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Construction of human Fab library and screening of a single-domain antibody of amyloid-beta 42 oligomers☆

Zuanning Yuan[1, 2], Minge Du[1, 2], Yiwen Chen[3], Fei Dou[1, 2]

1 *Key Laboratory of Cell Proliferation and Regulation Biology, Ministry of Education, Beijing Normal University, Beijing 100875, China*
2 *Key Laboratory of Developmental Genes and Human Disease, Ministry of Education, Southeast University, Nanjing 210009, Jiangsu Province, China*
3 *Geriatric Internal Department of Beijing Tongren Hospital, Capital Medical University, Beijing 100730, China*

Research Highlights

(1) We constructed a naïve human Fab library and obtained a human Fab antibody and a single-domain antibody that specifically recognized amyloid-beta (Aβ)42 oligomers.
(2) The Fab library was constructed using a parallel insertion strategy, which increased library capacity, variety and screening rate of positive clones.
(3) We screened beta-amyloid oligomer single-domain antibodies using a protein-on-membrane technique, which removed interference due to binding of amyloid-beta single-domain antibodies, and increased the possibility of obtaining anti-beta-amyloid oligomer conformation-dependent antibodies.

Abstract

Screening humanized antibodies from a human Fab phage display library is an effective and quick method to obtain beta-amyloid oligomers. Thus, the present study prepared amyloid-beta 42 oligomers and constructed a naïve human Fab phage display library based on blood samples from six healthy people. After three rounds of biopanning *in vitro*, a human single-domain antibody that specifically recognized amyloid-beta 42 oligomers was identified. Western blot and enzyme-linked immunosorbent assay demonstrated this antibody bound specifically to human amyloid-beta 42 tetramer and nonamer, but not the monomer or high molecular weight oligomers. This study successfully constructed a human phage display library and screened a single-domain antibody that specifically recognized amyloid-beta 42 oligomers.

Key Words

neural regeneration; amyloid-beta; Alzheimer's disease; oligomer; single-domain antibody; phage display; antibody library construction; alpha-synuclein; Parkinson's disease; humanized antibody; immunotherapy; grants-supported paper; neuroregeneration

Zuanning Yuan, Master.

Corresponding author: Fei Dou, Ph.D., Professor, Key Laboratory of Cell Proliferation and Regulation Biology, Ministry of Education, Beijing Normal University, Beijing100875, China; Key Laboratory of Developmental Genes and Human Disease, Ministry of Education, Southeast University, Nanjing 210009, China, douf@bnu.edu.cn.

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INTRODUCTION

Alzheimer's disease is a major neurodegenerative disease. Histopathological changes including senile plaques and neurofibrillary tangles are considered the primary cause of Alzheimer's disease and may induce the progressive death of neurons and synaptic dysfunction. However, the inconsistent relationship between plaque load and degree of cognitive impairment has led to the hypothesis that earlier amyloid-beta (Aβ) assemblies may be one of the most important causes of Alzheimer's disease[1-2]. Increasing evidence has shown that amyloid-beta oligomers exist naturally and inhibit long-term potentiation, impair synaptic functions, disrupt cognition and cause memory loss[3-7]. Hence, oligomers are considered an important therapeutic target for Alzheimer's disease.

Since Schenk first reported that immunization of the platelet-derived growth factor B promoter diving human amyloid precursor protein V717F (PDAPP) transgenic mice with synthetic, pre-aggregated amyloid-beta 42 reduced the extent and progression of Alzheimer's disease pathology[8], much progress has been made in designing a vaccine appropriate for human use[9]. However, using amyloid-beta active immunotherapy resulted in meningoencephalitis in approximately 6% of patients in a phase II clinical trial, thus hindering its further application[10-15]. However, passive immunization of anti-amyloid-beta antibodies reduced amyloid-beta burden and improved memory performance in various Alzheimer's disease transgenic mice[9]. However, the mouse antibody used has the disadvantage of triggering human anti-mouse antibody immune responses[16], which may decrease its therapeutic efficacy or cause serious side effects. Hence, a human origin antibody against amyloid-beta oligomers would be more promising for Alzheimer's disease research and clinical therapy. Phage antibody library technology is a relatively simple and quick way to obtain a considerable amount of humanized antibodies[17].

