Selecting DNA aptamers for the open conformation of Transglutaminase-2 protein

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Introduction:

DNA **Aptamers** are short oligonucleic acid units (approximately 15-120 nucleotides) that bind to a specific target molecule, such as a protein. They are capable of forming complex structural interactions with the proposed target, to which they are capable of binding with both, a high degree of specificity and affinity.

Aptamers were selected for the target protein transglutaminase-2 (***TG-2***) locked in the open conformation with an irreversible inhibitor. ***TG-2*** in human tissue is responsible for cross-linking proteins by their glutamine and lysine residues[3]. The open conformation is 150 nanometers larger than the closed form. The open conformer of the enzyme is the active form and catalyzes the acyl-transfer reaction[3]. The frequency of conformers observed in the human body is dynamic, but in equilibrium. ***TG-2*** is responsive to calcium ions, which bind to the enzyme switching the conformation open; absence of calcium ions leads to the closure of the enzyme via a conformational shift. A physiological block on one conformer may be possible using this method of **aptamer** binding conjugated with an inhibitor in order to provoke an equilibrium response (according to Le Chatelier's Principle) by the body's internal systems.

Selecting for ***TG-2*** is essential for its elevated levels in cells have been recognized as a factor in the promotion of undesired autoimmune inflammation and degenerative diseases, including but not limited to metastatic cancer. The method employed is the Systematic Evolution of Ligands in Exponential Enrichment (SELEX). The incubated oligonucleotides with the locked open conformers are collected and then subsequently incubated with the locked closed conformers. Only the desired doubly specific **aptamers** selected for the open conformation are collected and subject to amplification using Polymerase Chain Reaction (PCR). Thus, the evolved pool is used for the succeeding selection round. The repetition of this procedure is what gives SELEX the potential to generate a pool of **aptamers** with the highest binding affinity to the targeted ***TG-2***. In addition, the use of a fluorescence assay will be used to confirm the presence of a successful selection process.

Aptamers	Small Molecules	Antibodies
Higher degree of stability: –Can withstand long term storage –Can be transported at room temperature –If denatured, they can be regenerated	Variable degree of stability: –Storage is variable –Temperature ranges are variable depending on the functional groups –Denatured molecules can either be inert or decompose into dangerous derivatives	Lower degree of stability: –Limited window of opportunity for use –Temperature sensitive –Denatured antibodies are unusable
Avoids "batch-to-batch" synthesis variation	Can have (detrimental) impurities	Have "batch-to-batch" variation
Does not require the use of animals (*in vitro*)	Can be synthesized in reaction flasks, even in non-sterile environments	Animals are essential for generating antibodies (*in vivo*)
Can label aptamers without affecting binding affinities	Can add side chains to avoid compromising the labelling potential of the molecule	Labels decrease the relative binding affinity of the antibody to the antigen
The specific on/off parameters of aptamer kinetics can be changed at will by the experimenter	Kinetics can be manipulated by the use of catalysts	The parameters surrounding the interactions of the antibody and the antigen are fixed

Table 1[5]: Rational for choosing **aptamers** over small molecules or antibodies as the vector to be used for the selection of ligand to bind to ***TG-2***. Green text symbolizes a positive point; red text symbolizes a negative point; brown text is a neutral point or midpoint between extremes.

Methodology:

In vitro SELEX **aptamer** selection[2] shown in **Figure 1** was performed and repeated for five rounds. A fluorescence assay was performed at the very end of all experimental rounds of selection, and a gel was run after every round of double incubations and amplification (to ensure purity and presence of the **aptamers**).

