Binding of apolipoprotein E inhibits the oligomer growth of amyloid beta in solution as determined by fluorescence cross correlation spectroscopy*

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Background: ApoE is the most significant risk factor for Alzheimer’s disease, with known effects on Aβ deposition in the brain.

Results: ApoE binds to aggregating Aβ peptides and maintains a faster diffusion rate for the Aβ peptide over time.

Conclusion: Binding of apoE to Aβ slows the oligomerization of Aβ.

Significance: FCCS measurements quantify isoform-dependent differences in apoE binding to Aβ in solution.

ABSTRACT

One of the primary neuropathological hallmarks of Alzheimer’s disease is the presence of extracellular amyloid plaques resulting from the aggregation of amyloid beta (Aβ) peptides. The intrinsic disorder of the Aβ peptide drives self-association and progressive re-ordering of the conformation in solution, and this dynamic distribution of Aβ complicates biophysical studies. This property poses a challenge for understanding the interaction of Aβ with apolipoprotein E (apoE). ApoE plays a pivotal role in the aggregation and clearance of Aβ peptides in the brain, and the ε4 allele of APOE is the most significant known genetic modulator of Alzheimer’s risk. Understanding the interaction between apoE and Aβ will provide insight into the mechanism by which different apoE isoforms determine Alzheimer’s disease risk. Here we applied alternating laser excitation fluorescence cross correlation spectroscopy to observe the single molecule interaction of Aβ with apoE in the hydrated state. The diffusion time of freely diffusing Aβ in the absence of apoE shows significant self-aggregation, whereas in the presence of apoE, binding of the protein results in a more stable complex. These results show that apoE slows down the oligomerization of Aβ in solution, and provide direct insight into the process by which apoE influences the deposition and clearance of Aβ peptides in the brain. Furthermore, by developing an approach to remove signals arising from very large Aβ aggregates, we show that real-time single particle observations provide access to information regarding the fraction of apoE bound and the stoichiometry of apoE and Aβ in the complex.

INTRODUCTION

Alzheimer’s disease (AD) is a neurodegenerative disorder of aging that affects the cognitive ability of the brain. AD is characterized by two histopathological features of the brain: insoluble extracellular plaques comprised of amyloid beta (Aβ) peptides, and intracellular neurofibrillary tangles formed from hyperphosphorylated tau, a microtubule-associated protein. Although the primary cause and progression of AD are still not
well understood, they are thought to be linked to the aggregation of Aβ peptides. The Aβ peptides are generated as cleavage fragments by the action of γ and β secretases on the amyloid precursor protein, a constitutively expressed transmembrane protein. Due to their inherently disordered and “sticky” nature, the resulting Aβ peptides easily aggregate into oligomers, then fibrils, and finally, mature plaques in the brain.

To date, the ε4 allele of the apolipoprotein E (APOE) gene is the strongest known risk factor for the late-onset form of AD (1-4). The apoE protein is involved in lipid transport throughout the body, and is the principal lipid transport protein in the central nervous system. There are three apoE isoforms: E2, E3, and E4, and studies have demonstrated increased risk of AD and earlier age of onset in individuals carrying the ε4 allele. While the ε4 allele is linked to both sporadic and familial late-onset forms of AD, the mechanism of this association remains unknown. However, studies have revealed the presence of apoE in the amyloid fibrils and plaques of Alzheimer’s brains, which strongly suggests that apoE plays a critical role in the pathogenesis of AD through its interaction with aggregating Aβ peptides (5-8). It has also been established that apoE plays an important role in the homeostasis of Aβ in the brain, through its influence on both the deposition and clearance of the peptide (9-13). Emerging in vivo techniques in humans and other animal models further strengthen our understanding of the distributions of and relationships between Aβ and apoE in the brain (14,15). However, the interaction of Aβ with apoE is still poorly understood, with conflicting evidence with respect to differences in isoform interaction with Aβ (11). In addition, many previous studies were also designed with particular attention on isoform influence on amyloid burden rather than Aβ toxicity, and therefore focused on apoE associations with fibril/plaque species rather than the oligomeric forms of Aβ that are now recognized as the pathogenic species. As oligomeric Aβ represents a dynamic intermediate along the fibrilization pathway, it is very difficult to investigate this interaction and determine the affinity of apoE with Aβ directly in solution, particularly at the single molecule level. We therefore require insights into the oligomeric state of Aβ, its binding with apoE, and the distribution of these species across the system to understand how apoE influences Aβ deposition and clearance in the brain.

