

## Preparation of Monovalent Circular ssDNA Aptamer Using Rolling Circle Amplification Method

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Nucleic-acid aptamer is a promising therapeutic reagent. The poor stability of aptamer *in vivo* limits its application in the field of clinical therapy. Chemical modification can improve the stability of aptamer in serum; however it will bring a risk by the toxic nucleic acid metabolites. Circular aptamer is an attractive choice to improve the stability of aptamer. Here we developed a novelty rolling circle amplification (RCA) method to produce the monovalent circular ssDNA aptamer. In this RCA assay, the digestion reaction mediated by EcoR I was applied to ensure that the monovalent ssDNA aptamer molecules were obtained, the ligation reaction mediated by heat-resisted Taq DNA ligase was also applied to constructed circular aptamer template (caApt), and the digestion reaction mediated by Exonuclease I and III was used to detect and purify the circular ssDNA aptamer. A real-time RCA also was designed to estimate the efficiency of the cyclization. The result showed that our RCA method can efficiently produced monovalent circular ssDNA aptamer with exonuclease-resistant properties. The RCA method is a simple, fast, low-cost, and easy-to-automated method to prepare monovalent circular ssDNA aptamers.

**Key words:** Aptamer, Stability, Circularization, Rolling circle amplification.

Aptamer is an oligonucleotide selected from DNA or RNA libraries by iterative rounds of selection and amplification using a process called systematic evolution of ligands by exponential enrichment (SELEX). Aptamer has similar functions as antibody to specifically recognize and bind its ligand with a high affinity. Many of small molecules can become aptamer's ligand, such as metal ion, amino<sup>1</sup> acid, protein, and even whole organisms (Zhou *et al.*, 2009, Ng *et al.*, 2006, Kurreck *et al.*, 2003). Aptamer has a great potential to develop a new type of nucleic acid drugs and molecular diagnostic reagents. For example Macugen, a RNA

aptamer drugs, is becoming the first treatment to target the underlying disease process and has been proven to help preserve visual acuity in patients with wet AMD (European Commission Approves Macugen for Treatment of All Types of Wet Age-Related Macular Degeneration; First Treatment to Target Underlying Disease Process of Wet AMD. Information on <http://www.prnewswire.com/news-releases/55173912.html>). Aptamer are also be used as a infectious disease therapeutic agent, and are being researched to treat the diseases caused by HIV, HCV (Chou *et al.*, 2005, Soledad *et al.*, 2012, Liang *et al.*, 2011).

It is reported that an RNA fragment's stability is no more than one minute without modification. For example, the anticoagulant aptamer is only 108 seconds (Ng *et al.*, 2006). Modification of exposed termini of the aptamer molecules is an effective way to improve their

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stability in vivo (Kurreck *et al.*, 2003). Modification methods reported previously mainly concentrated on chemical process. LNA (Locked nucleic acid) (Schmidt *et al.*, 2004), PNA (Peptide nucleic acid) (Fabien *et al.*, 2006; Floch *et al.*, 2006) and unusual bases, such as, F, Br, OMe (Yan *et al.*, 2009), thiol (Pieve *et al.*, 2010), were introduced into the aptamer structure. However, the potential risk of genetic mutation and influence on nucleic acid circulation can not be ignored since unusual bases replacement.

Circularization of the aptamer molecules provides an attractive alternative because the circular aptamers displays a better stability characteristic than that of linear aptamer with canonical chemical modification. For example, the multivalent circular GS-522 improved anticoagulant potency with EC50 values 2-3-fold better than linear GS-522 (Giusto *et al.*, 2004). On the other hand, the circular aptamer can be constructed by nature nucleotide, which excludes unsafe factors that could impact on the nucleic acid metabolism in vivo.

In this assay, an efficient RCA method was used to produce monovalent circular ssDNA aptamer. As an auxiliary, the ligation-assisted

targets for DAN aptamer molecules were designed to improve the cyclization efficiency of the monovalent DNA molecules mediated by Taq DNA ligase. To monomerize the RCA products, a digested-assisted target was designed. We also designed a real-time RCA reaction to detect the cyclization efficiency produced by the RCA assay.