List of materials and apparatus:

- TAE buffer
- DPBS
- 30kDa cut-off filters
- Centrifuge
- 3% agar
- PCR Buffer
- Hot Start Polymerase
- $MgCl_2$
- dNTPs
- Multimode Microplate Reader – Filter Max F5
- Forward Primer (FAM-labelled and unlabelled): 5'-CTC CTC TGA CTG TAA CCA CG-3'
- Reverse Primer (unlabelled): 5' -GGC TTC TGG ACT ACC TAT GC-3'
- Sepharose 2B resin
  - –NC9 (open inhibitor): GSH-GST-TG2
  - –NC9 + GDP solution (closed conformer)

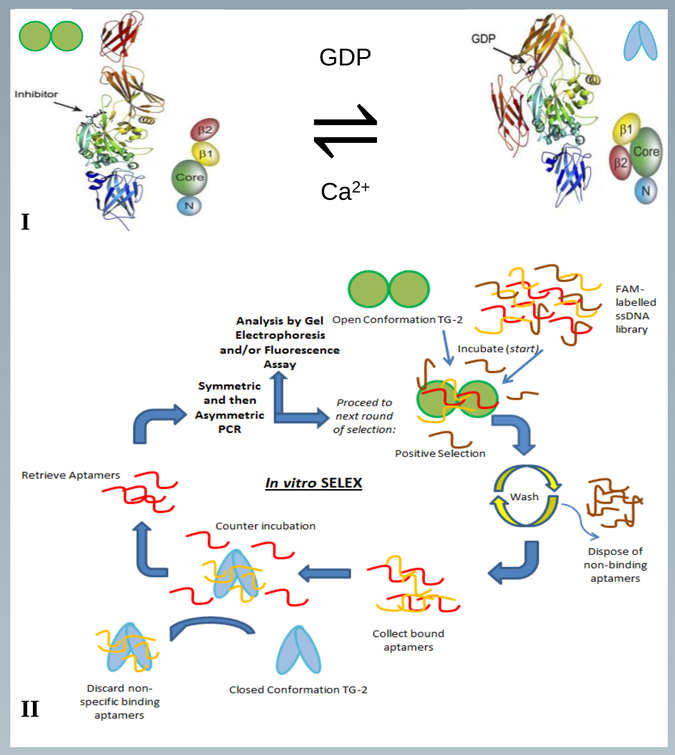

Figure 1. I: Shown in the top left corner is the inhibited/locked open form of ***TG-2***; the top right corner displays the closed GDP-bound form of ***TG-2***[4] including the equilibrium reaction of the conformer change. II: SELEX procedure for selecting **aptamers** for the open conformation of the ***TG-2*** protein.

Results:

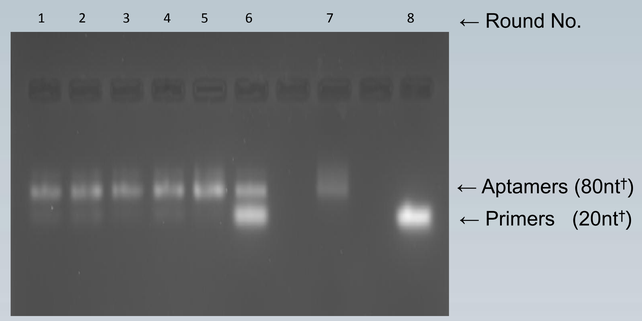

Figure 2. Picture of the gel electrophoresis from the final amplification (symmetric followed by asymmetric PCR) of the purified and evolved **aptamer** pools before analysis with the fluorescence assay. Lanes 1-5 are the evolved amplified **aptamers** pools and residual primers from Rounds 1 through5, respectively. Lane 6 is the positive control (FAM library DNA - diluted sample) for the PCR amplification experiment unpurified; Lane 8 demonstrates the negative control (DPBS) for the PCR; Lane 7 is the 200 nM DNA standard. † Nucleotides

Table 2: Key concentration and percent values from the final round of amplification of the open conformation aptamers.

