Published in final edited form as:

Alzheimers Dement. 2013 March ; 9(2): 99–112. doi:10.1016/j.jalz.2012.11.005.

New ELISAs with high specificity for soluble oligomers of amyloid β -protein detect natural A β oligomers in human brain but not CSF

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Abstract

Background—Soluble oligomers of amyloid β -protein (A β) have been increasingly linked to synaptic dysfunction, tau alteration and neuritic dystrophy in Alzheimer's disease (AD) and mouse models. There is a great need for assays that quantify A β oligomers with high specificity and sensitivity.

Methods—We designed and validated two oligomer-specific (o-) ELISAs using either an A β aggregate-selective monoclonal for capture and a monoclonal to the free N-terminus for detection or the latter antibody for both capture and detection.

Results—The o-ELISAs specifically quantified pure oligomers of synthetic A β with sizes from dimers up to much larger assemblies and over a wide dynamic range of concentrations, whereas A β monomers were undetectable. Natural A β oligomers of similarly wide size and concentration ranges were measured in extracts of AD and control brains, revealing >1,000-fold higher concentrations of A β oligomers than monomers in the soluble fraction of AD cortex. The assays quantified the age-related rise in oligomers in hAPP transgenic mice. Unexpectedly, none of 90 human CSF samples gave a specific signal in either o-ELISA.

Conclusions—These new o-ELISAs with rigorously confirmed specificity can quantify oligomer burden in human and mouse brains for diagnostic and mechanistic studies and for AD biomarker development. However, our data raise the likelihood that the hydrophobicity of A β oligomers makes them very low or absent in aqueous CSF.

Keywords

Alzheimer's disease; amyloid β -peptide; oligomers; cerebrospinal fluid; brain extracts; ELISAs

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1. Introduction

The growing recognition of diffusible A β oligomers as early neurotoxic species has made these assemblies a topic of special interest in the search for biomarkers of AD. A lowering of A β 42 monomer levels in human CSF has been widely validated as a robust biomarker for the diagnosis of AD, including in its earliest clinical stages (e.g., [1–4]). Mechanistically, the progressive accumulation of insoluble β -amyloid deposits in brain parenchyma has been postulated to explain the decline in the highly self-aggregating A β 42 monomer in both CSF and brain interstitial fluid [2, 5, 6]. But beyond monomers, the pathogenic activity of soluble oligomers makes it particularly desirable to measure their levels in brain tissues and biological fluids using assays with high specificity and sensitivity. Oligomer-specific ELISAs performed on CSF or plasma could prove useful for diagnosing AD, following disease progression, and monitoring responses to amyloid-lowering therapeutics. Moreover, such assays would be useful in analyses of biochemical pathology, e.g., to quantify the regional contributions of A β oligomers to pathogenic mechanisms in AD and other late-life dementias.

Assays with apparent selectivity for soluble oligomers over monomers and large insoluble assemblies of synthetic A β have been reported, but most have not been rigorously validated on natural human oligomers in biological samples [7–15]. Here, we describe and extensively validate two sandwich ELISAs with very high specificity for oligomeric A β and sensitivity sufficient to allow detection of low concentrations of natural A β oligomers in human brain samples. We demonstrate their utility for quantifying such species in extracts of AD and non-AD cerebral cortex and APP transgenic mouse brains. Unexpectedly, we detected no specific A β oligomer signals in 90 human CSF samples from AD and age-matched control subjects. The implications of the latter finding and the potential advantages of these ELISAs over earlier oligomer assays are discussed in detail.

2. Methods

2.1. Antibodies and ELISAs

Sandwich ELISAs were performed using the MSD MULTI-ARRAY® 96-Well Plate platform (Meso-Scale Discovery, Gaithersburg, MD) following the manufacturer's protocol. For the standard A β 1-x ELISA, samples were loaded in duplicate onto plates pre-coated with 30 μ l of mAb 266 to the A β mid-region (residues 13–28) or mAb 3D6 (to residues 1–5), then detected respectively with biotinylated 3D6 or biotinylated mAb 4G8 (to residues 18–22; Covance) and SULFO-TAG Streptavidin (MSD). For the A β o-ELISAs, we used 3D6 or mAb NAB61 [14] as capture antibodies and biotinylated 3D6 for detection. Light emitted from the SulfoTAG at the electrode surface was quantified with SECTOR® Imager 2400A. 266 and 3D6 were kindly provided by Elan, plc (South San Francisco, CA), and NAB61 was a gift of Dr. V.M. Lee (U. Penn.). Standard curves were generated with wt synthetic A β 1-40 for the A β 1-x ELISA, and with synthetic A β 1-40-S26C crosslinked dimers ((A β S26C)₂) [16] purified by size-exclusion chromatography (SEC) for the o-ELISAs.

2.2 Preparation of A β monomers, S26C dimers and aggregates thereof

Oxidized (disulfide crosslinked) A β S26C dimers were separated by SEC from higher aggregates and residual S26C monomers immediately before use [17]. Wt A β 1-40 monomers were also isolated by SEC and used directly. For certain experiments, each of the peptides was aggregated at 10 μ M concentration by shaking in 25 mM ammonium acetate, pH 8.5, at 37°C (Labnet Vortemp 56, 700 rpm) for 18 hr.

2.3. Size exclusion chromatography

One ml aliquots of TBS-soluble extracts of human (AD or control) cerebral cortex were injected onto a Superdex 75 10/30 high-resolution SEC column (GE Healthcare) and eluted with 50 mM ammonium acetate, pH 8.5, at 0.6 ml/min into 1 ml fractions. We lyophilized 500 μ l of each fraction, reconstituted it in 15 μ l of 2X LDS sample buffer (i.e., 4% Lithium dodecyl sulfate; Sigma) and heated it at 65°C for 5 min. Samples were subjected to Western blotting as described[18] using a mixture of 1 μ g/ml of mAbs 2G3 (to A β 40) and 21F12 (to A β 42) (gifts of Elan, plc) and 0.5 μ g/ml of mAb 6E10 (to A β 3-8; Covance) and imaged on a LiCor Odyssey Infrared System. The remaining 500 μ l of each SEC fraction was used for the ELISAs.

