Specific recognition of biologically active amyloid-β oligomers by a new Surface Plasmon Resonance-based immunoassay and an in vivo assay in Caenorhabditis elegans*

Matteo Stravalaci1, Antonio Bastone1, Marten Beeg1, Alfredo Cagnotto1, Laura Colombo1, Giuseppe Di Fede2, Fabrizio Tagliavini2, Laura Cantù3, Elena Del Favero1, Michele Mazzanti2, Roberto Chiesa1,5, Mario Salmona1, Luisa Diomede1, and Marco Gobbi1

1 Department of Molecular Biochemistry and Pharmacology, Istituto di Ricerche Farmacologiche “Mario Negri”, Via La Masa 19, 20156 Milan, Italy.
2 Fondazione IRCCS - Istituto Neurologico "Carlo Besta", Via Celoria 11, 20133 Milano, Italy.
3 Department of Medical Chemistry, Biochemistry and Biotechnology, University of Milan, Segrate, 20090 Milan, Italy.
4 Department of Biomolecular Sciences and Biotechnology, University of Milan, Via Celoria 26, 20133 Milan, Italy.
5 Dulbecco Telethon Institute and Department of Neuroscience, Istituto di Ricerche Farmacologiche “Mario Negri”, Via La Masa 19, 20156 Milan, Italy.

*Running title: Specific recognition of toxic Aβ oligomers

To whom correspondence should be addressed:
Marco Gobbi, Laboratory of Pharmacodynamics and Pharmacokinetics, Department of Biochemistry and Molecular Pharmacology, Istituto di Ricerche Farmacologiche "Mario Negri", Via La Masa 19, 20156 Milano, Italy.
email: marco.gobbi@marionegri.it ; tel. +39-02.39014.570

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Background: Aβ oligomers are major players in Alzheimer’s disease but the tools for their detection are not satisfactory.

Results: We developed a SPR-based immunoassay and a test in C.elegans, specifically identifying toxic oligomers.

Conclusion: These methods allow to study the effects of mutations or drugs on Aβ oligomerization.

Significance: The SPR-based immunoassay provides new opportunities for the detection of toxic oligomers in biological samples.

SUMMARY

Soluble oligomers of the amyloid-β (Aβ) peptide play a key role in the pathogenesis of Alzheimer’s disease, but their elusive nature makes their detection challenging. Here we describe a novel immunoassay, based on surface plasmon resonance (SPR), that specifically recognizes biologically active Aβ oligomers. As capturing agent, we immobilized on the sensor chip the monoclonal antibody 4G8, which targets a central hydrophobic region of Aβ. This SPR assay allows to specifically recognize oligomeric intermediates which rapidly appear and disappear during the incubation of synthetic Aβ1-42, discriminating them from monomers and higher-order aggregates. The species recognized by SPR generate ionic currents in artificial lipid bilayers and inhibit the physiological pharyngeal contractions in C. elegans, a new method for testing the toxic potential of Aβ oligomers. With these assays we found that the formation of biologically relevant Aβ oligomers is inhibited by epigallocatechin gallate and increased by the A2V mutation, previously reported to induce early-onset dementia. The SPR-based immunoassay provides new opportunities for detection of toxic Aβ oligomers in biological samples and could be adapted to study misfolding proteins in other neurodegenerative disorders.

Aggregation of β-amyloid (Aβ) peptides, eventually leading to brain deposition of
Specific recognition of toxic Aβ oligomers

amyloid plaques, represents a major and well-known hallmark of Alzheimer’s disease (AD). Although fibrillar amyloid may contribute to AD pathogenesis, different lines of evidence point to smaller and soluble Aβ assemblies (termed Aβ oligomers) as the main neurotoxic species (1,2). Aβ oligomers may also represent important biological markers of AD.

The development of reliable tools and sensors to selectively detect the toxic oligomeric species still represents a major need in AD research (3). The recognition that morphologically similar oligomers have very different toxicity points to the existence of subpopulations of biologically active oligomers with specific structural features (4-6). Most of the techniques currently used to visualize Aβ aggregates, or to determine their size, are not suited for the identification of the toxic oligomers. Conformation- or epitope-specific antibodies may provide a more suitable means for a structure-dependent recognition of specific oligomer subpopulations (5). However, classical immunoassays such as ELISA, Western blot, dot-blot, etc., are time-consuming and require indirect readouts, with long incubation steps and washing procedures which could affect the recognition of transient and unstable species. Here we describe a novel immunoassay based on surface plasmon resonance (SPR), which specifically detects biologically important oligomers of synthetic Aβ. SPR is a powerful method widely used to study interactions between two macromolecules in real time without labelling the molecules (7,8). Typically, one of the two interacting partners is immobilized on a sensor chip surface and the other is flowed through a microfluidic system in contact with the chip surface. Binding is measured in real time as a change of mass at the surface, and the interaction can be characterized in terms of on- and off-rates (kinetics) and binding strength (affinity).

We immobilized different antibodies on the sensor chip and injected Aβ solutions at different times during the aggregation. We identified an antibody, 4G8, which permitted a direct, real-time determination of the binding signal specifically due to transient oligomers, which could not be obtained with common immunoassays (ELISA or dot-blot).

We then assessed the toxicity of the Aβ oligomers detected by SPR (3) using two new assays suitable to study transient oligomeric species with a relatively short half-life (few hours). We used an electrophysiological assay in model membranes and a behavioral test in the nematode C. elegans, applied here for the first time to test the toxic potential of Aβ oligomers.

For the present studies we used the “depsi-peptide” technique for Aβ synthesis (9,10), improved in our laboratory (11,12), which consistently produces seed-free starting solutions. This is a prerequisite for any analysis of the properties of highly aggregating peptides (13).

EXPERIMENTAL PROCEDURES

Anti-Aβ antibodies 4G8 and 6E10 were from Covance; anti-oligomers antibodies A11 and OC (5) were from Invitrogen and Millipore, respectively. Epigallocatechin gallate (EGCG) was a kind gift from INDENA, Italy.

Aβ preparation

Depsi-Aβ1–42, depsi-Aβ1–40 (WT) and depsi-Aβ1–46A2V peptides were synthesized as previously described (10,11). The depsi-peptide is much more soluble than the native one and has a much lower propensity to aggregate, so preventing the spontaneous formation of seeds in solution. Aβ peptides were obtained from the corresponding depsi-peptide by a “switching” procedure involving a change in pH (11), and used immediately. The switched solutions were diluted in 10 mM phosphate buffered solution, containing 150 mM NaCl, pH 7.4 (PBS) and incubated as indicated.

