc group can easily be deprotected at high temperature, all the following reactions were carried out below 45 °C. In the next step, the bulky dimethoxytrityl (DMT) group could only be incorporated to the 5′-position of the sugar moiety due to steric hindrance and thus provided exclusively the product **P2**. On the other hand, acetic anhydride was used to protect the 2′-hydroxyl group of the ribose sugar to get **P3** due to its high reactivity and the small size of the acetyl group. After the DMT group was removed by mild protic acids, the 5′-hydroxyl moiety on **P4** was the only reactive nucleophile capable of participating in the subsequent derivatization reaction. To obtain the final phosphoramidite product, pyridine was used as solvent with a catalytic amount of DIPEA (*N*,*N*-diisopropylethylamine). Finally, the 5′-phosphoramidite puromycin **P5** was synthesized after five simple steps with the yield of 66%.



Scheme 1 Synthesis of the puromycin modifier. Reagents and conditions: a. Fmoc–OSu, Et_3N–CH_3CN; b. DMTrCl, Et_3N–pyridine; c. $(Ac)_2O$, DMAP–pyridine; d. $Cl_2CHCOOH$–CH_2Cl_2; e. Phosphorus reagent, DIPEA–pyridine.

With the puromycin phosphoramidite in hand, we have carried out the synthesis of the modified primer on the DNA synthesizer (Fig. S1, ESI†). Before coupling the final puromycin modifier, one PEG modifier (Spacer-18) and two reverse deoxy-cytidines (dC-5′–CE phosphoramidite) were added in between. The puromycin modified DNA primer was purified by reverse phase HPLC and verified with mass spectrometry (Fig. S2 and S3, ESI†). The full primer (**Pu16-L1**) was constructed by the ligation of the puromycin modified DNA primer and the sequence that is complimentary to the 3′-end sequence of mRNA (Fig. 2A). Further investigations by using the 5′-end puromycin-tethered oligonucleotide as a captured primer for the *in vitro* selection of peptides were carried out. After PCR (Polymerase Chain Reaction) and *in vitro* transcription, a full-length mRNA including a RBS (Ribosome Binding Site) and a ORF (Open Reading Frame) was used as the template for peptide expression. The fusion between peptides and **Pu16-L1** was formed

