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Targeting of Antibodies using Aptamers

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Targeting of Antibodies Using Aptamers

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1. Introduction

Antibodies are traditionally viewed as targeting entities that are used to specifically recognize and target other molecules, antigens, or receptors. However, there are occasions in which cognate ligands are generated against the antibodies themselves. The number of useful applications that rely on the recognition and targeting of antibodies extends to various diseases, including cancer, inflammation, and autoimmune disorders. For example, anti-idiotypic antibodies have been generated to target surface immunoglobulins on neoplastic B-lymphocytes and plasma cells for the treatment of lymphomas and leukaemias *(1,2)*. Such idiotypic determinants are tumor-specific, and have been exploited in a number of immunotherapeutic approaches, either in the form of vaccines *(3–11)* or as the target of anti-idiotypic antibodies *(12–24)*. The latter, in particular, have been used successfully both in the unconjugated mono- and bi-specific forms *(13,14,17)*, or as conjugates to other agents such as interleukin-2 *(18–20)*, cytotoxic drugs *(23)*, or radioisotopes *(12,22)*. In addition to haematological malignancies, antibodies are also implicated in autoimmune disorders and transplant rejection, and they could become possible targets in the management of these conditions. On the other hand, antigen-mimics, whether structural, functional or both, can be produced against a target antibody. Such mimics have been used in raising antibodies *(25)* and as anti-inflammatory and anti-tumor agents *(26)*. In addition, RNA aptamers have recently been used as antigen mimics to elude patient autoantibodies from binding to acetylcholine receptors in the control of myasthenia gravis *(27)* Finally, molecules with binding specificity for antibodies could also be used in the generation of immunoaffinity matrices for the purification of antibodies. To fulfill the promise of antibody targeting, significant interest has emerged in the generation of peptide ligands against antibody targets using phage-display gen-

erated peptide libraries *(28)*. However, the structural freedom of peptides and the resulting entropic cost upon target binding limit the use of peptide libraries in which high-affinity and specificity are required *(29, 30)*. Moreover, amino acids are not interactive with each other in the way that nucleotides are, causing most small peptides to be unstructured in solution, whereas structurally stable proteins are large *(31)*.

Aptamers are a novel and particularly interesting targeting modality, with the ability to bind a variety of targets including proteins, peptides, enzymes, antibodies, various cell-surface receptors, and small organic molecules with sub-nanomolar and even picomolar affinities and great specificity *(32)*. Aptamers are single-stranded DNA or RNA oligonucleotides that vary in size between 25 and 50 bases and are derived from combinatorial libraries through an in vitro selection process known as Systematic Evolution of Ligands through Exponential enrichment (SELEX). As compared to other targeting agents, they offer unique benefits because they bind with high affinity and selectivity, are not immunogenic or toxic and have good circulation clearance, are easily and quickly synthesized using in vitro techniques, and robust, stable and consistent, with no batch-to-batch variation *(33)*. These characteristics make them extremely attractive as alternatives to peptides *(29,30)* for use in assays, or as diagnostic *(34)* and therapeutic agents. Considerable success in the use of aptamers as inhibitors of cellular pathways has resulted in clinical trials of aptamers as angiogenic inhibitors for the treatment of cancer *(35)*. Furthermore, current research in the development of aptamers against idiotypic antibodies on the surface of B-lymphocytes may lead to the development of novel reagents for the treatment of B-cell lymphomas. These aptamers are specifically selected against B-cell receptors from individual patients, and thus, they play an important role in the design of patient-specific therapies. Finally, the significantly higher affinity presented by aptamers for their targets (K_{ds} in the sub-nanomolar to picomolar range), compared to those of peptides (K_{ds} in the micromolar and sub-micromolar range) as well as their increased stability, make them a preferred modality compared to peptide libraries currently in use. Aptamer recognition has been shown to rely strongly on the exceptional propensity of the nucleic acids to assume secondary and tertiary structural elements. The number of possible thermodynamically stable structural variants available for an oligonucleotide sequence is much higher than the number of variants available for a peptide sequence of the same length *(36)*. This is simply based on the ability of nucleotide bases to interact with each other through canonical Watson-Crick as well as unusual base pairing. The existence of oligonucleotide sequences that could assume a myriad of shapes within a random sequence library is the basis for the remarkable success that has been found in generating aptamers to a wide variety of target molecules. In fact, this

is done by a technique that involves generating a highly diverse library of oligonucleotide sequences and selecting the molecules that contain the structural features required for binding. The sampling of "shape space" in such libraries has proven to be effective for the isolation of aptamer ligands to targets that are not known to interact with nucleic acids physiologically *(37)*. Moreover, antibodies and proteins usually exhibit extensive surfaces with ridges, grooves, projections, and depressions, all covered with numerous H-bond donors and acceptors, making them excellent targets for aptamer selection. In fact, high-affinity aptamers against IgE antibodies have already been selected with implications in allergic diseases *(38)*.

