Truncation of PDGF-BB Aptamer by Secondary Structural Analysis and Immunoassay

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***Abstract*—Aptamers demonstrate high binding affinity and specificity to their targets and contribute to a number of applications which require recognition molecules. Typically, original sequences of the aptamers comprised of 80 to 100 nucleotides (nt). Only certain nucleotides in each aptamer sequence play a key role in the binding functionality of the aptamers. Thus, each aptamer sequence comprised of two oligonucleotide regions: essential region and non-essential region. In many cases, the non-essential region causes a reduction of binding affinity of the parent aptamer sequence. It was, therefore, necessary to identify the essential region after aptamer screenings. This work aimed to truncate the PDGF-BB aptamer. The strategy relied on analyses of the secondary structure generated by RNAstructure and mfold. Then the truncated sequences were experimentally verified their bindings by enzyme-linked immunosorbent assay (ELISA). The results indicated that RNAstructure showed the high probability for predicting the secondary structure of aptamer and the truncated 36Apt exhibited an excellent binding capability to the target comparing to the binding capability of the full-length aptamer. Within the results, the secondary structural analysis was a promising strategy not only for aptamer truncation but also for the prediction of oligonucleotide structures.**

***Index Terms*—secondary structure, PDGF-BB aptamer, ELISA**

I. Introduction

Nucleic acid aptamers are short single-stranded oligonucleotides (RNA, DNA or chemically modified RNA, DNA) which are systematically isolated from a randomly combinatorial oligonucleotide library by a well-defined process accomplished in vitro selection. Through the selection, several high-affinity and high-specificity aptamers have been selected against a wide range of targets including small inorganic and organic molecules (K^+, Hg^{2+}, ATP, ethanolamine, etc.), large biomolecules (peptides and proteins), and even supramolecular complexes (viruses, bacteria or cells) [1], [2]. Aptamers had tremendously contributed to a wide range of application due to their advantages as following. First, due to the robustness of the phosphodiester backbone, aptamers could be able to withstand harsh biological or chemical conditions while antibodies or proteins are denatured easily and irreversibly [3]. Second, the aptamers exhibit high binding affinity, and their dissociation constants could be consequently achieved to picomolar – femtomolar scales [4]. Third, because the aptamer generation is accomplished in vitro selection, the aptamers have been claimed as less immunogenic or toxic molecules [5]. Possession of these characteristics makes the aptamers as rising-star molecules in several fields, especially therapeutic [6]-[8] and diagnostic [9]-[11].

The aptamers were normally screened by a well-established process called Systematic Evolution of Ligands by Exponential Enrichment (SELEX). To perform the SELEX, a library containing a number of single-stranded oligonucleotides with random sequences was used. Typically, each oligonucleotide sequence comprised of a randomized nucleotide region and two fixed nucleotide regions. The randomized region demonstrated a diversity in the library sequences. The fixed regions were used as primer sites in enzymatic amplification process (i.e. polymerase chain reaction) [12]. After the SELEX, original sequences of the screened aptamer were comprised of 80 to 100 nucleotides including the primer sites. In principle, different nucleotide regions of the screened aptamers played different roles in binding to their targets [13]. In addition, the primer regions would not play roles in binding. Thus, not all of nucleotides in a full-length aptamer played a critical role for binding capabilities. Only certain nucleotides in the full-length aptamers were essential for molecular folding and responded to the binding functionality of the aptamers [14]. The essential nucleotides interacting to the targets were typically 10 – 15 nt in length and displayed as following structures: hairpin loops, G-quartet loops, bulges, or pseudoknots [15]. Other essential nucleotides which did not directly contact to the target also played an important role in supporting the interactions and remaining the structural conformation of the contacting nucleotides which relatively indicated the binding capability. In general, the number of essential nucleotides was approximately 25 –

Manuscript received December 21, 2015; revised February 21, 2016.

doi: 10.18178/ijpmbs.5.1.86-90

40 nt [16]. Consequently, the other nucleotide regions were therefore called non-essential nucleotide regions. In many cases, the non-essential nucleotides might reduce the binding affinity of the full-length aptamer. Therefore, cutting off those nucleotides either does not change or can significantly enhance the binding affinity. Truncation of the aptamer sequences provided additional merits. First, short aptamer sequences were easy to be encapsulated or modified for novel nanostructures and nanomaterial [17]. Second, the aptamer sequences whose lengths were shorter than 60 nt were feasible to synthesize or modify, chemically [18]. It was, therefore, necessary to identify the essential region and to truncate the full-length aptamers after the SELEX.

