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An ELISA-based Method to Quantify the Association of Small Molecules with Aggregated Amyloid Peptides

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Abstract

This paper describes a simple ELISA protocol for quantifying the binding of small molecules to aggregated Amyloid- β (A β) peptides. Amyloid-targeting small molecules have attracted wide interest as potential agents for the treatment or diagnosis of neurodegenerative disorders such as Alzheimer's disease. The lack of general methods to evaluate small molecule-amyloid binding interactions, however, has significantly limited the number of amyloid-targeting molecules that have been studied to date. Here, we demonstrate a general method to quantify small molecule-amyloid binding interactions via a modified quantitative ELISA protocol. A key feature of this protocol is the treatment of commercial ELISA plates with an air plasma to help maintain the desired β -sheet content of the aggregated A β upon immobilization of these peptides on to the polystyrene surface. We developed an ELISA-based competition assay on these air plasma-treated plates and evaluated the binding of five previously known amyloid-binding small molecules to aggregated A β . We show that this general ELISA-based competition assay can be used to quantify small molecule-amyloid binding interactions in the low nanomolar to low micromolar range, which is the typical range of affinities for many amyloid-targeting diagnostic agents under current development. This simple protocol for quantifying the interaction of small molecules with aggregated A β peptides overcomes many limitations of previously reported spectroscopic or radioactivity assays, and may, therefore, facilitate the screening and evaluation of a more structurally diverse set of amyloid-targeting agents than had previously been possible.

INTRODUCTION

Alzheimer's Disease (AD) is a progressive neurodegenerative disorder characterized by the deposition of senile plaques (along with neurofibrillary tangles) in the brain.¹ These senile plaques are primarily comprised of a 40–42 amino acid peptide called β -amyloid (A β) peptide. Currently, AD is diagnosed through the clinical evaluation of symptoms, and can only be confirmed post mortem by the presence of amyloid deposits in brain tissue.¹ Methods for both early diagnosis and for monitoring the progression of AD are critical for the development of effective treatments to combat this debilitating disease.² The clinical development of PET (positron emission tomography) and SPECT (single photon emission computed tomography) imaging agents that target deposits of aggregated A β peptides *in vivo* show tremendous promise for detecting changes in the accumulation of senile plaques in living patients.^{3–7} These imaging techniques, therefore, provide a potentially useful means to follow the pathological progression of AD.

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Supporting Information Available. Detailed experimental protocol for analysis of A β samples using specular FTIR, experimental protocol for the control study to assess non-specific binding of Thioflavin T to the anti-A β IgG, and ELISA curves for determining the binding of all molecules to aggregated A β . This material is available free of charge via the Internet at <http://pubs.acs.org>.

Methods to rapidly identify and quantify the association of small molecules to aggregated A β are critical for the development of new and improved imaging agents for diagnosing and monitoring AD. Currently, the association of small molecules with aggregated A β is most often quantified by radioactivity- or fluorescence-based assays.^{8–10} These analytical methods, however, limit the type of molecules that can be developed to those that are either inherently fluorescent or to those that are labeled with a radioisotope. Here, we describe a new ELISA-based assay to quantify the binding of small molecules to aggregated forms of Alzheimer's-related A β peptides. This method has the advantage that it does not require inherent radioactivity or fluorescent properties of the molecules being analyzed, consequently making it possible to quantify the binding of A β to a significantly more diverse set of molecules compared to current methods.

We previously reported a protein inhibition assay that could be used to qualitatively identify whether a small molecule could associate with aggregated forms of A β .¹¹ Since the molecules and antibodies were not introduced under competitive conditions in these previous studies, this inhibition assay, while simple, rapid, and inexpensive, was not capable of revealing quantitative information on interaction between the small molecules and A β . In order to generate a similarly accessible assay that could be used for quantification of dissociation constants, we developed an ELISA-based competition assay that prevents significant denaturation of A β upon adsorption to hydrophobic ELISA plates, which can be an inherent general problem with quantitative ELISA assays.^{12,13}

EXPERIMENTAL SECTION

Materials

A detailed list of materials used in this research can be found in the supporting information.