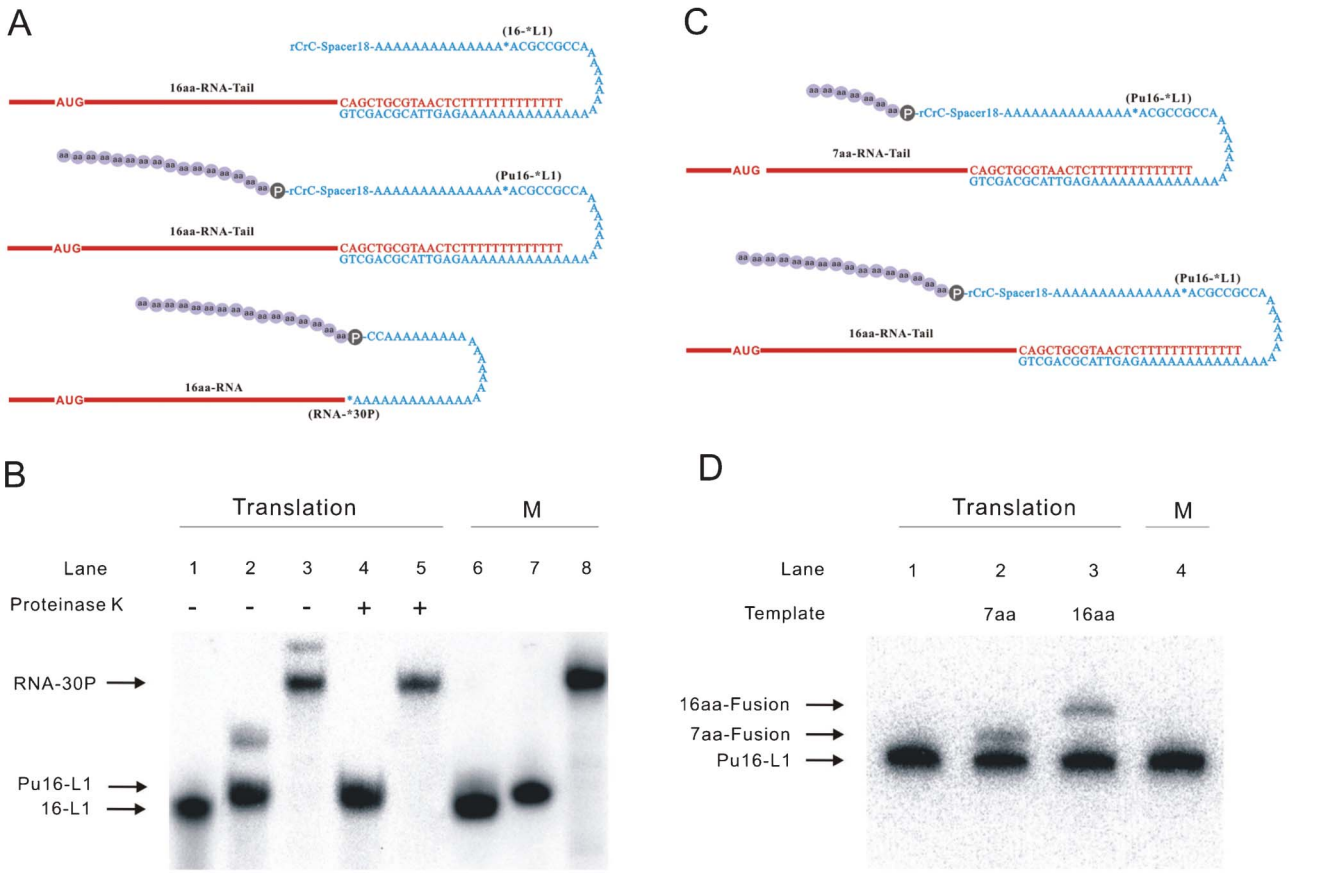


Fig. 2 A) From top to bottom: hybridization of the DNA primer (**16-L1**) without puromycin with mRNA–DNA (**16aa-RNA-Tail**); peptide displayed on the puromycin modified primer (**Pu16-L1**); mRNA display based on Szostak's strategy. The asterisk represents ^{32}P. B) *In vitro* translation and proteinase K degradation. Lane 1: *in vitro* translation with primer **16-L1**; lane 2: *in vitro* translation with primer **Pu16-L1**; lane 3: mRNA display with **RNA-30P**; lanes 4–5: the same samples from lanes 2 and 3 were treated with proteinase K; lanes 6–8: markers of **16-L1**, **Pu16-L1** and **RNA-30P**. C) *In vitro* translation with different lengths of the mRNA template (encoding peptide with 7 and 16 amino acids). D) Lane 1: *in vitro* translation without any mRNA template; lane 2: *in vitro* translation containing **16aa-RNA-Tail**; lane 3: *in vitro* translation containing **7aa-RNA-Tail**; lane 4: marker of **Pu16-L1**.

initially (Fig. S4, ESI†), but this result was not consistent with following repeated experiments. The situation did not improve even when a stop codon was inserted between the ORF and the duplex domain (data now shown). After excluding the possibility that the DNA primer was not long enough to get into the ribosome, we suspected that the ribsome was not completely stopped by the RNA and DNA hybridized region when it reached the junction of single stranded RNA and double stranded DNA because of ribosome helicase activity. Therefore, the 3′-end region of the RNA tail was replaced by a DNA sequence (Fig. 2A), and more than 10% of a DNA–peptide fusion product was formed. Afterwards, it was proved that only a DNA sequence can reduce the helicase activity of the ribosome to support the 5′-tethered puromycin on the primer **Pu16-L1** to attack nascent polypeptides. The 5′-end puromycin-tethered with a short oligonucleotide (30 nt) was also designed for translation. The autoradiography results show that the tether was still long enough for puromycin to capture resultant peptides in our system (data not shown). Furthermore, the fraction of DNA–peptide fusion increased significantly to 20% when potassium chloride and sodium chloride[17] were added into the *in vitro* translation reaction mixture under optimized conditions (Lane 2, Fig. 2B). This result is comparable to the yield of mRNA display (Lane 3, Fig. 2B) based on Szostak's strategy (**RNA-30P**, Fig. 2A). However, no DNA–peptide fusion was detected under the same conditions by using the similar primer **16-L1** that was devoid of puromycin on the 5′-end compared to primer **Pu16-L1**. This result indicates that the puromycin is indispensable in our DNA display strategy. To confirm that the higher molecular weight product (upper band in Lane 2, Fig. 2B) was the DNA–peptide fusion, proteinase K was applied to digest the peptides in the fusion product. As expected, the products of the DNA display and mRNA display disappeared upon treatment with proteinase K (Lane 4 and 5, Fig. 2B).

