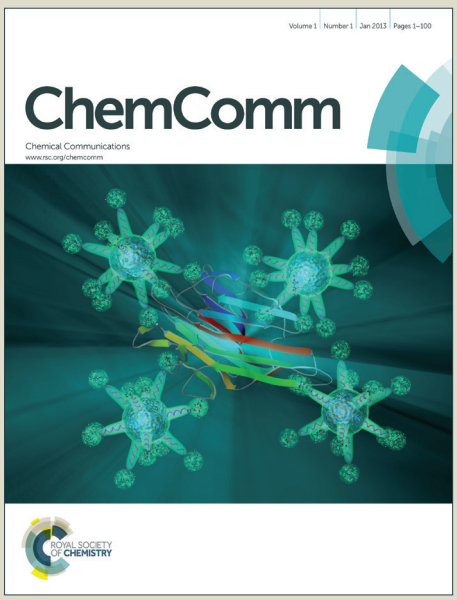
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Selection of Aptamers Targeting the Sialic Acid Receptor of Hemagglutinin by Epitope-Specific SELEX

Yeh-Hsing Lao,[a,b] Hui-Yu Chiang[a], Deng-Kai Yang[a], Konan Peck[b] and Lin-Chi Chen*[a]

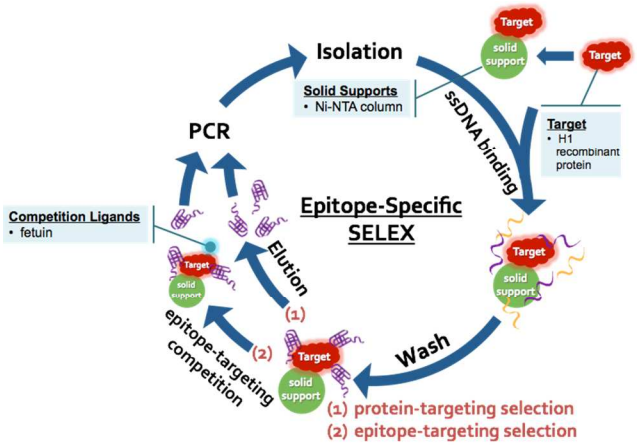
Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

A new SELEX scheme is proposed for selection of aptamers targeting a specific epitope of a native protein. Anti-sialic acid receptor (SAR) aptamers that inhibit H_1 hemagglutination at a low pico-mole dose are selected accordingly.

Aptamers are promising antibody mimics for small molecules, proteins, and cells[1-3] owing to high specificity, high stability, low immunogenicity[1] and low cost. Besides diagnostics, they are of the curing niches for anti-tumor proliferation, anti-coagulation, and viral inhibition[4]. SELEX (Systematic Evolution of Ligands by Exponential enrichment)[5, 6] is a common practice to generate "protein-specific" aptamers, but "epitope-specific" aptamers that have both greater diagnostic and therapeutic potentials are still lack of an efficient approach to obtain. Our colleagues developed immunoprecipitation-based SELEX[7] and post-SELEX microarray optimization to yield agonistic aptamers against Toll-like receptor 2. Their method was not for selecting epitope-specific aptamers, but their result suggested that antibody could be used to enhance SELEX's specificity. Inspired, we developed a new method for selecting aptamers targeting a specific protein epitope with the aid of a known, specific ligand competitor and report the method as "epitope-specific SELEX" (ES-SELEX, Scheme1 and S1, ESI) here. Hemagglutinin (HA) activating the binding and invasion of influenza viruses to the host cells[8] was chosen as the model target, since ordinary SELEX was widely adopted to generate aptamers against HAs of serotypes H1[9], H3[10], H5[11] and H9[12] very recently, reflecting the rising roles of aptamers for influenza prevention.

A domain-targeted SELEX[13] was reported to obtain aptamers aiming at a therapeutic domain on a cell surface protein, but it required protein denaturation to expose the domain for SELEX. In contrast, ES-SELEX directly selects aptamers aiming at the sialic acid receptor (SAR) of a native HA protein subunit (HA1). The SAR epitope recognizes the sialyllactoses on the host cell's surface[14, 15]. Natural and artificial molecules containing SA also interact with this epitope[16, 17]. In this work, we used fetuin, a SA-containing protein[18], as a specific ligand competitor to elute the DNA ligands bound to the SAR epitope during ES-SELEX, so SAR-specific aptamers rather than HA-binding aptamers were be evolved. To test anti-SAR aptamers, a glycosylated, recombinant HA of human influenza serotype H1 (A/New Caledonia/20/99) from a baculovirus-infected insect cell was used. The serotype H1 is a major group of seasonal flu[19], and this work produces the first DNA aptamers for H1. Besides, *in vitro* and *in silico* structural evidences for aptamer's epitope specificity are reported here, too.