It has been reported that RNAstructure and mfold software gave out the information of oligonucleotide structures. Since the structures also related to the binding functionality of those oligonucleotides, it was feasible to use this two software as a strategy for aptamer truncation. The prediction of the structure was performed to generate the secondary structure based on dynamic calculation [19], [20]. In order to identify the binding segment, a comparison of similar structures between a given full-length aptamer and shortened sequences was carried out. Then the information relating to the binding region could be evaluated. This technique was successful to identify the binding region of the anti-hPTK7 aptamer [21].

Herein, the truncation of the full-length aptamer that could bind to PDGF-BB was performed by secondary structural analysis using RNAstructure and mfold. After analysis the structural conformation, the full-length sequence was truncated. Those truncated sequences were experimentally verified their binding capabilities by using enzyme-linked immunosorbent assay (ELISA).

II. Materials and Methods

A. Reagents

All of the DNA molecules were purchased from Integrated DNA Technologies (Coralville, IA). Recombinant human platelet-derived growth factor BB (PDGF-BB, MW = 24.6 kDa), anti-human PDGF-BB antibodies, streptavidin-HRP, substrates were purchased from R&D Systems (Minneapolis, MN). Phosphate buffered saline (PBS) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO). Tween 20 was purchased from Bio-Rad laboratories (Hercules, CA).

B. Analysis of Secondary Structure

The secondary structures were generated by using RNAstructure version 5.7 (http://rna.urmc.rochester.edu/software.html) and the mfold online web server (http://unafold.rna.albany.edu). The most stable structures with the lowest free energy were reported.

C. Investigation of the Binding Functionality

To investigate the binding capabilities of truncated aptamers, the competitive assay was performed by using ELISA. Briefly, capture antibodies were immobilized on the surface of ELISA strips. Any unoccupied sites on the surface sites were blocked by BSA. Meanwhile, the aptamers incubated with PDGF-BB. After incubation, the solutions containing aptamer and PDGF-BB were transferred into the ELISA strips coated by the capture antibodies. Then, detection antibodies, streptavidin-HRP, and substrate were added into the strip, respectively (as shown in Fig. 1). The optical density of the solutions was measured by the plate reader (Chameleon, Hidex). The λ_{max} was set at 472 nm. The experiments were performed in triplicate.

Finally, the percentage was calculated by using equation:

$$Percentage\ (\%) = \frac{OD - OD_0}{OD_c - OD_0} \times 100$$

Whereas: OD is the optical density of samples; OD_0 is the optical density without PDGF-BB (blank); OD_c is the optical density with PDGF-BB only (control).

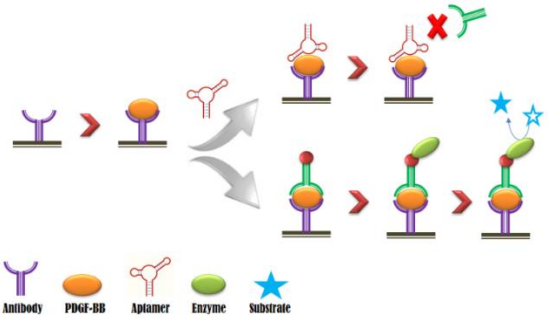

Figure 1. Schematic illustration for testing the binding functionality by ELISA [4].

