Dissertation Title-Development of Systematic Evolution of Ligands by Competitive enrichment-SELCO for highly selective and multiplex *in vitro* selection of DNA aptamers
Degree-Doctoral Course
Laboratory-Takamura Laboratory
Student Number-s1540201
Student Name-Ankita Kushwaha

Nucleic acids have been considered as units of genetic information, for inheritance and transfer from gene to protein. However, nucleic acids can also perform diverse functions such as enzymatic catalysis and transcription regulation. Further, the discovery of oligonucleotides able to bind various target molecules with high-affinity and high-specificity has been a valuable contribution for the multifunctional nature of nucleic acids. The conventional method for aptamer engineering is known as SELEX (Systematic Evolution of Ligands by Exponential enrichment). This technique mainly includes selection of aptamers from large pools of combinatorial library by binding with target molecule at indicated conditions. The non-binding ligands are removed and the binding candidates are amplified and proceed for the next round of selection. Such multiple rounds of selection are perform to yield a pool of few sequences with characteristic binding properties to target. Further, after cloning and sequencing, aptamer characterization and post-SELEX optimization are done in case of successful selection experiments. For selection of specific aptamers various approaches such as negative-, counter- and/or subtractive-SELEX are currently employed. The development of the aptamer selection technology, was considered to be a revolutionary start into solving several problems associated with diagnostics and therapy of diseases. However, the general hit rate has been low and only few aptamers have found place in the clinical trials despite the enormous number of publications in this field. So, to expand the range of selection for fit candidate, alternative strategies need to be considered.

In this research, a novel approach called 'Competitive non-SELEX' (and termed as 'SELCOS' (Systematic Evolution of Ligands by COmpetitive Selection)) for readily obtaining aptamers that can discriminate between highly similar targets has been developed. This approach is based on the theoretical background presented here (Figure 1), in which under the co-presence of two similar targets, a specific binding type can be enriched more than a nonspecifically binding one during repetitive steps of partitioning with no PCR amplification between them. Here, two subtype-H1N1 and H3N2 of influenza virus have been used to demonstrate this work.

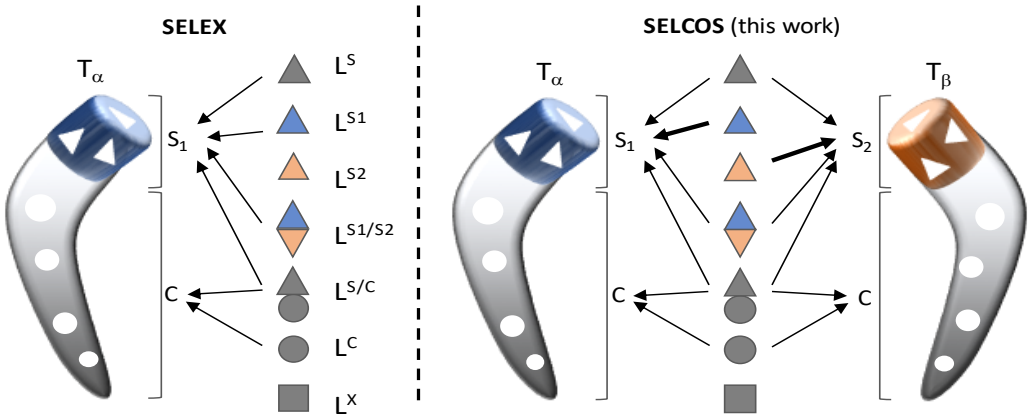

Figure 1. Schematic drawing of SELCOS (competitive non-SELEX). Comparison of (conventional) SELEX and SELCO in the ligand binding mode to the target protein. A pool of ligands is classified into 7 types in their binding mode to two different targets (T_α and T_β), which are composed of the common site (C) and the specific site (S_1 or S_2) as follows: L^S, L^{S1}, L^{S2}, $L^{S1/S2}$, $L^{S/C}$, L^C, and L^X. As shown in the figure, each ligand binds to its own binding site(s). For example, L^S is a ligand that can bind to the specific site of both targets (T_α and T_β), while L^{S1} and L^{S2} bind to the S_1 or S_2 sites only, respectively. This result indicates that the same site can be recognized differently depending on a ligand. $L^{S1/S2}$ binds to both S_1 in T_α and S_2 in T_β. $L^{S/C}$ binds to both site S (i.e., S_1 and S_2) and site C. L^C binds to the common site of T_α and T_β. L^X does not bind to either T_α or T_β.