Procedure for quantitative ELISA

All incubation steps were carried out at 25°C unless stated otherwise. Phosphate buffered saline (PBS, 10 mM sodium phosphate, 138 mM sodium chloride, 2.7 mM potassium chloride, pH 7.4) was prepared fresh for each experiment.

Growth of aggregated A β —A β aggregates were grown from synthetic A β (1–42) peptides by reconstituting the lyophilized peptide in ultrapure H₂O (final concentration 111 μ M) and incubating the peptides at 37 °C for 72 h. A β peptides were incubated in H₂O rather than in PBS to facilitate straightforward analysis of A β conformation by specular FTIR (see supporting information for additional details). We previously reported that this procedure produces mostly fibrillar structures that possess similar morphology to A β grown in PBS (as previously determined by AFM and TEM imaging experiments).^{11,14–17} A β aggregates were characterized by circular dichroism (CD) for the presence of β -sheet secondary structure. CD measurements were performed on an AVIV spectropolarimeter at room temperature. Spectra were recorded from 260 nm to 190 nm in a 1-mm cuvette pathlength, 1 nm stepsize (see Figure S1 of supporting information).

Air plasma treatment of polystyrene plates—Polystyrene (PS) plates were treated with air plasma for a duration of 60 seconds, rendering them hydrophilic. The air plasma was generated under partial vacuum (<1800 mTorr) in a Harrick Plasma Cleaner/Sterilizer (Model PDC- 32G). Plates were used in the assay immediately after treatment. Hydrophilicity was assessed qualitatively, by observing the behavior of water at the surface. Water droplets spread out on hydrophilic surfaces, whereas on untreated hydrophobic PS, water droplets bead together to minimize interactions with the hydrophobic surface.

ELISA protocol for competition of anti-A β IgG-A β interactions using small molecules—The wells of air plasma-treated or untreated 96-well plates were coated with aggregated A β peptide by incubating each well for 6 h with 50 μ L of a 1.4 μ M solution of aggregated A β in PBS (obtained by diluting A β in PBS from a 111 μ M solution of A β aggregates grown in ultrapure H₂O). After removal of solutions containing excess A β , 300 μ L of protein-free blocking buffer was added to each well and incubated for 1 h. The blocking buffer was removed and the wells were washed three times with PBS. The wells were then incubated for 12 h at 4°C with 50 μ L solutions containing various concentrations of small molecule (obtained by diluting from a 1 mg/mL stock solution of each molecule dissolved in PBS) and a fixed concentration of anti-A β IgG (clone 6E10, Lot 08BC00306, 1 nM in Protein-Free Blocking Buffer) [Note: In order to evaluate whether a 12 h incubation period was sufficient to reach equilibrium for competitive binding of small molecules and the anti-A β IgG, we incubated the small molecules and IgG with the aggregated A β in the wells for 3 h instead of the 12 h incubation period. We found that the K_i values obtained from this quantitative ELISA protocol was the same with either the 3 h or 12 h incubation periods, suggesting that 3 h was a sufficient duration of time to reach equilibrium. All reported values for K_i were estimated using an incubation time of 12 h for consistency.] The amount of bound monoclonal IgGs was quantified by removing the excess solution, washing the wells three times with 300 μ L of PBS, and incubating for 45 min with 50 μ L of a polyclonal secondary rabbit IgG (anti-mouse IgG, 6.8 nM in Protein-Free Blocking Buffer) conjugated with alkaline phosphatase. The wells were then washed five times with 300 μ L of PBS. The relative amount of secondary IgG bound in each well was quantified by adding 50 μ L of a solution containing *p*-nitrophenyl phosphate (*p*-NPP, 2.7 mM, in 0.1 M diethanol amine/ 0.5 mM magnesium chloride, pH 9.8) to each well. The plates were incubated until a color change was observed under visual inspection. The concentration of *p*-nitrophenoxide was quantified at 405 nm using a UV-Vis microplate reader. Note: the color change upon developing the plasma-modified polystyrene (PMPS) plates should not take more than one hour.