Fluorescence correlation spectroscopy (FCS) is a statistical technique to detect chemical reactions and determine translational and rotational diffusion coefficients of molecules and complexes (16-18). It is based on monitoring intensity fluctuations emitted from fluorescent molecules diffusing through a tightly focused laser excitation volume (~1 femtoliter). By subjecting these fluctuations to an autocorrelation analysis,

\[ C_2(τ) = \frac{⟨I(I+1)⟩}{⟨I⟩^2} \]  

the molecular diffusion time, sample concentration, and photophysical properties can be extracted. With precise knowledge of the diffusion time, τ₀, and beam waist, w₀, of the excitation laser spot, the diffusion coefficient, \( D = \frac{w₀^2}{4τ_D} \) can be determined which is proportional to the Einstein-Stokes hydrodynamic radius \( R_H = \frac{k_BT}{6πμD} \).

If two differently labeled species are in the sample, their colocalization can be monitored using fluorescence cross correlation spectroscopy (FCCS), originally developed by Schwille (19). FCCS has been applied to study binding events (20), enzyme kinetics such as oligonucleotide cleavage (21) and protease cleavage (22), and to monitor calcium activity in cells containing calmodulin (23). The addition of alternating laser excitation (ALEX) eliminates spectral cross-talk between fluorophores and reduces the possibility of false positives, because the different fluorophores are not excited simultaneously and their signals can be temporally separated. ALEX was first developed by Kapanidis et al. for fluorescence resonance energy transfer (FRET) measurements to determine the stoichiometry between biomolecules (24), and later extended to FCCS to eliminate cross-talk between two fluorescent proteins in cells (25), to monitor single molecule interactions (26,27), and also to antibody-based protein detection (28).

We apply ALEX-FCCS to investigate the interaction of apoE with Aβ in the hydrated state. The ability of this technique to report on the...
distribution of Aβ species, along with the binding of Aβ to other proteins, provides a powerful tool for studying the peptide’s interaction with apoE in the oligomeric state. To probe the molecular basis of apoE’s role in the development of Alzheimer’s disease, the E3 and E4 isoforms were selected as representative examples for this study. Because the fluorescent labeling required for this study takes advantage of thiol binding chemistry at a cysteine residue, it was necessary to avoid binding to the native cysteine residue found at position 112 in apoE3. Thus, we utilized the apoE3-like (apoE3L) protein, in which a serine is substituted for the cysteine at position 112. A thiol-reactive fluorescent label was then introduced to the C-terminal domain of apoE3L or apoE4 by replacing Trp264 with a cysteine residue. It has been shown that the cysteine substitution and subsequent modification of the W264C mutation of apoE with the thiol-specific label does not alter its predicted distribution among plasma lipoproteins, and circular dichroism analysis of the labeled protein is indistinguishable from the wild-type apoE (29-31).

EXPERIMENTAL PROCEDURES

Materials
Hexafluoro-2-propanol (HFIP) was purchased from Sigma-Aldrich (St. Louis, MO). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Pittsburgh, PA). Alexa Fluor 488 C5-maleimide was obtained from Invitrogen Molecular Probes (Carlsbad, CA) and Atto 647N NHS ester was obtained from Fluka Analytical, Sigma-Aldrich (St. Louis, MO).

Preparation of Amyloid β
Amyloid-β(1-40) peptide was purchased from Bachem (catalog number H-1194, Torrance, CA). The peptide was dissolved in HFIP and incubated at room temperature with gentle rocking for 48-72 hours. SpeedVac or evaporation was then used to remove the HFIP, resulting in a monomeric Aβ pellet. To direct preferential labeling of the N-terminal amine group of Aβ, a 0.1 mg aliquot of peptide was dissolved in 10 μL DMSO and reacted at pH 7.0 with 3 μL Atto 647N NHS ester label (10 mM stock in DMSO) and 500 μL phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.0). The mixture incubated for 1 hour at room temperature, after which it was washed 6 times with fresh PBS. After the final PBS wash was removed, HFIP was added to the labeled peptide and allowed to evaporate. The resulting pellet was stored at -20°C until use. Immediately before the experiment, the pellet was warmed to room temperature and dissolved in fresh DMSO to achieve a stock solution of 1 mM Aβ. To generate oligomers, the Aβ solution was then diluted into PBS buffer to a final concentration of 10 μM. The 10 μM solution was allowed to incubate at room temperature for 0-3 hours to produce oligomers. As demonstrated previously (29,32,33), these oligomeric preparations are A11-positive, prefibrillar oligomers (34), with a 10 μM solution producing particles of ~10 nm by AFM imaging.