Furthermore, only antibody fragments are produced by this technique, which may have potential advantages over whole antibodies for therapeutic purposes, since they are small and can better penetrate organs and tissues[18-19].

The present study describes the construction of a naïve human Fab library and identification of a human Fab antibody and a single-domain antibody that specifically recognized amyloid-beta42 oligomers, but not monomers or fibrils. Such antibodies may have potential for Alzheimer's disease diagnosis as well as accelerating central nerve regeneration and preventing lasting memory loss.

RESULTS

Identification of amyloid-beta 42 oligomers

1,1,1,3,3,3-hexafluoro-2-propanol (HFIP)-treated amyloid-beta 42 solutions were subjected to oligomerization. To characterize the aggregation state of the accumulated products, aliquots of amyloid-beta 42 oligomers were assayed by western blot with anti-amyloid-beta antibody (6E10).

Amyloid-beta 42 oligomers were detected after 24 hours. After 48 hours of incubation, oligomers formed robustly in the molecular weight range of 8 to 180 kDa (Figure 1).

Construction of phage antibody library

Total RNAs were isolated from lymphocytes of healthy human peripheral venous blood. The integrity of RNAs was confirmed by alkaline denaturing agarose gel electrophoresis. cDNAs coding for immunoglobulin heavy chains and light chains were amplified by a variety of primer combinations designed to amplify a majority of the known human antibody sequences (Table 1), with suitable restriction sites for later cloning into the p3MH vector. The size of the amplified fragments of heavy chain and light chain was about 680 bp (Figure 2).

The Fab library was constructed using a parallel insertion strategy. In the first round,

the κ light chain library, λ light chain library and heavy chain library were generated with sizes of about 2.00 × 10^7, 2.00 × 10^6 and 8.00 × 10^6 colony forming units (cfu), respectively. In the second step, heavy chain fragments were excised from the heavy chain library and inserted into the light chain library to construct the Fab phage library with a size of about 3.15 × 10^7 colony forming units (Table 1).

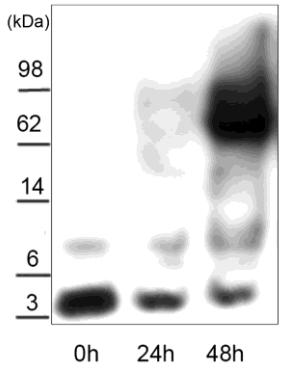

Figure 1 Amyloid-beta (Aβ) oligomerization.

1,1,1,3,3,3-hexafluoro-2-propanol-treated Aβ42 solutions were subjected to oligomerization and analyzed by western blotting with the anti-Aβ antibody 6E10.

Aβ42 oligomers were detected after 24 hours, and after 48 hours of incubation oligomers formed robustly in the molecular weight range of 8 to 98 kDa. h: Hour.

Table 1 Insert percentage and size of heavy chain, light chain and Fab libraries

Library	Insert (%)	Size (cfu)
V_H	85	8.00×10^6
$V_λ$	80	2.00×10^6
$V_κ$	70	2.00×10^7
Fab	70	3.15×10^7

The insert percentage of each library was generated by double digestion of restriction enzymes. The size of the library was estimated by plating several dilutions.

cfu: Colony forming units; V_H: variable region of heavy chain; $V_λ$: variable region of λ light chain; $V_κ$: variable region of κ light chain; Fab: antigen-binding fragments.