This chapter presents a protocol for the generation of oligonucleotide aptamers, using a variation of the SELEX methodology, as novel targeting entities against antibodies and an alternative to currently used peptide ligands and antigen-mimics.

2. Materials

1. DNA oligonucleotide library with a 25-base degenerate sequence flanked by primer sites on either side to facilitate amplification. The library sequence used here is the following: **5′-GGGAGACAAGAATAAACGCTCAA-(25N)-TTC-GACAGGAGGCTCACAA CAGGC-′3,** but any other primer sequence flanking the 25-base degenerate region (25N) could be used. All four bases (A, T, C, and G) are represented at each of the degenerate positions, and both the libraries and primers are HPLC-purified (*see* **Note 1**).
2. Library amplification primers (for library sequence in **step 1**):
   a. Forward primer: 5′-GGGAGACAAGAATAAACGCTCAA-3′;
   b. Reverse primer: 5′-GCCTGTTGTGAGCCTCCTGTCGAA-3′.
3. Target antibody, store frozen in aliquots at –20°C.
4. Sodium carbonate buffer (0.05 *M*), pH 9.6: dissolve 1.59 g of Na_2CO_3 and 2.93 g of $NaHCO_3$ in distilled water to 1 L final volume.
5. Phosphate-buffered saline (PBS): dissolve one PBS tablet (Sigma; cat. #P4417) in 200 mL of distilled water to yield 0.01 *M* phosphate buffer, 0.0027 *M* KCl, and 0.137 *M* NaCl, pH 7.4. Sterile-filter and store at room temperature.
6. Blocking buffer: PBS containing 0.1% (w/v) casein.
7. Washing buffer: PBS containing 0.05% (v/v) Tween 20.
8. 10X PCR buffer with 15 m*M* $MgCl_2$, deoxynucleotide 5′ triphosphate (dNTP) mix (25 m*M* of each nucleotide) and 15 m*M* $MgCl_2$ solution (MBI Fermentas).
9. Taq DNA polymerase, native form (Sigma; cat. #D1806).
10. TOPO TA cloning kit (Invitrogen).

3. Methods

The method presented here is for the selection of aptamers against purified antibodies. The procedure is based on a one-pot experiment in which all selection and amplification rounds take place within a single PCR tube and utilizes

the antibody's biophysical properties and characteristics *(39)*. Using the methodological approach that permits the rapid identification of an aptamer(s) for an antibody, in the first instance, the antibody is adsorbed directly to the surface of the PCR tube, prior to the addition of the oligonucleotide library. Unbound aptamers are washed away, PCR reagents are added to the tube, and binding aptamers are amplified using PCR. High temperature during the first PCR cycle unfolds the antibody, thus releasing the selected aptamer and allowing for its amplification sin successive PCR cycles. The entire process is then repeated 10× to effect affinity maturation. The use of this one-pot procedure eliminates a number of chromatographic or filter separation steps that would otherwise be necessary. In so doing, it provides a rapid isolation of high affinity and selectivity aptamers against antibody molecules.

3.1. Selection of Aptamers Against Antibodies: The One-Pot Experiment

1. Synthesize the aptamer library at a concentration of 40 µ*M* (40 nmols in 1 mL). This would allow for about 20 copies of each possible sequence to be present in the library.
2. Synthesize the forward primer at 1 m*M* (1 µmol in 1 mL) and the reverse primer at 40 µM (40 nmols in 1 mL).

3.1.1. Amplification of Aptamer Library

1. Prepare the following PCR mix in a thin-walled PCR tube:

Aptamer library	100 µL (*see* **Note 2**)
Forward primer	100 nmol (100 µL)
Reverse primer	4 nmol (100 µL)
10X PCR buffer with 15 m*M* MgCl$_2$	40 µL
dNTP mix	40 µL
15 mM MgCl$_2$	18 µL
Taq DNA polymerase, native form	10 U (2 µL)

2. Perform 99 rounds (instrument limit) of PCR at 94°C (1.5 min), 56°C (0.5 min) and 72°C (1.5 min) in a thermocycler. Include a final extension step at 72°C for 0.5 min and soak at 4°C (*see* **Note 3**).

3.1.2. Immobilization of Antibody for Aptamer Selection

1. Dilute the target antibody in 0.05 *M* sodium carbonate buffer, pH 9.6, to a final concentration of 10 µg/mL (*see* **Note 4**) and add 100 µL of the antibody solution to a 500-µL PCR tube. Incubate at 4°C overnight.
2. Remove the excess unbound antibody using a vacuum pump or a pipet. The use of a pipet allows the recovery of excess antibodies for further use, and also enables the quantification of the level of antibody immobilization.
3. Wash the tube 4× with 100 µL of washing buffer.