Cloning, Purification, and Labeling of Apolipoprotein E
Human apoE4 contains no endogenous Cys, so site-specific Alexa Fluor 488 incorporation was achieved by substituting the native tryptophan at position 264 with a cysteine and reacting the purified protein with Alexa Fluor 488 C5-maleimide. In order to specifically target the fluorophore label to a –SH group in the C-terminal region of the apoE3 protein, a cysteine-free version of apoE3 was first generated by substituting the native Cys residue at position 112 with a Ser as described previously (29). This apoE3-like (apoE3L) gene was then used as a template for introducing a cysteine substitution at position 264 by PCR mutagenesis. Multiple lines of evidence indicate this apoE3-like protein serves as a reasonable mimic of apoE3. These include structural studies (reduced domain interaction (29)), LPS binding properties (35), and formation of a SDS-resistant complex with Aβ unique to native apoE3 (36). The gene encoding human apoE3L-W264C or apoE4-W264C was then cloned, expressed, and purified (29), with an addition of a single pass through a His-bind Ni(II) chelating column prior to the size exclusion. Labeling of apoE was accomplished by incubating the sample with 200 μM Alexa Fluor 488 C5-maleimide for 1 hour at room temperature in the presence of 100 μM TCEP to maintain reduced disulfides. Excess dye was removed by running the sample through a Bio-Spin 6 column (Bio-Rad, Hercules, CA). The labeled apoE was stored at 4°C and diluted into PBS buffer (pH 7.4) to obtain
the desired concentration immediately before the experiments.

**Instrumentation**

We conducted our experiments using a MicroTime 200 confocal fluorescence spectroscopy system (PicoQuant GmbH, Berlin) equipped with two pulsed diode lasers (470 nm and 640 nm wavelengths, ~80 ps pulse width) operating at a repetition rate of 20 MHz. The 640 nm laser pulse was delayed by 25 ns with respect to the 470 nm laser to produce alternating laser excitation (Figure 1A). The lasers were coupled into a polarization-preserving single mode optical fiber, recollimated and then focused to a diffraction-limited spot of ~250 nm diameter by an Olympus 1.45 NA 100x oil objective to a height of 5 µm above a glass coverslip surface. The average power of each laser was 50 μW at the sample. The fluorescence emission was split by a dichroic mirror (600DCXR, Chroma Tech. Corp., Bellows Falls, VT), spectrally filtered with emission bandpass filters (HQ520/40 m and HQ680/75 m, Chroma Tech. Corp., Bellows Falls, VT), and detected by two avalanche photodiode detectors (SPCM-AQR-14, PerkinElmer, Waltham, MA). The signals were processed by a time-correlated single-photon counting board (TCSPC board, PicoHarp300, PicoQuant, Westfield, MA), operating in time-tagged time-resolved (TTTR) mode. The TTTR mode of the data acquisition records the photon arrival time from the last excitation pulse (micro-time) with 50 ps relative time resolution, and the photon arrival time from the start of the experiment (macro-time) with 100 ns absolute time resolution. TCSPC of separate detection channels allows for the temporal analysis of all detected photons. In autocorrelation analysis, the number of molecules N in the excitation volume is inversely proportional to the amplitude of the autocorrelation function G(0), whereas in cross correlation analysis, the number of bound molecules N_{XY} is proportional to G_{XY}(0) in the volume. By analyzing the auto- and cross correlations, we can resolve signals from only the truly bound species. Time traces of both Aβ and apoE3L are shown in Figure 1B.