Scheme 1 ES-SELEX for direct screening of antagonistic aptamers against the SAR epitope of a native influenza HA subunit (HA1).

In experiments, a 62-mer ssDNA library (diversity = 10^{15}) with a 30-mer randomized region flanked by two 16-mer PCR priming sites was incubated with His-tagged glycosylated, recombinant HA1 subunit (1 μg; HEK293 cell-expressed) in a selection buffer (50 mM Tris-HCl, pH 7.5, 4 mM KCl, 140 mM NaCl, 1 mM $MgCl_2$ and 2.5 mM $CaCl_2$) for 30 minutes at room temperature. After incubation, ssDNA-protein complexes were collected by a Sigma HIS-Select® affinity column. Then several washes with the selection buffer were carried out to remove non-specific binding events. Two selection routes, protein (HA1)-targeting and epitope (SAR)-targeting, were performed for the first to fifth round and the sixth to ninth round, respectively. In HA1-targeting selection, ssDNA-protein complexes were eluted from the column matrixes by imidazole (500 mM in the selection buffer). By contrast, the ssDNA ligands bound to the SAR epitope of HA1 were eluted by fetuin competition in the SAR-targeting selection. In round 9, fetuin solutions with a concentration gradient (33 nM to 20.6 μM) were used to partition SAR-bound ssDNAs with various affinities.

The amount of specific binding ssDNA ligands in each round was monitored by quantitative PCR (qPCR) analysis of the eluted sample. Two factors, (i) the percentage of ssDNA bound and (ii) the specific binding ratio, were used to assess the efficiency of the SELEX ligand enrichment and the evolution of ssDNA pool's specificity. The first factor was defined by the ratio of the amount

of ssDNA bound to the target with respect to the amount of input ssDNA molecules. The second factor was defined by the ratio of the first factor to the percentage of nonspecific ssDNA bound in the no-target control experiment. As can be seen in Fig. 1A, only 0.038 % of the 10^{15} randomized ssDNA molecules bound to the HA protein in the first SELEX round, and no significant specific binding ratio was observed for this round (Fig. S1, ESI). After five HA-targeting selection rounds, the percentage of ssDNA bound increased to 2.99 %, and the specific binding ratio also increased from 0.80 to 23.2. (Fig. S1A, ESI).

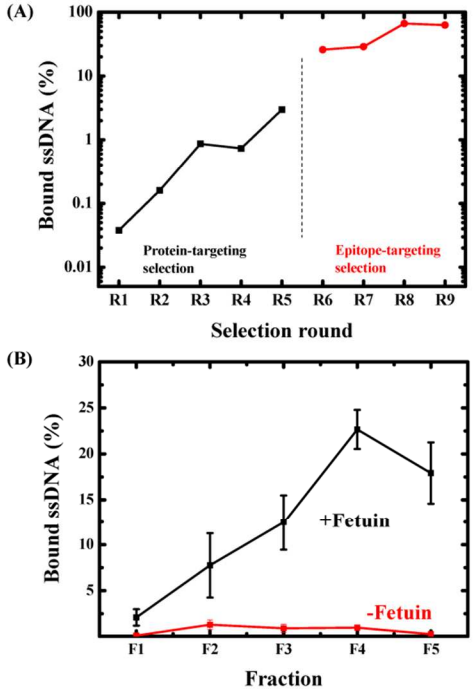


Fig. 1 (A) The percentage of specific ssDNA ligands bound to the target versus the selection round number. **(B)** The percentage of SAR specific ligands from the 9th round in different fetuin-eluted fractions.

