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METALLODITHIOLATE LIGANDS FOR REVERSING METAL ION INDUCED AGGREGATION OF BETA AMYLOID

A Honor’s Thesis

by

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Major Subject: Health Sciences
ALZHEIMER’S DISEASE (AD) is the sixth leading cause of death in the United States with no known methods to cure, reverse or halt disease progression. The “metal hypothesis” states that Cu$^{II}$, Fe$^{III}$, Zn$^{II}$ and Al$^{III}$ bind to truncated beta amyloid (Aβ) peptides and form soluble oligomers which deposit as senile plaques. These plaques play a crucial role in AD pathogenesis. For example, metallated-Aβ aggregates are hypothesized to disrupt membranes or generate a reactive oxygen species (ROS) through redox cycling in the presence of Cu$^{III}$ or Fe$^{III/II}$ and a reducing agent. ROS can lead to weakened synaptic signaling and neuronal cell death. In addition, the ratio of the metal:Aβ can form a variety of metallated-Aβ species which are both soluble and neurotoxic. Neurotoxicity can depend on the size of the metallated-Aβ species as well as the type of metal present. For example, some studies suggest that Cu$^{II}$ ions inhibit aggregation and fibrillation reactions induced by Zn$^{II}$ ions and may therefore be neuroprotective in this scenario. Regardless to the contradicting roles of metals, the most convincing piece of evidence supporting a link between metal homeostasis and the two pathological processes (Aβ$_{1-42}$ aggregation and oxidative damage) comes from chelation studies showing solubilization of Aβ$_{1-42}$ deposits and clinical improvement of patients with AD. Studies suggest control of metal imbalances as well as Aβ$_{1-42}$ concentrations are important.
“disease-modifying strategies”. Here we will present studies utilizing well-known metal ion capture agents, metallo-dithiolates or NiN2S2 ligands, as chelators to reverse Aβ1-42 aggregation. Fluorescence anisotropy, UV-Vis spectroscopy, and atomic force microscopy studies will show that interactions of Aβ with metal ions are reversible with metallo-dithiolate ligands. These studies will show that metallo-dithiolates might be a new class of ligands for reversing metal-induced Aβ aggregation and a potential disease modifying strategy.

Key Words: metallo-dithiolate, metal hypothesis, beta amyloid
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CHAPTER 1

INTRODUCTION

Alzheimer’s Disease (AD) impacts over 5.7 million people in the U.S. each year, costing over $225 billion in patient care. AD is characterized by the presence of neurofibrillary tangles (NFTs) and senile plaques (SPs) from increased concentrations of amyloid-b-peptide (AB). There are many studies that support the amyloid cascade hypothesis and its role in the progression of AD(1-4). The hypothesis states that the deposition of misfolded AB leads to NFTs, calcium dysregulation, cell death and dementia. The postmortem effects can be witnessed from large areas of cell death correlated to the same areas where Aβ is found. Among the many species of Aβ, Aβ_{1-42} has been shown to be the most toxic strain to humans. Reversing or slowing the build ups of Aβ_{1-42} has the potential to slow or reverse AD pathogenesis ( ). In vitro, the cascade begins by monomeric Aβ species experiencing changes in pH, temperature or interacting with the metal ions in the brain. There are many studies surrounding metal ion interactions and the effects they have on aggregation. Aside from their involvement in many neurodegenerative diseases, these ions such as Cu^{2+}, Zn^{2+} and Fe^{2+} play an important role in protecting against free radicals, enabling communication between neurons and helping to maintain homeostasis (https://onlinelibrary.wiley.com/doi/10.1002/9781118553480.ch02).
Figure I. Crystallographic structural representation of metallodithiolate forming stable metal complexes with Cu, Fe, Ni and Pb (1).

In this study the interactions between Aβ, NiN2S2 and metal ions are studied. Fluorescence intensity fluorescence anisotropy are used to build a high throughput screening assay and study the changes in molecular rotation of Aβ with the addition of metal ions and metallodithiolate interactions. UV-Visual Spectroscopy is used to confirm binding interactions and Atomic Force Microscopy is utilized to study changes in particle size and shape upon interaction.
CHAPTER 2
EXPERIMENTAL SECTION

Reagents and Buffer Solutions

Solution of 95% hexafluoroisopropanol (HFIP) is purchased from Sigma Aldrich. Beta-amyloid, Aβ1-42 and TAMRA-Aβ1-42 is purchased from Anaspec as lyophilized powders and stored at -20°C in HFIP. The Aβ1-42 is modified with a TAMRA dye at the N-terminal end of aspartic amino acid residue of the Aβ1-42 sequence Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-Gly-Val-Val-Ile-Ala-OH.