III. Results and Discussion

A. Analysis of Secondary Structure

The original sequence of PDGF-BB aptamer was 86 nucleotides comprising of 40 randomized nucleotides and 46 nucleotides for the primer region [22]. At 5'-end, there were 21 nucleotides while at 3'-end, there were 25 nucleotides as the primer sites, respectively. The primer regions were possible points to be deleted out so that after the deletion remaining sequences were able to bind to PDGF-BB. The full-length aptamer, denoted as Full-Apt, was therefore truncated either each side of 5′ -end or 3′ -end or both ends to generate truncated sequences named as 5N21Apt, 3N25Apt and 40Apt, respectively. The structural analysis suggested that if the essential nucleotides region did not change the conformation, the truncated aptamers would remain the binding functionalities. Furthermore, the binding functionalities were significantly reduced or completely lost when the structural conformation of the essential region was changed. To study the structural analysis, the lowest free energy of Full-Apt, 5N21Apt, 3N25Apt and 40Apt is shown in Fig. 2. Both RNA structure and m fold indicated that the essential nucleotides still remain the initial conformation. It suggested that the binding functionality of these truncated sequences should be comparable to that of the full-length aptamer.

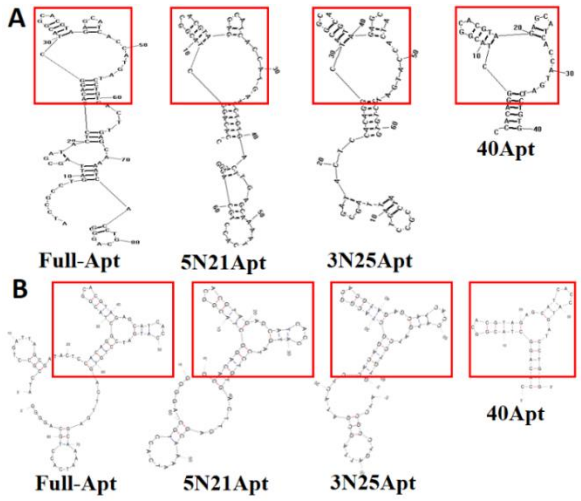

Figure 2. Represented secondary structures of Full-Apt, 5N21Apt, 3N25Apt, and 40Apt aptamers. (A) Using RNAstructure. (B) Using mfold. These structures were generated and displayed the lowest free energy. Rectangular boxes indicate the randomized nucleotides.

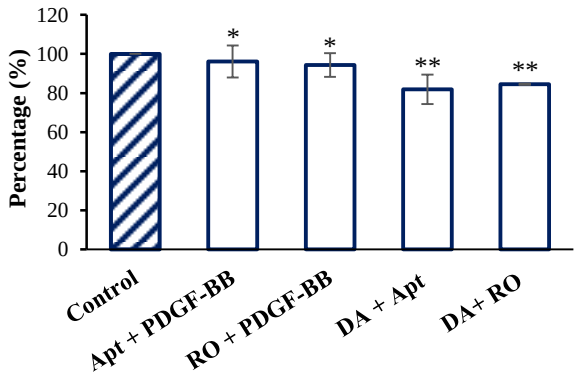

Figure 3. The effect of ELISA procedure on aptamer–protein interaction. 20 pM of PDGF-BB was sequentially incubated with different components. Control displays PDGF-BB (20pM). DA indicates detection antibody. RO indicates random oligonucleotide. Error bars indicate S.D (n=2). *P < 0.05; **P < 0.01 vs. control (two-tailed Student's t-test).

B. *Study of the Effect of ELISA Procedure on the Aptamer-Protein Interaction*

Prior to performing ELISA, effect of antibodies used in ELISA on the interference of aptamer-PDGF-BB interaction has been investigated (Fig. 3). To investigate the role of capture antibody on the interference, a certain amount of aptamer (Apt) or random oligonucleotide (RO) was incubated with the antibody. Then PDGF-BB was added and ELISA was performed normally. The results indicated that neither Apt nor RO interrupted interaction between PDGF-BB and capture antibody. The effect of detection antibody (DA) on the interference was also investigated by incubating the DA with the nucleotides and then performing ELISA normally. The results indicated that both Apt and RO did not interact with DA, therefore, the optical density of these two samples was 85% of the control. It suggested that there was no Apt-DA interaction. Within these results, the ELISA procedure could be utilized for investigating the binding interaction between aptamers and PDGF-BB.