Furthermore, when we used different lengths of mRNA for translation (Fig. 2C), different bands of DNA–peptide products formed with migration rates according to their corresponding molecular size (lanes 2 and 3, Fig. 2D). All these results indicate that the peptides can be captured efficiently by the 5′-end puromycin-tethered oligonucleotides in our novel system.

To encode nascent polypeptides, the DNA primer must be re-transcribed on the mRNA template after *in vitro* translation. Three different DNA primers (perfect matched primer **L1**, full primer

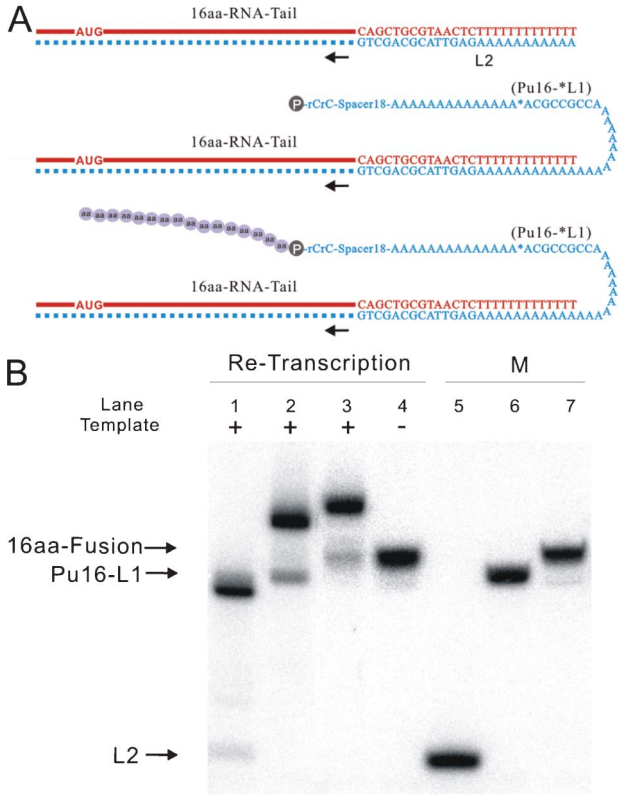


Fig. 3 A) Reverse transcription on mRNA templates with different primers. From top to bottom: perfect matched primer **L2**, full primer **Pu16-L1** and full primer **Pu16-L1** with peptide. B) lane 1: reverse transcription with perfect matched DNA primer **L2**; lane 2: reverse transcription with primer **Pu16-L1**; lane 3: reverse transcription with peptide tethered primer **Pu16-L1**; lane 4: reverse transcription with peptide tethered primer **Pu16-L1**, but without mRNA template; lanes 5–7: markers of **L2**, **Pu16-L1** and **Pu16-L1** with a peptide of 16 amino acids.

Pu16-L1, and full primer with peptide, Fig. 3B) were used in the reverse transcription experiment (Fig. 3). As illustrated in the autoradiographic results, all those primers have elongated well (lanes 1, 2 and 3, Fig. 3), which implies that the extra section, either the DNA tail or the tethered peptide, do not affect the re-transcription process. Based on our DNA display method, a peptide library containing as many as 10^{12}–10^{13} unique sequences could be constructed in one 1.5 ml Eppendorf tube in one day.

In summary, we have developed a new DNA display strategy based on a puromycin modifer that can be tethered on the 5′-end of a DNA primer by using standard DNA synthetic protocols. The DNA primer can hybridize with the mRNA to attack the nascent polypeptide to obtain a DNA–peptide fusion during the translation process. Due to the covalently bound DNA–peptide sequence, the fusion molecule is rather robust compared to an mRNA–peptide molecule, and may tolerate harsher *in vitro* drug selection processes than other display strategies that rely on biological systems. Moreover, the DNA sequences encoding displayed peptides in our strategy are hybridized with the corresponding mRNAs to form duplex molecules that avoid the undesired ssDNA aptamer in the drug selection process. Due to its feasible application, together with the highly stable nature of the protein–DNA complexes, our procedure proves to be a useful tool for *in vitro* display technology for the selection of peptide drug candidates. Follow-up screening experiments of specific target proteins by using this technology are currently under way in our laboratory.

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