The cross correlation signal from freely diffusing fluorescent molecules illuminated by two excitation lasers is:

\[ G^{(2)}(\tau)_{XY} = \frac{\langle C_{XY}\rangle \langle D_{XY}(\tau) \rangle}{\langle D_{XY}(0) \rangle} \]

where the term:

\[ D_{XY}(\tau) = \left(1 + \frac{\tau}{\tau_{D,XY}}\right)^{-1} \left(1 + \frac{\tau^2}{\tau_0^2} \right)^{-\frac{1}{2}} \]

denotes the temporal decay of the cross correlation function by the bound molecule with diffusion time \(\tau_{D,XY}\). \(C_X\) and \(C_Y\) are the concentrations of free X and Y molecules, and \(C_{XY}\) is the concentration of bound molecules.

At lag time \(\tau = 0\), equation (1) can be rewritten as (21):

\[ G_{XY}(0) = N_{XY} \left[ G_x(0) * G_y(0) \right] \]

where \(G_x\) and \(G_y\) are the autocorrelations of channels x and y. In autocorrelation analysis, the number of molecules N in the excitation volume is inversely proportional to the amplitude of the autocorrelation function G(0), whereas in cross correlation analysis, the number of bound molecules \(N_{XY}\) is proportional to \(G_{XY}(0)\) in the volume. By analyzing the auto- and cross correlations, we can resolve signals from only the truly bound species. Time traces of both Aβ and apoE3L are shown in Figure 1B.
correlation amplitudes, the number of bound molecules can be determined.

Aggregate Removal Algorithm
One problem encountered in taking accurate FCS measurements of Aβ is the presence of extremely large aggregates resulting in huge fluorescent bursts. These aggregates are most likely from Aβ that has formed large oligomers and are not necessarily a true representation of the average particle size in our sample. The large aggregates may also result from the tendency of Aβ to stick to glass surfaces, such as those used for the experimental measurements. Regardless of the cause, the large fluorescent bursts detected can skew the results of our analysis. As an example, Figure 1C shows a large Aβ aggregate with a burst size of almost ten times the average signal. To eliminate these aggregates from our data, we implemented a custom algorithm that cuts a portion of the intensity time trace when photon burst counts larger than five times the average signal are observed. The remaining portion of the time trace is then stitched back into the original time trace for photon correlation analysis.

RESULTS AND DISCUSSION

Kinetics and stoichiometry of the binding reaction-
Although it is not as toxic as the Aβ(1-42) species, Aβ(1-40) induces a similar, albeit attenuated pathology in neurons. We limited our initial study to Aβ(1-40) because it demonstrates more predictable behaviors, including lower surface affinity, and has a slower aggregation rate in solution. To establish the binding interactions between Aβ and apoE3L, we used a sample solution consisting of 10 μM Aβ and 10 μM apoE3L. Because micromolar Aβ in solution undergoes a process of oligomerization (33,37,38), a series of time measurements were performed at time zero, 15 minutes, 30 minutes, 1 hour, 3 hours, and 4 hours after introducing Aβ into solution with and without apoE3L protein. For each FCCS measurement, a small volume of this sample was diluted to less than 1 nM in PBS to ensure that the excitation volume contained at most one molecule per laser pulse, which gives a high signal to noise ratio. The data were recorded for five minutes at each time interval. To obtain an accurate statistical error distribution, the whole time series experiment was repeated five times with five independent Aβ-apoE3L samples.

The measurement taken immediately following the dilution (time zero) reveals very little correlated signal from the two probes, reflecting initially weak binding between Aβ and apoE3L. This is evident by the flat black cross correlation curve in Figure 2A. As the reaction is monitored over time, the amplitude of the cross correlation slowly increases from a value of G(0) = 0 during the initial measurement to G(0) ≈ 0.55 after 4 hours, clearly indicating binding between Aβ and apoE3L. Additional measurements were taken over a course of 48 hours, but no significant change in the correlation amplitude was observed, indicating that equilibrium was established.