The SAR-targeting selection started at the sixth round. From this round, ssDNA ligands displaced by fetuin-competition and bound still on HA1 after competition (eluted by imidazole) were both collected and analysed by qPCR. A significant enrichment of bound ssDNA was found (Fig. S1B, ESI), and most of them were likely to bind to the SAR epitope. Through the selection process, the ssDNA bound to the desired epitope was further enhanced. In the eighth round, we collected 66.5 % of the input ssDNA ligands by the fetuin-competition elution (Fig. 1A), and the amount of the specific ligands became 265-fold higher than that of nonspecific ligands (Fig. S1A, ESI). This demonstrates that the ssDNA ligands aiming at the SAR epitope on HA1 were effectively enriched during the ES-SELEX. In order to obtain the ligands with higher affinity against the SAR, we fractionated the final SELEX pool by elution with five different concentrated fetuin solutions. Thirty-three nano-molar to 20.6 μM of fetuin were utilized to generate five fractions of aptamer pools with different mean affinities against the SAR epitope. In parallel, five counterpart fractions (the ssDNAs eluted by the selection buffer without fetuin) were also generated for comparison (Fig. S2, ESI). The percentages of ssDNA bound in the five fractions and the counterpart fractions are compared in Fig. 1B. We observe that more ssDNA ligands are eluted (from 2.1% to 22.7%) with a higher concentration of fetuin, and the sum of the percentages of ssDNA bound eluted in the ninth round is 62.9%, similar to that of the eighth round. In comparison, simple buffer washes do not elute significant amounts of tightly bound ssDNAs (ranging between 0.16% and 1.29%). This implies that the ssDNA ligands eluted in the ninth round are highly specific to the SAR epitope. A binding assay was performed to assess the mean dissociation constant (K_D) of the fifth fetuin-displaced ssDNA fraction with respect to HA. The mean K_D is determined to be 1.16 ± 0.20 nM (Fig. S3, ESI), which is in the same order of the ideal K_D (0.65 nM) according to Cheng and Prussoff correction[20] and hence suggests a strong and specific binding.

We cloned the PCR products of the fifth fetuin-eluted ssDNA fraction of round 9 and arbitrarily sequenced 96 clones. We found that 96 aptamer candidates could be classified into five distinct groups according to the similarities in sequences and secondary structures. One representative candidate was chosen from each group based on the minimal free energy (Fig. S4, ESI) and was assessed if the ES-SELEX led to antagonistic aptamers capable of blocking the SAR epitope by hemagglutination inhibition (HAI) assays. In the HAI assays, one micro-molar of each aptamer was incubated with the HA (H1) protein (A/New Caledonia/20/99; expressed by a baculovirus system) for 30 mins in the selection buffer at room temperature. Then the aptamer-HA mixtures were interacted with fresh human RBCs (type O; 0.75 % in the selection buffer). Among the five aptamers tested, four (CP9P526, CP9P536, CP9P554, and CP9P596) of them showed complete or partial HA inhibition (Fig. 2). This suggests the efficiency of our method in selecting epitope-specific aptamers and provides the first dimension of evidence of the aptamer's SAR specificity.

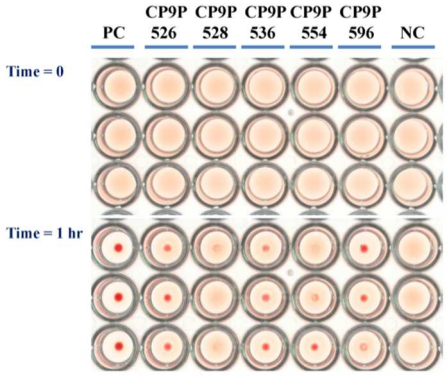


Fig. 2 The HAI assay was done by following the WHO standard protocol. Each anti-HA aptamer (100 pmol) was incubated with HAs for 30 mins and then mixed with 0.75% human RBCs to visualize the functionality.

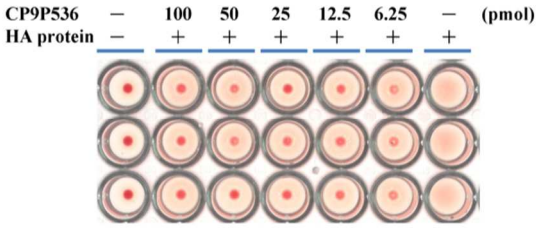


Fig. 3 Seeking the aptamer's minimal effective dose for HAI. Aptamer CP9P536 (with different doses) was tested in triplicate, and the HAI assays were carried out with the same protocol of Fig. 2.