Selection of a Fab phage antibody library specific for amyloid-beta42 oligomers

Phage screening was performed using three rounds of panning. The percentage of phage yield increased from 3.75 × 10^{-8} to 9.67 × 10^{-4} (Table 2), representing an enrichment of about 2.57 × 10^4-fold (Figure 3A) as a result of successive removal of low affinity and nonspecifically bound phages. Following each round of biopanning, enzyme-linked immunosorbent assay (ELISA) was performed to analyze the phage polyclonal reactivity with amyloid-beta 42 oligomers (Figure 3B).

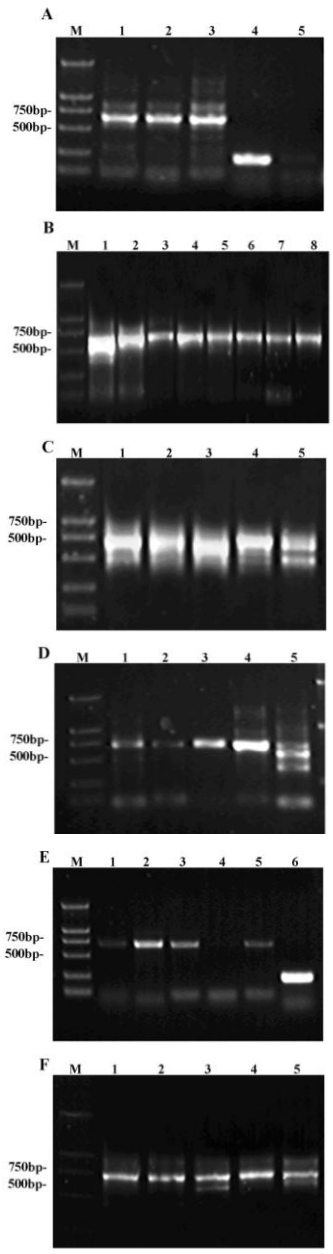

Figure 2 PCR amplification of heavy chain and light chain of human antibodies.

(A) Lanes 1–3: κ light chain of immunoglobulin; lane 4: β-actin; lane 5: negative control.

(B) Lanes 1–8: PCR product of λ light chain of immunoglobulin.

(C) Heavy chain of IgG1 family: lanes 1–5: H1a, H2a, H3a, H4f, H6a.

(D) Heavy chain of IgG2 family: lanes 1–5: H1a, H2a, H3a, H4f, H6a.

(E) Heavy chain of IgG3 family: lanes 1–5: H1a, H2a, H3a, H4f, H6a; lane 6: β-actin.

(F) Heavy chain IgG4 family: lanes 1–5: H1a, H2a, H3a, H4f, H6a. M = size marker: 2 000, 1 000, 750, 500, 250, and 100 bp.

Table 2 Positive clones are enriched after three rounds of panning

Round of panning	Input phage (cfu)	Eluted phage (cfu)	Yield (%)	Wash time
First	3.20×10^{14}	1.20×10^{5}	3.75×10^{-8}	5
Second	4.00×10^{13}	5.00×10^{6}	1.11×10^{-5}	10
Third	6.00×10^{12}	5.80×10^{7}	9.67×10^{-4}	10

The percentage of phage yield increased 2.57 $\times 10^{4}$-fold after three rounds of panning. Yield (%) = (eluted phages/input phages) × 100%. cfu: Colony forming units.

