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A hybrid structural method for investigating low molecular weight oligomeric structures of amyloid beta

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Abstract

Spurred in part by the failure of recent therapeutics targeting amyloid β plaques in Alzheimer's Disease (AD), attention is increasingly turning to the oligomeric forms of this peptide that form early in the aggregation process. However, while numerous amyloid β fibril structures have been characterized, primarily by NMR and cryo-EM, obtaining structural information on the low molecular weight forms of amyloid β that presumably precede and/or seed fibril formation has proved challenging. These transient forms are heterogeneous, and depend heavily on experimental conditions such as buffer, temperature, concentration, and degree of quiescence during measurement. Here, we present the concept for a new approach to delineating structural features of early-stage low molecular weight amyloid β oligomers, using a solvent accessibility assay in conjunction with simultaneous fluorescence measurements.

Graphical Abstract

Concept for a new hybrid characterization assay: Use inline fluorescence collection immediately prior to X-ray exposure to simultaneously characterize the evolution of residue-specific interactions and the evolution of global conformation along the amyloid aggregation timeline.

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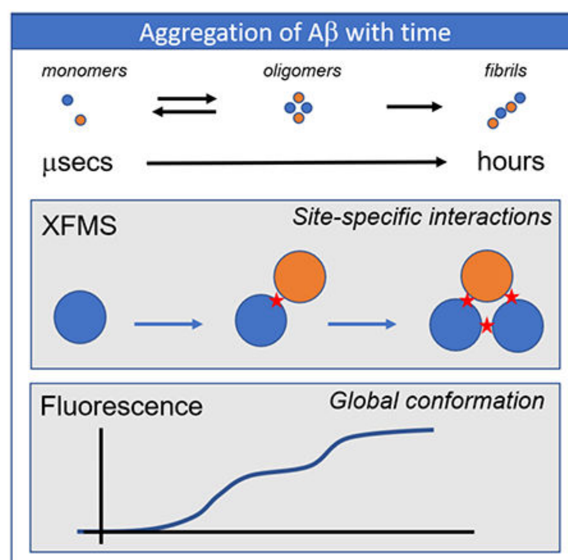

Keywords

amyloid beta; Alzheimer's Disease therapeutics; hydroxyl radical footprinting; hybrid methods; oligomeric structure

Background and motivation

Amyloid β (Aβ) peptides of various lengths are endogenous human peptides formed from the enzymatic cleavage of amyloid precursor protein (APP).[1] Excess production of this peptide has been extensively studied as a potential causal agent in cognitive decline in Alzheimer's Disease (AD) progression, since the end-products of Aβ peptide aggregation, commonly referred to as fibrillar forms, are found in AD brain plaques.[2] Both fibrils and soluble oligomers demonstrate cell toxicity *in-vitro*; however, a link has not yet been established between Aβ fibrils and progression of AD. Further, to date no therapeutics targeting amyloid fibrils have been successful in mitigating the cognitive decline observed in the progression of AD.[3] Aggregation of the Aβ peptide follows a pathway in which very low molecular weight oligomers presumably form early in the aggregation process and precede the formation of higher molecular weight oligomers, protofibrils and insoluble fibrils or plaques. These oligomeric forms are sometimes referred to as "on-pathway" or "off-pathway" to indicate whether they end in a trapped fibrillar state or dissociate back into monomer form.[4] Several fibril structures have been solved using cryoEM,[5] NMR,[6] microED,[7] and in some cases for truncated peptide versions, crystallography.[8] Solution state NMR has been applied with some success to the study of oligomeric forms of the peptide, albeit at relatively high concentrations,[9] and biophysical assays such as fluorescence, dynamic light scattering and CD can yield global structural information on larger oligomeric forms in solution.[10] Atomic Force Microscopy (AFM) has been used to characterize the morphology of multi-peptide structures within the first several minutes of aggregation;[11] these studies yield valuable information but typically require immobilization or dehydration steps and so may not reflect the dynamic nature of Aβ aggregation. To