Each data point (Figures S3 and S4 in the supporting information) from this assay represents the average of four independent measurements. Error bars represent one standard deviation. Graphs were normalized, plotted, and fitted with the sigmoidal curve fitting option in Origin 7.0 (Microcal Software, Inc., Northampton, MA) to obtain IC₅₀ values. K_i values for these compounds were calculated using the Cheng-Prusoff equation for competitive binding:¹⁸

$$K_i = \frac{IC_{50}}{1 + \frac{[C]}{K_c}}$$

where [C] is the concentration of anti-A β IgG used in this assay and K_c is the dissociation constant of the anti-A β IgG to A β . The conversion of IC₅₀ to K_i for estimating the binding of small molecules to A β in competition assays is the same as reported by others.^{10,19}

RESULTS AND DISCUSSION

Many binding interactions between small molecules and A β are dependent on the secondary structure of A β . It is widely accepted that the β -sheet secondary structure common to amyloids is necessary for binding of histological agents such as Thioflavin T.²⁰ The conformation of the peptide is, therefore, an important consideration in assays that attempt to quantify the interaction between small molecules and aggregated A β peptides.

Since a typical ELISA protocol includes the deposition of a protein (here, aggregated A β) to the hydrophobic surface of a polystyrene 96-well plate, we developed a protocol that accounts for the effect of the surface on the conformation of A β . The influence of hydrophobic and hydrophilic surfaces on the conformation of A β peptide has been reported previously.^{21–25} Giacomelli *et al.*,^{21,22} for instance, found that aggregated A β adsorbed to hydrophilic surfaces retained the β -sheet conformation that is typically characteristic of A β aggregates in aqueous solution; adsorption of aggregated A β on hydrophobic surfaces generally lead to an increase in the α -helix content of aggregated A β . Based on these observations, we hypothesized that we would need to use hydrophilic materials in the development of a quantitative ELISA protocol in order to mimic an aqueous solution environment and maintain the desired conformation (as estimated by β -sheet content) of the A β aggregates during analysis of small molecule-amyloid interactions.

ELISA assays routinely employ hydrophobic 96-well plates made from polystyrene (PS). We examined the influence of PS on the secondary structure of pre-aggregated A β (1–42) by dissolving a PS ELISA plate in toluene, spin-coating the PS onto a gold surface, evaporating the residual solvent from the PS, and examining aggregated A β peptides deposited on this thin film of PS by specular FTIR (see supporting information for details). The amide I region of the IR spectrum (1600–1700 cm⁻¹) is associated with the C=O stretching vibration and, thus, can reveal information on the backbone conformation (e.g., β -sheet content) of the peptide.^{26–30} Figure 1A shows the FTIR spectrum (black line) of A β (1–42) adsorbed on PS. Two major peaks of similar intensity centered at 1633 cm⁻¹ and 1676 cm⁻¹ appear in the amide I region of this spectrum. Second derivative spectral analysis of the amide I region^{29,31} resolved the overlapping bands and revealed component bands centered at 1630 (red line), 1660 (green line), 1680 (blue line), and 1695 (cyan line) cm⁻¹, which correspond approximately to the relative abundance of β -sheet, α -helix/unordered, β -turns, and antiparallel β -sheet content, respectively (the bands at 1695 cm⁻¹ may also contain absorbance contributions from the Arg, Asn, Gln sidechains).³² Subtracting the absorbance contribution of PS (at 1601 cm⁻¹ corresponding to the C=C stretch of the aromatic ring of PS) prior to peak fitting analysis and subsequent peak integration of the component band centered at 1630 cm⁻¹ revealed that the aggregated A β (1–42) adsorbed to PS contained ~37% β -sheet content. This value is significantly lower than the percentage of β -sheet content that is typically found in solution samples of aggregated A β peptides.^{32,33}

