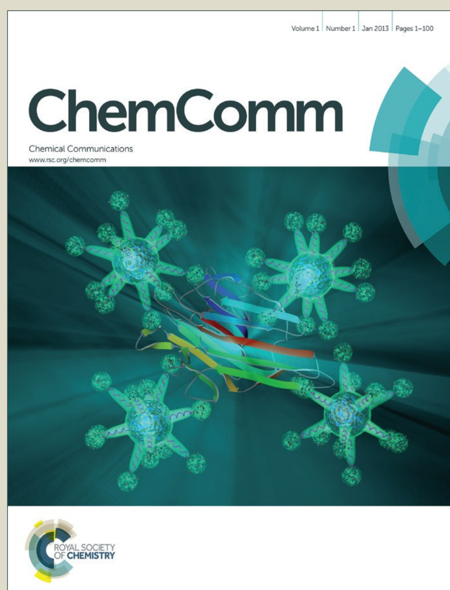
ChemComm

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Chemical Communications RSCPublishing

COMMUNICATION

Cite this: DOI: 10.1039/x0xx00000x

Generation of a pair of independently binding DNA aptamers an in single round of selection using proximity ligation

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

O. Chumphukam[a], T. T. Le[a,b], S. Piletsky[c] and A. E. G. Cass†[a,b]

The ability to rapidly generate a pair of aptamers that bind independently to a protein target would greatly extend their use as reagents for two site ('sandwich') assays. We describe here a method to achieve this through proximity ligation. Using lysozyme as a target we demonstrate that under optimal conditions such a pair of aptamers, with nanomolar affinities, can be generated in a single round.

Hundreds of aptamers have been generated to meet the demands in analytical and therapeutic applications, the general majority of them using SELEX (systematic evolution of ligands by exponential enrichment).[1, 2] This method involves several rounds of aptamer-target complex formation, partitioning, and amplification, which is both time-consuming and requires significant amounts of target for selection. In order to shorten the selection process, a number of modifications have been made including capillary electrophoresis for more efficient partitioning, microfluidics, automation and next generation sequencing to identify enriched sequences in early rounds. [3-6] Despite these developments, there are very few examples of pairs of aptamers that bind to a single target. The use of two site or sandwich immunoassays is very widespread due to the advantages that it offers in terms of specificity. Examples include sandwich ELISA, dual antibody microarrays and lateral flow assays *e.g.* pregnancy tests. The generation of suitable aptamer pairs would mean that these powerful assay formats could then be used with aptamers as well as antibodies.[7-11] We describe here an approach, proximity ligation selection (PLS) that is designed to generate such aptamer pairs and to do so in a single round of selection. This method is based on the use of DNA ligase to join two separated pieces of ssDNA together when the ends are in sufficiently close proximity to be bridged by a connector strand as shown in Figure 1.

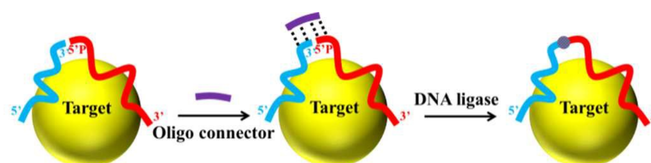


Figure 1: A schematic diagram of the ligation using an oligo connector.

Fredriksson *et al.* used this idea to join together two aptamers that were known to bind at different sites on human α-thrombin in order to design a fusion aptamer that bound to both sites simultaneously.[12] In the same paper these authors also described ligating two molecules of the same aptamer through their binding to a dimeric target (PDGF-BB). In the work reported in the current paper we use proximity ligation to generate a pair of aptamers that bind independently to a protein target, in a single round of selection. This opens the way to rapidly generate aptamer pairs for use in sandwich assays. To achieve this we designed two libraries with appropriate extended sequences to facilitate both ligation and subsequent specific amplification of the ligated products.