This principle was experimentally confirmed by the selection experiment for influenza virus subtype-specific DNA aptamers. The preliminary findings were supportive for this study when observed by studying the kinetic parameters for random library, pool selected by SELEX and pool selected by SELCO by SPR (Figure 2) and the selection products (pools of DNA aptamers) obtained by SELCOS were subjected to a DEPSOR-mode electrochemical sensor, enabling the method to select subtype-specific aptamer pools (Figure 3).

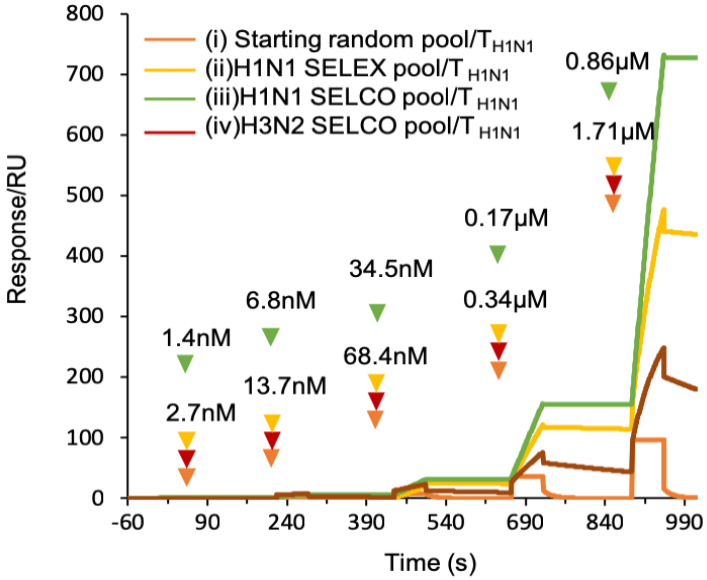

Figure 2. SPR analysis of the selection products with ligand T_{H1N1}. Selected aptamer DNA pools were anlayzed by single-cycle kinetics SPR using a BiacoreX100. For DNA pools, a successive injection of five increasing concentrations (0.0299, 0.149, 0.746, 3.73, and 18.66 μg/mL for analyte sample (i) and 0.0592, 0.296, 1.48, 7.4, and 37 μg/mL for analyte samples (ii), (iii), and (iv) were used. The target protein binding capacity on the sensor chip surface was in levels of 2500-3000 RU (response unit). The X-axis and Y-axis represent the response (RU) and time (s) of the single-cycle kinetics sensogram, respectively. The sensograms were obtained by fitting the data using a 1:1 binding model (BioEvaluation software).

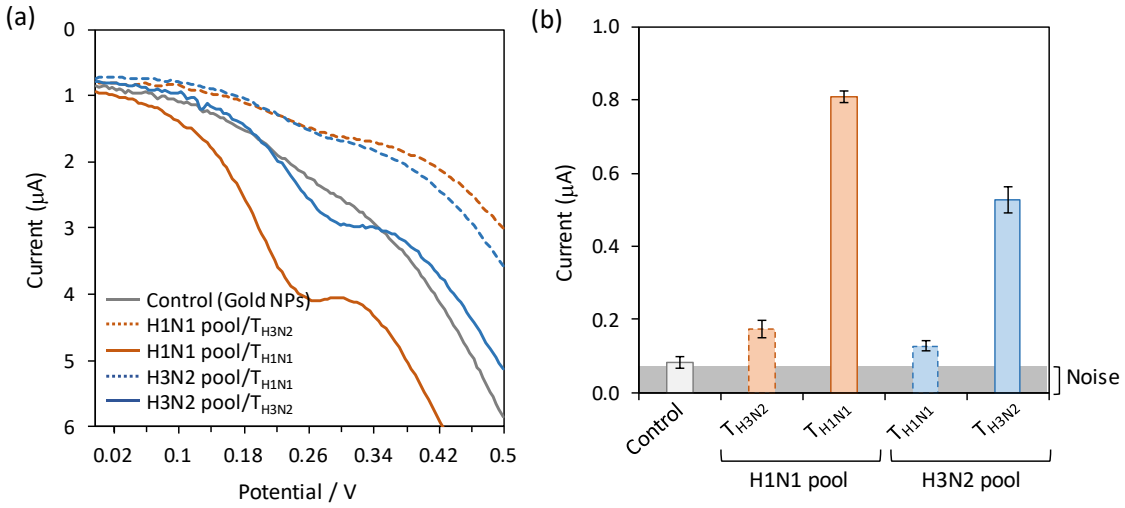

Figure 3. SELCO products. Aptamer pools obtained against T_{H1N1} (i.e., target H1N1, in red) and T_{H3N2} (blue) were subjected to the electrochemical measurement using Apta-DEPSOR. (a) For each sample, the DPV was measured against both T_{H1N1} and T_{H3N2}. (b) The I_{pc} (current for the signal peak) data are presented in a bar chart (using the average taken from 3 independent experiments).