SPR studies

The SPR apparatus used for the present study (ProteOn XPR36 Protein Interaction Array System, Biorad) has six parallel flow channels that can be used to uniformly immobilize strips of six ligands on the sensor surface. The fluidic system can automatically rotate 90° so that up to six different analytes can be injected, allowing simultaneous monitoring of up to 36 individual molecular interactions in a single run on a single chip (14). The antibodies were immobilized in parallel-flow channels of GLC sensor chips (Biorad) using amine-coupling chemistry, as previously described (14). Briefly, after surface activation, the antibodies (30 µg/ml in 10 mM acetate buffer pH 5.0) were injected for 5 min at a flow rate of 30 µl/min, and the remaining activated groups were blocked with ethanolamine, pH 8.0. The final immobilization levels were about 5000 resonance units (1 RU=1 pg protein/mm²), for all the antibodies tested. A “reference” surface was always prepared in parallel using the same immobilization procedure but without addition of the antibody.
After rotation of the microfluidic system, aliquots of Aβ₁₋₄₂ or Aβ₁₋₄₀ (WT or A2V) were injected over the immobilized antibodies for 2 min at a flow rate of 30 µl/min. Dissociation was measured in the following 11 min. The running buffer, also used to dilute the samples, was 10 mM PBS containing 150 mM NaCl and 0.005% Tween 20 (PBST). All these assays were done at 25°C. The sensorgrams (time course of the SPR signal in RU) were normalized to a baseline value of 0. The signal observed in the surfaces immobilizing the antibody was corrected by subtracting the nonspecific response observed in the reference surface. The resulting sensorgrams were globally fitted by a two-binding sites model (heterogeneous ligand model, ProteOn analysis software) to obtain the corresponding kinetics parameters of the fast-dissociating and the slow-dissociating component (k_on and k_off) and the equilibrium dissociation constant (K_D).

Lysates from CL4176 and CL802 C. elegans strains (15,16) were diluted in PBST to a final protein concentration of 0.05 µg/µl, and injected over immobilized 4G8 for 3-5 min at a flow rate of 30 µl/ml.

Size exclusion chromatography (SEC)

Synthetic Aβ₁₋₄₂ (100 µM) was incubated at 25°C for different times (0, 5 and 24h). Aliquots were then diluted with 10 mM PBS to a final concentration of 10 µM. SEC was performed using an FPLC apparatus (Biologic DuoFlow FPLC system, Biorad) equipped with a precision column pre-packed with: i) Superdex 75 HR 10/30 (separation range of 3-70 kDa), or ii) Superdex 200 HR 10/30 (5-5000 kDa) (GE Healthcare). The mobile phase (PBS) was set at 0.5 ml/min and the elution peak was detected at 214 nm UV absorbance. The columns were calibrated using appropriate MW standard proteins (see legend to Fig S2). The void volume was determined by Blu dextrane 2000 (2000 kDa).

Lysates from CL4176 C. elegans were fractionated in a Superdex 200 HR 10/30, as described above.

Atomic force microscopy (AFM)

Aliquots of synthetic Aβ₁₋₄₂ were diluted with 10 mM PBS to a final concentration of 10 µM, and incubated for 0.5-2 min on a freshly cleaved mica disk. The disk was washed with water and dried under a very gentle nitrogen stream. The sample was then mounted on a Nanoscope V Multimode AFM (Veeco/Digital Instruments, Santa Barbara, CA) operating in tapping mode using standard phosphorus-doped silicium probes (Veeco). The scan speed was 1 Hz. For every sample 3 pictures from distinct regions were taken. Plane corrected and background removed pictures were obtained with the open source software tool Gwyddion (17).

Dynamic laser Light Scattering (DLS)

Aliquots of synthetic Aβ₁₋₄₂ were diluted with 10 mM PBS to a final concentration of 1 µM and 10 µM. DLS measurements were taken on each sample at 25°C with a homemade apparatus which includes a diode laser (λ=532 nm), a temperature-controlled cell and a digital correlator (Brookhaven Instruments Co) (18,19). Dynamic measurements of the scattered intensity correlation function yielded the translational diffusion coefficient of particles and then, via the Stokes-Einstein relation, the average hydrodynamic diameter of the particles in solution was calculated. Several runs were performed on each sample to check for data reproducibility. Data were analyzed by the CONTIN method, suitable for polydisperse systems (20).

Native gels and SDS–PAGE, Western Blot and dot-blot analysis.

Synthetic Aβ₁₋₄₂ (100 µM) was incubated at 25°C for different times (0-24h). Aliquots were then diluted with 10 mM PBS to a final concentration of 10 µM and the Aβ species were evaluated. For native gels, equal amounts of protein were loaded onto 4–16% (w/v) Bis-Tris native gels (Invitrogen) and electrophoresed at 150 V. After blotting, membranes were probed with 4G8 (1:1000 dilution). NativeMark standards (Invitrogen) were run in parallel. Equal amounts of protein were fractionated by 16% Tris-Tricine SDS-PAGE and, after blotting, were probed with 4G8 (1:1000 dilution). For dot-blot analysis, 50 ng of synthetic Aβ₁₋₄₂ was spotted onto nitrocellulose membranes (Millipore), blocked with PBST (PBS, pH 7.4 plus 0.1% (v/v) Tween 20), and incubated for 1 hour with 4G8 (1:1000 dilution). Anti-mouse IgG peroxidase conjugate (1:2000 dilution, Sigma) was used as a secondary antibody for 4G8. Immunoreactive bands were detected by ECL chemioluminescence and quantified by Quantity One Image Software (Biorad).

Electrophysiological assay

Single-channel recordings from lipid bilayer systems (20) were obtained using the Tip-Dip method, as previously described (21). Experiments carried out using only ionic solutions and pure lipid
bilateral do not show any ionic flow for more than 1 h continuous current recordings (n=5). Aβ samples were made up to a final concentration of 100 nM, alone and in presence of 4G8 or EGCG.

*C. elegans studies*

Wild type N2, the transgenic Aβ muscle expressing strain CL4176 and its control CL802 (15) were obtained from the Caenorhabditis Genetic Center (University of Minnesota, USA). In CL4176, the expression of human Aβ1-42 depends on raising the temperature from 16 to 24°C and caused the progressive worm paralysis (15). All nematode strains were propagated on solid Nematode Growth Medium (NGM) seeded with E. coli (OP50) for food.

Pharyngeal pumping assay. N2 nematodes, L3-L4 larval stage, were collected by washing plates with M9 buffer, transferred to tubes, centrifuged and washed two times with 5 mM PBS, pH 7.4, to eliminate bacteria. Synthetic plates with M9 buffer, transferred to tubes, allowed to lay eggs overnight. Isolated hatchlings from the synchronized eggs (day 1) were cultured at 16°C on fresh NGM plates and, when reaching maturity at 3 days of age, nematodes were transferred to fresh NGM plates (35x10 mm culture plates, 100 worms/plate) seeded with E. coli. Transgene expression was determined by multi-angle laser light scattering coupled to SEC (24). SPR binding due to the minor fraction dissociated with Aβ1-42 incubated for 0, 24 and 72h dissociated almost completely, whereas only a minor fraction dissociated with Aβ1-42 incubated for 2-8 hours. Qualitatively identical results were obtained by diluting Aβ1-42 to 10 µM before the injection in the SPR apparatus (data not shown).

None of the sensorgrams could be adequately fitted by the equation modelling a simple bimolecular interaction, but required a complex model. All the curves could be fitted well (white lines in Fig. 1a) by assuming there were two binding species (Fig. 1b-c and Fig. S1) with markedly different dissociation rate constants (k_off): one (pointed lines) dissociates relatively fast (k_off : 6.3x10^{-3} s^{-1}) and the other (dotted lines) had a > 50-fold slower rate (k_off: 1.1x10^{-4} s^{-1}), approaching pseudo-reversible binding.