ChemComm Accepted Manuscript

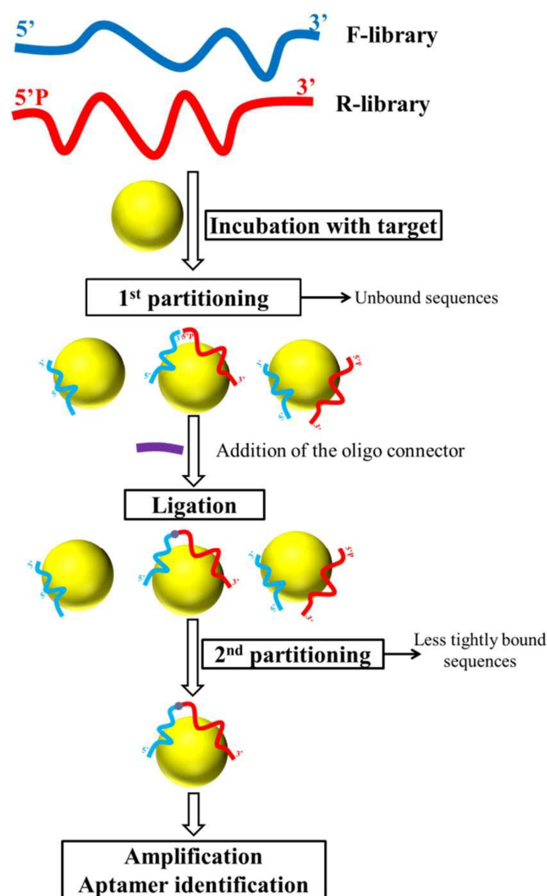


Figure 2: A schematic diagram of PLS using a pair of F- and R-libraries. When a sequence from the F-library and another sequence from the R-library bind simultaneously to a target molecule allowing hybridization of the connector to both F- and R- sequence and therefore the 3'-end of the F-library are sufficiently close to the 5' phosphate of the R-library, ligation of these two sequences can occur.

As illustrated in Figure 2, PLS consists of (1) incubation of the two DNA libraries with target in solution, (2) 1st partitioning (adsorption of the protein-DNA complex to nitrocellulose membrane and removal of unbound and weakly bound DNA), (3) incubation of the protein-DNA complexes with the oligo connector and subsequent ligation of DNA sequences that are in close proximity, (4) 2nd partitioning, (5) DNA amplification and aptamer identification. Compared to the conventional SELEX this procedure has an added ligation and an extra partitioning step. If the 1st partitioning step was omitted we observed a very high background of non-specific ligation.

Apart from the factors that contribute to the selection efficiency in conventional SELEX two more parameters are critical for PLS: design of the DNA libraries and optimisation of the ligation reaction. To facilitate ligation and selective amplification of ligated products in PLS we designed a pair of Forward (F)- and Reverse (R)-libraries as shown in Figure 3.

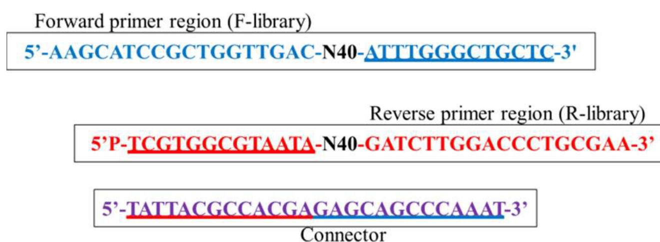


Figure 3: Schematic diagram of designing a pair of F- and R- libraries for PLS. This design will allow the ligated sequence to be amplified exponentially whilst F-library is not amplified and R-library is only amplified linearly. The underline regions are for hybridization with the oligo connector to facilitate the ligation. N40 represents the 40-nucleotide random sequence.

Each library contained a random sequence region flanked by defined sequences. The F-library has a forward priming region at the 5'-end and a connecting region at the 3'- end. The R-library instead has a connecting region at the 5'-end and a reverse priming region at the 3'- end. The R-library has a 5'-phosphate to allow the ligation reaction to occur. A pair of DNA libraries designed for PLS selection is shown in Figure 3.