## MATERIAL AND METHOD

### Synthesis and Phosphate modification of aptamer molecules

An 86bp aptamer template (aApt) and aptamer molecules (Apt) were designed according reference (Hasegawa *et al.*, 2008) and bioinformatics analysis. The ligation-assisted targets for aApt (aLAT) and Apt (LAT) and the digested-assisted targets (DAT) were designed to improve the production efficiency of the monovalent ssDNA molecules; a pair of primers (Pfapt, Prapt) were designed for double primed RCA reactions. All of the oligonucleotides were chemically synthesized by Invitrogen Inc. (Shanghai, China), and 5' end of the aApt and Apt molecules were modified by Phosphate using T4 polynucleotide kinase (New England Biolabs Inc.).

**Table 1.** Sequences of the aptamer, LAT, DAT and primers

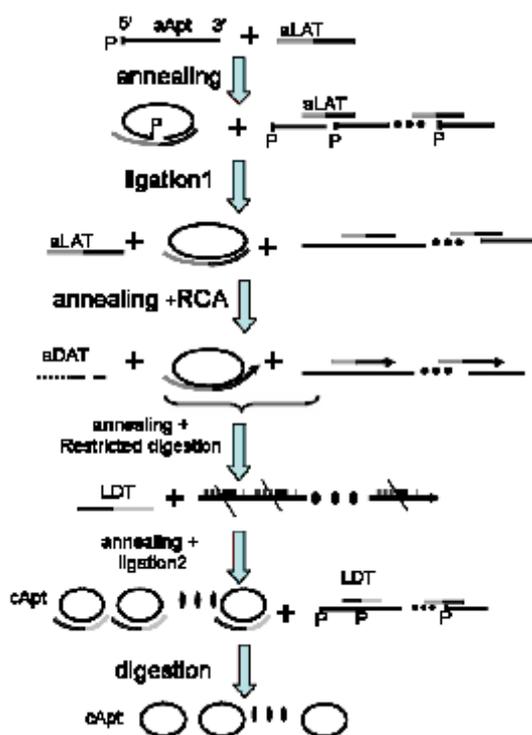
Name	Sequence(5'-3')
aApt	<u>TTAACCCGGGCAG</u> <sup>a</sup> GCCTACCACCCCTTAAGCCAAGCTTTTTTTTTTTCGAA AAAAAAAA ATATCATACACGTATGGTCAGATAAG
Apt	CCTATCTGACCATACGTGTATGATATTTTTTTTTTTCGAAAAAAAAAAGC <u>TTGGCTTAAG</u> <sup>a</sup> GGGTGGTAGGCCTGCCCGGGTTAA <sup>b</sup>
aLAT	<u>CTGCCCGGGTTAA</u> <sup>b</sup> CCTATCTGACCATACGTG
LAT	CACGTATGGTCAGATAAGTTAACCCGGGCAG
DAT(Pfapt)	GCCTACCACCCCTTAAGCCAA
Prapt	CTGCCCGGGTTAACTTATCTGAC

<sup>a</sup>The sequences of the LAT annealing with Apt were marked by dark gray and underline respectively.

### Preparation of the monovalent circular ssDNA aptamer

The preparation protocol of the monovalent circular ssDNA aptamer contains ligation by Taq DNA ligase, RCA reaction, restricted digestion by EcoR I, ligation by Taq DNA ligase, digestion by exonuclease I and exonuclease III, and so on (See Fig. 1).

First the aLAT molecules were annealed to phosphate mediated aApt molecules at the condition: 94°C 5min, ice bath for 5min. After annealing, Taq DNA ligase (TaKaRa Inc.) and buffer was added to incubate at 95°C 5min, then 15 cycle were carried out: 94°C 30s/45°C 5min. Then added aLAT into the reaction system again, the Bst DNA polymerase, Bst DNA polymerase buffer, dNTPs



**Fig. 1.** Scheme of monovalent circular aptamer preparation by RCA method. The protocol of the production of the circular anti-VEGF165 aptamer (cApt) by RCA method mainly includes the followed steps: first the 5' end of the Apt molecule was modified by phosphate groups, and then a RCA cycle was carried out: one RCA reaction, two Enzyme-linked reactions, two digestion reactions, and the produce of the cApt molecules increased in a high efficiency

were also added; the mono-primered RCA reaction was carried out: 65°C 2.0h; 80°C, 20min to inactivate the Bst DNA polymerase. To monomerize the RCA products, 1fmol products of RCA and 100pmol DAT molecules were annealed in EcoR I buffer, and then incubated at 37 °C for 3h; the EcoR I was inactivated at 65°C for 20min. After this, the monomerized molecules were purified by agarose gel. The monomerized molecules and LAT molecules were annealed in the buffer of Taq DNA ligase, then the Taq DNA ligase was added and 15 cycle were carried out: 94°C 30s, 45°C 5min.