Aptamer Concentrations		Primer Concentrations	
Band	**Concentration (nM)**		**Concentration (nM)**
DNA Standard	200	Average of positive and negative controls	756
Round 1	238	Average of the selection rounds	137
Round 2	270		
Round 3	253	Percent of primers purified from solution	82%
Round 4	312	Average percent	
Round 5	396	mass of the primers remaining in solution	32%

Five rounds of **aptamer** selection were performed yielding usable pools of the oligonucleotides for future use. The PCR results indicate that the **aptamers** selected in each round had the majority of the leftover primers being purified out from the DPBS buffered solution using 30kDa cut-off filters (**Table 1**). Each one of the five rounds of SELEX performed produced aptamers that were both selected for open conformation and against closed conformation (as a negative control).

The fluorescence assay revealed higher fluorescence intensity from the hybrid open conformation selected **aptamers**–open conformation ***TG-2*** resin than from the open conformation selected **aptamers**–closed conformation ***TG-2*** resin after the five rounds of selection.

Conclusion:

The purpose of this research was to select *in vitro* **aptamers** for the protein transglutaminase-2 from a FAM-labelled library **aptamer** pool. After the five rounds of selection, this objective was achieved, although more rounds of selection can be completed to improve both, the statistical significance and quality of the results[1], for the potential of a highly evolved **aptamer** pool to have greater binding constants is practically certain.

This objective was achieved as the analysis of the fluorescence assay performed on the open and closed conformation beaded resins of the target ***TG-2*** protein confirms the increasing trend of the detection of fluorescence levels after 5 SELEX rounds from the **aptamers** selected for the open ***TG-2***, as opposed to the decreasing fluorescence level measurements observed in the assays including the closed form of the enzyme incubated with the open-conformation selected **aptamer** pool (**Figure 3**).

As for the fluorescence assay, the result is a mixed blessing. For one, it can be interpreted that **aptamers** were selected for the targeted open conformation of the protein. Unfortunately, the validity of the specification of the **aptamers** is in question due to the wide range of fluorescence values recorded in a relatively unpredictable pattern (**Figure 3**).

While the selection did reduce the level at which **aptamers** were selected in the negative form (i.e.: fewer **aptamers** were generated capable of binding to the closed after being engineered positive), the selected **aptamers** have not exceeding the binding capacity of the FAM-library.

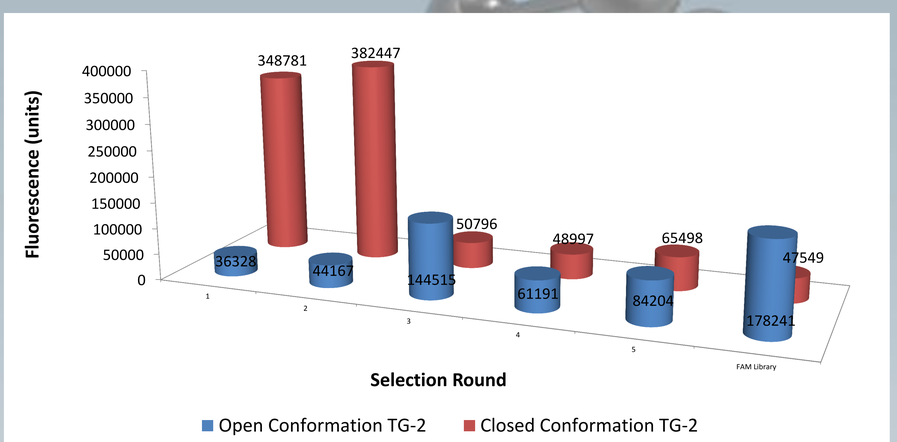

Figure 3. Corrected (using DPBS fluorescence measurement) fluorescence unit levels for the open and closed ***TG-2*** resin bound proteins incubated with the selected **aptamers** across the five rounds of the SELEX procedure. Greater fluorescence is indicative of higher concentrations/quantities of target FAM-labelled aptamers bound to the ***TG-2*** resin above background signal noise. The trend of the data indicates the slight increase of the SELEX-selected open conformation **aptamers**' affinity for the desired open ***TG-2*** target compared to the closed unwanted target. This can be interpreted as a positive sign that the **aptamers** being selected demonstrate a higher binding affinity to the open ***TG-2*** in comparison to non-specific binders.

References:

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