PLS selection is based on the assumption that, after unbound and weakly bound sequences from the F- and R-libraries are removed, pairs of aptamers that have the 3'-end of the F-library in sufficiently close proximity to the 5'phosphate-end of the R-library are brought together through hybridization with an oligonucleotide connector that can form an F-R fusion in the presence of DNA ligase. The critical advantage in designing a pair of F- and R-libraries for PSL selection over the use of a single library is that when PCR amplification is performed, only ligated sequences are expected to be amplified exponentially. Un-ligated sequences from the F-library are not amplified due to the absence of the reverse primer sequence whilst un-ligated ones from the R-library are only amplified linearly as shown in Figure 4. This design favours PCR amplification of the ligated sequences formed specifically from the two halves of the libraries. Moreover only independently binding aptamer pair should be exponentially amplified.

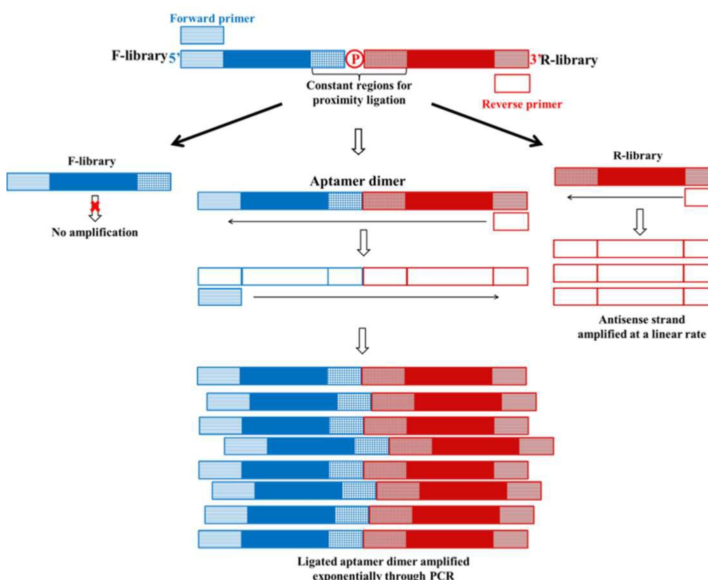


Figure 4: Schematic diagrams of PCR amplification in PLS. Using a pair of F-and R-libraries results in dual-sequences as a majority.

ChemComm Accepted Manuscript

 This journal is © The Royal Society of Chemistry 2012

Another critical step is the ligation reaction, which needs to be optimized in order to achieve efficient specific ligation of the two bound sequence whist giving a minimum background of non-specific ligation. Optimal parameters include the concentration of the oligonucleotide connector, the ligation time, the amount of ligase and concentration of the target.