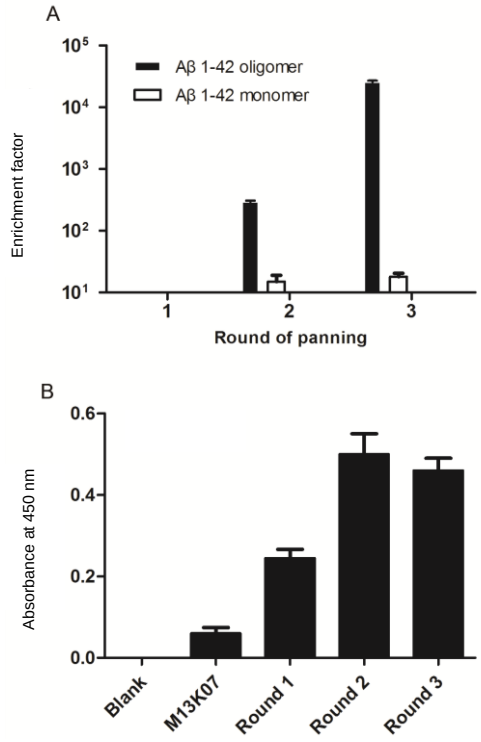

Figure 3 Biopanning of the phage antibody library against amyloid-beta (Aβ) 42 oligomers *in vitro*.

(A) Phage elutes from each round were analyzed by plaque assay to determine enrichment before phage amplification.

(B) Polyclonal phage enzyme-linked immunosorbent assay after 1–3 rounds of library panning against the Aβ42 oligomer.

Data are presented as the mean of duplicate wells. Phages with specificity to Aβ42 oligomer were enriched after three rounds of biopanning.

After the final round of panning, 90 monoclonal antibodies were tested by ELISA against amyloid-beta42 oligomers. Three positive clones (10A1, 11F5, 1A1) that met the criteria (absorbance (A) $_{450nm}$ > 0.5, and the ratio of A_{450nm} for positive control to A_{450nm} for negative control > 3) were selected. Importantly, there was no increase in eluted phages and enrichment factors after three rounds of panning the antibody library against the amyloid-beta42 monomers (Figure 3A). The failure to isolate anti-amyloid-beta monomers antibody indicates that the natural antibodies in the human blood preferentially recognize amyloid-beta oligomers or fibrils compared with monomers, consistent with previous studies[21].

Analysis of Fab antibodies by western blot and DNA sequencing

Western blot demonstrated the specific binding of Fab antibodies to amyloid-beta42 oligomers; 10A1 and 11F5 could specifically bind to amyloid-beta42 oligomers bands with molecular weight of about 17 kDa and about 34 kDa corresponding to amyloid-beta 42 tetramers and octamers, but not monomers and fibrils (Figure 4A). Subsequent sequence analysis showed that clone 10A1 consisted only of a heavy chain and shared 67% homology with IGHV4-59*01. In addition, we determined that clone 10A1 could not recognize oligomers formed by alpha-synuclein (Figure 4B).

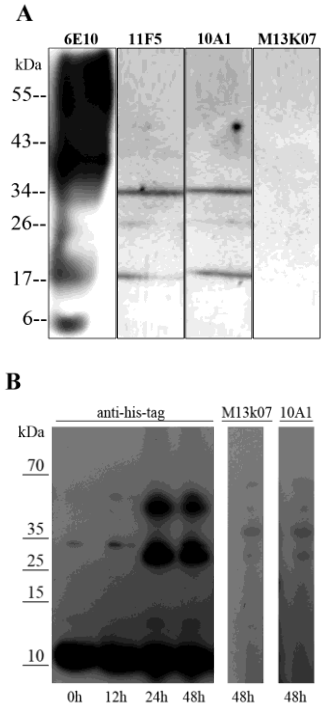

Figure 4 Western blot analysis of single-chain antibody binding to beta-amyloid (Aβ)42 and alpha-synuclein.

(A) Anti-Aβ monoclonal antibody 6E10 could recognize all types of Aβ42, but clone 10A1 and 11F5 only bound to tetramers and octamers. Helper phage M13K07 was used as a negative control.

(B) Western blot was used to analyze whether clone 10A1 could recognize oligomers formed by alpha-synuclein. Aggregated alpha-synuclein was detected (left panels) using anti-his-tag mouse monoclonal antibodies. However, the 10A1 phage antibody could not recognize these oligomers.