Aβ Oligomer and Monomer Preparation Sample Preparation for Aβ Binding Studies

Aβ oligomers are prepared by adding Aβ1-42 (10 μL 222 μM) in HFIP solvent to 90 μL in 10 mM HEPES for pH 6.5 or 10 mM PBS. The Aβ1-42 peptide solutions are incubated at 4 °C for 24 hours to allow for oligomerization. Aβ monomers are prepared by adding Aβ1-42 (10 μL 222 μM) in HFIP solvent directly to buffered hybrid lipid-coated AuNP solutions.

UV-vis Spectroscopy Studies

Au-SO-PC-HT and Au-SO-PC-Chol-HTs (1:1) are buffered with 10 μL of 1 M HEPES at pH 6.5 or PBS at pH 8.0. Aβ monomers or oligomers are then introduced to the lipid coated AuNP solutions to yield final concentrations of 2 μM for monomers or 2 μM for oligomers. The UV-vis spectra or DLS is taken before and 15 min after the introduction of Aβ. To probe the effect of Aβ on membrane integrity, samples are exposed to cyanide and UV-Vis spectra is taken. Cyanide (10 μL 307 mM in H2O) is added to the samples and UV-Vis spectra taken
after 1 h of incubation. The final concentration of cyanide in the sample is 3 mM. UV-vis spectra is taken with an Ocean Optics USB4000 UV-vis-NIR spectrophotometer using a 1 cm quartz cuvette. Dynamic Light Scattering measurements are recorded with Horiba LB-550 particle size analyzer with a quartz cuvette.

**Fluorescence Anisotropy**

TAMRA-Aβ<sub>1-42</sub> (47 nM monomer) is added to 1000 µL 10 mM PBS at pH 8.0 or 10 mM HEPES at pH 8.0. The anisotropy of the TAMRA-Aβ<sub>1-42</sub> is monitored for 10 min before the metal ions ( ) are added to the solution. The sample is incubated for 10 min before the anisotropy is recorded for an additional 10 min. Fluorescence measurements are conducted on a PTI spectrophotometer using Felix32 software. Measurements are taken using a quartz cuvette at an excitation of 544 nm, an emission of 580 nm, and a 12 nm bandpass on both monochromators. The steady-state anisotropy (r) is calculated from equation 1:

\[
r = \frac{I_{VV} - G I_{VH}}{I_{VV} + 2GI_{VH}} \tag{1}
\]

where \( I_{VV} \) and \( I_{VH} \) are the fluorescence intensities measured, the subscripts indicate the orientation of the excitation and emission polarizers, and \( G = I_{HV}/I_{HH} \) is the wavelength dependent sensitivity of the instrument.\(^{16}\) Reported Δr values are the average of three independent samples each averaged over 10 min.

**Atomic Force Microscopy Preparation and Measurements.**

AFM samples were prepared using 10 µL of the 10.25 µM HiLyte-Aβ<sub>1-42</sub> stock solution in 100µL of 10 mM PB pH 8 for a final concentration of .1025 µM HiLyte-Aβ<sub>1-42</sub>. 5 µL of this
solution was deposited dropwise onto freshly cleaved mica. The sample was dried under a clean flux of nitrogen gas, rinsed with NANOpure water and again dried under nitrogen. Imaging was performed using Digital Instruments Veeco AFM/LFM Instrument (Veeco Metrology group). Rotated monolithic, uncoated silicon AFM probes were used with 125 µm tip length, 300 kHz resonant frequency and a 40 Newton/meter spring constant model Tap300-G (Tedpella). Machine was operated in tapping mode keeping drive amplitude to a minimum, collecting scans of 2 µm x 2 µm at a slow scan frequency of 1.5-2.5 Hz and 512 x 512 scans per line. AFM images were analyzed using Digital Instruments software. Aβ1-42 peptide size was determined using the cross section analysis tool and size distribution histogram of each image.
CHAPTER 3

BETA-AMYLOID AND METAL ION BINDING STUDIES

Figure II i, ii. Representative fluorescence intensity studies of metal chelation via EDTA using 47 nM TAMRA-Aβ_{1-42} in 10 mM PBS buffer pH 8.0 after addition of 52 μM Cu^{2+} and 2 mM EDTA (i). Fluorescence intensity measuring interactions in same conditions with Cu^{2+}, Zn^{2+} and Fe^{2+}.