2.4. Human and mouse brain samples

Homogenates of human brains (n=28; demographics and diagnoses in Supp. Table S3) were prepared as described[16, 18]. Frozen cortices were provided by Dr. M. Frosch (MGH/Harvard NeuroDiscovery Center) under IRB-approved protocols and by Dr. M. Farrell (Beaumont Hospital, Dublin, Ireland) under Ethical Review Committee/IRB approval. Frozen temporal or frontal cortices were Dounce-homogenized (setting 10, 25 strokes) in ice-cold TBS (20 mM Tris HCl, 150 mM NaCl, pH 7.4) at 4:1 TBS volume/brain wet weight. Homogenates were spun at 175,000g in a TLA100.2 rotor on a Beckman TL100 ultracentrifuge and supernatants (called TBS extracts) aliquoted and stored at -80°C. TBS extracts of wt or J20 hAPP tg mice[19] were prepared identically.

2.5. CSF sample preparation

For lumbar puncture (LP), an IRB-approved published protocol was used[20]. Tubes were pre-cooled, and LP used an atraumatic technique with needle bevel in parallel to dura fibers. After collecting CSF into one 14 ml polypropylene tube and mixing gently by inverting 3–4 times, 500 μ l were aliquoted for cell count and 500 μ l for glucose and protein levels. CSF was spun at 400g for 10 min; aliquots of the crystal-clear sup were stored at -80°C.

2.6. Immunoprecipitation (IP) and western blotting (WB)

Our IP/WB protocol was described previously [21]. To detect A β in TBS extracts of human brain and in CSF, we IPed them with one of our polyclonal A β antisera AW8, AW7 or R1282 (1:75) and Protein A Sepharose (PAS; Sigma) or else mAbs 3D6, NAB61 or, as a negative control, 3F3 to the APP C-terminus (each at 3 μ g/ml) and Protein G agarose (PGA; Roche) plus PAS. After washing, precipitates were eluted with 10 μ l 4% LDS sample buffer, heated at 65°C for 5 min and spun at 8,500g for 5 min. Sups were electrophoresed on 26-well 4–12% bis-Tris gels using MES running buffer (Invitrogen)[18]. Proteins were transferred to 0.2 mm nitrocellulose, and filters were boiled and reacted with a mixture of 1 mg/ml each of 2G3 and 21F12 and 0.5 mg/ml of 6E10 and visualized by LiCor. Some gels were visualized by silver staining[22].

2.7. A β immunodepletion of human brain samples and spike-in assays on human fluids

We performed IP of A β species in brain TBS extracts or CSF with the indicated antibodies and transferred the immunodepleted sups to new tubes for further experiments. We also prepared serial dilutions of our A β peptide standards spiked into human CSF or plasma to search for interference of these fluids with A β detection by the ELISAs. Simultaneous serial dilutions of the A β standards in regular dilution buffer (1% Blocker A for the MSD® ELISA) served as the control.

2.8. Statistical Analyses

ELISA data on human brain samples were analyzed by nonparametric tests without assuming Gaussian distributions (Mann-Whitney test), followed by two-tailed P values to determine significance.

3. Results

3.1. Design of the 3D6/3D6B and NAB61/3D6B ELISAs and validation of specificity and sensitivity for A β oligomers

We first tested several combinations of certain anti-A β antibodies to develop sandwich ELISAs specific for A β oligomers. For some of the combinations tested, we utilized the same antibody in a sandwich for both antigen capture and detection, a format that should quantify A β assemblies containing at least two exposed copies of the same epitope [7, 10]. For all ELISAs, we employed a highly sensitive electrochemiluminescence detection system (MesoScale Discovery), which uses “SULFO-TAG” [(Ru(sulfo-2,2'-bipyridine)₃)-Streptavidin] for detection of the primary biotinylated detection antibody by emitting light upon electrochemical stimulation initiated at the electrode surfaces of 96-well plates.

To establish the specificity of the ELISAs for oligomeric forms of soluble A β (oA β), we used a synthetic human A β 1-40 peptide in which Ser26 was changed to cysteine (A β 40-S26C) [18]. A β 40-S26C forms disulfide cross-linked dimers when oxidized. Using SEC on a Superdex 75 column, we sized the A β 40-S26C assemblies and separated A β 40-S26C dimers from monomers under non-reducing conditions. SDS-PAGE of SEC fractions showed that Fraction 9 contained almost exclusively dimers (hereafter called F9, or (A β S26C)₂), and Fraction 11 contained almost exclusively monomers (called F11, or A β S26C monomers) (Supp. Fig. S1). The A β concentration in F9 was estimated to be 90 μ M, as judged from quantitative WBs of increasing amounts of synthetic wt A β 40. F9 was then serially diluted to serve as a standard to develop the oligomer-specific ELISAs. We documented that (A β S26C)₂ did not aggregate to produce Thioflavin T positive material at high concentrations (12 μ M) and at the temperatures and conditions used in our ELISAs; 12 μ M is higher than the maximal concentration of (A β S26C)₂ we routinely used for our o-ELISA standard.

All of the five mAb combinations we initially tested displayed large dynamic ranges, with detection limits as low as ~39 pg/ml and as high as 2,133,000 pg/ml of the F9 synthetic dimers (Supp. Table S1). Among the different antibodies and combinations tested, the two most sensitive combinations were NAB61/3D6B (with a lower detection limit of ~39 pg/ml of the dimeric peptide) and 3D6/3D6B (with a lower detection limit of ~197 pg/ml) (Supp. Table S1). The 3D6/3D6B ELISA employs 3D6 for both capture and biotinylated detector, and the NAB61/3D6B ELISA uses NAB61 for capture and 3D6 as biotinylated detector. 3D6 recognizes a linear epitope of residues 1–5 (DAEFR) having an intact N-terminal aspartate. Importantly, it does not recognize holoAPP and its secreted APPs- α or - β ectodomains [23]. The NAB61 mAb is reported to recognize preferentially a conformational epitope within the N-terminal region of A β oligomers; it is not specific for the free N-terminal aspartate [14]. We also tested and rejected (due to less sensitivity) sandwiches of monoclonal antibodies 82E1 (to the free Asp-1 at the A β N-terminus; Immuno-Biological Labs, Inc.) and 6E10 (to an A β epitope within residues 3–8 (EFRHDS); Covance) (Supp. Table S1).