Following the kinetic assessment of the binding reaction between Aβ and apoE3L over time, we determined the fraction of Aβ and apoE3L that bind to each other to form the complex with both molecules added at a concentration of 10 μM. At time 4 hours, the autocorrelation was fitted to values of G_{Aβ}(0) ≈ 3.7 and G_{apoE3L}(0) ≈ 5.2, which are inversely proportional to the number of Aβ and apoE3L molecules in the excitation volume, or N_{Aβ} ≈ 0.27 and N_{apoE3L} ≈ 0.19. The cross-correlated value is G_{Aβ/apoE3L}(0) ≈ 0.59 (Figure 2B). Solving equation 2 with these values yields N_{Aβ/apoE3L} ≈ 0.03, the number of fully bound particles detected in the excitation volume. This implies that approximately N_{fraction bound Aβ} = \frac{N_{Aβ/apoE3L}}{N_{Aβ}+N_{Aβ/apoE3L}} ≈ 10.0 ± 3% of the total Aβ concentration and N_{fraction bound apoE3L} = \frac{N_{Aβ/apoE3L}}{N_{apoE3L}+N_{apoE3L/Aβ}} ≈ 13.6 ± 3% of the total apoE3L concentration form a binary-complex species. It should be noted that although Aβ and apoE have a single fluorophore attached, the diffusion bound complex has variations in its number of apoE and Aβ proteins, particularly the latter from self-aggregation. Due to the way the cross correlation is calculated, any such inhomogeneity can result in a lower apparent fraction bound as the free Aβ available for binding becomes much lower than the free apoE. It should also be noted that the amount of oligomeric Aβ in
the system is significantly lower than the total peptide added. Thus the values for apparent dissociation constants (below) should be considered as upper limits.

Diffusion rate of the binary complex-

Next, we compared the diffusion time of the unbound Aβ and apoE3L to the bound species (Figure 2C) by analyzing the normalized correlation data. In principle, if every Aβ molecule binds to every apoE3L molecule, then the autocorrelation curves for the two channels would be identical. However, because the red channel measures both free and bound Aβ (Figure 2C, red) and the green channel contains data for the mixture of free and bound apoE3L (Figure 2C, green), at equilibrium we expect the two autocorrelation curves to be similar but not identical. Analysis of the autocorrelation signal at 4 hours provides average diffusion times of 110 μs for apoE3L and 100 μs for Aβ. This corresponds to a hydrodynamic radius of ~1.6 nm for both apoE3L and Aβ. By using cross correlation spectroscopy to analyze the signals from both channels arriving within a very short time interval, signals from the free proteins can be separated from that of the bound complex. The cross-correlated signal, representing a complex of Aβ-apoE3L, had a diffusion time of approximately 2 ms (Figure 2C, black) with an average hydrodynamic radius of 27 nm, which suggests that the complex in solution forms from the cooperative association of more than one apoE3L and Aβ oligomer. The formation of large, multimeric complexes of apoE and Aβ is consistent with our previous observations of apoE structure upon Aβ binding (29).

Comparison of apoE isoforms-

As described earlier, the relevance of the Aβ-apoE interaction was first recognized due to the increased AD risk for individuals carrying the ε4 isoform allele of apoE. We therefore investigated the binding of Aβ as a function of apoE concentration for both the E3L and E4 proteins by FCCS (Figure 3). In these experiments, we fixed the concentration of Aβ at 10 μM while adjusting the concentration of apoE3L or apoE4 to 5 μM, 10 μM, 20 μM, and 40 μM. The correlation spectroscopy data were acquired at uniform incubation times (2 hours) for all mixtures. The fraction of Aβ bound to either apoE3L or apoE4 was calculated from the correlation data as described in the previous section. The results indicate the E3L protein has a substantially higher affinity for oligomeric Aβ compared to apoE4. The slightly cooperative concentration-dependence is consistent with the notion that apoE3L binding to Aβ results in a complex that involves more than one apoE molecule.

Previously, we used surface plasmon resonance (SPR) and EPR spectroscopy of site-directed spin labels to explore the affinity of these two principal isoforms with oligomeric Aβ (29). While SPR utilizes an immobilized substrate, both species are free in solution with measurements by FCCS and EPR. The Aβ-apoE association by EPR can be observed on the basis of (i) changes in the rotational diffusion (both local and global) and (ii) dipolar coupling via increased self-association of apoEs C-terminal domain in the presence of oligomeric Aβ. However FCCS has clear advantages over EPR for detecting the Aβ-apoE complex as both rotational and translational diffusion can be measured and also well separated as these two effects occur at very different timescales. Furthermore, the sampling of single particles in FCCS provides superior resolution regarding the heterogeneity of the species in solution.