A representative fluorescence anisotropy plot of the TAMRA-Aβ_{1-42} in the absence and presence of Cu^{2+}, Zn^{2+} and Fe^{2+} is shown in Figure 2. An increase in the anisotropy (Δr = 0.1) is observed at 10 min after addition of Au-SO-PC-HT to the TAMRA-Aβ_{1-42}, which suggest the hydrodynamic radius is increased and the molecular rotation is slowed, indicating binding to the AuNP surface (Figure III.2, (ii)). The beginning of the experiment (time = 0) begins with 47 nM TAMRA-Aβ_{1-42} in 10 mM PBS buffer pH 8.0, which has an intrinsic anisotropy of 0.12 in 10 mM PBS buffer at pH 8. The anisotropy of monomeric TAMRA-Aβ_{1-42} alone does not increase over 30 min in the absence of lipid-coated AuNPs. Therefore, the observed Δ in r is due to TAMRA-Aβ_{1-42} binding to the lipid bilayer of the AuNPs. Furthermore, the Δ in r is indicative of the binding efficiency (% of TAMRA-Aβ_{1-42} binding to the lipid-coated AuNPs) and the relative size of the resulting Aβ-lipid-coated AuNP conjugate. This could be because the stronger the affinity Aβ has for the lipid membrane, the
more monomers that will bind to its surface, and the greater the hydrodynamic radius of the TAMRA fluorophore on its surface.

**Figure III.** Representative fluorescence anisotropy of 47 nM TAMRA-Aβ₁₋₄₂ in 10 mM PBS buffer pH 8.0 after addition of 52 μM Cu²⁺ and 2 mM EDTA (i). Fluorescence anisotropy interactions in same conditions with Cu²⁺, Zn²⁺ and Fe²⁺.

A representative fluorescence anisotropy plot of TAMRA-Aβ₁₋₄₂ in the absence and presence of CuCl₂ and EDTA is shown in (Figure III, (i)). An increase in anisotropy ($\Delta r_{\text{avg}} = 0.59 \pm 0.07$) is observed at 10 min after addition of CuCl₂ to the TAMRA-Aβ₁₋₄₂ indicating binding (Figure 2, (ii)). The starting point of the experiment (time = 0) begins immediately after metal addition to the TAMRA-Aβ₁₋₄₂ in 10 mM sodium phosphate buffer pH 8.0. The average intrinsic anisotropy of monomeric TAMRA-Aβ₁₋₄₂ alone is $0.15 \pm 0.03$ in 10 mM sodium phosphate buffer pH 8.0 over the course of 1 h. Therefore, the observed $\Delta$ in r of TAMRA-Aβ₁₋₄₂ in the presence of metal ions is not due to self-induced peptide aggregation but to the bridging of peptides by the metal ions. The $\Delta$ in r is a function of binding efficiency (% of metals bound to TAMRA-Aβ₁₋₄₂ and % of TAMRA-Aβ₁₋₄₂ bound to Mⁿ⁺-[TAMRA-Aβ₁₋₄₂] from bridging interactions) and the relative size of the resulting metallated-conjugate.
Addition of EDTA at 20 min results in a significant drop in r or slow molecular rotation is observed. This suggests that the overall hydrodynamic radius has decreased and indicates that the peptides are monomeric upon metal capture by EDTA. The exact size of the aggregate is not known and the metal:peptide ratio is not known since excess metal is present in the sample. This confirms that metals cause the peptides to aggregate and that aggregation is reversible with chelators that have a higher affinity for the metal.

**Figure IV.** Comparison of the change in anisotropy in 10 mM phosphate buffer pH 8.0 of i) TAMRA-AB 1-42 ii) after addition of 47 uM of metal ions and iii) after the addition of 5 mM NiN2S2.

To determine if the aggregate was reversible dependent on metal type, anisotropy studies were performed in the presence of CuII, FeIII, and ZnII ions at pH 8.0 (Figure 2). The Δr of Hilyte-Aβ1-42 in the presence of CuII is 0.8 ± 0.03 (Figure 2). The average
anisotropy in the presence of FeIII and ZnII were $\Delta r = 0.64 \pm 0.02$ and $0.45 \pm 0.05$ respectively. The initial hydrodynamic radius of the CuII species is larger (slower molecular rotation) indicating that it has the highest affinity for the AB1-42 peptide. With the addition of NiN$_2$S$_2$ there is a 67% decrease in the hydrodynamic radius of the CuII + AB1-42 aggregate. There is a ___% increase in the FeIII + AB1-42 aggregate and a ___% decrease in the ZnII + AB1-42 aggregate.

