Atomic Force Spectroscopy for Testing Synthetic Amyloid-β Aggregation Inhibitors

University of Waterloo

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Introduction

Alzheimer's Disease Facts and Figures:

In 2010, estimated 36 million cases worldwide with 6 million in US and 500,000 in Canada. Resulting in an estimated economic cost of $608 billion.

Increases in AD are expected to double every 20 years, 110 million by 2050 for a total projected cost of 20 trillion dollars (US only figures).

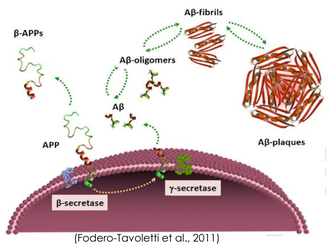
No cures, therapeutics to slow disease or conclusive early diagnostic techniques

The cause is not known, although several hypothesis have been proposed and may be at work in tandem.

Due to failures of many AD clinical trials in the last year, new drugs designed to prevent AD, before onset of symptoms appear to be the way forward.

Amyloid-β Hypothesis

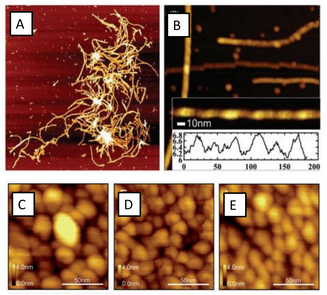
The appearance of amyloid plaques in AD patients implicated Aβ as the primary cause of AD pathology, and led to the first formulation of the Amyloid Cascade Hypothesis. It is now believed that an imbalance between amyloid production and clearance is responsible for the deposition of amyloid in the brain. This results in accumulation of toxic amyloid beta oligomers, fibrils and plaques. Initially it was believed that reducing total amyloid beta burden is crucial for reversing or slowing disease progression. However after more research it was found that plaques and fibrils(A & B below) are less toxic, while early oligomers (C-E below) are the more toxic species. This modification of the amyloid cascade theory presents early aggregates as the prime target in treating AD.



(Fodero-Tavoletti et al., 2011)

Aβ is a protein fragment from the native membrane spanning protein called the Amyloid Precursor Protein(APP). APP is cleaved by specific enzymes important for various neurophysiological functions to produce the Aβ monomer. Aβ itself is believed to be important for healthy brain function and is present in normal individuals, with one study suggesting that Aβ is neuroprotective at low concentrations. Aβ is its own ligand; it misfolds, and self aggregates to form toxic soluble species that cause neurodegeneration. Amyloid oligomers are known to interact with lipid domains which disrupt membrane integrity, cause oxidative stress, disrupt synaptic plasticity and eventually cause cell death.

The amyloid cascade hypothesis has yet to be verified through human clinical trials which target Aβ. The failures of these Aβ targeting drugs in clinical studies have led to speculation. Despite their ability to remove amyloid burden they have not been able to improve cognition in AD patients. Although it is expected that Aβ neurotoxicity must be addressed for the effective treatment of AD, it is not clear that Aβ is the primary insult and some speculate that it may be a secondary cause of neurodegeneration. This problem is one of the largest issues that must be resolved in AD research.



Drolle et al., 2014; Moores et al., 2011

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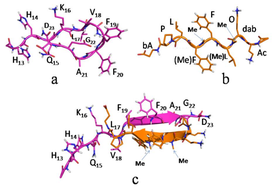
Results

Amyloid-β Aggregation inhibitors:

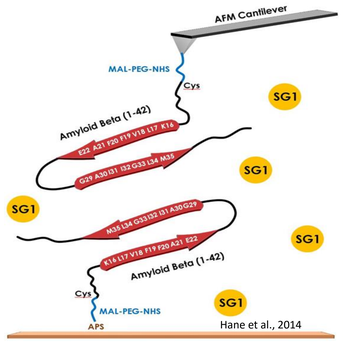
Inhibitor drugs are designed to bind to the amyloid beta self-recognition site Aβ(16-23) using Molecular Dynamics simulations. Ideally inhibitors will prevent dimerization, a critical step in preventing toxic aggregation. Leading clinical trials that engage Aβ as a target use monoclonal antibodies (MAb) for increasing AB clearance, however with limited success so far. Peptide inhibitors are an alternative strategy with important advantages (such as improved safety, smaller size, and ease of modification) as clinical paradigms shift to prevention of amyloid neurotoxicity in pre-dementia patients.



Single Molecule Force Spectroscopy:

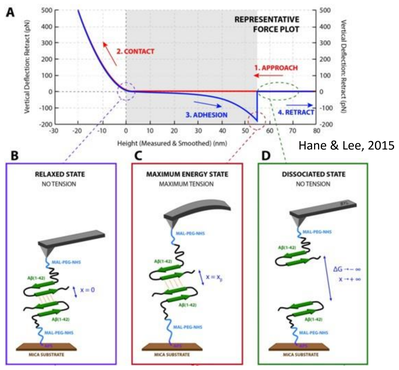
One can use an Atomic Force Microscope (AFM) to measure binding events between two peptides by functionalizing the tip and mica substrate with Aβ. This allows for a direct measure of AB dimerization, the critical step in Aβ aggregation. By adding molecules which mediate or affect Aβ oligomerization we can test their effects on dimer formation. Peptide inhibitors designed to bind specifically to amyloid are expected to block dimer formation therefore they should reduce the total yield of binding events over the course of many force measurements.

