

Title	DNAアプタマの高選択性多重 in vitro セレクションのための競争的濃縮によるリガンド系統的進化法SELCOの開発
Author(s)	Kushwaha, Ankita
Citation	
Issue Date	2019-06
Type	Thesis or Dissertation
Text version	ETD
URL	<a href="http://hdl.handle.net/10119/16071">http://hdl.handle.net/10119/16071</a>
Rights	
Description	Supervisor:高村 禪, マテリアルサイエンス研究科, 博士(マテリアルサイエンス)

氏名	KUSHWAHA, Ankita		
学位の種類	博士(マテリアルサイエンス)		
学位記番号	博材第 473 号		
学位授与年月日	令和元年 6 月 24 日		
論文題目	Development of Systematic Evolution of Ligands by Competitive enrichment-SELCO for highly selective and multiplex <i>in vitro</i> selection of DNA aptamers		
論文審査委員	主査	高村 禅	北陸先端科学技術大学院大学 教授
		塚原 俊文	同 教授
		平塚 祐一	同 准教授
		筒井 秀和	同 准教授
		保川 清	京都大学 教授

## 論文の内容の要旨

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 performed 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.

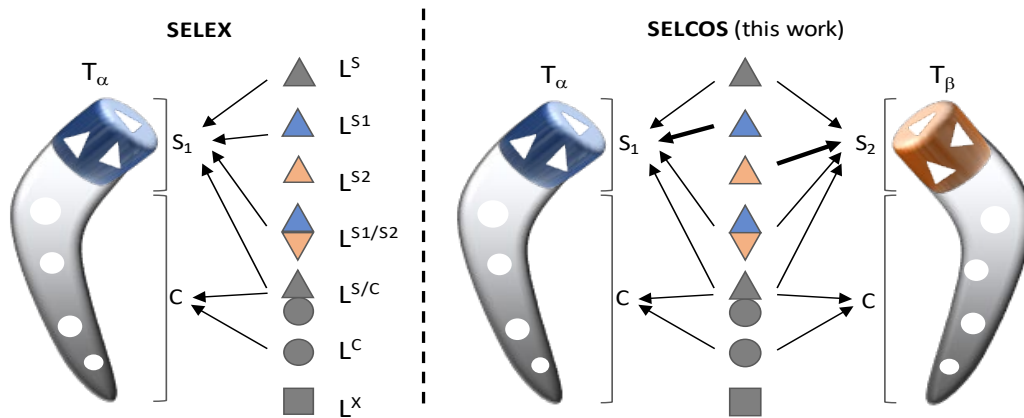


Figure 1. Schematic drawing of SELCOS (competitive non-SELEX). Comparison of (conventional) SELEX and SELCO in the ligand binding mode to the target protein. A pool of ligands is classified into 7 types in their binding mode to two different targets ( $T_\alpha$  and  $T_\beta$ ), which are composed 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 ( $T_\alpha$  and  $T_\beta$ ), 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  $T_\alpha$  and  $S_2$  in  $T_\beta$ .  $L^{S/C}$  binds to both site S (i.e.,  $S_1$  and  $S_2$ ) and site C.  $L^C$  binds to the common site of  $T_\alpha$  and  $T_\beta$ .  $L^X$  does not bind to either  $T_\alpha$  or  $T_\beta$ .

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).

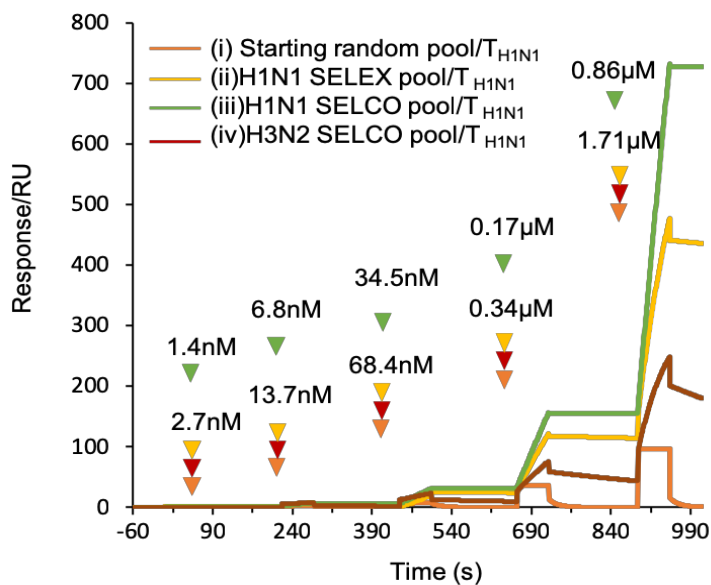


Figure 2. SPR analysis of the selection products with ligand TH1N1. Selected aptamer DNA pools were analyzed by single-cycle kinetics SPR using a BiacoreX100. For DNA pools, a successive injection of five increasing concentrations (0.0299, 0.149, 0.746, 3.73, and 18.66  $\mu\text{g}/\text{mL}$  for analyte sample (i) and 0.0592, 0.296, 1.48, 7.4, and 37  $\mu\text{g}/\text{mL}$  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).

