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# Non-covalent, electrostatic interactions induce positively cooperative binding of small molecules to Alzheimer's and Parkinson's disease-related amyloids

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**ABSTRACT:** Amyloids are self-assembled protein aggregates that represent a major hallmark of many neurologic and systemic diseases. Among the common features of amyloids is the presence of a high density of multiple binding sites for small molecule ligands, making them an attractive target for design of multimeric binding agents. Here, we demonstrate that non-covalent, intermolecular interactions between a 1:1 mixture of oppositely charged benzothiazole molecules enhances their binding to two different amyloid aggregates: Alzheimer's-related Amyloid-β (Aβ) peptides or Parkinson's-related α-Synuclein (αS) proteins. We show that this mixture leads to positively cooperative binding to amyloid targets, with up to 10-fold enhancement of binding compared to the uncharged parent compound. The observed enhancement of amyloid binding using non-covalent interactions was similar in magnitude to a benzothiazole dimer to aggregated Aβ. These results represent a novel strategy for designing amyloid-targeting molecules with enhanced affinity, which could aid in the development of new diagnostic or treatment strategies for amyloid-associated diseases.

**Keywords**: Amyloid-β • Cooperativity • Benzothiazole • Aggregation • α-Synuclein

The conversion of soluble proteins into amyloids—proteinaceous aggregates with high cross-beta sheet structure—is associated with a broad range of neurodegenerative disorders[1,2] such as Alzheimer's disease[3] (AD) or Parkinson's disease[4] (PD), as well as non-neuropathic diseases such as type II diabetes[5] and sexually transmitted diseases such as human immunodeficiency virus (HIV).[6] As the list of amyloid-associated diseases continues to grow,[7] methods to target these natural materials with small molecules for disease prevention, treatment, and/or diagnosis continues to be of increasing fundamental importance.

We previously reported the design and synthesis of several oligo(ethylene glycol) derivatives of benzothiazole aniline (BTA) that are capable of targeting amyloid aggregates with mid to high nanomolar affinity.[8-10] In addition to demonstrating the capability of these molecules to protect neuroblastoma cells from the toxicity of Alzheimer's-related Amyloid-β (Aβ) aggregates,[10,11] we showed that these molecules were also capable of targeting the naturally abundant amyloid Semen-derived Enhancer of Virus Infection (SEVI) and neutralizing its capability to enhance HIV transmission in cervical cells.[8] While these studies reveal some exciting potential opportunities for amyloid targeting agents in disease treatment and prevention, novel approaches to further improve amyloid

binding affinities are still needed to improve potency.



a **Multivalent binding to amyloids**

BTA monomer (**1**)

BTA dimer (**2**)
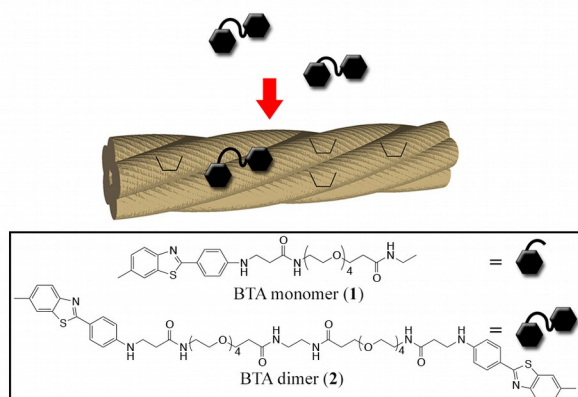
b **Enhanced amyloid binding due to non-covalent, intermolecular interactions**

BAM1-EG$_6$
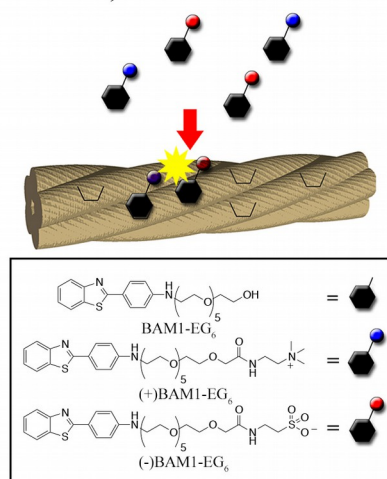
(+)BAM1-EG$_6$

(-)BAM1-EG$_6$

**Figure 1.** Comparison of strategies that use either multivalency (a) or non-covalent electrostatics (b) to improve the binding of various BTA derivatives to amyloid fibrils.