We then compared the hydrodynamic radius of the bound particle to free Aβ incubated in the absence of apoE3L and apoE4 (Figure 4). Aβ is known to form soluble oligomers that are conformationally and pathologically distinct (34,37). Under the conditions employed here, Aβ oligomers assemble into relatively disordered peptides defined as prefibrillar oligomers (37). Monomeric Aβ has a hydrodynamic radius of approximately 0.7 nm as measured by FCS. Rapid aggregation of 10 μM Aβ over the course of the first 2 hours resulted in many large particles approaching 100 nm hydrodynamic radius, which then dissociated afterwards to an average size of 60 nm at around 4 hours. This is consistent with previous measurements of Aβ aggregation (37,38). Although the basis for this partial disassembly is unclear, it may be related to a reorganization of the
prefibrillar oligomer, including the elimination of antiparallel interactions (38).

The change in hydrodynamic radius is markedly different when an equal amount of apoE is present (Figure 4). When mixed with the apoE3L protein, the average bound complex particle size slowly approaches 27 nm with no observable dissociation into smaller particles. This suggests that apoE3L, when bound to Aβ, forms a more stable complex and interferes with Aβ’s ability to form larger oligomers. Inclusion of apoE4 also results in smaller hydrodynamic radii over time, although to a lesser extent than is achieved by apoE3L. As shown in Figure 4, incubation of Aβ+apoE4 results in an average particle size of 46 nm at 4 hours, a size midway between Aβ+apoE3L and Aβ alone. The basis of this distinction is not clear, however the lower Aβ affinity for apoE4 may relate to a diminished ability to arrest Aβ oligomerization.

Although the interaction of apoE and Aβ as detected by FCCS does not reflect submicromolar affinity, apoE is known to form strong, SDS-resistant complexes with Aβ under conditions similar to those employed here (36,39). As mentioned above, we estimate apoE affinity for Aβ based on the total peptide in the system, although the concentration of oligomeric species is considerably lower. The dissociation constants calculated here represent relative upper limits and provide a useful mechanism to compare the affinity for Aβ between apoE isoforms. Equilibrium binding measurements are further hindered by the increased aggregation encountered with employing higher levels of Aβ and/or longer incubation times. Due to the inherent self-assembly of Aβ, FCCS measurements of Aβ binding are most useful for determining differences in initial rates, rather than steady states. Nevertheless, the reduced Aβ binding and polymerization rate seen with apoE4 is significant in a physiological context. Low affinity interactions have significant physiological relevance, as competition among relatively weak interactions can have a profound consequence in age-dependent diseases. For example, the balance between low affinity metal ions has been proposed to influence the degree of Aβ toxicity over a period of decades (40). In addition, elevated local concentrations of apoE draw physiological connections to our findings. In response to injury neurons rapidly release large amounts of apoE (41), where its accumulation in the extracellular matrix (42) results in effective concentrations exceeding the levels applied here. Future FCCS work will also consider the interaction of lipid-bound apoE isoforms and Aβ using FCCS, as previous measurements have shown a higher affinity for oligomeric Aβ when the protein is assembled in HDL-like lipoprotein particles (29). Such measurements will demand additional analysis, as Aβ has the potential to interact with the lipid phase alone in such particles. However previous experience (43,44) in using FCS and photon antibunching to determine lipoprotein particle size and protein stoichiometry will be helpful in this pursuit.

Consequences of apoE association-
We have shown that the presence of the apoE3L protein retards the progression of Aβ monomers into oligomers. If such an effect occurs in vivo, it may correlate with a packaging of Aβ to facilitate its clearance from the brain (45). We and others have previously postulated that reduced binding of apoE4 to oligomeric Aβ may correlate with a loss of apoE protection with regard to Aβ clearance (29,45-48). The ability of ALEX-FCCS to provide a quantitative measure of apoE binding to Aβ and also Aβ assembly will be helpful in not only elucidating the molecular mechanism of Aβ pathogenicity and protection, but also to identify factors that influence these processes.