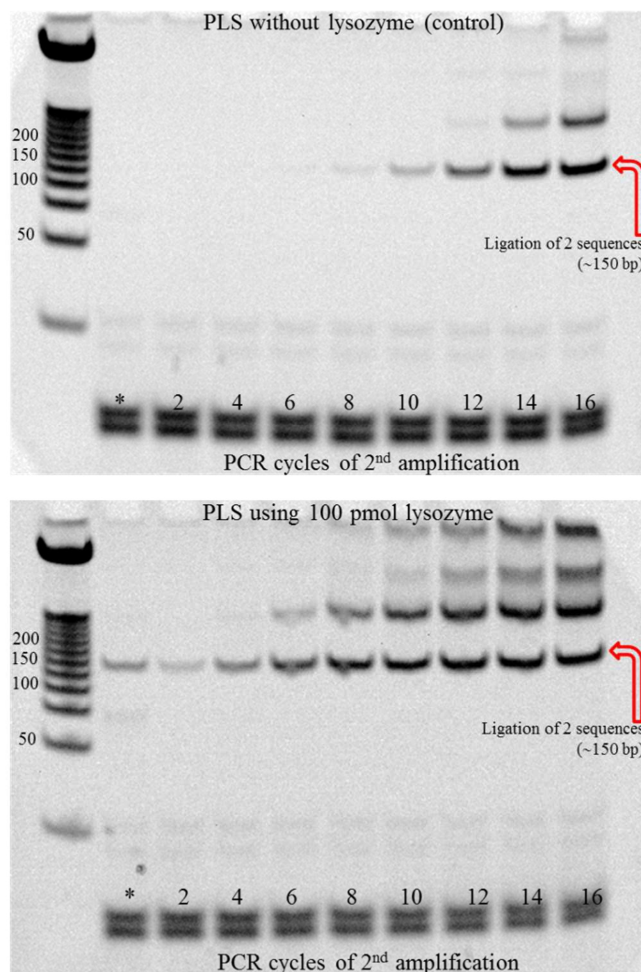


Figure 5: Proximity ligation DNA products in the presence and absence of lysozyme. The selections were performed in parallel. Amplification was carried out twice. The first amplification product was used as a template for the second PCR amplification that was performed with sample being taken every 2 cycles for the subsequent gel analysis. The bands that appeared at the molecular weight expected for the ligated products were compared. In the absence of lysozyme, the ligation product appeared at cycle number 8 in the second amplification; compared to the presence of lysozyme where the band occurred at the first cycle (bottom).

In this system the best conditions were found to be selection with 10^{-9} moles mixed F- and R- libraries and 10^{-10} moles of lysozyme in 100 μL requiring ligation time of 5 minutes, 0.05 unit of the T4 ligase, 10^{-12} moles of the oligonucleotide connector. These optimal parameters were then used to carry out PLS for aptamers against lysozyme. As a control, a parallel reaction was performed without the target. Figure 5 shows that the number of PCR cycles required for amplification of the ligated aptamers is fewer than in the control consistent with the specific ligation of DNA bound to the target.

Two independent selections were performed to assess the reproducibility of the method. The PCR products of the PLS selection in the presence of lysozyme were inserted into pCR2.1 vector and sequenced. Two sequences, confirmed to be formed from the two parts in which one was from the F-library and the other was from the R-library based on the sequences of the 5'- and the 3'- ends which corresponded to the priming sequences of the respective libraries were assayed for affinity towards lysozyme.

One sequence (PLS4) appeared twice in the sequenced pool of 50 clones, which confirmed that it came from the two libraries joined in a 5'-F-R-3' order. One disadvantage of cloning and sequencing is that only a small number of sequences are sampled. Recently the use of Next Generation Sequencing has allowed much deeper analysis of enrichment[6] especially in early stage of selection and it is likely that PLS would further benefit from this technology, although the analysis can be time consuming. This aptamer was selected for affinity determination. The diversity of the pool (shown in the Supplemental Information) is unsurprising given only a single round of selection was performed. The 5'-end half from the F-library, PLS4F, has sequence AAGCATCCGC TGGTTGACTT GAGAGTGAAT CTATCG TGAC GTACTG AACCGAATAA AGAATTTGGG CTGCTC whilst the 3'-end half from R-library, PLS4R, has sequence TCGTGGCGTA ATATCATCTC GCGAGTAATG GTTATAAGTC GCTCCTCATA GTGGATCTTG GACCCTGCGAA. These binding affinities of these two separate sequences were determined using ELONA (Enzyme-Linked Oligonucleotide Assay) along with those of published aptamers reported to bind to lysozyme.