The fast-dissociating binding species predominant in the freshly prepared Aβ1-42 solution (t=0, Fig. 1b) likely correspond to monomers. Thus, SEC analysis at t=0 showed a single peak at an elution time corresponding to an apparent MW of 12-13 kDa (Fig. S2a,d,e, green traces). It has been shown that this elution position corresponds to Aβ1-42 monomers, as determined by multi-angle laser light scattering coupled to SEC (24). SPR binding due to the fast-dissociating binding species (monomers) decreased with the incubation time (Fig. S1a-f, blue pointed lines), indicating evolution towards other molecular species.

Slow-dissociating species made a negligible contribution in freshly prepared Aβ1-42 solutions (t=0, Fig. 1b) and in solutions incubated for 24-72h (Fig. S1e,f), but it was up to 20 times higher at 2-8h of incubation (Fig. 1e and Fig. S1b-d). Figure 1d illustrates the binding signal due to these species in relation to Aβ1-42 incubation, with a bell-shaped kinetics and a peak at t=5h.
SEC analysis at this time (Fig. S2a,d,e, red traces) showed the appearance of a peak in the void volume. Dot-blot analysis of the void volume with either 4G8 and 6E10 confirmed a marked increase of signal at t=5h (data not shown). SPR analysis on the fraction corresponding to the void volume showed that it contained the slow-dissociating species only (Fig. S2b), whereas the fraction corresponding to the monomeric peak contained the fast-dissociating species only (Fig. S2c). AFM at t=5h (Fig. S3b) confirmed the appearance of aggregates, and analysis of the void-volume (Fig. S3d-f) highlighted the presence of species with different morphologies, including globular aggregates and short protofibrils. DLS analysis at t=5h (Fig. S4) indicated that most of the aggregates have a hydrodynamic diameter of 10-30 nm, although larger species were also observed. DLS volume distributions were similar when diluting Aβ1-42 to 1 μM (i.e. the concentration used for SPR studies) or to 10 μM (i.e. the concentrations needed for AFM or SEC) (Fig. S4). Native gel confirmed the appearance, at t=5h, of high order aggregates (Fig. S5a), which were SDS-labile and migrated on SDS-PAGE as monomers and dimers (Fig. S5b).

The SPR signal due to the slow-dissociating species decreased with incubation time ≥8h until negligible values after 24-72h incubation (Fig. 1d). This finding is likely due to the concomitant evolution of Aβ1-42 towards different aggregated species, losing the property to bind immobilized 4G8. In agreement, SEC analysis showed that the peak in the void volume markedly decreased after 24 hours (blue traces, Fig. S2a,d,e), suggesting that the aggregates formed at t=24h do not enter the columns. At this time, DLS analysis showed bigger species, the majority of which had a hydrodynamic diameter of around 500 nm (Fig. S4, lower panels). Larger aggregates were also detected by AFM (Fig. S3c) and native gel (Fig. S5a). Western blot analysis, carried out under denaturing conditions, showed a decrease of monomers, an increase of dimers and the appearance of higher MW SDS-stable species (Fig. S5b). Dot-blot analysis showed that 4G8 recognized in a similar way the assemblies present at t=5h and those formed at t=24h (Fig. S5c,d), indicating that this technique cannot be used to characterize transient oligomeric species.

In summary, the SPR-based immunoassay specifically detects a transient subpopulation of Aβ1-42 oligomers, characterized by a pseudo-irreversible binding to immobilized 4G8.

SPR studies using 6E10 as capturing antibody showed low dissociation rates for both Aβ1-42 monomers and oligomers (Fig. S6). Therefore, the contribution of monomers and oligomers could not be investigated with this antibody. We also did not detect any binding of Aβ1-42 (tested at concentrations up to 10 μM) on immobilized A11 (not shown).

Another set of SPR studies was designed to investigate the direct binding of A11 and OC antibodies (5) to the Aβ1-42 oligomers which had been previously captured by immobilized 4G8. We found a concentration-dependent binding of OC (Fig. S7) indicating that the 4G8-binding oligomers are also OC-positive. No reliable data could be obtained with A11 since we found a very high non-specific binding to immobilized 4G8, even in the absence of captured oligomers.

We then used the 4G8-based SPR immunoassay to examine how the incubation conditions affects the kinetics of appearance/disappearance of Aβ1-42 oligomers. Figure S8 shows that raising the temperature from 25°C to 37°C shifted the peak time from 5h to 1h, indicating much faster oligomer formation. The disappearance of the oligomers was also faster, and was almost complete after 8 hours of incubation. In addition, the peak time was affected by the peptide concentration, being 1, 3 and 5h at 100, 50 and 25 μM Aβ1-42, respectively. The maximum resonance signal was also affected, with lower concentrations producing fewer oligomers. Finally, the rate of disappearance of the oligomers was directly proportional to the concentration of Aβ1-42 (Fig. S8). In summary, higher temperatures or higher concentrations of Aβ1-42 resulted in faster oligomerization and faster evolution towards species not recognized by 4G8.

To investigate whether the SPR-based immunoassay could be applied to study molecules with potential anti-oligomeric effect, we tested the small polyphenolic green tea constituent (-)-epigallocatechin gallate (EGCG), whose effects on Aβ aggregation and fibrillogenesis have been reported (25,26). We incubated Aβ1-42 (100 μM) for 5 hours with or without 3-100 μM EGCG. Samples were then diluted in PBS and flowed over immobilized 4G8. Preincubation with EGCG dose-dependently reduced Aβ1-42 binding to 4G8 (Fig. 2a). The reduction was specifically due to the slow-dissociating component (Fig. 2b), which completely disappeared with equimolar concentrations of EGCG. The binding of
preformed Aβ$_{1-42}$ oligomers to 4G8 was not affected by co-injection of equimolar concentrations of EGCG (data not shown), suggesting that EGCG inhibits the oligomer formation. Thus, the SPR assay can be used to monitor the effect of potential anti-oligomeric compounds.

**SPR studies with native Aβ$_{1-42}$**

Next we tested whether the SPR-based immunoassay could detect natural (i.e. not synthetic) Aβ$_{1-42}$ oligomers. We employed the transgenic CL4176 strain of the nematode *C. elegans* engineered to express human Aβ$_{1-42}$ peptide in body-wall muscles when the temperature is raised (15). As previously reported, about 60% of the worms were paralyzed 44 hours after temperature induction, in parallel with the deposition of oligomeric Aβ assemblies in the body-wall muscle cells (16). Non-induced CL4176 and CL802 nematodes, which do not express the Aβ$_{1-42}$ transgene, were used as controls. SPR analysis showed a clear binding signal only with lysates from induced CL4176 worms (Fig. 3a). The shape of the sensorgram, particularly the slow dissociation rate, suggests that the binding signal is due to the Aβ$_{1-42}$ oligomer species. To substantiate this finding, lysates from CL4176 worms were eluted by SEC and fractions were collected, in particular at time points corresponding to the elution of “monomers” and “oligomers” (void volume). The binding signal was only present when injecting the oligomeric fraction (Fig. 3b), consistently with the results obtained with the whole lysate. It is likely that monomers are not detectable under these conditions for sensitivity reasons (see discussion).

These data indicate that the SPR assay can be used to detect the presence of native oligomers.

**Biological effects of synthetic Aβ$_{1-42}$**

To examine the biological effects of the short-lived oligomers detected by SPR we used *in vitro* and *in vivo* tests.