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date, structural information on the earliest stage oligomeric forms of the peptide have been obtained using solvent accessibility assays, and these are the methods on which the conceptual method presented here is based. Specifically, application of fast photochemical oxidative footprinting (FPOP) determined solvent accessibility of specific residues within Aβ42 over a timeline of 2 minutes to 48 hours during aggregation, and was used to propose a multistage nucleation model of fibril formation.[12] Another solvent accessibility assay, hydroxyl radical footprinting, was used to investigate the relative protection of specific residues in the prefibrillar and the fibril states of Aβ40 peptide, yielding a model of peptide packing in the fibrillar state that validated solid state NMR models.[13] Hydrogen deuterium exchange (HDX), based on solution state accessibility of peptide backbone hydrogens, has also been successfully applied to the study of Aβ40 and Aβ42 conformation changes during aggregation, yielding information on stretches within the peptides that become solvent protected at different rates during aggregation.[14] Despite these important studies, there remains very little detailed structural information on low molecular weight oligomers of this peptide in the solution state, such as might be used for therapeutic development. The conceptual hybrid structural assay that we present here builds on previous solvent accessibility studies and could be applied to the characterization of low molecular weight oligomeric forms of peptide in low concentrations. The proposed method integrates a global structure fluorescence assay with the residue-specific structural characterization X-ray hydroxyl radical footprinting assay. The method could be used over a wide range of peptide concentrations, could allow characterization of aggregation timepoints in the millisecond regime, and would enable direct comparison of label-free peptide aggregation with ThT or other fluorescent probe preparations.

The structural footprinting assay

Protein footprinting is a structural biology method based on determining the relative changes in the degree of solvent accessibility of regions or single residues within proteins from one state to another. This change in solvent accessibility, in turn, is used to determine structural information. For example, ligand binding in a protein pocket often occludes water, and detecting the residues that become inaccessible to water during ligand binding pinpoints the ligand-protein interaction region. Similarly, bound water in a protein membrane channel will shift position as the channel activates, and those shifts can be used to infer channel opening/closing or other internal structural rearrangements.

Solvent accessibility maps can be determined through several different means, including protease-based footprinting,[15] hydrogen-deuterium exchange,[16] or hydroxyl radical (·OH) based modifications.[17] Of these methods, an ·OH-based assay offers several advantages. The small size of the ·OH molecule means that the “footprint” occurs at the single-residue level. The modifications made are irreversible and covalent, and can thus withstand a range of buffer conditions post-processing, in contrast to exchange-based methods, in which dynamics of water exchange must be preserved using temperature or pH. The high reactivity and short lifetime of ·OH in solution enables short exposure to the radical without major perturbation to the protein. This enables fast timescale kinetics experiments, down to a microsecond, and allows a fast liquid sample delivery method, which can be leveraged to design hybrid methods, as described below. Hydroxyl radicals can be produced

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through various means, including chemical methods such as Fe-EDTA chemistry,[18] or radiolytic methods such as electron,[19] gamma[20] or UV laser methods,[21] and relatively recently through plasma-based[22] and synchrotron X-ray based generation.[23] All these methods also require downstream LCMS analysis to determine locations of modification. Here, we use the term X-ray Footprinting with Mass Spectrometry (XFMS) to refer to X-ray generation of hydroxyl radical in the footprinting assay. With this method, the synchrotron X-ray energy deposited in aqueous buffer is absorbed by water molecules, generating ·OH, electrons and secondary radical species.[24] The ·OH molecule and electrons are the most reactive of these to protein sidechains, and ·OH in particular generates covalent modifications to sidechains, with the most common modifications including an -OH or carbonyl addition, with some dependency on dissolved oxygen content in solution.[24–25]

Simultaneous global and local structural characterization

Because sufficient, measurable hydroxyl radical modification can occur within microseconds of exposure to an X-ray beam, we recently developed a liquid jet sample delivery system capable of microsecond exposures.[26] With this system, a syringe pump connected to a jet nozzle pushes sample with a controlled velocity past an X-ray beam, and the speed of the liquid determines the length of X-ray exposure. The microsecond exposure times limit secondary damage, yield significantly better signal-to-noise data than with longer exposure times, and allow interrogation of nanomolar to micromolar concentration samples. With the further implementation of a second syringe pump and a mixing cell, this configuration could be used to initiate aggregation and collect timepoints along an aggregation pathway starting at hundreds of microseconds. Further, the liquid stream delivery can be leveraged to introduce laser excitation and measurement of fluorescence inline immediately prior to X-ray irradiation (Figure 1).