From the clonal analysis of these pools, only a few rounds of *in vitro* selection were sufficient to achieve the surprisingly rapid enrichment of a small number of aptamers with high selectivity, which could be attributed to the SELCOS principle and the given selection pressure program. The subtype-specific aptamers obtained in this manner had a high affinity (e.g., K_D = 82 pM for H1N1; 88 pM for H3N2) and negligible cross-reactivity. Also, the kinetic parameters of aptamer selected for subtype H1N1 showed close resemblance with respective monoclonal antibody (Figure 4), thus showing the efficiency of aptamers similar to antibodies and their replacement in future. By making the H1N1-specific DNA aptamer a sensor unit of the DEPSOR electrochemical detector, an influenza virus subtype-specific and portable detector was readily constructed, indicating how close it is to the field application goal (Figure 5).

Ligand	k_{on} ($M^{-1}s^{-1}$)	k_{off} ($M^{-1}s^{-1}$)	K_D (M)
mAb	2.331×10^{5}	5.903×10^{-8}	0.253×10^{-12}
apta03	3.736×10^{4}	3.075×10^{-6}	82.34×10^{-12}

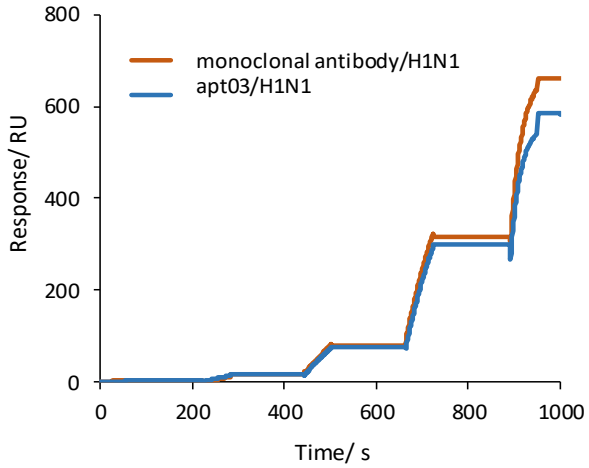

Figure 4. SPR analysis of selected aptamer vs. antibody. Comparison of selected aptamer Apt03>T_{H1N1} by SELCOS (blue) with monoclonal antibody (orange) for target H1N1 showing comparable kinetics. Single-cycle kinetics conditions for the study of interaction analysis used are as follows: Immobilization of ligand-target protein H1N1 on the sensor surface to level 2000 RU, Analyte (Apt03>T_{H1N1} and mAb) solutions were prepared in the order 26.66, 5.33, 1.07, 0.213 and 0.0427 µg/ml and sequentially injected starting with lowest concentration at a flow rate of 30 µl/min and the fitting was done by 1:1 binding model by BiacoreX100 Evaluation software.

The competition-driven selection of DNA aptamers using multiple targets (termed as SELCOS, Systemic Enrichment of Ligands by COmpetitive Selection) was first introduced in this study. The experimental results confirmed our success in obtaining influenza virus subtype-selective aptamers using SELCOS, which could be readily monitored with an electrochemical sensing tool (Apta-DEPSOR) as introduced here. By loading a selective aptamer as obtained (Apt03>T_{H1N1}) on its sensor unit, the feasibility of detecting the virus subtype was examined, and the detectability of subtype H1N1 ranged from 0.4 – 100 µg/mL. Although the situation in which the apta-DEPSOR can be useful is limited at present due to its sensitivity in the sub-µg/mL range, its portability (a merit of DEPSOR) enables us to collect important data at the POC (point of care). The theoretical consideration of SELCOS revealed its potential difference relative to conventional SELEX. In particular, its methodological advantages will be reinforced by multiple target selection, with the simultaneous acquisition of multiple aptamers of high selectivity. SELCOS, which is PCR-free, has appropriate properties for wider categories of selection such as the *in vitro* selection of peptides/proteins and the DNA-encoded library (DEL) selection of small molecules. For these selections, the PCR-free nature of SELCO is very convenient because the troublesome retagging process (such as puromycin-linker ligation to mRNA) required for those technologies, can thus be discarded (SELEX is, conveniently, free from this tagging process).