It has been reported that soluble oligomers from several types of amyloid boosted lipid bilayer conductance regardless of the sequence, while fibrils and monomeric species did not (27). Electrophysiological Tip-Dip experiments (21) were used to measure the effects of the different Aβ$_{1-42}$ species on the ionic conductance of a lipid bilayer. Samples of synthetic Aβ$_{1-42}$ were analyzed at t=0 (monomers), t=5h (4G8-binding oligomers) and t=24h (when 4G8-binding oligomers are no longer detected). Pure lipid bilayer exposed to vehicle did not show any ionic current. The Aβ$_{1-42}$ solution incubated for 5 hours had more effect on ion conductivity than solutions incubated for 0 and 24 hours (Fig. 4a). Analysis of the corresponding current/voltage relationships (Fig. 4b) showed conductance values of 33±3.6, 82±5.0 and 26±2.5 pS at 0, 5 and 24h of incubation, respectively. The oligomer-associated increase of conductance was completely abolished by 4G8 or EGCG (Fig. 4c). These data indicate that the 4G8-binding oligomeric species specifically affect membrane permeability.

Next we tested the effect of Aβ$_{1-42}$ on the pharyngeal pumping rate in *C. elegans*. This is the rhythmic contraction and relaxation of the pharyngeal muscle responsible for the ingestion and transport of food from the mouth to the intestine, and is rapidly reduced by sublethal doses of chemical stressors (28). N2 ancestral nematodes were fed with Aβ$_{1-42}$ taken after 0, 5, 24 or 48 hours incubation. Two hours later the worms were transferred onto fresh NGM agar seeded with *E. coli*, and the pumping rate was measured. The pumping rate was significantly impaired only in nematodes fed with the Aβ$_{1-42}$ solution incubated for 5 hours, corresponding to the peak of 4G8-binding Aβ$_{1-42}$ oligomers (Fig. 5a). The solutions containing monomers only (t=0) or lacking 4G8-reactive oligomers (t=24-48h) had no effect. The effect of Aβ$_{1-42}$ oligomers was dose-dependent (Fig. 5b) and reversible, the pumping rate returning to baseline 16 hours after exposure to Aβ$_{1-42}$ (data not shown). Pre-incubation with 4G8 antagonized the effect on the pumping rate (Fig. 5c), as did EGCG (Fig. 5d), suggesting that it was due to the 4G8-reactive oligomeric forms recognized by SPR.

**Studies with wild-type and A2V-mutated Aβ$_{1-40}$**

Finally, we applied the SPR-based immunoassay and the pumping rate test to study the A2V mutated form of Aβ$_{1-40}$ previously found to be more amyloidogenic and more cytotoxic than the WT peptide (19). This peptide was selected on the basis of the neuropathological studies conducted on the brain of the homozygous proband which indicated that the deposition of Aβ$_{1-40}$ species were overrepresented and that the effects of A2V genetic mutation might be more pronounced on Aβ$_{1-40}$ than on Aβ$_{1-42}$ (29). During incubation at 37°C in quiescent conditions, Aβ$_{1-40}$ A2V formed transient 4G8-binding species whereas Aβ$_{1-40}$ WT did not (Fig. 6a-b). The maximum binding signal was found after 8 hours incubation (Fig. 6c).
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6c). Studies in C. elegans at this time point indicated a significant decrease in the pumping rate with Aβ1-40/A2V but not Aβ1-40 WT (Fig. 6d).

DISCUSSION

We describe here a new SPR-based immunoassay which selectively recognizes a subpopulation of biologically active Aβ oligomers. The method exploits the fact that these soluble aggregated species bind immobilized 4G8 in a pseudo-irreversible manner (very slow dissociation rates), whereas monomers bind 4G8 with a significantly faster dissociation rates. The possibility to accurately determine the binding constants is the key feature that allows the use of 4G8 in the SPR immunoassay to distinguish between different Aβ species.

SPR studies were also done with the anti-Aβ antibody 6E10 which, however, proved unsuitable for distinguishing monomers from oligomers because both species bound the antibody with strong affinities, and very slow dissociation rates (30). It might be interesting to test other antibodies, particularly those raised using Aβ oligomers as antigens (31,32).

Recently, SPR data have been reported employing an “oligomer-specific” IgM anti-Aβ antibody (OMAB) (33). In that study, the binding of Ap1-40/2 monomers/dimers to OMAB had fast off-rate kinetics, while binding of SEC-enriched Ap1-40/2 oligomers resulted in a strong complex with an exceptionally slow k_off. OMAB might thus serve as another capturing antibody useful for recognition of oligomers by SPR. It will be interesting to see whether 4G8 and OMAB recognize the same species.

In principle the SPR assay might employ conformation-dependent antibodies that recognize oligomers of different proteins or peptides, such as A11 or OC (34)(5). A reliable immobilization of OC requires a purified antibody, which is currently not available. The immobilization of A11 did not result in any binding signal. Whether this antibody is unable to recognize transient Aβ1-42 oligomers, or its activity is abolished by the immobilization on the SPR sensor chip, remains to be established.

The aggregated species which bind immobilized 4G8 in a pseudo-irreversible manner rapidly appear and disappear during the incubation of synthetic Aβ1-42. With 100 µM peptide at 25°C, the oligomer-dependent SPR signal was maximal after 5 hours incubation decreasing thereafter to negligible values at ≥24h. Analysis of the solution incubated for 5 hours (by SEC, DLS, AFM and gel electrophoresis) showed the presence of a heterogeneous population of SDS-labile aggregates, including globular species and short protofibrils, with a main hydrodynamic diameter of 10-30 nm and eluting in the void volume even with SEC columns suitable for high MW molecules. However, the morphology of Aβ aggregates, as well as their interaction with column matrix, can significantly alter migration times, and may result in overestimated dimensions (24,35). The solution incubated for 24 hours mainly contained greater and SDS-stable species, which do not even enter in the SEC columns. Thus, the 4G8-based SPR immunoassay selectively recognized a specific population of soluble aggregates. This population is also recognized by OC, a conformation-specific antibody proposed to selectively target fibrillar oligomers (5), as suggested by an SPR assay in which OC was flowed onto 4G8-captured oligomers. This “sandwich” SPR format (Fig. S7) could be applied for identifying interactors of captured oligomers.

When tested in a dot-blot assay, 4G8 recognized the oligomeric assemblies present at t=5h and t=24h in a similar way, in line with published evidence (36). There are two possible explanations for the discrepancy between the dot-blot and SPR results: i) it is possible that steric hindrance or specific features of the SPR technology do not allow detection of the species at t=24h, for example because of impaired diffusion of large aggregates in the microfluidic channels (37); ii) the fact that SPR measures the binding events in a dynamic manner (time-scale of seconds) could provide a much higher sensitivity to discriminate between species with different affinity values, which cannot be achieved by dot-blot in which long-incubation steps may level out differences in affinity.

The pseudo-irreversible binding observed by SPR at t=5h suggests multivalent interactions between different epitopes on a single oligomeric assembly and different immobilized 4G8 antibodies (38). The affinity of the oligomers for 4G8 could not be determined because of the lack of information about their actual concentration, a consequence of the uncertainty about the precise MW. However, assuming (from SEC data) that after 5 hours of incubation ~40% of monomers have assembled into oligomers, and assuming a mass ranging...
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from 90 to 400 kDa (39), it follows that the concentration of oligomers is in the low nanomolar range (4-20 nM) and their affinity for immobilized 4G8 is exceptionally high ($K_D<1$ nM). The fact that monomers have a much lower affinity ($K_D=75$ nM) and mass, could explain why monomers are recognized with a much lower sensitivity than oligomers. Thus, the data in Fig. S2a-c shows that the SPR signal found using monomeric fractions was much lower than found with oligomeric fractions, although the SEC peaks were similar.