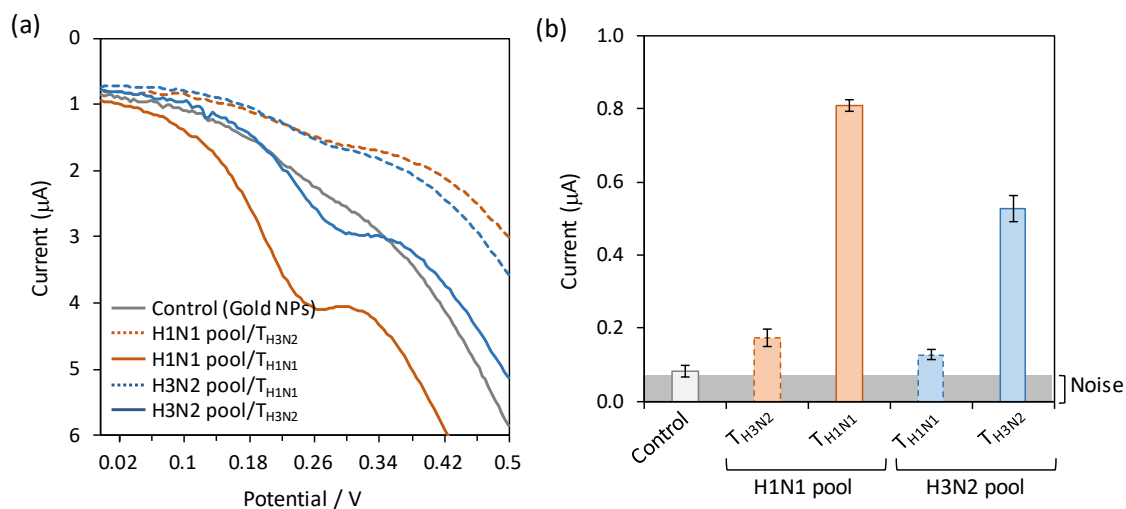


Figure 3. SELCO products. Aptamer pools obtained against  $T_{H1N1}$  (i.e., target H1N1, in red) and  $T_{H3N2}$  (blue) were subjected to the electrochemical measurement using Apta-DEPSOR. (a) For each sample, the DPV was measured against both  $T_{H1N1}$  and  $T_{H3N2}$ . (b) The  $I_{pc}$  (current 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	$2.331 \times 10^5$	$5.903 \times 10^{-8}$	$0.253 \times 10^{-12}$
apta03	$3.736 \times 10^4$	$3.075 \times 10^{-6}$	$82.34 \times 10^{-12}$

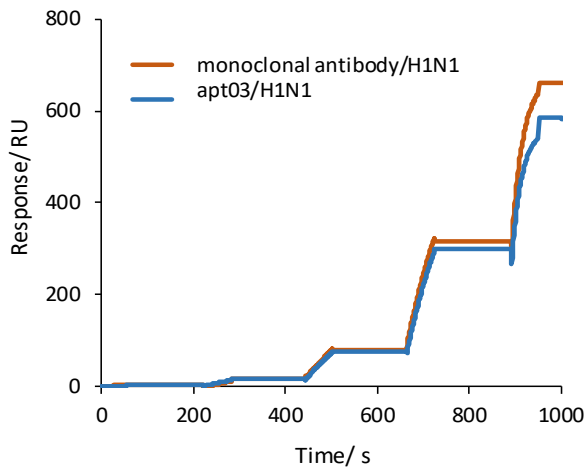


Figure 4. SPR analysis of selected aptamer vs. antibody. Comparison of selected aptamer Apt03> $T_{H1N1}$  by SELCOS (blue) with monoclonal antibody (orange) for target H1N1 showing comparable kinetics. Single-cycle kinetics conditions for the study of interaction analysis used are as follows: Immobilization 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  $\mu$ g/ml and sequentially injected

starting with lowest concentration at a flow rate of 30  $\mu\text{L}/\text{min}$  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> $T_{\text{H1N1}}$ ) on its sensor unit, the feasibility of detecting the virus subtype was examined, and the detectability of subtype H1N1 ranged from 0.4 – 100  $\mu\text{g}/\text{mL}$ . Although the situation in which the apta-DEPSOR can be useful is limited at present due to its sensitivity in the sub- $\mu\text{g}/\text{mL}$  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).