C. *Examination of the Binding Functionality*

The secondary structural analysis suggested that after cutting off the primer sites, PDGF-BB aptamer could maintain the binding functionality. To confirm this hypothesis, a competitive assay based on ELISA was carried out (Fig. 4). 20 pM of PDGF-BB was incubated with 0.1 and 100 pM of Full-Apt and 40Apt. The results showed that truncation of primer sites could remain the binding capability of the aptamer. However, the truncated 40Apt exhibited a four-fold lower binding capability than the full-length aptamer. The possible explanation was that some remaining nucleotides were non-essential nucleotides and they caused the reduction of the binding affinity. Therefore, the truncation of aptamer should be further performed.

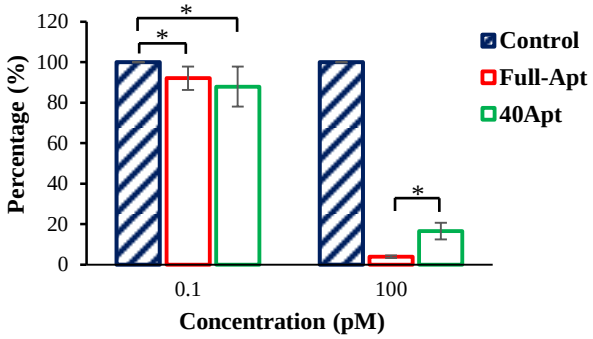

Figure 4. Effect of Full-Apt and 40Apt on PDGF-BB function measured by ELISA. The competitive binding assay, the low percentage indicates the binding capability; 20 pM of PDGF-BB incubated with different concentrations of these sequences. The control displays PDGF-BB (20pM). Error bars indicate S.D (n=3). *P < 0.05 (two-tailed Student's t-test).

D. *Truncation of PDGF-BB Aptamer*

To further study the ability of structural analysis for aptamer truncation, a nucleotide at both ends of 40Apt has been eliminated to generate 38Apt, 36Apt, 34Apt, and 32Apt. These sequences were analyzed the secondary structure by using RNAstructure and mfold (Fig. 5). Interestingly, there was a different structure prediction between two software. The RNAstructure predicted that the sequence conformation was changed after the truncation was over 4 nucleotides while mfold suggested that the conformation was changed once the truncation was over 2 nucleotides. The ELISA was then carried out to validate the binding of those sequences generated by the software.

Based on the structural prediction by mfold and RNAstructure, 36Apt, 34Apt, and 32Apt demonstrated different structures. The binding capability of two representative sequences, 36Apt and 32Apt, is shown in Fig. 6. The results showed that the binding capability of truncated 36Apt was greater than that of Full-Apt. The binding inhibition of 100 pM of 36Apt was approximately a hundred percent. More important, 36Apt displayed an excellent binding even at 0.1 pM. Meanwhile, Full-Apt did not bind to the target at this concentration. It indicated that the binding capability of the aptamer could be enhanced even though a number of

non-essential nucleotides has been removed. Not surprisingly, the 32Apt did not bind to the PDGF-BB. In this case, random 31Apt showed no binding.