Based on their sensitivity for the synthetic (A β S26C)₂ oligomers, the 3D6/3D6B and the NAB61/3D6B ELISAs were selected for further validation; henceforth, we will refer only to these two as the oligomer-ELISAs (o-ELISAs). We found that these o-ELISAs using the MSD ELISA platform showed excellent reproducibility across many runs, with consistently

low standard errors of the mean (SEMs) in each run (SEMs are included in all graphs herein, but most are too small to be visible at the wide signal ranges obtained).

Two lines of evidence suggested that the signals in the two o-ELISAs came from oligomeric, not monomeric, A β . *First*, the NAB61/3D6B and the 3D6/3D6B o-ELISAs gave markedly lower signals for the F11 (A β S26C monomer) than the F9 ((A β S26C)₂) SEC fractions (Figs. 1A, B: black and blue traces, respectively; all graphs are plotted on log-log scales because of the very large dynamic range of the assays). Any oxidation of the abundant A β 40-S26C monomers in F11 will yield small amounts of dimers, especially detectable at high concentrations, and we could indeed detect these by WB, even in the presence of a reducing agent (β ME) (Fig. 1E: compare F11 50 ng vs. 10 ng). Accordingly, very high concentrations (i.e., 10 ng/ml or higher) of F11 (S26C monomers) gave detectable, dose-dependent signals in the two o-ELISAs (Fig. 1A, B: blue traces); however, these signals were just 1–3% of the o-ELISA signals that F9 (A β S26C)₂ gave at the same concentrations (Supp. Table S2). *Second*, dissociation of the disulfide bond of (A β S26C)₂ into monomers almost abolished its signal in the two o-ELISAs. Here, we first verified that almost all of the peptide from F9 (A β S26C)₂ ran as SDS-stable dimers on WB, and that treatment with 3% β ME quantitatively reduced the dimers into monomers, although a small amount of residual dimer remained (Fig. 1E: WB with 3D6; Fig. 1F: silver-stained gel). We then serially diluted F9, either with or without β ME treatment, and assayed it by the two o-ELISAs (Figs. 1A, B: black and red traces). β ME treatment markedly reduced the signals of F9 in both o-ELISAs by more than 97% at most concentrations tested (Supp. Table S2). To exclude the possibility that β ME itself interfered with the ELISA method, we applied the same β ME treatment to a F11 (S26C monomer) fraction and to wt synthetic A β 1-40 monomers. β ME (which would be present at 0.1% or less final concentration in our ELISAs) had no effect on the measurement of these monomers as detected by two different A β 1-x ELISAs: we observed no significant difference between the signal curves of F11 (monomers) with and without β ME, and these two curves completely overlapped those of the wt A β 1-40 peptide with and without β ME (Figs. 1C, D; and Supp. Figs. S2A, B).

To confirm that the small signals detected by our o-ELISAs at high concentrations (0.01 μ g/ml – 2.13 μ g/ml) of the F11 (A β 40-S26C monomer) fraction came from the small amount of oxidized dimers remaining in that fraction and not from the monomers themselves, we tested a SEC fraction of wt synthetic A β 1-40 monomers in the two o-ELISAs. Neither the 3D6/3D6B nor the NAB61/3D6B o-ELISAs gave a signal above background for the wt A β 1-40 monomers, even at a very high concentration of 2.13 μ g/ml. (Supp. Figs. S2C, D). These results strongly suggest that the two o-ELISAs are specific for A β oligomers (and see also below). The same wt synthetic A β 1-40 peptide gave a large dynamic range of signal when assayed by the 266/3D6B (1-x) ELISA or by another conventional ELISA that detects monomers (3D6/4G8B), and β ME had no effect whatsoever on the wt A β 1-40 monomer signals read by these two conventional ELISAs (Supp. Figs. S2A, B). Finally, we tested the o-ELISAs with the SEC dimer fraction (F9) of a tyrosine-10 synthetic A β dimer, which is covalently crosslinked and can never dissociate. This dityrosine A β dimer only aggregates into species higher than dimers at high μ M concentrations. Our two o-ELISAs showed comparable standard curves using the di-tyrosine dimer (data not shown). Taken together, all of the above results strongly indicate that the 3D6/3D6B and the NAB61/3D6B ELISAs specifically measure the levels of *bona fide*, SEC-purified A β dimers in a linear fashion but are insensitive to monomers.

Next, we asked whether the two o-ELISAs can recognize oligomeric A β assemblies larger than dimers. To this end, SEC fractions F9 and F11 of A β 40-S26C and the wt A β 1-40 monomer SEC fraction were each incubated by shaking at 10 μ M concentrations at 37°C overnight. SEC and WB of these peptides post-incubation showed that most of the A β had

now shifted to higher MW aggregates, i.e. they now eluted principally in the void volume (F4 and F5) of the Superdex 75 column (Fig. 2A–C). When we tested the incubated A β peptides in the NAB61/3D6B and 3D6/3D6B o-ELISAs, we found that all three A β preparations now gave markedly increased signals (>2 log units higher than the original signals before incubation) (Figs. 2D, E). We conclude that the two o-ELISAs recognize multiple assembly sizes of still water-soluble A β oligomers, ranging from dimers to much higher MW pre-fibrillar (non-pelletable) oligomers.

3.2. The o-ELISAs do not detect APPs- α or APPs- β

As APPs is a prominent secretory product in biological fluids, we asked whether our o-ELISAs show any cross-reactivity with APPs- α and - β . 3D6 antibody was previously shown not to recognize holoAPP or APPs but to sensitively recognize A β species with a free N-terminal aspartic acid[23]. Likewise, NAB61 antibody has been reported not to recognize either holoAPP or its C99 fragment[14]. We confirmed that neither o-ELISA detected recombinant APPs- α and APPs- β , even at highly supraphysiological concentrations of ~2.13 μ g/ml (Supp. Figs. S3A, B). As a positive control, (A β S26C)₂ (F9) was assayed simultaneously and again gave an excellent dynamic range in each o-ELISA (Supp. Figs. S3A, B).