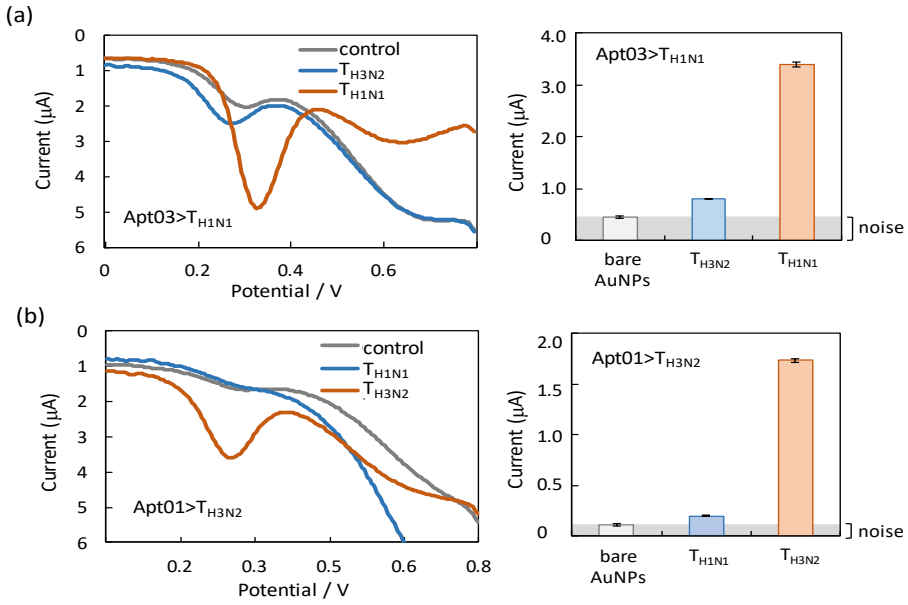

Figure 5. Validation of selected aptamer molecules by Apta-DEPSOR. (a) The aptamer, Apta03>T_{H1N1} (namely, aptamer #03 selected against the target H1N1 protein (T_{H1N1})), was measured against T_{H1N1} and T_{H3N2}. The DPV curves (left) and the corresponding bar graph (right) are shown. (b) The aptamer, Apt01>T_{H3N2}, was used here. "Control" (gray) indicates the signal from bare gold nanoparticles (AuNP). The concentrations of the target proteins, T_{H1N1} and T_{H3N2}, were both 250 μg/mL.

Journal Paper:

1. Competitive non-SELEX for the selective and rapid enrichment of DNA aptamers and its use in electrochemical aptasensor. Ankita Kushwaha, Yuzuru Takamura, Koichi Nishikagi and Manish Biyani (accepted in Scientific Reports).

Book Chapter:

2. Book title: Immunodiagnostic technologies from laboratory to point-of-care testing. Ankita Kushwaha, Yuzuru Takamura, Manish Biyani, "Alternative analyte-binding compounds for immunosensor-like point-of-care application" (submitted)

Conference:

1. Ankita Kushwaha, Yuzuru Takamura, Manish biyani; Multiplex Selection: On chip based inter-species selection of high affinity aptamers for multiple targets in parallel; *Bio-wakate Symposium*, Kanzawa (2016).
2. Ankita Kushwaha, Yuzuru Takamura, Manish Biyani; Two-plex *in vitro* selection of DNA aptamer for multiple subtypes of Influenza A viruses, *The Electrochemical Society of Japan*, Toyama (2016).
3. Ankita Kushwaha, Yuzuru Takamura, Manish Biyani; AptaArray: Ultraspecific co-detection of multiple biomarkers using multiplex *in vitro* selection, *The 39th Annual Meeting of the Molecular Biology Society of Japan (MBSJ 2016)*, Yokohama (2016).
4. Ankita Kushwaha, Yuzuru Takamura, Manish Biyani; Development of multiplexed in vitro selection of aptamers for multiplexed protein sensing, *JAIST Japan-India Symposium on Material Science*, Japan (2017).
5. Ankita Kushwaha, Yuzuru takamura, Manish Biyani; Ultra specific co-detection of multiple types of Influenza viruses using multiplex in vitro selection and electro-aptasensor; *5th International Conference on Bio-Sensing Technology*, Riva Del Garda-Italy (2017).
6. Ankita Kushwaha, Yuzuru Takamura, Manish Biyani; Creation of smart aptameric reagents for the global antigenic diversity of Influenza viruses; *8th Annual Symposium-Bridging Nature and Technology ISAJ*, Tokyo (2017) (Oral Presentation)
7. Ankita Kushwaha, Yuzuru Takamura, Manish Biyani; Highly selective and sensitive detection of emerging subtypes of Influenza viruses using SELCO and DEPSOR; *JAIST Japan-India Symposium on Material Science*, Japan (2018).

Keywords: Aptamer, SELEX, SPR, electrochemical sensor, influenza virus