The formation of 4G8-binding oligomeric species was completely prevented by the co-incubation of $\beta_1$-42 in the presence of equimolar concentrations of EGCG, a prototypical inhibitor of oligomerization (25,26). A previous study showed that EGCG prevented the conversion of $\beta_1$-42 into toxic intermediates and favoured the formation of unstructured non-toxic oligomers (25). Moreover, we found that the A2V mutation, which enhances the aggregation and fibrillogenic properties of $\beta$ and results in early-onset dementia (19), boosts the formation of $\beta_{1,40}$ transient oligomeric species recognized by 4G8.

4G8 recognizes a central region of $\beta$ (amino acids 17-21, LVFFA) which is believed to be implicated in the hydrophobic interactions underlying the formation and elongation of amyloid fibrils (40), but not in $\beta$ oligomerization (41). Our SPR data indicate that the short-lived $\beta_{1,42}$ oligomers expose this 4G8-binding hydrophobic sequence. Very recent data (4,6) suggest that the exposure of hydrophobic motifs is the main conformational determinant of oligomer toxicity, whereas size and secondary structures are probably less important (6).

Thus, the 4G8-based SPR immunoassay has the potential to specifically recognize the toxic oligomeric species of $\beta_{1,42}$. To demonstrate this, we used two assays that require short-term exposure to $\beta_{1,42}$ solutions, a pivotal condition for studying the biological effects of short-lived species.

Several reports suggest a direct interaction between $\beta$ and the plasma membrane as one possible mechanism of toxicity (21,42-45). Electrophysiological experiments employed the Tip-Dip technique, which accurately studies single ion permeability in artificial lipid bilayers (21). The results showed that $\beta_{1,42}$ solutions preincubated for 5 hours (i.e. those containing 4G8-binding oligomers) had a much greater effect than freshly prepared solutions (monomers) or solutions preincubated for 24h (lacking 4G8-binding oligomers). 4G8 and EGCG counteracted the oligomer-induced increase in conductance, but had no effect on the conductance associated with monomers.

In vivo we used *C. elegans*, an attractive model for several studies, including toxicological investigations. The rhythmic contraction and relaxation of the *C. elegans* pharynx is fundamental for the worm’s feeding and is affected by a variety of chemical stressors, such as alcohol, heavy metals and sulfhydryl-reactive compounds, which induce the production of cellular stress proteins (28). Stress-induced inhibition of feeding was in fact suggested as an important survival mechanism that limits the intake of toxic solutes. We found, for the first time, that $\beta_{1,42}$ oligomers, but not monomers or larger aggregates, act as “stressors” in *C. elegans*, significantly inhibiting their pharyngeal pumping. This innovative in vivo model may serve as a rapid and convenient marker of the toxic potential of $\beta$ oligomers, as also suggested by the significant effects found with the toxic $\beta_{1,40}A2V$ mutant. The correspondence between inhibition of the pumping rate in *C. elegans* and the peculiar binding signal in SPR studies (the pseudo-reversible binding to 4G8) is also noteworthy; this was true when using $\beta_{1,42}$ after different incubation times, when comparing $\beta_{1,40}WT$ with $\beta_{1,40}A2V$, and when incubating with EGCG. These findings strongly suggest that the oligomers recognized by 4G8 in the SPR immunoassays are the same as those that are toxic in the *C. elegans* pharyngeal pumping test. In fact, 4G8 antagonized the effect of $\beta_{1,42}$ oligomers on pharyngeal behavior, emerging as an antibody recognizing and antagonizing the toxic $\beta$ oligomers. Consistently, we previously found that 4G8 also antagonizes the memory impairment induced by intracerebroventricular injection of synthetic $\beta_{1,42}$ oligomers in mice (22).

The present study also shows that the SPR immunoassay can recognize natural oligomers formed in transgenic *C. elegans* expressing $\beta_{1,42}$. The ability of this assay to detect native toxic oligomers in other biological samples, including those from human AD patients, is under investigation.
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REFERENCES

1. Lambert, M. P., Barlow, A. K., Chromy, B. A., Edwards, C., Freed, R., Liosatos, M., Morgan, T. E., Rozovsky, I., Trommer, B., Viola, K. L., Wals, P., Zhang, C., Finch, C. E., Kraft, G. A., and Klein, W. L. (1998) Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins. *Proc Natl Acad Sci U S A* 95, 6448-6453

2. Walsh, D. M., and Selkoe, D. J. (2007) A beta oligomers - a decade of discovery. *J Neurochem* 101, 1172-1184

3. Holtzman, D. M., Goate, A., Kelly, J., and Sperling, R. (2011) Mapping the road forward in Alzheimer's disease. *Sci Transl Med* 3, 114ps148

4. Campioni, S., Mannini, B., Zampagni, M., Pensalfini, A., Parrini, C., Evangelisti, E., Relini, A., Stefani, M., Dobson, C. M., Cecchi, C., and Chiti, F. (2010) A causative link between the structure of aberrant protein oligomers and their toxicity. *Nat Chem Biol* 6, 140-147

5. Glabe, C. G. (2008) Structural classification of toxic amyloid oligomers. *J Biol Chem* 283, 29639-29643

6. Ladiwala, A. R., Litt, J., Kane, R. S., Aucoin, D. S., Smith, S. O., Ranjan, S., Davis, J., Vannostrand, W. E., and Tessier, P. M. (2012) Conformational differences between two amyloid beta oligomers of similar size and dissimilar toxicity. *J Biol Chem* doi:10.1074/jbc.M111.329763

7. Cooper, M. A. (2002) Optical biosensors in drug discovery. *Nat Rev Drug Discov* 1, 515-528

8. Rich, R. L., and Myszka, D. G. (2007) Higher-throughput, label-free, real-time molecular interaction analysis. *Anal Biochem* 361, 1-6

9. Sohma, Y., Hayashi, Y., Kimura, M., Chiyomori, Y., Taniguchi, A., Sasaki, M., Kimura, T., and Kiso, Y. (2005) The 'O-acyl isopeptide method' for the synthesis of difficult sequence-containing peptides: application to the synthesis of Alzheimer's disease-related amyloid beta peptide (Abeta) 1-42. *J Pept Sci* 11, 441-451

10. Taniguchi, A., Sohma, Y., Hirayama, Y., Mukai, H., Kimura, T., Hayashi, Y., Matsuzaki, K., and Kiso, Y. (2009) "Click peptide": pH-triggered in situ production and aggregation of monomer Abeta1-42. *ChemBioChem* 10, 710-715

11. Beeg, M., Stravalaci, M., Bastone, A., Salmona, M., and Gobbi, M. (2011) A modified protocol to prepare seed-free starting solutions of amyloid-beta (Abeta)(1-40) and Abeta(1-42) from the corresponding depsipeptides. *Anal Biochem* 411, 297-299

12. Stravalaci, M., Beeg, M., Salmona, M., and Gobbi, M. (2010) Use of surface plasmon resonance to study the elongation kinetics and the binding properties of the highly amyloidogenic Abeta(1-42) peptide, synthesized by depsipeptide technique. *Biosens Bioelectron* 26, 2772-2775