3.3. The o-ELISAs detect multiple oligomeric forms of A β , including dimers, in soluble extracts of human brain

To assess whether the o-ELISAs can accurately quantify natural A β species from human tissue samples, we first prepared TBS-soluble (post-175,000g) extracts of confirmed AD brains (called AD-TBS; see Methods), subjected them to SEC on the Superdex 75 SEC column, and assayed the resultant fractions for A β using WB and three ELISAs: the conventional A β 1-x ELISA (266/3D6B) and the NAB61/3D6B and 3D6/3D6B o-ELISAs. When we analyzed the SEC fractions by WB, we observed that >90% of the A β -immunoreactive signal eluted in the void volume fractions (i.e., F4–5) that size at >70 kDa (designated HMW A β hereafter) (Figs. 3A–C), which then ran as A β monomers and dimers on denaturing SDS-PAGE gels of the SEC fractions (Fig. 3D), just as reported previously using these methods[18]. (NB: The MW sizing of our Superdex 75 A β -rich fractions had been established previously using linear polydextran standards, not inappropriate globular protein standards[24]). There was very little A β immunoreactivity in the SEC fractions whose elution corresponds to the sizes of dimers (~8 kDa) and monomers (~4 kDa) (Fig. 3A–D). With the goal of attempting to disassemble these HMW A β species, we subjected the major void volume fraction (F4) to incubation at 37°C for 2 days in 50 mM ammonium acetate (pH 8.5). A WB showed that this 2-day incubation of the HMW A β had induced its dissociation into dimers and monomers; this was proven by a second SEC run that showed a dramatic decrease in the A β WB signals in the void volume fractions and a parallel increase in the dimer and monomer fractions (data not shown). ELISA analyses of the first-run SEC fractions by our o-ELISAs showed that the void volume fraction (F4) from the first run (i.e. before the 2-day incubation in buffer) produced robust signals in both o-ELISAs. However, after the 2-day incubation, the A β signals detectable by the two o-ELISAs in this void volume fraction from the first SEC run were dramatically decreased, while the monomeric A β signals detectable by the conventional 1-x monomer ELISA were correspondingly increased (Table 1). These experiments in which buffer-soluble A β species from AD cortex were separated and measured by ELISA confirm that a) the o-ELISAs are specific for oligomeric, not monomeric, A β species in human brain extracts, and b) that the o-ELISAs and a conventional A β ELISA can be used together to detect their inter-conversion.

We next assayed serial dilutions of AD TBS cortical extracts with the two o-ELISAs and the A β 1-x ELISA. All three ELISAs showed highly linear dose-response curves (Figs. 4A, B).

To confirm that the ELISA signals derived specifically from the A β species in the human brain extracts, we performed immunodepletion assays (Fig. 4C). An AD-TBS extract was first subjected to two sequential pre-clearing steps using protein A plus protein G agarose beads. Then, the supernatants of this pre-cleared AD-TBS were subjected to immunodepletions by the A β antibodies 3D6 or NAB61 or, as a negative control, by 3F3 (to the APP C-terminus). After removal of the antibody-bead complex by centrifugation, the resultant sups were assayed with the two o-ELISAs. We observed no significant decrease in the o-ELISA signals between the start material and the sups from the first and second pre-clearing steps (Fig. 4C). However, there were marked decreases in signal after immunodepletion with either 3D6 or NAB61; in contrast, there was a negligible decrease with the control 3F3 antibody. A single immunodepletion of the AD-TBS extract with the 3D6 antibody left just 8–9% of the starting A β signal as measured by either o-ELISA, whereas a single immunodepletion with the NAB61 antibody left ~28% of the starting signal (Fig. 4C). We checked these ELISA results by performing IP-WB on the same sups and their corresponding pellets. The IP-WB analysis agreed with the ELISA results: A β dimers and monomers were markedly decreased after pulling down with 3D6 or NAB61 and were accordingly recovered in the pellets, whereas no significant changes were seen after pre-clearing with protein A+G agarose beads or after precipitation with the control antibody 3F3 (Fig. 4C, bottom). As expected, immunodepletion of AD-TBS extracts with anti-human-Tau or anti-a synuclein antibodies did not decrease the o-ELISA signals, even though these IPs successfully precipitated their respective proteins (data not shown). Therefore, our o-ELISAs cannot detect oligomers of tau or α -synuclein in AD-TBS homogenates. We systematically performed A β immunodepletion assays on TBS extracts of 27 of 28 AD brain samples we acquired, i.e., all except the brain of a carrier of the APP D694N (“Iowa”) mutation, which was in low supply (Supp Table S3 lists clinical information on the 28 brains). The AD-TBS extracts were IPed with A β antiserum AW8 and the sups measured with the o-ELISAs and the 1-x ELISA; the mean signals were dramatically ($97 \pm 3\%$) decreased in all three ELISAs. Taken together, all of the above results indicate that the two o-ELISAs can detect natural oligomers present at pathophysiologically relevant concentrations in AD brain samples and that the signals are derived specifically from A β .

3.4. The o-ELISAs detect rising signals with age in APP transgenic mouse brains

To assess the utility of our assays in an AD mouse model, we applied the o-ELISAs to an analysis of thirty hAPP transgenic mice of increasing age (2–28 months) (J20 line: FAD mutations KM670/671NL + V717F)[6, 19]. The o-ELISA signals in the TBS brain extracts rose in an age-dependent fashion in the NAB61/3D6B and 3D6/3D6B assays (Supp. Fig. S4A, B). Wild-type mouse brain TBS extracts gave a very low signal in the NAB61/3D6B assay and no signal at all in the 3D6/3D6B assay (Supp. Fig. S4A, B). This age-related rise in o-ELISA signals in the J20 mice is consistent with our recently published IP/WB and conventional A β ELISA analyses of TBS extracts from the same mouse line[6, 25].