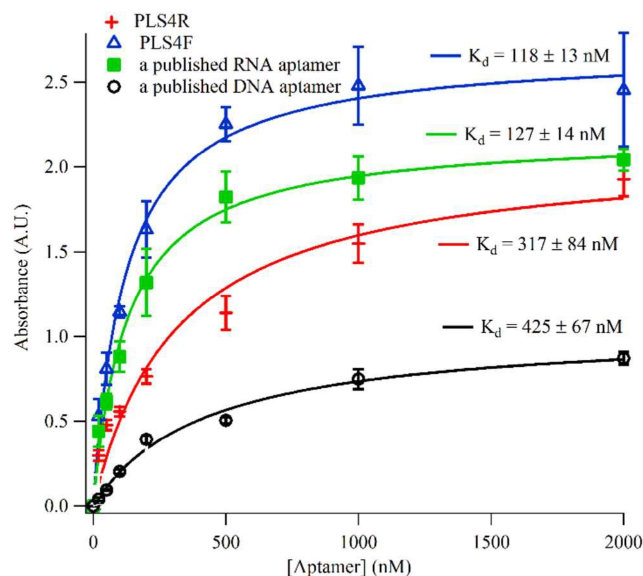


Figure 6: Binding affinity of aptamers to lysozyme by ELONA. The binding of the two aptamer constituents, PLS4F and PLS4R, that formed PLS4. Binding of F-library (5'-end) half PLS4F (green) had a K_d = 118 ± 13 nM and binding of the R-library (3'-end) half PLS4R (blue) had a K_d = 317 ± 84 nM for whilst an published RNA aptamer against lysozyme[13] showed a K_d of 127 ± 14 nM and a published DNA aptamer for lysozyme[14] showed a K_d value of 425 ± 67 nM.

Competitive binding assays were also carried out with one biotinylated and the other non-biotinylated aptamer and it was confirmed that there was no interference of the binding of one aptamer by the other, consistent with these two aptamers binding lysozyme at different sites and thus are suitable for use as a pair in sandwich assays.

As shown in Figure 6 the affinities of these two aptamers for lysozyme are comparable to that of the RNA aptamer obtained from

ChemComm Accepted Manuscript

12 rounds of automated selection, which had a K_d of 127 ± 14 nM in our ELONA measurements. This is similar to the reported K_d value of 31 nM determined using a nitrocellulose membrane filter binding assay with radioisotope labelling, a standard assay for aptamer-protein binding. The other published sequence, a DNA aptamer was reported to have been selected against lysozyme after 5 rounds of capillary electrophoresis [11] selection and showed a K_d value of 425 ± 67 nM in our ELONA measurements which is around two order of magnitudes lower in affinity compared to the reported K_d value of 3 nM using kinetic capillary electrophoresis measurements.[14] This difference may be due to the solution-based nature of CE compared with the solid-phase nature of ELONA as well as between kinetic and static measurements in determining affinity of the two approaches. An ELONA assay for the linked PLS4 aptamer showed an increase in affinity with a K_d value of 9 ± 2 nM compared to the two halves separately (see Supplemental Information).

The M-fold structures[15] of the F- and R- sequences are presented in Figure 7.

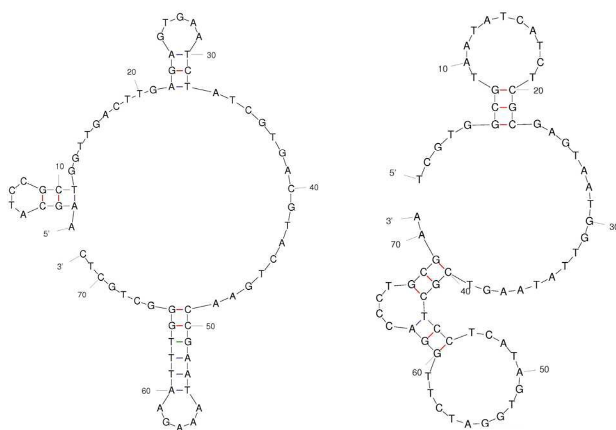


Figure 7: Mfold structures of aptamers PLS4F (left) and PLS4R (right).

Avidity effects would be expected result in tighter binding of the dual aptamer sequence compared with the two individual aptamer sequences. A similar phenomenon has been widely employed in ‘fragment based drug discovery’.[16, 17] However a longer sequence will probably have more internal base pairing so forming a more stable ‘stem’ structure (see Supplemental Information) that could have the effect of reducing binding affinity.[18] Furthermore ligated PLS sequence is probably not desirable as a single aptamer as its size (almost 150 nucleotides) would be more expensive to produce.

