Development of DNA aptamers targeting low molecular- weight amyloid-β peptide aggregates *in vitro*.

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1. Procedure for developing nucleic acid aptamers to amyloid beta peptide 1-40.

1a. Materials and methods

Biotin-labelled $A\beta_{1-40}$ (MW: 4556.2 g/mol) was synthesized by MIMOTOPES Pty Ltd, Victoria, Australia. Invitrogen Dynabeads® M-280 Streptavidin was purchased from ThermoFisher Scientific. Heparin and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) were purchased from Sigma-Aldrich. Aptamer binding buffer was composed of 10 mM Tris, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM KCl, pH 7.5.[1] Phusion high fidelity DNA polymerase was purchased from New England Bio lab and supplied by Genesearch Australia. NucleoSpin® Extract II for DNA isolation was sourced from Macherey-Nagel and supplied by Scientifix Life Australia. Single stranded DNA library with 40 random bases and primers (F: ACAAAGCGACACACAGGAGCC, R: GGACAGGACCACACCCAGCG-FAM) were synthesized by Integrated DNA Technologies, Coralville, Iowa, USA.

1b. $A\beta_{1-40}$ preparation and immobilization

A stock solution of biotin-labelled $A\beta_{1-40}$ (438.96 µM) was prepared by suspending 1 mg of $A\beta_{1-40}$ with 0.5 mL of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) followed by sonication for 5 min to prevent rapid aggregation of $A\beta_{1-40}$.[2] Aliquots of the $A\beta_{1-40}$ solution was stored at -80 °C and only freshly thawed aliquots were used for each selection round. Freshly thawed $A\beta_{1-40}$ aliquots were sonicated again for 5 min followed by total evaporation of HFIP. The dried $A\beta_{1-40}$ was immobilized with 500 pmols of streptavidin-coated magnetic bead (Dynabeads®) in PBS solution. To increase stringent conditions for aptamer selection, the amount of immobilized $A\beta_{1-40}$ was reduced as the selection progressed (Table S1).

Table S1: Ratio of immobilized $A\beta_{1-40}$ to Dynabeads® used for each selection round.

Magnetic beads (pmol)	$A\beta_{1-40}$ (pmol)	$A\beta_{1-40}$:Dynabeads® ratio	Selection rounds
500	10974	22:1	1, 2, 3
500	4380	9:1	4,5
500	3285	7:1	6,7,8
500	1095	2:1	9,10

1c. SELEX

The magnetic-bead assisted selection procedure consisted of 10 rounds of ssDNA library incubation with bead-immobilized $A\beta_{1-40}$, separation of unbound ssDNA and extraction of the $A\beta_{1-40}$-bound ssDNA, and amplification of the target ssDNA with PCR. For the first round of aptamer selection, 1 nmol of randomized ssDNA library was used to maximize enrichment of aptamer candidates, later rounds of aptamer selection used 100 pmol of enriched ssDNA library. For each selection round, the ssDNA library was heated at 95 °C for 3 min followed by gradual cooling to 4 °C to allow stable structural folding. The refolded ssDNA was incubated with the immobilized $A\beta_{1-40}$ in a microcentrifuge tube in selection buffer (10 mM Tris, 150 mM NaCl, 5 mM $MgCl_2$, and 5 mM KCl) for 40 minutes (Table 2). Heparin was also used in the selection buffer to limit any charge-based interactions between the ssDNA and the $A\beta_{1-40}$. These conditions allow aptamer candidates to bind to the bead-immobilized $A\beta_{1-40}$. To separate unbound ssDNA from $A\beta_{1-40}$-bound ssDNA, a magnetic source was introduced to aggregate the bead-immobilized $A\beta_{1-40}$ along with their bound ssDNA, leaving the unbound ssDNA free in solution for aspiration. After removal of the unbound ssDNA, the bead-immobilized $A\beta_{1-40}$ was washed with selection buffer to remove residual unbound ssDNA and low affinity $A\beta_{1-40}$-bound ssDNA. As the selection progressed, the total washing volume was increased to further remove low affinity aptamers and select for high affinity aptamers (Table S2). In addition, H_2O was introduced in the washing process in the final 4 selection rounds to increase stringency. After the washing step, the bead-immobilized $A\beta_{1-40}$ was suspended in 100 µL H_2O and heated at 95 °C for 5 min to denature the bound ssDNA, allowing its separation from the bead-immobilized $A\beta_{1-40}$ by using a magnetic source. The dried Dynabeads® were once again suspended with H_2O and heated at 95 °C for 5 min to ensure the extraction of high affinity aptamers. To avoid selecting non-specific ssDNA, a counter selection was performed using the same selection procedure but in the absence of the $A\beta_{1-40}$ peptide. 50 pmol of Dynabeads® were incubated with 100 pmol of ssDNA in a microcentrifuge tube for 4 min at room temperature. After incubation, the reaction was subjected to a magnetic field to separate bound and unbound ssDNA. Unbound ssDNA was collected and used in selection rounds with bead-immobilized $A\beta_{1-40}$.