3.5. Quantifying the levels of soluble A β oligomers in brain tissues of AD patients vs. age-matched non-AD humans

Once the specificity of the o-ELISAs for natural and synthetic A β oligomers had been validated by all of the studies described above, we asked whether these assays could be used routinely to quantify such species in many human brain and CSF samples. We used the two o-ELISAs and the conventional A β 1-x ELISA to quantify relative amounts of A β species in TBS-soluble extracts of cortex from 28 subjects, 13 of whom were clinically and neuropathologically diagnosed with AD (mean age 78, range 55–92 years) and 15 of whom were non-AD controls whose ages spanned the human lifespan (mean age 57, range 0.8–93 years) (Supp. Table S3). Notably, one of the 13 patients had AD caused by the APP D694N (“Iowa”) mutation and the other 12 were “sporadic” late-onset cases. We focused on TBS-

soluble extracts, because the presence of dementia in AD has been documented to correlate best with the levels of aqueously soluble A β species[26–29]. The mean A β levels measured by the three ELISAs for the group of 15 controls and the group of 13 AD subjects are provided in Supp. Table S4.

Using the conventional A β 1-x ELISA (266/3D6B), we obtained mean total A β levels of 165 ± 48 pg/g wet tissue in the 9 controls whose ages matched those of our AD patients (i.e., >55 years old) vs. $1,995 \pm 1,217$ pg/g in the 13 AD patients (Fig. 5B; see Supp. Table S3 for individual values). Despite a ~13-fold difference in these average A β levels, statistical significance was $p=0.159$ due to the large variability in the AD group and the relatively small sample sizes. We also assayed A β 1-x as a function of age across all 28 subjects but observed no significant age-dependent trend in this mixed population (not shown).

We next assayed the 28 TBS-soluble cortical extracts by the two o-ELISAs. In the 13 AD brains, the NAB61/3D6B assay had a mean $\pm$ SEM of $4,555,869 \pm 1,617,730$ pg/g wet tissue, and the corresponding 3D6/3D6B assay values were $2,251,638 \pm 863,175$ pg/g wet tissue. In the 9 age-matched control cases, the respective values were $89,195 \pm 37,691$ pg/g and $57,425 \pm 24,017$ pg/g (Fig. 5C, D; see Supp. Table S3 for individual values). On average, the levels of o-A β in the 13 AD cases were ~50 times higher than those of the 9 age-matched controls using the NAB61/3D6B assay ($p=0.0173$), and ~39 times higher using the 3D6/3D6B assay ($p=0.0259$).

We correlated the o-ELISA results with those obtained by IP-WB. TBS extracts were IPed with antiserum AW8 to A β and blotted with combined mAbs 6E10 (to A β 3-8), 2G3 (A β 40 end-specific) and 21F12 (A β 42 end-specific). This IP-WB analysis revealed A β monomers and SDS-stable dimers in the TBS-soluble cortical extracts from all AD subjects (Fig. 5A). Most TBS extracts from controls showed very little or no monomers and negligible amounts of dimers at the level of detection of our IP/WB assay. In general, the individual IP/WB results correlated well with those of the ELISAs (compare Fig. 5A with Supp. Table S3).

3.6. Assaying cerebrospinal fluid for the presence of A β oligomers

CSF samples from 89 humans [plus a commercially obtained CSF pool from numerous humans with unknown diagnoses (CSF#12)] were measured using the two o-ELISAs and the conventional A β 1-x, 1-40 and 1-42 ELISAs (Supp. Table S5). The 266/3D6B, 2G3/3D6B and 21F12/3D6B ELISAs readily detected A β 1-x, 1-40 and 1-42 peptide levels, respectively, in all CSF samples. In sharp contrast, both the 3D6/3D6B and the NAB61/3D6B o-ELISAs did not detect any specific A β signal in all of the 90 CSF samples, even when certain CSF samples (#6 and #12) were concentrated 5-fold, except in one subject (CSF#27). However, the apparent signal from CSF#27 could not be diluted when diluting the sample two-fold, indicating we were not detecting true oA β species (Supp. Table S5). This result nicely confirms the oligomer-specific nature of our o-ELISAs, since all CSF samples contained abundant monomers.

The lack o-A β signals by both o-ELISAs in all 90 CSF samples suggested several possibilities: 1) that A β oligomers are absent in these samples; 2) that the levels of oligomers are below the limit of detection of the o-ELISAs; or 3) that other proteins in CSF interfere with the o-ELISAs, thereby masking any oligomers from detection. To clarify this issue, we IPed 2 ml of CSF from each of 6 of our subjects with A β antiserum 1282 (Fig. 6A) and from each of 18 with A β antiserum AW7 (not shown) and blotted the precipitates with A β mAbs 6E10+2G3+21F12. This IP-WB method revealed that all 24 samples contained robust A β monomers but had no detectable SDS-stable dimers. Both the lack of A β signal by the o-ELISAs and the lack of A β dimers upon IP-WB suggest that there are very low (< 39 pg/ml) or no A β oligomers in human CSF. To make low sensitivity a less likely

explanation, we increased the CSF volume from 2 ml to 5 ml and performed a first IP with 3D6. Even then, we failed to see any dimers by IP/WB (Fig. 6B). Note that in contrast to the absence of dimers, the A β monomers present in the 5 ml CSF samples gave a robust signal even in a second IP with an A β antiserum (Fig. 6B). We likewise failed to detect dimers or monomers in 5 ml of CSF IPed with NAB61, and a strong A β monomer band was brought down by a second IP with the A β antiserum (not shown). This result confirms the oligomer specificity of NAB61. To rule out the possibility that the failure to detect A β oligomers by the o-ELISAs was due to other molecules in CSF interfering with the ELISA method, we spiked the (A β S26C)₂ SEC fraction (F9) into human CSF, performed serial dilutions and assayed them by the two o-ELISAs (Figs. 6C, D). The ELISA curves generated from these spiked-in CSF samples overlapped perfectly with the curves from the same F9 fraction in standard specimen diluent in both o-ELISAs. An amount of (A β S26C)₂ as low as ~39 pg per ml of human CSF could be detected by the assays. Finally, aliquots of AD-TBS extracts containing natural human A β dimers and monomers were spiked into 1 ml of either artificial CSF or human CSF (#12). IP/WB analysis on these spiked-in samples revealed indistinguishable dimer and monomer bands when spiked into human CSF or into artificial CSF (Supp. Fig. S5). Taken together, these results argue strongly against the possibility of other proteins in human CSF interfering with A β oligomer detection in our o-ELISAs.