DISCUSSION

The use of phage display to screen potential antibodies, antigen-binding fragments (Fab) or single-chain variable fragments that recognize specific antigens is well established and has high efficiency.

As amyloid-beta oligomers are important in Alzheimer's disease pathology, and immunotherapy is a promising therapeutic approach for Alzheimer's disease, currently many studies are attempting to generate conformational dependent antibodies against amyloid-beta oligomers for the prevention of neuron apoptosis. Furthermore, these antibodies will play an important role in accelerating central nerve regeneration and preventing lasting memory loss.

In general, two kinds of libraries can be constructed: immune or naïve. Immune libraries are generated after an immunization step or from infected patients; naïve libraries derived from natural unimmunized humans are intended to be unbiased, so antibodies can be selected against any antigen. Several other groups have successfully isolated anti-amyloid-beta antibodies from both kinds of libraries, such as the synthetic camelid VHH domains library, immunized alpaca phage display library, naïve human single-chain variable fragment library and immune mouse library[20-26].

As specific antibodies against these different amyloid-beta conformations can develop spontaneously as natural antibodies present in the plasma, and serum from healthy age-matched controls has higher levels of natural anti-amyloid-beta antibodies than serum from elderly patients with AD[27], we constructed a naïve library from the lymphocytes of healthy humans to screen human anti-amyloid-beta oligomers antibodies to avoid the use of human anti-mouse antibodies or human anti-camelid antibody reactions.

After the human Fab library was successfully generated, we used amyloid-beta 42 oligomers as targets for selection. Compared with the ELISA-based biopanning assay, which coats the plate with a mixture of amyloid-beta 42 monomers, oligomers and fibrils, we used a polyvinylidene difluoride membrane-based assay to separate amyloid-beta 42 oligomers from monomers and fibrils to increase the possibility of obtaining oligomer-specific antibodies. Two out of the three positive clones we obtained could bind amyloid-beta 42 tetramers and octamers.

By carefully reviewing previous studies results, we observed that about 17 kDa and about 34 kDa bands could be recognized by almost all phage display antibodies, whether they were human or camelid; single-chain variable fragments or VHH domains that imply these amyloid-beta 42 oligomers may have higher antigenicity than other amyloid-beta 42 forms. We also identified a clone (10A1) that encoded a single heavy chain binding domain devoid of a light chain. Single-domain antibodies can exist naturally in camelids and sharks and can retain antigen-binding activity.

In addition, single-domain antibodies have been detected in certain human diseases[25-26, 28]. In 1989, Ward *et al* [29] isolated a functional murine VH domain that bound lysozyme. Because of the small size and stability of single-domain antibodies, it is easier to obtain a large amount of active and soluble proteins compared with full size antibodies, Fabs and single-chain variable fragments.

Although we obtained a human origin small-sized antibody that may be potentially useful against amyloid-beta oligomers, our observations have some limitations. The data are largely based on *in vitro* assays such as enzyme-linked immunosorbent assay and western blot.

Future studies should focus on demonstrating antibody activity for recognizing natural species of amyloid peptides from clinical specimens or Alzheimer's disease mouse brain.

MATERIALS AND METHODS

Design

Single sample experiment.

Time and setting

The study was performed at the Key Laboratory of Cell Proliferation and Regulation Biology, Ministry of Education, Beijing Normal University in China, and the Key Laboratory of Developmental Genes and Human Disease, Ministry of Education, Southeast University, Nanjing, China from March 2007 to September 2010.

Materials

Aβ42 was purchased from Calbiochem (San Diego, CA, USA). After obtaining informed consent according to the *Declaration of Helsinki*, human peripheral venous blood was collected from three 60-year-old healthy male vo-

lunteers from Beijing, China. They were generally in good health, non-smokers, with no history of drug or alcohol abuse, no history of Alzheimer's disease or Parkinson's disease. The other chemicals, enzymes, and vectors were stored in our laboratory.