As the amyloid molecule attached to the probe approaches an amyloid molecule on the substrate it will interact and bind to form a dimer. In the presence of SG inhibitor molecules this interaction will be less likely to take place. After running several hundred scans it is possible to count a statistical yield of binding events and compare with control scans where no inhibitor is present. This technique is very sensitive as average forces are on the nanoscale and as such has a high error. Its speed, and simplicity are advantageous, allowing for a quick screen of effective inhibitors.



Hane et al., 2014

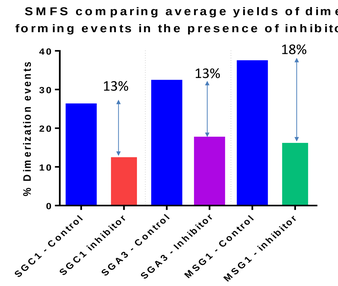
Data



Hane & Lee, 2015

- Cantilever deflection during the different portions of a force measurement are shown on the cartoon below.
- A positive binding event exhibits a worm-like chain followed by a steep vertical drop (unbinding event) and is depicted on the representative force plot below.
- For the amyloid-amyloid system we expect that the unbinding event should occur at 40-70 nm above the substrate.
- Interested in only dimer forming events with a single worm-like chain.
- Multiple unbinding events suggest higher order structure has formed, these interactions are more complicated and a model to analyze them has not yet been explored

Analysis



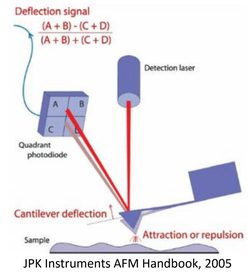
The SMFS experiments with surface modified amyloid shows reductions in dimerization events for 3 different inhibitors, at a concentration of 40nN. Controls, in buffer, followed by force measurements with inhibitors were performed for each tip sample trial, consisting of 625 individual force plots. Six trials for each inhibitor were performed in total.

It was typical to have 1 or 2 data sets per inhibitor that yielded inconsistent results, typically as a result of too few binding events or because of too much higher order structure interference. Although protocols are carefully establish to isolate individual amyloid peptides, this error highlights the sensitivity of single molecule biophysics which is especially challenging for studying peptide-peptide interactions when the surface area of the two ligands are comparable in size.

Methods

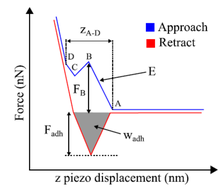
Atomic Force Microscopy:

Atomic Force Spectroscopy (AFM) utilizes a nano-sized probe tip attached to a flexible cantilever arm to measure the surface topology of a particular sample. As it scans across the sample the force of interaction between the surface and the tip is measured via a feedback system. A detection laser is deflected off the cantilever arm and strikes a photodiode, the deflection is then measured. In it's simplest mode of operation the piezo (which the cantilever is mounted on) is then fed a voltage to raise or lower the cantilever, keeping it at a constant height above the sample. This voltage is then converted into a distance using the known electromechanical properties of the piezo device.



JPK Instruments AFM Handbook, 2005

Force Spectroscopy:



Choi Y. & Attwood S.

One realized potential of AFM technology is the ability to measure the force which is exerted between the probe and the sample. This can be used to test the properties of various materials such as lateral friction, breakthrough and adherent forces (van der waals and adhesion). One very important set of systems that can be analyzed using force spectroscopy is biological ligand-ligand interactions, specifically binding/unbinding forces between them. This can be used to test receptor-ligand interactions, ligand-cell, and cell-cell interactions.

Surface Modifications:

Silicon nitride AFM tips and mica substrates were modified with PEG linked Aβ, covalently, in a stepwise fashion, with critical washing steps in between:

1. After washing tips and cleaving mica, they are silanized with amino propyl silatrane (APS) to form covalent bonds for subsequent modifications.
2. Next Mal-PEG-SVA is attached to APS, increasing the pull-off distance for single event detection. Specific ligand-ligand interactions are easier to detect at longer pull off distances then non-specific, tip-substrate adhesion.
3. Cysteine terminated Aβ is than conjugated to the other free maleimide (MAL) on the PEG linker.
4. Lastly, the system is quenched with beta-mercaptoethanol to saturate the unlinked MAL groups on the PEG linkers.

Conclusions

- We have demonstrated that SMFS can be used as a useful screen technique for testing the ability of inhibitors to prevent dimerization of amyloid species, this may be applied to other amyloidogenic peptide species for testing novel compounds.
- We have also shown that SG inhibitors block dimer formation as a step toward further molecular, cell and animal studies. These are necessary for realizing their clinical potential in treating AD.

Acknowledgements

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