In order to evaluate the effects of a hydrophilic material on the secondary structure of aggregated A β (1–42), we subjected the PS to an air plasma treatment to render the PS more hydrophilic. Figure 1B shows the FTIR spectrum of A β (1–42) adsorbed on plasma-modified PS (PMPS). Second derivative spectral analysis revealed that aggregated A β (1–42) adsorbed on PMPS contained a β -sheet content of ~61%, which is more consistent with the approximate β -sheet content that is typically found in solution samples of aggregated A β peptides.^{32,33} Thus, we conclude that deposition of the peptide on PMPS plates would result in better retention of the desired β -sheet content of aggregated A β (1–42), and, thus, might be more suitable for development of an ELISA protocol for quantifying dissociation constants between small molecules and aggregated A β peptides.

Figure 2 outlines a general procedure for a modified quantitative ELISA protocol that can be used to estimate dissociation constants (here, reported as K_i's since they are derived from a competition assay) for the binding of small molecules to aggregated A β (1–42) peptides. In this assay, the binding of small molecule to A β (1–42) is determined by its competition with a monoclonal anti-A β IgG (clone 6E10, K_d = 340 nM, see supporting information for details on determining the dissociation constant between the IgG and A β). We chose to use clone 6E10 as the competitor antibody in this assay because it is the most widely used antibody in AD research and we previously reported that this antibody effectively competes with

multiple, different binding sites for small molecules along the aggregated A β (1–42) peptide surface.¹¹ Although it may still be possible that not all amyloid-binding small molecules will effectively compete for binding with the anti-A β IgG (clone 6E10) used in this quantitative binding assay, we have previously shown that Thioflavin T could also effectively compete with a different clone of anti-A β IgG (clone AMY-33) for binding to aggregated A β .¹⁵ This result with clone AMY-33 suggests that other anti-A β antibodies could be used as a surrogate for clone 6E10, if needed. We estimated the binding constants (K_i 's) of small molecules to aggregated A β peptides from the IC₅₀'s obtained directly from the ELISA data (see Figures S3 and S4 in the supporting information).¹⁸ In order to provide a comparison for the K_i values obtained from this assay, we evaluated the binding of five molecules whose binding constants to aggregated A β (1–42) had previously been reported in the literature (Table 1).^{8,10,19,20,34,35} We performed the ELISA protocol on both air plasma treated and untreated PS plates to examine whether treating the plates with air plasma was necessary for accurate estimation of K_i 's.

The measured K_i 's for compound **1–5** are given in Table 1. In the case of fluorescent molecules Thioflavin T (**1**, ThT) and Congo Red (**2**), the K_i 's obtained from this ELISA assay on PMPS plates were similar to previously reported literature values obtained from fluorescence-based^{8,10,19,20,34,35} or radioligand assays.^{10,19,20,36,37} On untreated PS plates, compounds **1–5** exhibited a much lower affinity for aggregated A β than they did on PMPS plates. These results suggest that air plasma treatment of PS was necessary to afford reliable estimations of K_i values, which can be partly attributed to the ability of the plasma-modified surface to conserve the native secondary structure of aggregated A β .