We studied the minimal effective dose of aptamer CP9P536 (*TCCCT ACGGC GCTAA CCGAT AGGTG TAGCG TGGGG CACAT GTTCG CGCCA CCGTG CTACA AC*) for HAI. Figure 3 shows that CP9P536 inhibits the HA function at a low picomole dose (6.25 pmol). By contrast, a commercial antibody, even with a sub-nanomole dose, did not inhibit hemagglutination (Fig. S5, ESI). The HAI efficacy of CP9P536 is better than the other anti-HA aptamers[11, 12, 21, 22] obtained from ordinary SELEX (Table S1, ESI). Competitive enzyme-linked aptamer assay (ELAA) was used to further check the SAR epitope specificity of the aptamers selected. The HA protein solution (0.1 μM in carbonate buffer, pH 9.4) was coated in a microtiter plate at 4°C overnight. After washes with PBST buffer (0.05% Tween 20, pH 7.4), 1 μM CP9P536 in the selection buffer was incubated with the coated HA. Then 2,6-sialyllactose (SA), a standard ligand of SAR of H1, was utilized for competition-based elution of the bound CP9536, and simple buffer elution was done for comparison. The eluted aptamers were quantified by qPCR. The result in Fig. 4 gives the second dimension of evidence for the aptamer's SAR specificity.

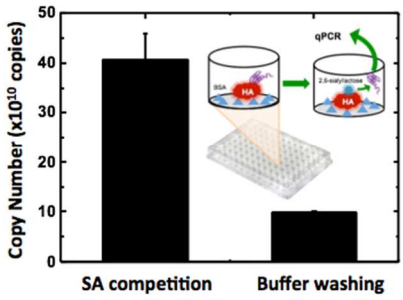


Fig. 4 qPCR quantitation of the HA-bound CP9536 aptamers of the ELAA experiments eluted by SA competition and simple buffer washes.

The third dimension of evidence for the SAR specificity of the aptamer CP9P536 was given by a high-density microarray and *in silico* molecular docking. The "base-by-base shift" design[7] was utilized to generate a variety of subsequences of CP9P536 with a length between 25 to 40 mers and synthesized on a microarray. The aptamer microarray was assayed with either HA or a virus-inoculated allantoic fluid following our previous method[23]. The subsequences with higher fluorescent signals than the full-length CP9P536 were picked out for alignment (Fig. S6, ESI). We find that the "CGATA GGTGT AGCGT GGGGC ACATG TTCGC GC" domain is responsible for SAR recognition, and the aptamer microarray showed highly correlated fluorescent responses for recombinant HA and real virus-inoculated allantoic fluid (Fig. S7, ESI). Then the *in silico* aptamer-HA docking was carried out for the first time. The 3D aptamer structure was predicted by the combined use of RNAstructure[24] and RNAcomposer[25], and the structure of hemagglutinin H1 was acquired from PDB (PDB ID: 1RUZ). The *in silico* DNA-protein docking was executed using PatchDock[26]. The result reveals that CP9P536 spontaneously and perfectly docks on the epitope of SAR of the HA (Fig. S8, ESI), and this *in silico* model also predicts a minimal binding region of CP9P536, "GCGTG GGGCA CATGT TC", which is centred in the binding domain of CP9P536 given by the microarray assay.

In conclusion, ES-SELEX is useful to obtain epitope-specific aptamers, and anti-SAR aptamers are found. Besides, a separate work indicates that ES-SELEX is also working for selecting anti-HA aptamers when real H1N1 virus is used as the SELEX target. By viral ES-SELEX, aptamer BR33 showing anti-HA specificity in ELAA (Fig. S9, ESI) was selected, whereas a standard viral SELEX did not result in such aptamers due to the complexity of surface protein composition on H1N1 virus.

We thank Ms. C.-C. Huang, Prof. C.-H. Chou, and Prof. H.-J. Tsai in NTU for viral ES-SELEX, Prof. J.-R. Liu in NTU for providing H1, and Dr. P.-Y. Lin, Dr. S.-C. Jao, Ms. S.-H. Wong in Academia Sinica for helpful aids. This work was supported by Ministry of Science and Technology & National Health Research Institutes of R.O.C. (Taiwan) (NSC 98-2221-E-002-099-MY3, 96-2321-B-194-001-MY2 & NHRI-EX103-10141EI).