Methods

Amyloid-beta 42 oligomers preparation

Amyloid-beta 42 was purchased from Calbiochem and amyloid-beta 42 oligomers were prepared as previously described with some modifications[18-19].

Briefly, each vial of lyophilized amyloid-beta 42 peptide (0.25 mg) was diluted in 55 μL 100% 1,1,1,3,3,3- hexafluoro-2-propanol and shaken at 37°C for about 1.5 hours. Then HFIP was evaporated under a vacuum. HFIP-treated aliquots were completely resuspended to a final concentration of 5 mmol/L in anhydrous dimethyl sulfoxide by pipette mixing. Immediately prior to use, amyloid-beta 42 oligomers were prepared by diluting 5 mmol/L amyloid-beta 42 to 100 μmol/L in ice-cold PBS, vortexing immediately for 30 seconds. After incubation at 37°C for 24–48 hours, the samples were centrifuged at 14 000 × *g* for 10 minutes. The supernatant was defined as amyloid-beta 42 oligomers, which comprised fibril-free solutions of oligomers, as well as monomers.

The formation of amyloid-beta 42 oligomers was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blot using mouse anti-human amyloid-beta monoclonal antibody (6E10; 1:5 000; Covance, Princeton, NJ, USA) and a secondary antibody rabbit anti-mouse IgG (1:10 000; Sigma, St. Louis, MO, USA).

Construction of a human phage antibody library

Total RNA from lymphocytes were extracted with Trizol according to the manufacturer's instructions (Invitrogen, Grand Island, NY, USA). To generate first-strand cDNA, an oligo(dT)18 was used as a primer, and 30 μg RNA was reverse-transcribed using the MMLV First Strand cDNA Synthesis Kit (Takara, Dalian, China).

To amplify the V_H, V_λ, V_κ and C regions of immunoglobulin, PCR amplification was performed using the primers described in Table 2. PCR products of amplified V_H-CH and VL-CL regions from cDNA synthesis were gel purified using QIAEXII Agarose Gel Extraction Protocols (Qiagen, Valencia, CA, USA).

Table 3 Primers for PCR amplification of human heavy chain and light chain genes

	Sequences (5′–3′)	Product size (bp)
Heavy chain F primers		
V_H1a	CAG GTG CAG CTC GAG CAG TCT GGG	24
V_H2a	CAG GTG CAG CTA CTC GAG TCG GG	23
V_H3a	GAG GTG CAG CTC GAG GAG TCT GGG	24
V_H4f	CAG GTG CAG CTG CTC GAG TCG GG	24
V_H6a	CAG GTA CAG CTC GAG CAG TCA GG	23
Heavy chain R primers		
IgG1	GCA TGT ACT AGT TTT GTC ACA AGA TTT GGG	30
IgG2	CTC GAC ACT AGT TTT GCG CTC AAC TGT CTT	30
IgG3	TGT GTG ACT AGT GTC ACC AAG TGG GGT TTT	30
IgG4	GCA TGA ACT AGT TGG GGG ACC ATA TTT GGA	30
κ Light chain F primers		
V_κ1a	GAC ATC GAG CTC ACC CAG TCT CCA	24
V_κ2a	GAT ATT GAG CTC ACT CAG TCT CCA	24
V_κ3a	GAA ATT GAG CTC ACG CAG TCT CCA	24
κ light chain R primer		
C_κ1	GCG CCG TCT AGA ATT AAC ACT CTC CCC TGT TGA AGC TCT TTG TGA CGG GCG AAC TCA G	58
λ light chain F primers		
V_λ1	AAT TTT GAG CTC ACT CAG CCC CAC	24
V_λ2	TCT GCC GAG CTC CAG CCT GCC TCC GTG	27
V_λ3	TCT GTG GAG CTC CAG CCG CCC TCA GTG	27
V_λ4	TCT GAA GAG CTC CAG GAC CCT GTT GTG TCT GTG	33
V_λ5	CAG TCT GAG CTC ACG CAG CCG CCC	24
V_λ6	CAG ACT GAG CTC ACT CAG GAG CCC	24
V_λ7	CAG GTT GAG CTC ACT CAA CCG CCC	24
V_λ8	CAG GCT GAG CTC ACT CAG CCG TCT TCC	27
λ light chain R primer		
C_λ1	CGC CGT CTA GAA TTA TGA ACA TTC TGT AGG	30