Table 1 also summarizes the K_i values of two non-fluorescent amyloid-binding molecules, (*S*)-Naproxen (**3**) and (*R*)-Ibuprofen (**4**).^{8,10,37} We included these molecules in the initial development of this ELISA protocol since their K_i 's for binding to A β (1–42) peptides had previously been reported from competition assays using known radiolabeled or fluorescent amyloid-binding molecules. Surprisingly, the literature K_i values for (*S*)-Naproxen binding to aggregated A β varied significantly (by ~ 4 orders of magnitude), presumably due to the use of different formats for analysis (i.e., fluorescence- vs radioactivity-based competition assays) or due to the significant difference in the structures of molecules used as competitors in these previously reported assays. This discrepancy in reported K_i values highlights the need for developing novel and general platforms for the quantitative and comparative study of small molecules that bind to aggregated amyloid peptides. We demonstrated that the quantitative ELISA protocol on PMPS could be used to estimate K_i values for **3** and **4**, even though these molecules are not inherently fluorescent or radioactive (since the binding of Naproxen to aggregated A β was previously estimated from competition assays using radiolabeled or strongly fluorescent competitors,^{8,19,37} any weak fluorescence that is inherent to Naproxen is presumably unsuitable for directly determining its binding to A β using standard fluorescence assays^{8,19}). The K_i value of 4.1 μ M for (*S*)-Naproxen that we measured is in better agreement with the low micromolar values reported by Lockhart *et al.*¹⁹ and Levine⁸ compared to the low nanomolar K_i value reported by Agdeppa *et al.*³⁷ Additionally, the low micromolar K_i value that we estimated for (*R*)-Ibuprofen was also similar (within an order of magnitude) to the reported literature values.^{8,37} Lastly, we estimated the K_i value for BTA-EG₄ (**5**) using this ELISA protocol, an amyloid-targeting molecule with potential therapeutic applications for AD.³⁸ The 20 nM K_i value that we measured for **5** is comparable to the binding constants reported for a number of benzothiazole aniline (BTA) derivatives, which have K_d values reported in the low to mid nM range.^{8,35}

The accuracy of this new ELISA-based method for quantifying the interaction of small molecules with amyloid targets is based on at least the following two assumptions: 1) The method assumes that the small molecules exhibit a 1:1 stoichiometry with the anti-A β IgGs

that compete for overlapping binding sites for A β . This same assumption is made for previously reported competition assays used to estimate the binding constants of amyloid-targeting molecules.^{8,10,19,37} Deviations from this 1:1 binding stoichiometry (e.g., if it requires more than one small molecule to competitively displace an antibody from the amyloid surface) could lead to reported K_i values that were weaker than the true value. The similar values between the K_i 's estimated for **1** and **2** and the literature K_d values for these molecules (which were not obtained through competition, and therefore, do not require the assumption of a 1:1 binding stoichiometry with a competitor), however, suggests that making the rough approximation of a 1:1 stoichiometry between the small molecules and the IgG leads to reasonably accurate values for binding constants. 2) The method assumes that the small molecules do not significantly interact with the monoclonal anti-A β IgG during the co-incubation step (step d in Figure 2) of the ELISA protocol, and, therefore, do not affect the estimation of K_i values. Since the primary antibody used in this assay is monoclonal and was raised specifically to recognize residues 3–8 of the A β peptide, we do not anticipate any significant interaction between small molecules and this antibody. To support this assumption, we did not observe a change in the absorbance or emission spectrum of ThT (**1**) when it was incubated with the anti-A β IgG alone in solution, suggesting that ThT (and, presumably, molecules **2–5**) does not significantly interact in a non-specific manner with the IgG (see Figure S2 in the supporting information) to affect the accuracy of the ELISA method.^{20,39,40}