Conclusion-
The ε4 allele of the APOE gene represents the most significant genetic risk factor for AD (49-51). The differential ability of apoE isoforms to interact and clear Aβ is likely key to the mechanism of the isoform influence on AD (12,29). An accumulating body of evidence demonstrates just how vital a role apoE plays in the aggregation and clearance of Aβ peptides in the brain (9,45,52). This dynamic process presents an intriguing point of intervention for rational therapies designed to prevent and/or delay the progression of AD pathology, but in order to approach this question it is first necessary to
understand the precise interactions of apoE with Aβ as they relate to the deposition and clearance of Aβ peptides. Although other methods can detect both Aβ binding and oligomerization (33,37) they are limited in their ability to describe the size and stoichiometry distribution of species in the system. We have shown using ALEX-FCCS that apoE inhibits the oligomerization of Aβ in the hydrated state. We have also demonstrated the ability of this method to report on the size and composition of biological complexes in solution, therefore providing a powerful tool for unraveling the molecular interaction of Aβ with apoE in Alzheimer’s disease. Furthermore, since the entropically-driven growth of Aβ oligomers is an indicator for a high-energy (and likely pathogenic) state of the peptide, ALEX-FCCS provides a quantitative approach for the real-time, in-solution evaluation of small molecules that can modulate apoE interaction and/or the stability of Aβ.

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Figure Legends

Figure 1. A) Alternating laser excitation with two pulsed diode laser sources. The 640 nm laser pulse was delayed by 25 ns with respect to the 470 nm laser to produce alternating laser excitation with a total repetition rate of 40 MHz for both lasers taken together. The emission of the red fluorophore (Atto 647) after 640 nm excitation is shown by the red decay curve, and similarly, emission of the green fluorophore (Alexa 488) after 470 nm excitation is shown by the green decay curve. Time gating (black dotted lines) allows us to remove leakage of the green fluorophore into the red channel. B) Intensity time traces recorded for two minutes produced by direct excitation of apoE3L with the 470 nm laser (top) and direct excitation of Aβ by the 640 nm laser (middle). Since all emitted fluorescence photons contain a time-tag with respect to which laser excitation produces them, only photons that overlapped in time (bottom) are used to calculate their cross correlation. C) Intensity time trace of a sample containing a large Aβ aggregate with photon burst count ten times larger than the average signal.
Figure 2. A) Progression of cross correlation curves for a mixture of 10 μM Aβ and 10 μM apoE3L over time. The degree of binding between the two molecules determines the amplitude of the cross correlation. The initial reaction of Aβ and apoE3L at time zero shows low correlation, indicating very weak binding. The degree of binding increases as time progresses, which is shown as a rise in the cross correlation amplitude up to time 4 hours. B) Amplitudes of the autocorrelations of the Aβ and apoE3L signals and their cross correlation at time 4 hours. C) Normalized auto and cross correlation at time 4 hours. Square dots denote raw data. Solid lines denote fitted data. (AC)-autocorrelation. (CC)-cross correlation.
Figure 3. Differential binding of the apoE isoforms to oligomeric Aβ in solution. Binding was determined by the fraction of soluble signal from particles bearing both Aβ and apoE as detected by FCCS. The fraction bound was calculated as described in the text with error bars representing the standard deviation of 5 measurements. Each datum represents the indicated concentration of either apoE3L or apoE4 combined with 10 µM Aβ and measured after a 2-hour incubation. Data were fit to a logistic equation $B = B_{\text{max}} \times ([L]_n^a/(K_n^a+[L]_n^a))$, giving an apparent dissociation constant (K) of 29.9 and 40.4 for apoE3L and apoE4 binding, respectively. The resulting Hill slope (n) and the $B_{\text{max}}$ for apoE3L are 1.4 and 0.81, respectively. $B_{\text{max}}$ and n for the apoE4 data are 0.48 and 1.1, respectively.

Figure 4. Bar chart of the hydrodynamic radii at different reaction times as measured by FCCS. At time 0, Aβ has a hydrodynamic radius of 0.7 nm, which increases to over 60 nm after a 4 hour reaction (blue). After a 4-hour reaction in the presence of apoE3L or apoE4, the bound complex has a size of 28 nm or 46 nm, respectively. Note that at time zero, there is no binding between the two molecules and therefore the red and blue bars have been omitted.
Binding of apolipoprotein E inhibits the oligomer growth of amyloid beta in solution as determined by fluorescence cross correlation spectroscopy
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