Restriction enzyme recognition sites are underlined. Primers were synthesized by Takara (Dalian, China). F: Forward primer; R: reverse primer.

After the PCR reaction, the phage antibody library was constructed using a parallel insertion strategy. First, the λ and κ light chain products of PCR were digested with restriction enzymes *Sac*I and *Xba*I (Takara) and ligated overnight with *Sac*I/*Xba*I-linearized p3MH vector at 16°C. Recombinant DNA was transformed into *E. coli* Top10 F' by electroporation. The insertion of target λ and κ fragments was detected by digestion with *Sac*I and *Xba*I (Takara). The culture was added to 100 mL of Super Broth media containing 50 μg/mL ampicillin and 10 μg/mL tetracycline and was cultivated overnight. Phagemids containing light chains were prepared from this overnight culture and named p3MH-LC.

For cloning, the heavy chain fragments, heavy chain products of PCR and the p3MH vector were digested with restriction enzymes *Xho*I and *Spe*I (Takara). Ligation and transformation were performed as described above. After amplification and preparation, heavy chain fragments were excised from phagemids p3MH-HC and inserted into p3MH-LC between *Xho*I and *Spe*I sites.

Insertion of Fab fragments was detected by digestion with *Sac*I and *Spe*I. M13K07 helper phage was added to Top10 samples containing Fab gene libraries and incubated overnight at 30°C with shaking. The cultures were centrifuged at 12 000 r/min for 10 minutes at 4°C. The supernatant was mixed with 20% PEG 8 000/ 2.5 mol/L NaCl (final concentration) and incubated on ice for 30 minutes. Phages were precipitated by centrifugation at 12 000 r/min at 4°C for 30 minutes. The supernatant was discarded and the pellet was drained. The phages were resuspended in 2 mL of PBS containing 1% bovine serum albumin, vortexed and centrifuged at 12 000 r/min for 5 minutes to remove debris. The supernatant was stored at 4°C or used directly for the next round of biopanning.

Bioscreening of the phage antibody library against amyloid-beta 42 oligomers

To separate amyloid-beta 42 oligomers from monomers, amyloid-beta 42 samples were prepared as described above and run on a 12% Bis-Tris NuPAGE gel, followed by electro-transfer onto polyvinylidene difluoride membranes in western transfer buffer (25 mmol/L Tris, 150 mmol/L glycine, and 20% methanol). The location of amyloid-beta 42 oligomers was determined by cutting one lane from the polyvinylidene difluoride membrane and staining with mouse anti-amyloid-beta monoclonal antibody (6E10; 1:5 000; Covance) and a secondary antibody, anti-mouse IgG. Bands corresponding to amyloid-beta 42 oligomers on the rest of membrane were excised and transferred to binding buffer (5% skimmed milk in PBS) containing 1 014 colony forming units of Fab phage antibody library, which was pre-incubated with bovine serum albumin-blocked polyvinylidene difluoride membrane to eliminate non-specific binding. After 1 hour at 37°C, the membrane was washed with gentle shaking five, 10 and 10 times with TBS plus 0.3% Tween-20 added for rounds 1, 2 and 3, respectively. Bound phages were eluted by adding elution buffer (0.1 mol/L HCl, adjusted to pH 2.2 with glycine and containing 0.1% bovine serum albumin). The eluted solution was neutralized with 2 mol/L Tris buffer, pH 8.0. Eluted phages were amplified by infecting log phase *E. coli* XL1-blue in the presence of M13K07 helper phage.