3.7. Assaying plasmas for the presence of A β oligomers

We searched for A β oligomers in human plasma. Samples from 6 patients with clinical AD and 5 normal subjects, plus three pooled plasma samples from numerous humans with unknown diagnoses (Supp. Table S6), were assayed by the o-ELISAs and the conventional A β 1-x ELISA. The A β 1-x ELISA suggested that all 11 plasma samples contained low amounts of A β 1-x peptides, with no significant differences between AD and controls (not shown). The 3D6/3D6B o-ELISA gave plasma readings that were on average much lower than those of the 1-x ELISA, and the NAB61/3D6B o-ELISA gave mostly zero signals, with the exception of two samples (one from subject #5 (AD) and one from subject #13 (non-AD control)). Importantly, diluting each of the two positive plasmas just two-fold caused a very marked (many-fold) decrease in the oELISA readings, indicating the assays were not detecting true oA β species (data not shown). To determine whether interfering molecules exist in plasma that preclude reliable detection of oligomers by the o-ELISAs, we spiked the (A β S26C)₂ SEC fraction (F9) into two human plasmas -- one from an AD patient and one from a control -- and performed serial dilutions (Supp. Figs. S6A, B). Compared to the standard curves generated from the same F9 fraction diluted into plain specimen diluent, the ELISA curves generated from the spiked-in plasmas showed a significant reduction in signal (note the log scale). The NAB61/3D6B o-ELISA showed up to a 93% reduction in the signal of F9 in AD plasma and a 78% reduction in control plasma, with great variability within any one concentration (Supp. Fig. S6A), and the 3D6/3D6B o-ELISA failed to display a dose-dependent linear curve (Supp. Fig. S6B). Repeating this experiment with 3 additional human plasmas again indicated that molecules in human plasma interfere with the o-ELISAs. To confirm further this interference in plasma, we spiked aliquots of AD-TBS extracts containing natural human A β dimers and monomers into human plasma. IP/WB analysis on these spiked-in samples revealed decreased detection of the dimer and monomer bands compared to spiking into TBS buffer. We conclude that any A β oligomers that might exist in human plasma cannot be detected by these o-ELISAs due to interfering substances.

4. Discussion

Oligomeric assemblies of A β induce altered neuronal structure and function and appear to play an important role early in the development of AD [30]. SDS-stable A β oligomers, including dimers, trimers, dodecamers and other relatively low MW assemblies, have been observed by western blotting in AD brains[18, 26, 27, 29, 31–33]. SDS-stable A β oligomers

can be detected in the brains of APP transgenic mouse models at or before the age at which deficits in learning and memory begin to emerge [25, 34, 35]. These and other studies encourage the inclusion of A β oligomers in the search for useful biomarkers of AD. Most A β ELISAs reported to date were designed to detect monomers, and their sensitivity for detecting natural oligomers has not been established. Methods that allow one to observe the quality (type) of A β species in a sample, such as Western blotting, usually require denaturation to see the peptides, and this could disassemble both large and small oligomers to monomers, since most A β oligomers in brain are not known to be covalently bonded. Further, Western blotting is not amenable to high-throughput measurement of many samples. Therefore, we sought to develop an assay that selectively measures natural A β oligomers of various sizes. Here, we report two o-ELISAs in which we fully validated specificity for oligomeric A β and then showed their utility when applied to human and mouse brain tissues.

Two general approaches have been used to develop ELISAs that could be specific for A β oligomers. One uses putatively aggregate-specific antibodies, i.e., antibodies directed against non-linear (non-contiguous) epitopes of A β that can selectively recognize assemblies of two or more monomers. The other uses a single monoclonal antibody for both antigen capture and detection. In the first category, a literature screen revealed several reports of antibodies believed to be specific for aggregated A β that were used in ELISAs [8, 12–15, 36]. However, only three such ELISAs [8, 15, 36] were tested on biological samples. Englund et al [8] used a mAb158 sandwich ELISA to analyze the media of transfected cells and transgenic mouse brains, and Lord et al [36] then reported that this ELISA detected A β protofibrils in brains of tg mice expressing the “Arctic” and “Swedish” hAPP mutations. Another study focused on highly aggregated forms of A β present in SDS-solubilized extracts of AD brain [15]. We are unaware that these or other ELISAs relying on aggregate-specific antibodies have been reported to measure aqueously soluble forms of A β (e.g., in simple buffer extracts of brain homogenates), which are thought to contain the most bioactive forms of A β , or in human fluids.

Regarding the second approach of using a single antibody for both capture and detection, we found only 4 published studies to date [8, 9, 11, 36]. Fukumoto et al. [9] used BAN50 (to A β residues 1–16) in a sandwich ELISA, but they reported that their assay could only detect very small amounts of high-molecular weight (HMW) oligomers of synthetic A β sized at ~40–200 kDa by SEC, the identities of which were not independently confirmed (e.g., by light scattering or native PAGE). Low MW oligomers (dimers through hexamers) were much more abundant in the synthetic oligomer mixture used as their ELISA standard, but these by themselves gave no signal in the assay. This ELISA was used to assay human CSF, and the levels of the putative HMW A β oligomers in each subject were expressed in arbitrary units based on monomer-equivalents in the oligomer standard rather than in actual units of defined A β oligomers, making comparisons of oligomer content among CSF samples problematic. This BAN50-BAN50 ELISA was not validated on AD and control brain extracts, nor was the A β specificity of the oligomer signal in CSF confirmed by immunodepletion studies.

As mentioned above, Englund et al [8] developed an ELISA using the same conformational antibody for capture and detection, i.e., mAb158 generated against synthetic protofibrils composed of the “Arctic” (E22G) A β peptide. The authors stated that the synthetic protofibrils serially diluted to establish a standard for the ELISA were of uncertain size and molarity, so they expressed their protofibril concentrations based on the MW of an A β monomer. This 158-158 ELISA detected synthetic protofibrils (estimated to be >100 kDa) much better than it did low MW A β mixtures (estimated to be 4–20 kDa). Englund et al used this ELISA to analyze the media of transfected cells and transgenic mouse brains

expressing the Swedish APP mutation or the Swedish plus Arctic APP mutations together. The ELISA detected the presence of A β protofibrils in both the cell media and mouse brains, and higher protofibril levels were seen in the Arctic-Swedish than Swedish samples. The authors showed that mAb158 does not bind to APP or to other types of amyloids. They did not report immunodepletions to confirm the specificity of the ELISA signals nor assays on human brain samples. A preliminary study from our group[11] described sandwich ELISAs using monoclonal antibodies 82E1 or 3D6. These assays detected signals in human brain homogenates, with higher levels seen in AD than control brains. Human plasmas were also assayed, but many were near or at the lower limit of detection. At that time, we derived only *relative* oligomer values, as our standard curve employed a synthetic A β 1-40 peptide that had been quantified by conventional monomer-directed ELISAs. Again, immunodepletion was not used to confirm the specificity of the signals.