CONCLUSION

We have, thus, developed a simple and accessible method for quantifying the binding of small molecules to aggregated A β peptides. We demonstrate that this ELISA method can estimate binding constants of small molecules from the low nanomolar to the low micromolar range, regardless of the inherent physical properties (i.e., spectroscopic properties) of the molecules. Although other analytical techniques such as surface plasmon resonance (SPR)^{41–45} and quartz crystal microbalance (QCM)^{46,47} have also previously been employed to study the interaction of small molecules with aggregated A β peptides, these techniques also require the immobilization of A β to a surface, either by amino coupling of A β to the surface, or by exploiting the strong, noncovalent interaction of an avidin-coated surface with modified, biotinylated A β . These immobilization techniques may, thus, also inadvertently alter the desired secondary conformation of the aggregated A β during analysis. A key step in this ELISA-based method is the air plasma treatment of the polystyrene surface of the ELISA plates, which helps maintain the native β -sheet content of the amyloid peptides during analysis. The relatively large size of the IgG⁴⁸ makes it possible for it to compete with multiple, different binding sites for small molecules along the surface of the amyloid,¹¹ thus, making it a general competitor for many classes of molecules. This ELISA method addresses a major limitation of previously reported binding assays by making it possible to evaluate small molecule-amyloid binding interactions without any obvious restrictions on the molecules that can be analyzed. Complementary techniques, such as linear dichroism (LD)^{49–51} and NMR,^{52,53} may also be useful to supplement this ELISA method since these techniques can provide further insight into the binding mode of small molecules to A β aggregates. This ELISA-based method only requires access to a UV-Vis microplate reader and an air plasma generator (both common and relatively inexpensive laboratory equipment), can be carried out with minimal training of laboratory personnel, and should be readily translatable to amyloidogenic peptides other than A β . This assay should, therefore, serve as a valuable tool in both industry and academic laboratories for developing novel diagnostics^{4–7,54} (and possibly therapeutics³⁸) for amyloid-associated neurodegenerative diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

The FTIR spectrum of aggregated Aβ(1–42) adsorbed on polystyrene (A) or on plasma-modified polystyrene (B). The black lines denote the raw FTIR spectra. The red, green, blue, and cyan lines denote the relative β-sheet, α-helix/unordered, β-turns, and antiparallel β-sheet content, respectively, as estimated by second derivative spectral analysis.³²

Figure 2.

Schematic representation for the experimental steps in a quantitative ELISA protocol for estimating competitive inhibition constants (K_i 's) for the interaction of small molecules with aggregated A β peptides. (a) We treat a commercial polystyrene 96 well plate with a 60 sec exposure to air plasma. (b) We deposit pre-aggregated A β (1–42) peptides into the wells. (c) We incubate the wells with a protein-free blocking buffer to minimize non-specific adsorption of the IgGs to the wells. (d) We co-incubate the wells with a fixed concentration of anti-A β IgG and increasing concentrations of small molecules. (e) After removal of all excess primary (1°) antibody, we incubate the wells with a secondary (2°) antibody conjugated to alkaline phosphatase followed by introduction of para-nitrophenyl phosphate (p-NPP). We quantify the relative abundance of 2° antibody remaining in each well using a UV-Vis microplate reader.

Table 1

Summary of the K_i values obtained for compounds **1–5** for binding to A β (1–42) peptides using a quantitative ELISA format on polystyrene (PS) or plasma-modified polystyrene (PMPS). The literature values for the binding of compounds **1–5** to A β (1–42) are also given.

Compound #	Name	K_i (μ M) on PS	K_i (μ M) on PMPS	Literature Values (K_d or K_i , μ M)
1	Thioflavin T	164 ± 59	1.7 ± 0.2	$2^{b,d}$ (ref. 18) $0.75^{b,d}$ (ref. 10)
2	Congo Red	3.1 ± 0.6	0.45 ± 0.14	$1.1^{b,h}$ (ref. 34) $1.5^{b,e}$ (ref. 36)
3	(<i>S</i>)-Naproxen Na	No Binding	4.1 ± 2.2	$0.0057^{c,e,g}$ (ref. 37) $>20^{c,d,f}$ (ref. 17) $>15^{c,d}$ (ref. 8)
4	(<i>R</i>)-Ibuprofen ^a	No Binding	2.3 ± 1.4	$44^{c,e,g}$ (ref. 37) $>15^{c,d}$ (ref. 8)
5	BTA-EG ₄	>1000	0.020 ± 0.008	$0.02^{b,d}$ (ref. 8) $0.13^{b,d}$ (ref. 35)

^a2.5% DMSO in buffer;

^b K_d ;

^c K_i ;

^ddetermined by fluorescence assay;

^edetermined by radioactivity assay;

^f10% EtOH used as a cosolvent;

^g1% EtOH used as a cosolvent;

^hmeasured by UV-Vis