13. Anonymous. (2011) State of aggregation (Editorial). *Nat Neurosci* 14, 399

14. Bravman, T., Bronner, V., Lavie, K., Notcovitch, A., Papalia, G. A., and Myszka, D. G. (2006) Exploring "one-shot" kinetics and small molecule analysis using the ProteOn XPR36 array biosensor. *Anal Biochem* 358, 281-288

15. Link, C. D. (2005) Invertebrate models of Alzheimer's disease. *Genes Brain Behav* 4, 147-156

16. Diomede, L., Cassata, G., Fiordaliso, F., Salio, M., Ami, D., Natalello, A., Doglia, S. M., De Luigi, A., and Salmona, M. (2010) Tetracycline and its analogues protect Caenorhabditis elegans from beta amyloid-induced toxicity by targeting oligomers. *Neurobiol Dis* 40, 424-431

17. Nečas, D., and Klapeček, P. (2012) Gwyddion: an open-source software for SPM data analysis. *Central Eur J Physics* 10, 181-188

18. Lago, P., Rovati, L., Cantù, L., and Corti, M. A. (1993) Quasielastic light scattering detector for chromatographic analysis. *Rev. Sci. Instrum.* 64, 1797-1802

19. Di Fede, G., Catania, M., Morbin, M., Rossi, G., Suardi, S., Mazzoleni, G., Merlin, M., Giovagnoli, A. R., Prioni, S., Erbetta, A., Falcone, C., Gobbi, M., Colombo, L., Bastone, A., Beeg, M., Manzoni, C., Francescucci, B., Spagnoli, A., Cantù, L., Del Favero, E., Levy, E., Salmona, M., and Tagliavini, F. (2009) A recessive mutation in the APP gene with dominant-negative effect on amyloidogenesis. *Science* 323, 1473-1477
Specific recognition of toxic Aβ oligomers

20. Provencher, S. W. (1982) A constrained regularization method for inverting data represented by linear algebraic or integral equations. *Comput. Phys. Comm.* 27, 213-227

21. Paulis, D., Maras, B., Schinina, M. E., Francesco, L., Principe, S., Galeno, R., Abdel-Haq, H., Cardone, F., Florio, T., Pocchiari, M., and Mazzanti, M. (2011) The pathological prion protein forms ionic conductance in lipid bilayer. *Neurochem Int* 59, 168-174

22. Balducci, C., Beeg, M., Stravalaci, M., Bastone, A., Selip, A., Biasini, E., Tapella, L., Colombo, L., Manzoni, C., Borsello, T., Chiesa, R., Gobbi, M., Salmona, M., and Forloni, G. (2010) Synthetic amyloid-beta oligomers impair long-term memory independently of cellular prion protein. *Proc Natl Acad Sci U S A* 107, 2295-2300

23. Lauren, J., Gimbel, D. A., Nygaard, H. B., Gilbert, J. W., and Strittmatter, S. M. (2009) Cellular prion protein mediates impairment of synaptic plasticity by amyloid-beta oligomers. *Nature* 457, 1128-1132

24. Hepler, R. W., Grimm, K. M., Nahas, D. D., Breese, R., Dodson, E. C., Acton, P., Keller, P. M., Yeager, M., Wang, H., Sloughhrue, P., Kinney, G., and Joyce, J. G. (2006) Solution state characterization of amyloid beta-derived diffusible ligands. *Biochemistry* 45, 15157-15167

25. Ehrnhoefer, D. E., Bieschke, J., Boeddich, J., Oppenheimer, M., Masino, L., Kurz, R., Engemann, S., Pastore, A., and Wanker, E. E. (2008) EGCG redirects amyloidogenic polypeptides into unstructured, off-pathway oligomers. *Nat Struct Mol Biol* 15, 558-566

26. Bieschke, J., Russ, J., Friedrich, R. P., Ehrnhoefer, D. E., Wobst, H., Neugebauer, K., and Wanker, E. E. (2010) EGCG remodels mature alpha-synuclein and amyloid-beta fibrils and reduces cellular toxicity. *Proc Natl Acad Sci U S A* 107, 7710-7715

27. Kayed, R., Sokolov, Y., Edmonds, B., McIntire, T. M., Milton, S. C., Hall, J. E., and Glabe, C. G. (2004) Permeabilization of lipid bilayers is a common conformation-dependent activity of soluble amyloid oligomers in protein misfolding diseases. *J Biol Chem* 279, 46363-46366

28. Jones, D., and Candido, E. P. (1999) Feeding is inhibited by sublethal concentrations of toxicants and by heat stress in the nematode Caenorhabditis elegans: relationship to the cellular stress response. *J Exp Zool* 284, 147-157

29. Giaccone, G., Morbin, M., Moda, F., Botti, M., Mazzoleni, G., Uggetti, A., Catania, M., Moro, M. L., Redaelli, V., Spagnoli, A., Rossi, R. S., Salmona, M., Di Fede, G., and Tagliavini, F. (2010) Neuropathology of the recessive A673V APP mutation: Alzheimer disease with distinctive features. *Acta Neuropathol* 120, 803-812

30. Ramakrishnan, M., Kandimmalla, K. K., Wengenack, T. M., Howell, K. G., and Poduslo, J. F. (2009) Surface plasmon resonance binding kinetics of Alzheimer's disease amyloid beta peptide-capturing and plaque-binding monoclonal antibodies. *Biochemistry* 48, 10405-10415

31. Barghorn, S., Nimmrich, V., Stiebing, A., Krantz, C., Keller, P., Janson, B., Bahrm, M., Schmidt, B., Bittner, R. S., Harlan, J., Barlow, E., Ebert, U., and Hillen, H. (2005) Globular amyloid beta-peptide oligomer - a homogenous and stable neuropathological protein in Alzheimer's disease. *J Neurochem* 95, 834-847

32. Lambert, M. P., Velasco, P. T., Chang, L., Viola, K. L., Fernandez, S., Lador, P. N., Khuon, D., Gong, Y., Bigio, E. H., Shaw, P., De Felice, F. G., Krafft, G. A., and Klein, W. L. (2007) Monoclonal antibodies that target pathological assembles of Abeta. *J Neurochem* 100, 23-35

33. Lindhagen-Persson, M., Brannstrom, K., Vestling, M., Steinitz, M., and Olofsson, A. (2010) Amyloid-beta oligomer specificity mediated by the IgM isotype--implications for a specific protective mechanism exerted by endogenous auto-antibodies. *PLoS One* 5, e13928

34. Kayed, R. (2003) Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. *Science* 300, 486-489

35. Benilova, I., Karran, E., and De Strooper, B. (2012) The toxic Abeta oligomer and Alzheimer's disease: an emperor in need of clothes. *Nat Neurosci* 15, 349-357

36. Perchiccia, J. M., Ladiwala, A. R., Bhattacharya, M., and Tessier, P. M. (2012) Structure-based design of conformation- and sequence-specific antibodies against amyloid beta. *Proc Natl Acad Sci U S A* 109, 84-89
37. Štěpánek, J., Vaisocherová, H., Piliarik, M., and Homola, J. (2006) Molecular Interactions in SPR Sensors. in *Surface Plasmon Resonance Based Sensors*, Springer Berlin Heidelberg. pp 69-91