Conclusions

We demonstrate a method, PLS, for the generation of DNA aptamer pairs that bind lysozyme. After determining the optimal conditions, a single round of PLS produced a pair of aptamers, with K_d values of 118 ± 13 nM and 317 ± 84 nM and which bind independently to their target. This pair of aptamers have affinities comparable to those of published sequences created after 12 rounds of automated-SELEX[13] or 5 rounds of CE-SELEX.[14]

Acknowledgements: AEGC and TTL would like to thank BBSRC for funding support and OC would like to thank the Thai government for a PhD studentship.

Notes and references

[a] Department of Chemistry, Imperial College London, UK.
[b] Institute of Biomedical Engineering, Imperial College London, UK
[c] Department of Chemistry, University of Leicester, UK
†Corresponding author (t.cass@imperial.ac.uk)

1. Ellington, A.D. and J.W. Szostak, *In vitro selection of RNA molecules that bind specific ligands.* Nature, 1990. **346**(6287): p. 818-822.
2. Tuerk, C. and L. Gold, *Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase.* Science, 1990. **249**(4968): p. 505-10.
3. Cox, J.C., P. Rudolph, and A.D. Ellington, *Automated RNA Selection.* Biotechnology Progress, 1998. **14**(6): p. 845-850.
4. Mendonsa, S.D. and M.T. Bowser, *In Vitro Selection of High-Affinity DNA Ligands for Human IgE Using Capillary Electrophoresis.* Analytical Chemistry, 2004. **76**(18): p. 5387-5392.
5. Hybarger, G., et al., *A microfluidic SELEX prototype.* Analytical and Bioanalytical Chemistry, 2006. **384**(1): p. 191-198.
6. Schütze, T., et al., *Probing the SELEX Process with Next-Generation Sequencing.* PLoS ONE, 2011. **6**(12): p. e29604.
7. Mir, M., M. Vreeke, and I. Katakis, *Different strategies to develop an electrochemical thrombin aptasensor.* Electrochemistry Communications, 2006. **8**(3): p. 505-511.
8. Haab, B.B., *Methods and applications of antibody microarrays in cancer research.* PROTEOMICS, 2003. **3**(11): p. 2116-2122.
9. Posthuma-Trumpie, G., J. Korf, and A. van Amerongen, *Lateral flow (immuno)assay: its strengths, weaknesses, opportunities and threats. A literature survey.* Analytical and Bioanalytical Chemistry, 2009. **393**(2): p. 569-582.
10. Gan, S.D. and K.R. Patel, *Enzyme Immunoassay and Enzyme-Linked Immunosorbent Assay.* J Invest Dermatol, 2013. **133**(9): p. e12.
11. Sosic, A., et al., *Human Thrombin Detection Through a Sandwich Aptamer Microarray: Interaction Analysis in Solution and in Solid Phase.* Sensors (Basel, Switzerland), 2011. **11**(10): p. 9426-9441.
12. Fredriksson, S., et al., *Protein detection using proximity-dependent DNA ligation assays.* Nat Biotech, 2002. **20**(5): p. 473-477.
13. Cox, J.C. and A.D. Ellington, *Automated selection of anti-protein aptamers.* Bioorg Med Chem, 2001. **9**(10): p. 2525-31.
14. Tran, D.T., et al., *Selection and Characterization of DNA Aptamers for Egg White Lysozyme.* Molecules, 2010. **15**(3): p. 1127-1140.
15. Zuker, M., *Mfold web server for nucleic acid folding and hybridization prediction.* Nucleic Acids Research, 2003. **31**(13): p. 3406-3415.
16. Hajduk, P.J. and J. Greer, *A decade of fragment-based drug design: strategic advances and lessons learned.* Nat Rev Drug Discov, 2007. **6**(3): p. 211-219.
17. Murray, C.W. and D.C. Rees, *The rise of fragment-based drug discovery.* Nat Chem, 2009. **1**(3): p. 187-192.
18. Le, T.T., O. Chumphukam, and A.E.G. Cass, *Determination of minimal sequence for binding of an aptamer. A comparison of truncation and hybridization inhibition methods.* RSC Advances, 2014. **4**(88): p. 47227-47233.

ChemComm Accepted Manuscript

This journal is © The Royal Society of Chemistry 2012