DNA amplification was done in two stages: small scale and large-scale amplifications. Small scale PCR was used to determine the number of PCR cycles required to amplify the target ssDNA while avoiding non-specific PCR products. Each selection ssDNA product was amplified in a single 100 µL PCR reaction (62.4 µL H_2O, 20 µL of 5x phusion buffer, 8 µL of 10 mM dNTP, 4 µL of 50 µM primers, 0.6 µL of Phusion polymerase enzyme, and 5 µL ssDNA template) for 24 cycles. The forward primer contained a biotin label to facilitate single strand separation. During the small-scale PCR, 9 µL of the reaction was aspirated every second cycle and visualized on a 3% agarose gel. The PCR cycle number that produced the sharpest target band with no non-specific bands was applied in the large-scale PCR. The final PCR products were purified using the NucleoSpin® Extract II kit following the manufacturer's protocol. To prepare the ssDNA library, the purified PCR products were mixed with Dynabeads® and heated at 74 °C for 5 min to separate the DNA strands. The solution was subjected to a magnetic field to allow the extraction of the target ssDNA library for aptamer selection.

Table S2: Binding reactions between ssDNA library and immobilized $A\beta_{1-40}$ peptides.

Selection round	ssDNA library (pmol)	$A\beta_{1-40}$:Dynabeads® ratio	Washing solution
1	1000	22:1	1.25 mL Selection buffer
2	100	22:1	3 mL Selection buffer
3	100	22:1	5 mL Selection buffer
4	100	9:1	7 mL Selection buffer
5	100	9:1	9 mL Selection buffer
6	100	7:1	12 mL Selection buffer
7	100	7:1	15 mL Selection buffer + 100 µL H_2O
8	100	7:1	17 mL Selection buffer + 200 µL H_2O
9	100	2:1	19 mL Selection buffer + 300 µL H_2O
10	100	2:1	21 mL Selection buffer + 400 µL H_2O

1d. Cloning and Sequencing:

Purified PCR products (50 ng) form the last round were cloned into pCR-Blunt (Invitrogen) according to the manufacturer‘s instructions. Plasmid DNA was extracted using QIAprep (Qiagen) and sequenced by the Australian Genome Research Facility (AGRF, Brisbane, Australia). After obtaining the data, the sequences were aligned to the actual library design, and repeating sequences after truncating the primer binding regions were considered for further evaluation (Table S3). Folding structure of each aptamer sequence was predicted using mfold, and only the structures with lowest energy (RNV95) was considered for β-Amyloid binding analysis in Alzheimer brain tissues.

Table S3: Amino acid sequences of $A\beta_{1-40}$ peptide and the sequences of the selected aptamer candidates.

Name	Clone Number	Sequences	Repeats
$A\beta_{1-40}$		Biotin-D A E F R H D S G Y E V H H Q K L V F F A E D V G S N K G A I I G L M V G G V V	
RNV91	76,74	GCGCCTAGCCAGGGACGGCCGCTGCAGATGAACCGAACGC	2
RNV92	14, 17	AGTATCGGCAGGGGGATAAGGGCACAACGACGAAGAGCGC	2
RNV93	75, 77	CACGAGGGGATGGGGGGCGACGGAGCACGGACGAGTAGTG	2
RNV94	29, 39	CAAGTAGAATGCGCAGGCCGTGTACCAAAGACCGTCAACG	2
RNV95	78, 25	TGGGGGGCGGACGATAGGGGCCCCCCGGTAGGATGGACG	2
RNV96	50, 32	TGGAAAGGGCAAGTCAAAGAGCGGGAAGAGCGCGCCCTC	2
RNV98	40, 47	AACACGAACTCGCGCAAGACCCCGGACGAGAGCCAGCGT	2
RNV99	42, 9	CACGCGAGGGACGGGGCAGTAGAGGCGTAGGAGGGGTAT	2
RNV100	53, 38	TGGGGAGCTGCGTCGACACAACGGCGGACAACGTGAGTC	2