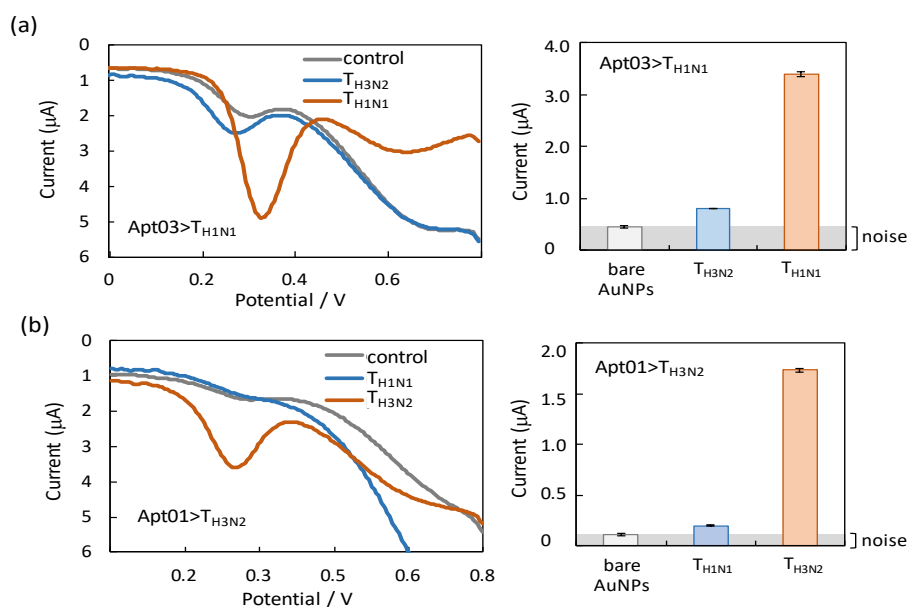


Figure 5. Validation of selected aptamer molecules by Apta-DEPSOR. (a) The aptamer, Apt03> $T_{\text{H1N1}}$  (namely, aptamer #03 selected against the target H1N1 protein ( $T_{\text{H1N1}}$ )), was measured against  $T_{\text{H1N1}}$  and

T<sub>H3N2</sub>. The DPV curves (left) and the corresponding bar graph (right) are shown. (b) The aptamer, Apt01>T<sub>H3N2</sub>, was used here. “Control” (gray) indicates the signal from bare gold nanoparticles (AuNP). The concentrations of the target proteins, T<sub>H1N1</sub> and T<sub>H3N2</sub>, were both 250 µg/mL.

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

## 論文審査の結果の要旨

本研究は、DNA アプタマーを進化分子工学的に開発する際に競合的な選択ステージを導入した新しい手法を考案し、その有効性を確かめたもので、特に構造のよく似た複数の分子を見分ける選択性の高い DNA アプタマーを効率よく求めることができる。

従来、DNA アプタマーの開発には SELEX 法等の進化分子法が用いられ、複数のターゲット分子に対するアプタマーを得るには、それをターゲットの数だけ独立に実行する必要があった。しかし、これではそれぞれのターゲットに対して独立に最適化されるため、構造のよく似た分子では双方に結合するアプタマーのみが得られたり、また十数世代を経ても結合力が上がらないこともしばしばあった。本研究では、同じ空間に複数のターゲット分子を配置し、競合的にセクションさせる具体的な方法を考案、これによりそれぞれのターゲットに選択的に結合するアプタマーを効率的に得ることに成功した。

第 1 章では、DNA/RNA アプタマーの特性とその有用性、将来性と従来のセクション法についてまとめ、その問題点を指摘し、本研究の課題を明確にした。

第 2 章では、本研究の主題である競合的な選択法 SELCO の概念と期待される効果について述べ、ビーズを用いたその具体的な実施方法を考案した。さらに、パンデミックリスクの高いインフルエンザウイルスのサブタイプである H1N1 と H3N2 を見分けるアプタマーの開発を例題として、同法と従来の SELEX 法の両方によりその開発を実施し、SELCO の優位性を実証した。SELCO では、高い選択性を持ち、かつ SELEX 法より 3 桁以上結合力の高い  $K_D=10^{-10}M$  レベルのアプタマープールを、1 ラウンドのセクションで得ることができた。

第 3 章では、第 2 章で得たアプタマープールより、性能の高いアプタマーを複数単離し、それぞれの配列および構造を決定し、また結合特性と選択性を表面プラズモン共鳴法と電気化学法を用いて明らかにした。

第 4 章では、第 3 章で単離されたアプタマーを用いて、印刷電極を用いた使い捨てモバイル型インフルエンザセンサーを開発している。競合法により、血清中で、H1N1 ウイルス由来のたんぱく質と、H3N2 ウイルス由来のタンパク質を検出することに成功した。

第 5 章では、以上による本論文の意義を総括した。

以上、本論文は、進化分子的 DNA アプタマー開発において、競合的な選択ステージを導入することで、複数のターゲット分子に対して高い結合力と高い選択性を持つアプタマーを容易に開発できる手法を提案、確立、実証したもので、学術的に貢献するところが大きい。よって博士（マテリアルサイエンス）の学位論文として十分価値あるものと認めた。