In order to obtain circular ssDNA aptamer, it is needed to remove linear ssDNA aptamer, for example the complex of aptamer and LAT. Exonuclease I and exonuclease III (TaKaRa Inc.) were added into the circularization reaction to digest

linear DNA molecules at 37°C for 2.5h. The samples were separated on 8% polyacrylamide gel with 7 M urea in 1×TBE buffer (45 mM Tris-borate, pH 8.3/1 mM EDTA) dying with Goodview and the gel was analyzed on the Ultraviolet Imager.

#### Estimating of the cyclization efficiency by real-time RCA

A real-time RCA reaction for the quantitative of cApt was carried out (StepOne™, ABI Applied biosystems): 2 μL template samples (a series of concentration of cApt sample were prepared: 5, 25, 50, 75, 100, 150, 200, 250fmol; two sample: caApt, the produce first cyclization reaction, and cApt, 1/5 concentration of the second ligation reaction products) were replicated in 2 μL Bst DNA polymerase (New England Biolabs) buffer respectively, 0.6 μM of Pfapt and Prapt, 0.3 μM dNTPs, 6 U Bst DNA polymerase (New England Biolabs), 1 × SybrGreen I (Shinegene real time PCR core kit, shanghai shinegene); were added into the reaction system; 65 °C for 120 min, and the amplification curve dates collected once every 2 min. The results were analyzed with software StepOne Software v2.1.

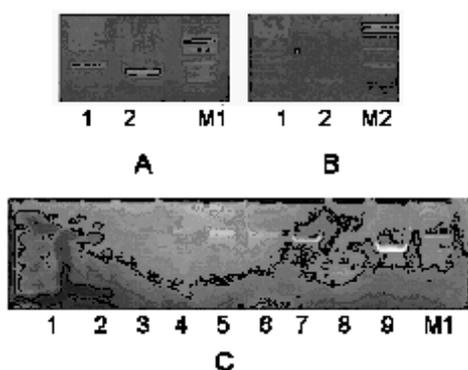
## RESULTS AND DISCUSSION

### Preparation of circular ssDNA aptamer

In order to obtain monovalent circular ssDNA aptamer, an effectively RCA protocol were designed. In the protocol, the monomerizing of the RCA products mediated by EcoR I is very important. The results of the lane3 and lane4 in Fig.2 showed that the the circle DNA molecules were successedly obtained in first ligation reaction, because linear DNA template have no products in RCA reaction. The results of lane1 and lane2 in Fig.2 showed that the monomerization reaction is succeeding for in the lane1 and lane2 monovalent DNA molecules being dominant.

When the protocol was finished, the monovalent circular ssDNA aptamer have to be detected and confirmed. In this assay, the agarose gel electrophoresis was used to directly detect the monovalent circular ssDNA aptamer sample and double-primered RCA method was also used to further detect monovalent circular ssDNA aptamer sample (see Fig.3). The position of the circular ssDNA aptamer in lane1 is lager than that of linear ssDNA

aptamer in line 2; and the double-primed RCA products (circular ssDNA aptamer as a template) in line 4 showed the ladder bands while the products (linear ssDNA aptamer as a template) in line 5 have no amplification. These results prove that the monovalent circular ssDNA aptamers were obtained through our assay, the above results showed that circular ssDNA aptamer were obtained after second ligation reaction. In the Fig. 3 C, we can find that the linear ssDNA aptamer molecules are easily digested by exonuclease I or exonuclease I+III. However, circular ssDNA aptamer molecules have a characteristic resistance against the degradation of exonuclease I and III, this can provide that the circular ssDNA aptamer will have a better stability in vivo, because the study showed that the exonuclease plays a major role in the serum or lymph to degrade the ssDNA or ssRNA aptamer molecules (Shaw *et al.*, 1991).