To address these drawbacks of published o-ELISAs, we undertook the current experiments to obtain ELISAs whose specificity and sensitivity on both synthetic and natural oligomers were validated in several ways. The o-ELISAs we report here can selectively quantify synthetic and natural oligomers of human A β over a wide analytical range. The following observations validate their oligomer specificity. *First*, the o-ELISAs detected increasing concentrations of pure synthetic (A β S26C)₂ in a linear and highly reproducible fashion. Treating these cysteine-bonded dimers with β ME quantitatively dissociated them to monomers and resulted in a marked (>97%) loss of the o-ELISA signal. The very small residual signal was shown to be due to trace remaining amounts of disulfide-bonded dimers. *Second*, the o-ELISAs give no signal with wt A β 40 monomers and gave 1–3% of the signal of (A β S26C)₂ with the S26C monomer fraction. *Third*, overnight incubation of (A β S26C)₂ or wt A β 40 peptides at 37°C, which polymerized them into higher aggregates (confirmed by SEC), markedly increased the o-ELISA signals, indicating that the assays detect large but still buffer-soluble aggregates far greater than dimers. *Fourth*, we confirmed that the o-ELISA signals emanate from A β and not other soluble cleavage products of APP that contain part or all of the A β sequence (APPsa, APPs β). *Fifth*, the o-ELISAs specifically detected natural A β oligomers in soluble human brain extracts. One round of immunodepletion of A β from the TBS extracts (confirmed by WB) reduced the o-ELISA signals to ~8% (3D6) or ~28% (NAB61) of their original values. *Sixth*, hAPP transgenic mouse brain extracts showed increasing signals over an age range of 2–28 months as brain oligomer levels rise, whereas brain extracts of wt littermates had no detectable signal. We conclude that the two o-ELISAs can specifically quantify a wide size range of oligomeric assemblies of soluble A β , even in complex biological samples. The absolute sensitivities of the assays for oA β were as low as ~6 pg/ml for 3D6/3D6B and ~12 pg/ml for NAB61/3D6B after optimization, based on quantifying the synthetic A β dimer standard by amino acid analysis, a preferred method to determine rigorously an absolute protein concentration, allowing us to establish accurate oligomer sensitivities of our ELISAs. It should be noted that our o-ELISAs, in which 3D6B detects A β species having a free N-terminal aspartic acid, cannot quantify N-terminally modified A β species such as pE3-42, which potentially have a pathogenic role in AD.

Having validated the specificity and sensitivity of the o-ELISAs, we applied them to samples of human brain tissue and biological fluids. Previously, dot blot assays suggested that the levels of soluble A β oligomers were on average up to ~70-fold higher in extracts of AD brains than age-matched controls[31]. We measured A β oligomer levels in TBS-soluble extracts of 13 AD and 15 non-AD control subjects (9 of the 15 were age-matched). Soluble oligomer levels were ~50-fold higher in our AD than control brains by NAB61/3D6B ($p=0.0173$) and ~39-fold higher by 3D6/3D6B ($p=0.0295$) (Fig. 5). One previous study [15] used a sandwich ELISA (either of two conformational antibodies were combined with a polyclonal A β 1-42 antibody) to assay A β oligomers in SDS-solubilized brain homogenates

and found that oligomer levels were significantly higher in AD than control cases. Semi-quantitative Western blotting has suggested that the levels of relatively low MW oligomers in buffer-soluble extracts of human brain correlate with the presence of AD dementia [18, 29, 37, 38]. Oligomer-selective ELISAs such as those we report could be useful for quantitative neuropathological analyses relevant to both diagnosis and mechanistic studies of AD.

We applied the two o-ELISAs to search for A β oligomers in human CSF but failed to detect any specific signals in >90 subjects. We verified in some of the samples that there were no detectable SDS-stable A β assemblies by IP-WB. Importantly, we excluded via spiking experiments that these negative results were due to molecules in CSF that interfere with the assays. Our finding of no measurable levels (i.e., lower than 6 pg/ml) of oligomeric A β in human CSF is in contrast to a few prior reports of higher A β oligomer levels in the CSF of AD than control subjects [9, 21, 39–43]. Vigo-Pelfrey et al. [39] identified three A β oligomer forms, stable trimers of (Asp¹-Met³⁵)₃ and (His⁶-Ala⁴²)₃ and a stable dimer of (Asp¹-Val⁴⁰)₂, by purifying A β by HPLC from 3 liters of pooled human CSF and performing laser atomic desorption mass spectrometry. Pitschke et al. [41] reported apparent A β aggregates in CSF by fluorescence correlation spectroscopy (FCS) that used labeled synthetic A β peptides as “seeds” for polymerization; they stated that aggregate formation could be detected at concentrations down to 100 nM. Georganopoulou et al. [42] used a nanoparticle-based biobarcode assay to detect oligomers, using as a calibration curve assemblies of synthetic A β 1-42 incubated at 7°C for 24 h (“ADDLs”). They observed very low amounts of ADDL signal in CSF (<4.3 pg/ml). The biobarcode assay they reported requires specialized equipment not available in most labs, and it used a polyclonal antiserum not available in quantities needed for large-scale, multicenter validation studies. Also, this assay used polyclonal and monoclonal antibodies raised against synthetic ADDLs that were not proven to be entirely specific to oligomers of A β . Santos et al. [43] reported an assay for detection of oligomeric and fibrillar structures based on multiparametric analysis of data obtained by flow cytometry and fluorescence resonance energy transfer (FRET). Its sensitivity was determined by titration of *in vitro*-assembled synthetic A β fibrils, and the units were FRET events. The authors reported detection of A β oligomers in CSFs from 174 non-demented individuals with various neurological disorders. The data showed a large variation in A β oligomers and a weak correlation between age and A β oligomer levels. Walsh et al. [21] found that 15 of 56 human CSF samples showed small amounts of SDS-stable dimers (~8 kDa) by IP-WB. Klyubin et al. [44] reported that 6 CSF samples containing SDS-stable A β dimers by IP/WB inhibited hippocampal LTP in living rats and that acute systemic infusion of a mAb to A β prevented this.