Notes and References

[a] *Department of Bio-Industrial Mechatronics Engineering, National Taiwan University, Taipei 10617, Taiwan. (E-mail: chenlinchi@ntu.edu.tw);* [b] *Institute of Biomedical Sciences, Academia Sinica, Taipei 11529, Taiwan.*

† Electronic Supplementary Information (ESI) available: Experimental details and supplementary data. See DOI: 10.1039/b000000x/

1. S. M. Nimjee, C. P. Rusconi and B. A. Sullenger, *Annu Rev Med*, 2005, **56**, 555-583.
2. D. H. J. Bunka and P. G. Stockley, *Nat Rev Microbiol*, 2006, **4**, 588-596.
3. R. Stoltenburg, C. Reinemann and B. Strehlitz, *Biomol Eng*, 2007, **24**, 381-403.
4. K. W. Thiel and P. H. Giangrande, *Oligonucleotides*, 2009, **19**, 209-222.
5. A. D. Ellington and J. W. Szostak, *Nature*, 1990, **346**, 818-822.
6. C. Tuerk and L. Gold, *Science*, 1990, **249**, 505-510.
7. Y. C. Chang, W. C. Kao, W. Y. Wang, W. Y. Wang, R. B. Yang and K. Peck, *Faseb J*, 2009, **23**, 3078-3088.
8. M. R. Hillman, *Vaccine*, 2002, **20**, 3068-3087.
9. S. C. B. Gopinath and P. K. R. Kumar, *Acta Biomater*, 2013, **9**, 8932-8941.
10. M. Wongphatcharachai, P. Wang, S. Enomoto, R. J. Webby, M. R. Gramer, A. Amonsin and S. Sreevatsan, *J Clin Microbiol*, 2013, **51**, 46-54.
11. S. Y. Park, S. Kim, H. Yoon, K. B. Kim, S. S. Kalme, S. Oh, C. S. Song and D. E. Kim, *Nucleic Acid Ther*, 2011, **21**, 395-402.
12. S. Choi, C. Lee, K. Lee, S. Y. Choe, I. Mo, R. Seong, S. Hong and S. Jeon, *Mol Cells*, 2011, **32**, 527-533.
13. B. Waybrant, T. R. Pearce, P. Wang, S. Sreevatsan and E. Kokkoli, *Chemical communications*, 2012, **48**, 10043-10045.
14. J. J. Skehel and D. C. Wiley, *Annu Rev Biochem*, 2000, **69**, 531-569.
15. C. McCullough, M. X. Wang, L. J. Rong and M. Caffrey, *Plos One*, 2012, **7**.
16. A. S. Gambaryan and M. N. Matrosovich, *J Virol Methods*, 1992, **39**, 111-123.
17. T. Matsubara, M. Sumi, H. Kubota, T. Taki, Y. Okahata and T. Sato, *J Med Chem*, 2009, **52**, 4247-4256.
18. J. U. Baenziger and D. Fiete, *J Biol Chem*, 1979, **254**, 789-795.
19. K. G. Nicholson, J. M. Wood and M. Zambon, *Lancet*, 2003, **362**, 1733-1745.
20. Y. Cheng and W. H. Prusoff, *Biochemical pharmacology*, 1973, **22**, 3099-3108.
21. S. H. Jeon, B. Kayhan, T. Ben-Yedidia and R. Arnon, *J Biol Chem*, 2004, **279**, 48410-48419.
22. S. C. B. Gopinath, T. S. Misono, K. Kawasaki, T. Mizuno, M. Imai, T. Odagiri and P. K. R. Kumar, *J Gen Virol*, 2006, **87**, 479-487.
23. Y. H. Lao, K. Peck and L. C. Chen, *Anal Chem*, 2009, **81**, 1747-1754.
24. J. S. Reuter and D. H. Mathews, *BMC bioinformatics*, 2010, **11**, 129.

25. M. Popenda, M. Szachniuk, M. Antczak, K. J. Purzycka, P. Lukasiak, N. Bartol, J. Blazewicz and R. W. Adamiak, *Nucleic Acids Res*, 2012, **40**.
26. D. Schneidman-Duhovny, Y. Inbar, R. Nussinov and H. J. Wolfson, *Nucleic Acids Res*, 2005, **33**, W363-W367.