38. Hong, S., Leroueil, P. R., Majoros, I. J., Orr, B. G., Baker, J. R., Jr., and Banaszak Holl, M. M. (2007) The binding avidity of a nanoparticle-based multivalent targeted drug delivery platform. *Chem Biol* 14, 107-115

39. Freir, D. B., Nicoll, A. J., Klyubin, I., Panico, S., Mc Donald, J. M., Risse, E., Asante, E. A., Farrow, M. A., Sessions, R. B., Saibil, H. R., Clarke, A. R., Rowan, M. J., Walsh, D. M., and Collinge, J. (2011) Interaction between prion protein and toxic amyloid beta assemblies can be therapeutically targeted at multiple sites. *Nat Commun* 2, 336 doi: 310.1038/ncomms1341

40. Tjernberg, L. O., Callaway, D. J., Tjernberg, A., Hahne, S., Lilliehook, C., Terenius, L., Thyberg, J., and Nordstedt, C. (1999) A molecular model of Alzheimer amyloid beta-peptide fibril formation. *J Biol Chem* 274, 12619-12625

41. Matsumura, S., Shinoda, K., Yamada, M., Yokojima, S., Inoue, M., Ohnishi, T., Shimada, T., Kikuchi, K., Masui, D., Hashimoto, S., Sato, M., Ito, A., Akioka, M., Takagi, S., Nakamura, Y., Nemoto, K., Hasegawa, Y., Takamoto, H., Inoue, H., Nakamura, S., Nabeshima, Y., Teplow, D. B., Kinjo, M., and Hoshi, M. (2011) Two distinct amyloid beta-protein (Abeta) assembly pathways leading to oligomers and fibrils identified by combined fluorescence correlation spectroscopy, morphology, and toxicity analyses. *J Biol Chem* 286, 11555-11562

42. Kagan, B. L., Azimov, R., and Azimova, R. (2004) Amyloid peptide channels. *J Membr Biol* 202, 1-10

43. Kawahara, M., Ohtsuka, I., Yokoyama, S., Kato-Negishi, M., and Sadakane, Y. (2011) Membrane Incorporation, Channel Formation, and Disruption of Calcium Homeostasis by Alzheimer's beta-Amyloid Protein. *Int J Alzheimers Dis* 2011, 304583

44. Quist, A., Doudevski, I., Lin, H., Azimova, R., Ng, D., Frangione, B., Kagan, B., Ghiso, J., and Lal, R. (2005) Amyloid ion channels: a common structural link for protein-misfolding disease. *Proc Natl Acad Sci U S A* 102, 10427-10432

45. Shirwany, N. A., Payette, D., Xie, J., and Guo, Q. (2007) The amyloid beta ion channel hypothesis of Alzheimer's disease. *Neuropsychiatr Dis Treat* 3, 597-612
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Abbreviations

1The abbreviations used are: Aβ: amyloid-β; AFM: atomic force microscopy; DLS: dynamic laser light scattering; EGCG: epigallocatechin gallate; k_{off}: dissociation rate constant; k_{on}: association rate constant; SDS: sodium dodecyl sulphate; SEC: size exclusion chromatography; SPR: surface plasmon resonance.

Footnotes

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Author Contributions

MG, MSt, AB, MSa, RC and LD contributed to conceptual and experimental design. MSt and MG did the SPR experiments. AB and MB carried out, respectively, SEC and AFM analysis; LCa and ED carried out DLS studies; MB, AC and LCo contributed to peptide synthesis and purification. MM designed and conducted electrophysiological experiments. LD designed and carried out assays on C. elegans. MSt, LD and MG wrote the manuscript and contributed to preparation of the figures. All authors discussed the results and manuscript.

Competing financial interests

The authors declare no competing financial interests.
FIGURE LEGENDS

FIGURE 1. SPR studies. (a) Synthetic Aβ₁₋₄₂ (100 μM) was incubated at 25°C and samples were taken at different times (from 0-72h), diluted to 1 μM in PBS and injected over immobilized 4G8 for 2 min (bar), followed by 11 min dissociation. The figure shows the sensorgrams (time course of the SPR signal expressed in resonance units, RU) obtained in a representative experiment (black lines). Fitting the sensorgrams (white lines) required a model assuming the presence of two binding species with different dissociation rates. (b) Experimental sensorgram obtained injecting the freshly prepared solution (t=0, continuous line) is shown together with the theoretical sensorgrams corresponding to the fast-dissociating binding species (pointed lines) and the slow-dissociating binding species (dotted lines). The experimental sensorgram is mainly accounted for by the fast-dissociating species, which bind to 4G8 with a K_D of 75±10 nM (n=5). (c) Experimental and theoretical sensorgrams obtained injecting the solutions incubated for 5 hours. Legends as in panel b. Note the increase of the signal due to the slowly-dissociating species. (see the complete sets of data in Fig. S1). (d) Time-course of the slowly-dissociating component. Each point is the mean±SD of 3-4 independent experiments, as a percentage of the signal found after 5 hours incubation.

FIGURE 2. Effect of (-) epigallocatechin gallate (EGCG). Synthetic Aβ₁₋₄₂ (100 μM) was incubated at 25°C in the absence or presence of different concentrations of EGCG (3, 10, 30 and 100 μM). Samples were taken after 5 hours incubation, diluted 100-fold and injected over immobilized 4G8 for 2 min (bar), followed by 11 min dissociation. Panel a shows the sensorgrams (time course of the SPR signal expressed in resonance units, RU), which could be dissected for the presence of fast-dissociating species (monomers) and slow-dissociating species (oligomers) (see also Fig. 1). Panel b shows the effects of EGCG on the SPR binding signal due to each of these components, highlighting a selective inhibitory effect on the oligomeric species.

FIGURE 3. SPR studies with native Aβ₁₋₄₂ from C. elegans. (a) Lysates obtained from CL4176 transgenic C. elegans strain 44 hours after the temperature rise (i.e. after induction of oligomeric Aβ₁₋₄₂ expression) were injected onto immobilized 4G8. Lysates from not-induced CL4176 and CL802 nematodes, which do not express the Aβ₁₋₄₂ transgene, were used as control. This experiment was carried out twice with similar results. (b) Lysates from induced CL4176 worms (i.e. 44 hours after the temperature rise) were eluted by SEC using a S 200 column. Fractions collected at time points corresponding to the elution of “monomers” and “oligomers” (void volume) were injected onto immobilized 4G8.