In this experimental configuration, the delay time between fluorescence excitation/detection and X-ray irradiation could be as short as 10 microseconds. This method can be applied to proteins along an aggregation pathway. For instance, Aβ can be run through the experimental set up at timepoints ranging from microseconds to hours. At each aggregation timepoint, fluorescence spectra can be collected immediately prior to X-ray irradiation, providing a near-simultaneous global structural readout from the fluorescence assay and local residue structural information from the XFMS assay. Intrinsic fluorescence from tryptophan can be measured this way,[10h] or extrinsic fluorescence from an added fluorophore, such as Thioflavin T (ThT), a standard assay for measurement of fibril formation during Aβ aggregation.[27] In practice, samples can be delivered alternately from two syringe pumps, one containing protein in buffer, and one containing protein plus ThT. Therefore, this allows parallel comparison between structural changes of Aβ with and without ThT in solution, as well as structural changes at earlier timepoints than is possible to measure with the ThT assay alone. Further, this ensures that all structural data are obtained under identical conditions, both in time and in the sample environment. This hybrid method to simultaneously collect global and residue-level structural information will be useful for many protein systems, but has particular relevance for studying aggregation of the Aβ peptide. Each time an Aβ peptide sample is prepared, conditions such as protein concentration, buffer constituents, and temperature will necessarily vary, and even small

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differences in these conditions affect aggregation kinetics and timeline. This new proposed hybrid approach circumvents these issues in batch-to-batch variability of aggregation which has made this peptide so difficult to study, and provides a method to combine residue-specific structural information nearly simultaneously with global protein structure readouts from either intrinsic protein fluorescence or fluorophores such as ThT.

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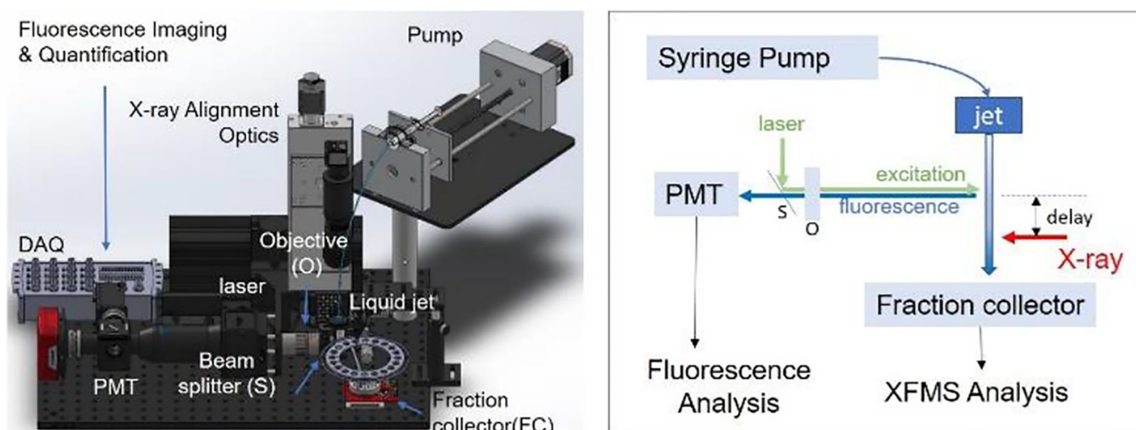

Figure 1.

Conceptual hybrid XFMS and fluorescence experiment for characterizing Aβ oligomers throughout the aggregation pathway. CAD design is shown on left, and conceptual model on right. The protein solution is delivered via a syringe pump to a liquid jet nozzle and into the fraction collector. As the sample streams into the collector, excitation light from a laser impinges on the sample, and fluorescence is collected back along the same optical path. A beam splitter (S) is used to reflect excitation light and transmit fluorescence. An objective (O) is used to focus the excitation onto the liquid stream, and to focus fluorescence onto the photomultiplier tube (PMT). PMT voltage is converted in a Data Acquisition module (DAQ) for fluorescence analysis, while collected samples are processed for downstream LCMS analysis.