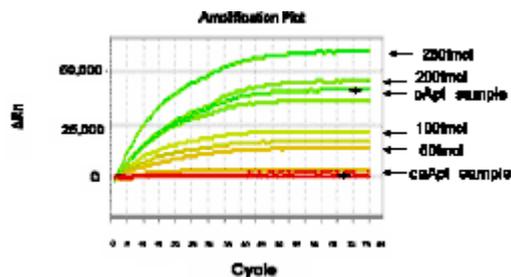


**Fig. 3.** The results of the agarose gel electrophoresis of circular ssDNA aptamer (cApt) sample and the linear ssDNA aptamer (Apt).  
 A. The results of circular ssDNA aptamer sample and the linear ssDNA aptamer. Lane 1, the sample of circular ssDNA aptamer (the products of cyclization reaction without purification); lane 2, the sample of linear ssDNA aptamer; lane M1, 20bp DNA Marker;  
 B. The results of circular ssDNA aptamer and the linear ssDNA aptamer by double-primed RCA. Lane 1, the sample of circular ssDNA aptamer (the products of cyclization reaction without purification); lane 2, the sample of linear ssDNA aptamer; M2, 200bp DNA Marker;  
 C. The results of circular ssDNA aptamer sample and the linear ssDNA aptamer digested by Exonuclease I, Exonuclease III. Line 1, the linear ssDNA aptamer sample digested by Exonuclease I+III; line 2, the linear ssDNA aptamer sample digested by Exonuclease I; line 3, the circular ssDNA aptamer sample digested by Exonuclease I+III; line 4, the circular ssDNA aptamer sample digested by Exonuclease I; line 5, the circular ssDNA aptamer sample; line 6, the circular ssDNA aptamer sample; line 7, the linear ssDNA aptamer sample; line 8, the DAT sample (25bp); line 9, the linear ssDNA aptamer sample (278bp); M1 20bp DNA Marker;

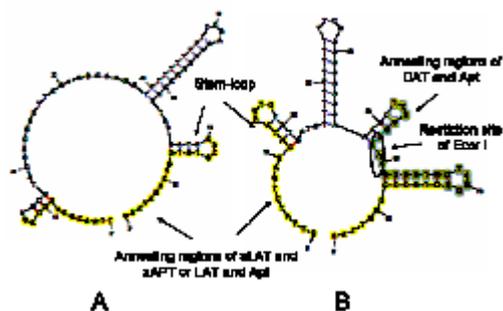
**Fig. 3.** The results of the agarose gel electrophoresis of circular ssDNA aptamer (cApt) sample and the linear ssDNA aptamer (Apt)

In order to estimate the cyclization efficiency of the aptamer molecules, we designed a real-time RCA reaction. According to the results of Fig. 4, we could estimate that the cApt molecules have a  $1.7 \times 10^3$  fold increasing corresponds to the beginning of the initial aptamer molecules within 10 hours, and through careful design, the protocol can achieve automated operation in a low cost. The amplification curves of the samples (caApt and cApt) were amplified by real-time RCA with forward primer (Pfapt), and reverse primer (Prapt). The caApt sample is the initial template molecules; and the cApt sample is the result of RCA cycle being cyclized by Taq DNA ligase and diluted 5-fold.

According to the results, there are a number of limiting factors to limit the improvement



**Fig. 4.** The analysis results of cyclization efficiency through real-time RCA method



**Fig. 5.** Prediction of secondary structures and annealing regions of Apt and aApt molecules. Secondary structures of Apt and aApt molecules were predicted by m-fold software.  
 A The prediction of secondary structures of aApt molecules, and the annealing regions of aLAT and aApt molecules;  
 B The prediction of secondary structures of Apt molecules, and the annealing regions of LAT, DAT and Apt molecules.

**Fig. 5.** Prediction of secondary structures and annealing regions of Apt and aApt molecules. Secondary structures of Apt and aApt molecules were predicted by m-fold software

of the reaction efficiency in our assay. Through analysis using bioinformatics methods, we found that there are stable stem-loop structures in the annealing regions of aLAT and aApt molecules, LAT or DAT and Apt molecules (see Fig 5). The reactions efficiency decreased and the cyclization efficiency of the aptamer molecules also decreased. So how to design the aptamer molecules, LAT, DNA molecules is important in our assay, and in the next study, we will dedicated to the design of new molecular aptamer to improve the production efficiency of the monovalent circular ssDNA aptamer.

### CONCLUSIONS

In this study, we developed a RCA assay with ligation and digestion steps to prepare monovalent circular aptamer and designed a real-time RCA method to detect circular aptamer production. The obtained circular ssDNA aptamer had stronger resistant to exonuclease than linear ssDNA aptamer. It laid foundation for drug or molecular diagnostic reagents research and development of nucleic-acid aptamer. The work of using RCA method to produce and quantity circular aptamer is studying to further perfect.

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