FIGURE 4. Membrane conductance changes induced by synthetic Aβ₁₋₄₂ species. (a) Representative current traces obtained after applying synthetic Aβ₁₋₄₂ solutions to an artificial lipid bilayer. Synthetic Aβ₁₋₄₂ (100 μM) was incubated at 25°C and sampled at different times (t=0, 5 and 24h), then diluted to 100 nM in 144 mM NaCl, 1.8 mM CaCl₂, 1.2 mM MgCl₂, 10 mM Hepes, pH 5.0, and applied to a lipid bilayer. Current traces were recorded at different potentials (+80, +40 mV and -40 mV, -80 mV). (b) The current/voltage relationship from a complete set of Tip-Dip currents. (c) Representative histogram comparing the conductance using freshly prepared Aβ₁₋₄₂ (t=0, monomers) and after 5 hours incubation (t=5h), the latter preincubated or not with 4G8 (1:500 vol:vol) or epigallocatechin gallate (EGCG, equimolar). Values are means±SD (n=5). **p<0.01 versus all the other groups (Bonferroni’s multiple comparison test following one-way ANOVA). 4G8 or EGCG alone had no effect on ionic currents, nor they did induce lipid bilayer ruptures (not shown).
FIGURE 5. Effects of Aβ1-42 species on pharyngeal pumping rate in C.elegans. (a) Synthetic Aβ1-42 (100 µM) was incubated at 25°C and sampled at different times (t=0, 5 and 24h), diluted to 10 µM in PBS and administered to N2 worms for two hours. Control worms were fed vehicle alone (dotted line). Only the samples preincubated for 5 hours (i.e. those containing the 4G8-binding Aβ1-42 oligomers) markedly inhibited the pumping rate. Values are means±SE (n=20), expressed as a percentage of the corresponding control. ** p<0.01 versus t=0, Bonferroni’s test after two-way ANOVA. (b) Dose-response effect of Aβ1-42 oligomers on pharyngeal pumping. Worms were fed Aβ1-42 (0.5-10 µM) previously incubated for 5 hours. Values are means±SE, expressed as a percentage of control worms fed vehicle. (c) The inhibitory effect of Aβ1-42 oligomers on pumping rate was antagonized by 4G8. Aβ1-42 was preincubated for 5 hours, added with 4G8 (1:500, vol:vol in vehicle) and incubated for further 30 min before administration to worms. Control worms were fed vehicle or 4G8 alone. Values are means±SE (n=16). Two-way ANOVA showed a significant interaction between Aβ1-42 and 4G8 (p<0.01); **p<0.001 versus vehicle and °°p<0.001 versus Aβ1-42 (Bonferroni’s test). (d) Epigallocatechin gallate (EGCG) antagonized the inhibitory effect of Aβ1-42 oligomers on pharyngeal pumping. Aβ1-42 (100 µM) was incubated at 25°C with or without EGCG (100 µM). After 5 hours, samples were diluted ten times and administered to worms. Control worms were fed vehicle or 10 µM EGCG alone. Values are means±SE (n=16). Two-way ANOVA showed a significant interaction between Aβ1-42 and 4G8 (p<0.01); **p<0.001 versus vehicle and °°p<0.001 versus Aβ1-42 (Bonferroni’s test).

FIGURE 6. Effect of A2V mutation of Aβ1-40 on the kinetics of 4G8-binding oligomers and on pharyngeal pumping rate in C.elegans. (a-b) Synthetic Aβ1-40 WT (a) or A2V (b) (100 µM) were incubated at 37°C and samples were taken at different times (from 0-48h), diluted to 3 µM in PBS and injected over immobilized 4G8 for 2 min (bar), followed by 11 min dissociation. The figure shows the sensorgrams (time course of the SPR signal expressed in resonance units, RU) (black lines) obtained in a representative experiment. Sensorgrams were fitted as described in Fig. 1 and S1, dissecting the experimental curve into its fast- and the slow-dissociating components (k off: 8.0±0.2x10^{-3} s^{-1} and 6.0±1.6x10^{-5} s^{-1}, respectively). (c) Time-course of the slowly-dissociating components. Each point is the mean±SE of 3 independent experiments. (d) Worms were fed for two hours Aβ1-40, WT or A2V, 50 µM, freshly prepared (t=0) or preincubated at 37°C for 8 hours. Control worms were fed vehicle alone. Values are means±SE (n=20 worms). **p<0.01 versus all the other groups (Bonferroni’s test after one-way ANOVA).
Figure 1

(a) Experimental data (binding species 1 + binding species 2)
(b) Binding species 1 (fast $K_{off}$)
(c) Binding species 2 (slow $K_{off}$)

(b) Binding species 1 (fast $K_{off}$)
(c) Binding species 2 (slow $K_{off}$)

(d) Experimental data (binding species 1 + binding species 2)

- Binding species 1 (fast $K_{off}$)
- Binding species 2 (slow $K_{off}$)
Figure 2

a) RU vs time (s) for different molar ratios of Aβ1-42 alone and with EGCG:
- Aβ1-42 alone
- Aβ+EGCG (1:0.03)
- Aβ+EGCG (1:0.1)
- Aβ+EGCG (1:0.3)
- Aβ+EGCG (1:1)

b) RU vs EGCG : Aβ1-42 (molar ratio) for fast-dissociating and slow-dissociating species:
- Fast-dissociating species (monomers)
- Slow-dissociating species (oligomers)
Figure 3

![Graph showing the time-course of CL4176 and CL802 in induced and not-induced conditions. The x-axis represents time in seconds (0-600), and the y-axis represents RU (RU). The graph shows two main peaks: one for CL4176 induced and another for CL802. There are also markers for void volume and monomer peak, with corresponding lysate fractions.]
Figure 4

(a) Current (pA) at different time points:
- t=0
- t=5h
- t=24h

(b) Voltage (mV) vs. Current (pA) for different time points:
- t=0
- t=5h
- t=24h

(c) Conductance (pS) for different time points:
- Aβ t=0
- Aβ t=5h
- Aβ t=5h + 4G8
- Aβ t=5h + EGCG

** indicates statistical significance.
Figure 5

(a) The graph shows the pumping rate as a function of the Aβ1-42 incubation time. The pumping rate is expressed as a percentage of the corresponding vehicle. The graph indicates a significant decrease in pumping rate over time, marked by **.

(b) The graph depicts the inhibition of the pumping rate in relation to the concentration of Aβ1-42. The inhibition increases significantly with higher concentrations, marked by **.

(c) The bar graph compares the pumping rate under different conditions: Vehicle, Aβ1-42, 4G8, and Aβ1-42+4G8. The pumping rate is measured in pumps/min. The graph shows a significant decrease in pumping rate for Aβ1-42 compared to the vehicle, marked by **.

(d) The bar graph compares the pumping rate under different conditions: Vehicle, Aβ1-42, EGCG, and Aβ1-42+EGCG. The pumping rate is measured in pumps/min. The graph shows a significant decrease in pumping rate for Aβ1-42 compared to the vehicle, marked by **.
Figure 6

a) RU vs. time (s) for Aβ₁-₄₀ and WT with time points 0, 2, 8, 48 h.

b) RU vs. time (s) for Aβ₁-₄₀ and A₂V with time points 0, 2h, 8h, 48h.

c) Binding (RU) due to the slow-dissociating component over incubation time (h).

A2V - Vehicle
WT - WT

The graph shows the relative units (RU) of binding over time for Aβ₁-₄₀ and WT.

The graph shows the relative units (RU) of binding over time for Aβ₁-₄₀ and A₂V.

Time points include 0, 2h, 8h, 48h.

d) Bar graph showing pumping rate (pumps/min) for different conditions:

- Vehicle
- WT t=0
- A₂V t=0
- WT t=8h
- A₂V t=8h

The graph indicates a significant difference (**) between the conditions.
Specific recognition of biologically active amyloid-β oligomers by a new Surface Plasmon Resonance-based immunoassay and an in vivo assay in Caenorhabditis elegans

Matteo Stravalaci, Antonio Bastone, Marten Beeg, Alfredo Cagnotto, Laura Colombo, Giuseppe Di Fede, Fabrizio Tagliavini, Laura Cantu', Elena Del Favero, Michele Mazzanti, Roberto Chiesa, Mario Salmona, Luisa Diomede and Marco Gobbi

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