2. Protocols for detection of amyloid beta peptides in Alzheimer brain tissues *in vitro*.

Aβ Western Blot Analysis: Neuropathologically confirmed, frozen Alzheimer brain tissues from the hippocampus was obtained from the Queensland Brain Bank and homogenised in 50 mM Tris, pH 7.5.[3] Protein from both AD and control samples were mixed with 2X Laemmli sample buffer (1 mL 1 M Tris-HCl, 4 mL 10% sodium dodecyl sulphate, 2 mL glycerol, 2.5 mL β-mercaptoethanol, 500 µL 1% bromophenol blue, pH 6.8) and heated at 95°C for 10 min. 25 µL of each sample and vehicle controls were run on a 10% Mini-PROTEAN TGX precast protein gels (BioRad Laboratories, Gladesville, NSW 2111, Australia) and run at a constant 200 V for 30 mins. The proteins on the gel were transferred to nitrocellulose membranes in a Trans-Blot® (BioRad) Semi-Dry Electrophoretic Transfer Cell according to Laemmli (1970), Towbin *et al.* (1979), and Dunn (1986).[4-6] The membranes were blocked for 2 h at room temperature in 4% dried skim milk powder in TBS, then washed in wash buffer (0.4% skim milk powder, 0.1% Tween20 in TBS) three times for 10 min each. After washing, the membrane was incubated overnight at 4°C in TBS containing 1% dried skim milk powder and 0.1% Tween20 w/v with a dilution of 1:1,000 v/v primary antibody, BAM90.1 (Sigma) or 0.1 µM biotinylated aptamer, RNV95with gentle agitation. The following day, the membranes were washed in wash buffer and incubated in either biotinylated goat anti-mouse IgG (Cat# RPN100V from Licor) to detect anti-Aβ antibody at a dilution of 1:250, or Alexa Fluor 680-conjugated streptavidin (ThermoFisher) to detect RNV95 at a dilution of 1:400 v/v in TBS containing 1% dried skim milk powder/PBST for 2 h at room temperature with agitation. The membranes were washed again in PBST, then purified, deionised, and distilled H_2O, then dried after a brief methanol rinse. The membranes were scanned at the 700 and 800 nm near-infrared channels on the Odyssey scanner system (Licor). Densitometry of the bands or measuring of the band intensity was performed using the Image J Software.[7] The band intensity of the different size bands for different samples were measured and the raw values were plotted on excel (Fig. 3C. and Fig. S1.).

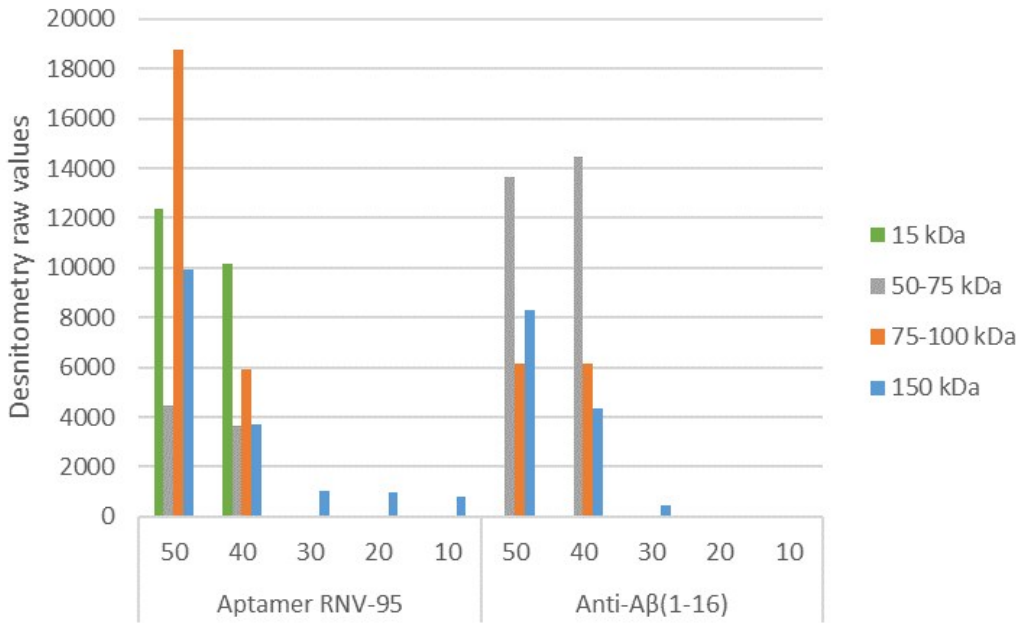

Fig. S1.Quantification of the Western Blot bands shown in Fig. 3B using densitometry through the Image J software. [7]

3. Enzyme-linked oligonucleotide assay (ELONA) for aptamer binding analysis to Aβ Oligomer.

Aβ oligomers were prepared using a similar method to that described by *Tsukakoshi et al.* $A\beta_{1-40}$ peptide was dissolved in hexafluoro-2-propanol (HFIP) at a concentration of 2.5 mg/mL and incubated in ice for 20 min and stored at -20 °C until use. A 50 µL sample of the $A\beta_{1-40}$ monomer solution was taken and the HFIP evaporated by blowing air into the tube. The tube was spun down in a speed vacuum for 15 mins to remove excess moisture. 900 µL of Milli-Q water was added to the tube and incubated at 25°C with 300 rpm shaking for 2.5 days. An enzyme-linked oligonucleotide assay (ELONA) was used to evaluate the binding capacity to β-amyloid oligomers. A 100 µL sample of Aβ oligomer was immobilized in a 96-well microtiter plate and incubated at 37°C for 2 h. The wells were blocked using 4% skim milk in TBS-T. Biotin-labeled aptamer candidates were added to the wells and incubated for 2 h at room temperature. The wells were washed three times using 100 µL TBS-T, and an anti-biotin antibody conjugated with HRP, diluted 1000-fold in TBS-T was added to the wells and incubated under the same conditions for 1h. After the wells were washed three times, the 100 µL of the TMB substrate (ELISA Technoclone TC) was added and incubated for 45 mins. For endpoint, 100 µL of stop solution (Sulfuric acid) was added to the wells and the chemiluminescence was measured at 450 nm using a FLUOstar Omega Microplate Reader (BMG Biotech).

4. References

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