After culture at 37°C overnight, phages were collected by centrifugation and resuspended in 3 mL of PBS containing 1% bovine serum albumin as described above. Input and output phages were titrated on SOB-ampicillin-tetracycline agar plates to calculate the enrichment ratios.

ELISA of polyclonal phage clones from each round

Polyclonal phage clones (100 μL) after each round of selection were incubated at 37°C for 2 hours in triplicate wells of an enzyme-linked immunosorbent assay plate coated with amyloid-beta 42 samples and blocked with 3% bovine serum albumin. After five washes with PBS/0.05% Tween-20, 100 μL horseradish peroxidase-conjugated anti-M13 antibody (GE Healthcare, Piscataway, NJ, USA) was added (1:2 000 in PBS/2% (v/v) bovine serum albumin) and incubated for 1.5 hours at 37°C. Following five washes, clones were developed with 100 μL 3,3′,5,5′-tetramethylbenzidine substrate, and the reaction was terminated with 50 μL of 2 mol/L H_2SO_4. In each ELISA, a negative control using M13K07 alone was used to assess background signals.

Screening of clones by monoclonal phage ELISA

A total of 90 clones from the third round screening were picked and grown in 96-well plates overnight at 37°C. On the next day, 15 μL overnight cultures were transferred to 1 mL of fresh Lysogeny broth medium with ampicillin and grown for another 4 hours before they were super-infected with M13K07 helper phage.

Monoclonal phages were obtained as described above. ELISA for screening of positive clones was performed

as described above. Clones were considered positive when the A_{450nm} was more than three times the signal seen in wells with M13K07 alone. Clones with higher absorbance, based on ELISA, were selected for further studies. The presence of heavy chain and light chain fragments inserted in a plasmid isolated from the selected clone was confirmed by PCR amplification and sequencing. Sequences from ompA leader region (5′-AAG ACA GCT ATC GCG ATT GCA G-3′) and from pelB leader sequence (5′-ACC TAT TGC CTA CGG CAG CCG-3′) were used as primers for sequencing.

Reactivity of Fab antibodies to amyloid-beta42 oligomers was analyzed by western blotting using 6E10 as a positive control and M13K07 as a negative control.

Expression and purification of alpha-synuclein

To identify whether the single-strand antibody specifically bound to amyloid-beta oligomers or whether it could also bind to alpha-synuclein oligomers, which are thought to be toxic in Parkinson's disease, we expressed the alpha-synuclein *in vitro* for binding assay.

Full-length alpha-synuclein cDNA was cloned by PCR into expression vector pET28b, with a His6 tag at the N-terminal. These constructs were transformed into *E. coli* strain BL21 DE3, and recombinant protein was induced with 1 mmol/L isopropyl β-D-1-thiogalactopyranoside at 37°C for 4 hours. Cell pellets were resuspended in lysis buffer (50 mmol/L Tris pH 7.5, 1.5 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT), and were lysed by high pressure homogenization. Cell debris was removed by centrifugation, and the soluble fusion protein was purified by His affinity chromatography (GE Healthcare), followed by gel filtration (GE Healthcare) chromatography.

Protein concentrations were calculated by measuring the absorbance at 280 nm. The purity of alpha-synuclein was assessed by Coomassie blue sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Oligomerization of alpha-synuclein

Alpha-synuclein was diluted to 1 mg/mL, and oligomerized under the conditions of pH 6.9–7.4, 37°C, about 100 mmol/L Na^+ for 1–2 days with or without the addition of Fe^{3+} and Cu^{2+}. Samples were collected at different time points to determine whether clone 10A1 could bind to the alpha-synuclein oligomers. Anti-his-tag (Sigma, St. Louis, MO, USA) was used as a positive control, and M13K07 (GE Healthcare) was used as a negative control. The oligomerization of alpha-synuclein was assessed by western blot.

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