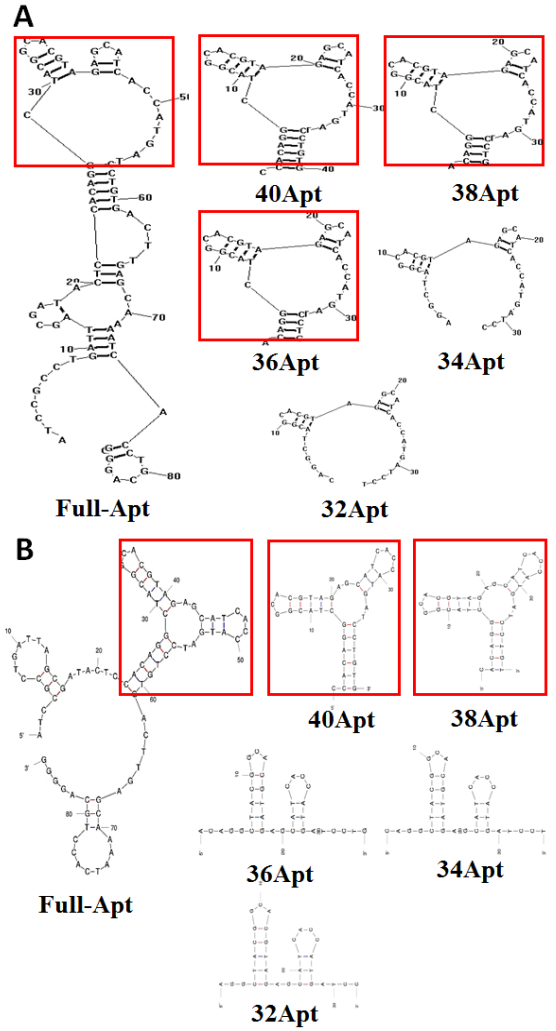

Figure 5. Secondary structures of Full-Apt and truncated aptamers (A) Using RNAstructure. (B) Using mfold. These structures were generated and displayed the lowest free energy.

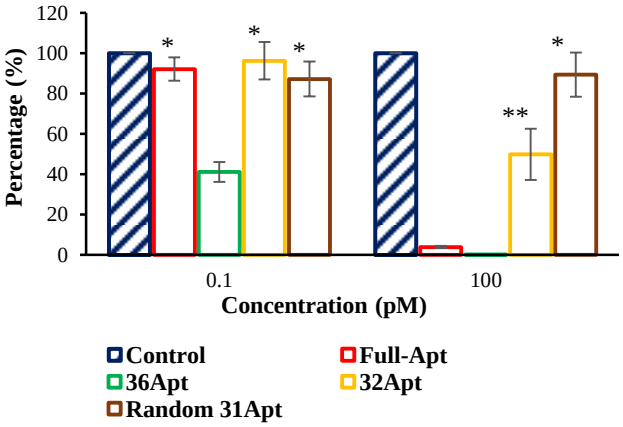

Figure 6. Binding verification of Full-Apt and truncated aptamers. The competitive binding assay. The control displays PDGF-BB (20pM). The random apt31 indicates no binding. Error bars indicate S.D (n=3). *P < 0.05; **P < 0.01 vs. control (two-tailed Student's t-test).

Our results demonstrated that the truncation was successful by analysis of the secondary structure. The existence of some non-essential nucleotides could induce the great interference to the binding capability of the aptamer. The RNAstructure software demonstrated high probability for predicting the secondary structure of the aptamers. It revealed an advanced understanding of the functionality of aptamers. However, there was a limitation. Since the secondary structure was theoretically generated through the algorithms which were based on the calculation of thermal parameters and free energy binding. In addition, the analysis has been done without the real biological environment or absence of target molecule. Consequently, there were numerous factors that could affect the binding functionality, including not only the changing of conformation but also the real biological media (pH, temperature, ions, etc.). However, the software provided an easy method for insight understanding of the binding functionality. Therefore, in the future work, investigation of aptamer interaction by the molecular dynamic simulation is a promising aspect. X-ray crystallography or nuclear magnetic resonance might be tools for the aptamer-target complex or interaction.

IV. Conclusion

In summary, the truncation of PDGF-BB aptamer has investigated by analysis the secondary structure generated by RNAstructure and mfold software. Our results demonstrated that the truncation was successful through the secondary structural analysis. The existence of some non-essential nucleotides could induce the great interference to the binding capability of the aptamer. In comparison with the full aptamer, the truncated 36Apt exhibited an excellent binding capability to the target. The RNAstructure software displayed the high probability for predicting the secondary structure of aptamer. Through this study, the secondary structural analysis was a promising tool not only for aptamer truncation but also for the secondary structural prediction of oligonucleotides.

Acknowledgment

The authors thank Faculty of Science and Technology, Thammasat University for the support of lab equipment. Mr. Cong was financially supported by Asean Economics Community (AEC) scholarship from Thammasat University. This work was granted by Research Grant for New Researcher from Thailand Research Fund (TRG5880201).

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