These results indicate that the hydrodynamic radius of the FeIII-Ab1-42 and the ZnII-Ab1-42 species is smaller than that of CuII-Ab1-42, indicating they have a lower affinity for TAMRA-Ab1-42 in comparison to CuII. Interestingly, in previous studies FeIII has a higher affinity for NiN2S2 than CuII so the result that CuII is aggregation may be reversible and the FeIII is not surfaces the question of whether the FeIII-AB1-42 species is experiencing a a change in formation. The difference in the hydrodynamic radius between CuII, FeIII, and ZnII suggest that the size of the resulting aggregates is likely due to the difference in coordination geometry preferences and ligand donor type on the peptide scaffold. These preliminary studies show that at pH 8.0 the change in the hydrodynamic radius is greater for CuII > FeIII > ZnII.
Evaluating the Effect of NiN₂S₂ on Aβ interaction with metal ions

**Figure V.** UV-Vis spectroscopy comparing change in absorbance of Aβ₁-₄₂ with 0.3 µM metal ions and 0.4 µM NiN₂S₂ A) CuSO₄ B) ZnBr₂ C) FeCl₃

In these UV-Visual studies, a solution of Hilyte-Aβ₁-₄₂ in phosphate buffer pH 8, next the metal ion was added and traced. Separately the NiN₂S₂ was traced on its own as well as when it was added to the Aβ and metal ion solution. The NiN₂S₂ can be characterized by the bands seen at 228 and 276 nm. Shown in A, as NiN₂S₂ is added to the solution of CuSO₄ and Aβ, the bands
shift to to 244 and 230, representative of a NiN₂S₂ and Cu²⁺ complex forming in a 2:4 ratio respectively. In B, the peaks shift upwards at the 228 and 276 characteristic bands when NiN₂S₂ is added to the AB and Zn²⁺ complex, showing a rise In C the bands shift to 267 and 297 with the addition of NiN₂S₂ representing a no interaction between the Zn²⁺ and the NiN₂S₂.

Figure VI. Atomic Force Microscopy
CHAPTER 4

DISCUSSION

Figure 5. AFM Analysis of 0.55 µM Aβ₁₋₄₂ solubilized in HFIP and added to PB pH 8 incubated at 0 hr with A) AFM image scan of 2 x 2-µm x-y, 5 nm total z-range i) Aβ₁₋₄₂ ii) Aβ₁₋₄₂ + Cu²⁺ iii) Aβ₁₋₄₂ + Cu²⁺ + EDTA iv) Aβ₁₋₄₂ + Zn²⁺ v) Aβ₁₋₄₂ + Zn²⁺ + EDTA vi) Aβ₁₋₄₂ + Fe³⁺ vii) Aβ₁₋₄₂ + Fe³⁺ + EDTA. Concentrations of 47 µM of metal ions and 2 mM of EDTA were used, added drop wise to a solution B) Histogram distribution analysis of images i-vii. To compare the results from the fluorescence anisotropy tests, AFM was used as a second assay to study the aggregation effects of pH and metal ions on Aβ₁₋₄₂. TAMRA-Aβ₁₋₄₂ HFIP solution was used for controlled aggregation studies. Pre-treatment in HFIP allows for a homogeneous solution of unaggregated Aβ-peptide for imaging (Figure 5A,i). The z-height value for individual peptides was ____ (± ____ ) nm shown in the histogram analysis (Figure 5B, i) which agrees with expected size of an Aβ monomer. AFM cannot determine molecular weight so it does not provide data to show that the weight of this solution is consistent with the Aβ monomer weight of 4 kDa. At pH 8.0 after incubating for 24 hours at 4° C in phosphate buffer (pH 8) peptide sizes were (____ (± ____ ) nm proving a ___% increase in size. After incubation of Aβ₁₋₄₂ with CuII for 10 minutes at 25° C there were ____% and ____% increases in z-height and radius respectively. When exposed to FeIII at pH 8 the peptide experienced a ____% and ____%. ZnII addition to the peptide resulted in a ____% and ____% increase in z-height and radius at pH 8. In comparison to the increase in hydrodynamic radius observed in the anisotropy studies, the values are consistent for all. The
size distribution of aggregated peptides with metal ions is Cu>Fe>Zn in pH 8 conditions.

Next, the addition of EDTA to these peptide-metal ion solutions was studied a second time with AFM imaging. Similar reversibility in peptide size can be witnessed with CuII and ZnII. A ____% and ___% decrease in z-height and radius at pH 8. The AFM analysis of EDTA addition to the FeIII-Aβ_{1-42} solution
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