In summary, we have established two novel o-ELISAs that selectively quantify soluble human A β oligomers over a wide range of sizes. The mean levels of oligomeric A β were markedly (>1,000-fold) higher than those of monomers in buffer-soluble extracts of AD cortex, signifying that the vast majority of soluble A β in AD brain is in oligomeric form. Soluble oligomer levels were significantly higher in AD than age-matched non-AD brains. We obtained no evidence of soluble A β oligomers in 90 human CSF samples, despite the fact that the o-ELISAs detected synthetic and natural (AD brain) human oligomers spiked into CSF at very low picogram concentrations. This unexpected finding provides a new biological insight that the relatively hydrophobic A β oligomers may have very brief or no dwell times in the aqueous environment of the CSF and may be rapidly adsorbed onto cellular membranes. As detailed above, we believe these o-ELISAs have significant advantages over previous assays for oligomeric A β and provide a useful and sensitive method to quantify it in multiple biological samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank members of the Selkoe and Walsh labs for helpful advice and comments. Supported by NIH grants AG027443 (DJS and DW) and AG012749 (DJS). DJS is a consultant and director of Elan and a consultant to Satori.

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Figure 1. 3D6/3D6B and NAB61/3D6B ELISAs specifically recognize Aβ dimers but not monomers

Both NAB61/3D6B (A) and 3D6/3D6B (B) show wide dynamic ranges for quantifying SEC-purified Aβ S26C dimer (F9; black squares), but do not detect F9 reduced to monomers by βME (red triangles) or Aβ S26C monomers (SEC F11; blue inverse triangle). Conventional Aβ ELISAs 266/3D6B (C) and 3D6/4G8B (D) detect F11 (blue inverse triangles), βME-treated F11 (purple diamond), and the wt synthetic Aβ40 (green circles) equally well, indicating that βME does not interfere. Data are means ± SEMs (error bars too small to see on this log scale). (E, F) βME effectively dissociates the SDS-stable S26C dimers of F9 into monomers, with small amounts of residual dimers (<10%). At high concentrations, the F11 monomer fraction can be seen to have small amounts of dimers. (E: 3D6 WB; F: silver stain).

Figure 2. The o-ELISAs recognize a large size range of synthetic Aβ oligomers, including dimers (A–C) WB (6E10+2G3+21F12) of the SEC elution profile of F9 (A), F11 (B) and wt synthetic Aβ40 monomer (C) that had each been incubated at 37°C overnight and then subjected to SEC (WB: 6E10+2G3+21F12). (D, E) Samples before and after 37°C incubation were serially diluted and assayed by NAB61/3D6B (D) or 3D6/3D6B (E); data are means ± SEM.

Figure 3. The o-ELISAs detect multiple oligomeric forms of Aβ, including dimers, in soluble extracts of human brain tissue

(A) NAB61/3D6B and (B) 3D6/3D6B o-ELISAs, and (C) 266/3D6B 1-x ELISA. By each method, ~80% of soluble Aβ species elute in the void volume of the Superdex 75 SEC column (i.e., F3–F5, corresponding to >70 kDa). (D) SEC elution profiles of AD-TBS extracts analyzed by WB (6E10+2G3+21F12); synthetic wt Aβ40 (2 ng) is a WB control.

Figure 4. The o-ELISAs specifically detect Aβ species in AD TBS brain extracts (A–B) AD-TBS was serially diluted and assayed with the two o-ELISAs (A) and the conventional Aβ1-x ELISA (B). All three ELISAs showed highly linear concentration curves. (C) After two sequential pre-clearing steps, AD-TBS was subjected to IP by antibodies 3D6 or 3F3 or NAB61. As numbered, the various supernatants were assayed by o-ELISAs (bar graph) and the corresponding immunoprecipitates by WB. (WB: 6E10+2G3+21F12).

Figure 5. Aqueously soluble A β oligomers are significantly increased in AD vs. control brains (A) IP/WB of TBS extracts (IP: AW8; WB: 6E10+2G3+21F12). Each lane represents an individual subject (black: control; red: AD). (B–D) Scatterplots for total A β (1-x) levels (B) and A β oligomer levels by NAB61/3D6B (C) or 3D6/3D6B (D) in human brain TBS extracts. Each dot represents data from 1 subject. Horizontal lines: means $\pm$ SEMs. p values from two-tailed Mann-Whitney t-tests.

Figure 6. Lack of Aβ oligomer signals in CSF of representative control and AD subjects by IP/WB and o-ELISAs

IP/WB analysis on (A) 2 ml CSF (IP: Aβ antiserum R1282; WB: 6E10+2G3+21F12), and (B) 5 ml CSF (1st IP: 3D6 (3ug/ml), 2nd IP: Aβ antiserum R1282; WB: 6E10+2G3+21F12). Clinical information is in Table 3. (C–D) S26C dimer fraction (F9) was spiked into a human CSF, serially diluted, and assayed by NAB61/3D6B (C) and 3D6/3D6B (D) o-ELISAs. Readings generated from F9 diluted in CSF (red squares) show a perfect overlap with those of F9 diluted in specimen diluent (black circles). Data are means ± SEMs.

Table 1

A β amount detected by the ELISAs before vs. after incubation

	F4 before incubation (pg/ml)	F4 after incubation (pg/ml)
266/3D6B	176 $\pm$ 16	2,578 $\pm$ 19
NAB61/3D6B	94,424 $\pm$ 4	58,740 $\pm$ 5
3D6/3D6B	97,926 $\pm$ 6	18,264 $\pm$ 12

F4, void volume of SEC with 200 μ l of AD-TBS.

SEC F4 was incubated at 37° overnight