4. Add 500 μL of blocking buffer to the tube and incubate at room temperature for 1 h. Ensure that the entire antibody-coated surface is covered by the blocking buffer.
5. Wash the tube once with 500 μL of washing buffer.

3.1.3. Aptamer Selection

1. Add the amplified aptamer library (400 μL, from **Subheading 3.1.1.**) to the antibody-coated and blocked PCR tube and incubate at room temperature (~~20°C), for 1 h.
2. Discard the library solution and wash the tube once with 500 μL of washing buffer. All unbound sequences will be washed out and only antibody-binding sequences will remain in the PCR tube.
3. Add PCR reagents to the tube as follows:

Forward primer	400 pmol (40 μL)
Reverse primer	4 pmol (10 μL)
10X PCR buffer with 15 mM MgCl$_2$	40 μL
dNTP mix	10 μL
15 mM MgCl$_2$	4.5 μL
Taq DNA polymerase, native form	7.5 U (1.5 μL)
Water	24 μL (18Ω)

4. Perform 99 rounds (instrument limit) of PCR at 94°C (1.5 min), 56°C (0.5 min) and 72°C (1.5 min) in a thermocycler include a final extension step at 72°C for 0.5 min and soak at 4°C (*see* **Note 5**).
5. Repeat **steps 2–4** 10×. In each round adsorb the antibody on a new PCR tube, add to it the PCR products of the reaction, discard the solution, add PCR reagents, and amplify. This allows for affinity maturation through competitive binding of the continuously amplified number of aptamers to the limited number of antibody molecules. In each round, the higher-affinity binding aptamers are enriched and thus displace the nonspecific binders or those that bind with lower affinity. At the end of the selection rounds, only one or two sequences with similar affinities remain.
6. Clone the PCR products from the last round using the TOPO TA cloning kit, according to the manufacturer's instructions. Please note that a standard PCR amplification procedure with equal amounts of both primers should be carried out during cloning in order to obtain double-stranded forms of the PCR products obtained in **steps 1–5.**
7. Isolate plasmid DNA from E. coli colonies harbouring the cloned aptamer inserts, prepare plasmid mini-preps using a standard plasmid purification kit, and send for DNA sequencing. You should receive back a single sequence or more than one with similar affinities. Generally, you will find that you get a single sequence or highly homologous sequences that span different parts of the total aptamer sequence.
8. Synthesize the aptamer sequences for further characterization or application. Modifications of the aptamer may be performed to suit particular applications (*see* **Note 6**).

4. Notes

1. Longer degenerate regions could also be used, and there have been examples of 50-75, or 100-base degenerate regions. However, 25 bases are the minimum length to provide all the possible combinations of secondary structures in the library. Additionally, using this length, it is easy to synthesize a complete library, without any possible sequence missing within the synthesis scale of a standard commercial oligonucleotide preparation.

2. This would allow at least two copies of all possible sequences to be present in the solution.

3. This amount of reagents allows the library to be amplified only once in the direction using the reverse primer, and thus generating one complementary c-DNA strand for every sequence. This subsequently gets amplified 99× on the other direction, thus generating 99 single-stranded sequences that are identical to the original DNA.

4. The antibody can be adsorbed on the surface of the PCR tubes, not only in carbonate buffer, but also in PBS. If there is a problem using carbonate buffer, pH 9.6, PBS, pH 7.4 can be used instead. However, the coating efficiency was deemed to be superior with the use of carbonate buffer with a variety of IgG, IgM, and immunoglobulins purified from B-cell lymphomas, especially at lower antibody concentrations (author's unpublished observations).

5. During this process, the following takes place. At the very first denaturation step at 95°C, the coated antibody is unfolded *(39)*, thus releasing the bound aptamers for amplification in subsequent thermocycling rounds. Since this is a unidirectional PCR, after 99 rounds of amplification (this is the limit in most PCR machines), 99 single-stranded copies of each binding sequence will be generated. In the early rounds, the binding sequences will include nonspecific binding aptamers, which are also amplified during the PCR process. However, these will be competed out by the enrichment of higher binding sequences in subsequent rounds.

6. One useful modification that may be considered is the introduction of 3′-amino modified group (3′-end-cap). This will allow the coupling of the aptamer to activated Sepharose for the generation of chromatographic materials to use in antibody affinity purification. Furthermore, a 3′-amino-modified sugar in the nucleotide will provide a primary amine for the coupling of the aptamer to a chelator carrying a radionuclide to generate a novel targeted radiopharmaceutical. However, 3′-amino modification also increases the resistance of aptamers to nucleases (the principal serum nuclease is a 3′-exonuclease) and their blood clearance properties, thus making them extremely useful for in vivo targeting applications *(40)*.

Acknowledgments

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