In order to develop a method to improve the binding of synthetic molecules to amyloid targets, we[12,13] and others[14] previously reported the development of oligomeric[12] and polymeric[13] BTA derivatives that were designed to simultaneously bind to multiple binding sites (i.e., multivalent binding) along the surface of amyloid fibrils (Figure 1a); adjacent binding sites on amyloid fibrils for BTA moieties have been estimated to be as close as 2 nm apart.[15] As expected, the oligomeric BTA derivatives exhibited a trend of increasing binding to aggregated Aβ peptides as a function of increasing valence number.[12] While this multivalent approach to the design of amyloid binding molecules led to significant improvements in overall binding compared to monomeric BTA molecules, some significant drawbacks[16,17] to this previous approach are: 1) the difficulty of synthesis,[12] 2) the poor aqueous solubility of the oligomers,[18] and 3) the large size of the oligomeric compounds that introduce potential challenges for biocompatibility.[19] In order to address these challenges while also building upon the promising use of cooperative interactions for improving amyloid binding of synthetic molecules, here we designed and synthesized two charged derivatives of the known amyloid- binding benzothiazole molecule, BAM1-EG$_6$ (Figure 1b),[10,20] which we hypothesized could introduce cooperative non-covalent interactions between molecules bound to adjacent binding sites along the surface of amyloids, thereby improving amyloid binding while eliminating the need for covalent linkages between amyloid-binding moieties.

In this proof-of-concept study, we used electrostatic interactions between small molecules as a simple demonstration of an approach to introduce cooperativity in the binding to amyloid targets (Figure 1b). We incorporated choline and sulfonate groups into the parent BAM1-EG$_6$ compounds since they have been widely used to install positive and negative charges, respectively, on molecules due to their capability to retain essentially permanent charges across a broad range of pH.[21] The negatively charged (-)BAM1-EG$_6$ and positively charged (+)BAM1-EG$_6$ (Figure 1b) were prepared from BAM1-EG$_6$[10,20] through standard S$_N$2 and amide coupling procedures (see Scheme S1 and the SI for details on the synthesis and characterization of these charged compounds). We used previously reported[12] BTA monomer (**1**) and dimer (**2**) (Figure 1a) as controls in this study to compare multivalent versus non-covalent interactions as strategies for designing high affinity binding agents to amyloid targets.
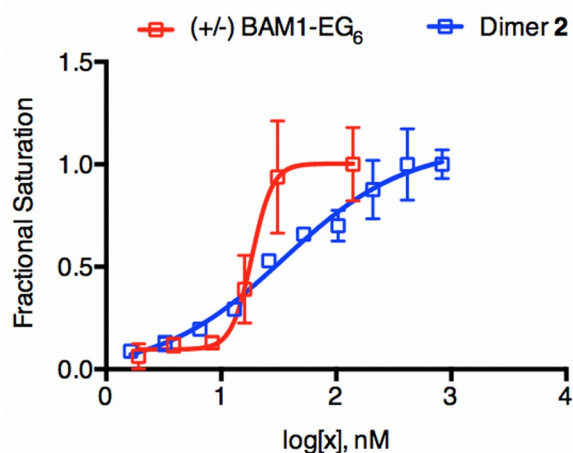
Table 1. Non-covalent interactions improve binding of BAM1-EG$_6$ derivatives to aggregated amyloid-β (1-42) peptides and α-synuclein proteins.

| Compound | Aβ | | | αSynuclein | | |
|---|---|---|---|---|---|---|
| | $K_d$ to Aggregated Aβ Peptides (nM) | Hill Coefficient (h) | Enhancement Factor (β) | $K_d$ to Aggregated αSynuclein (nM) | Hill Coefficient (h) | Enhancement Factor (β) |
| BAM1-EG$_6$ | 170 ± 30 | 2.1 ± 0.4 | NA | 1,100 ± 200 | 1.4 ± 0.2 | NA |
| (+/-)BAM1-EG$_6$ | 20 ± 2 | 3.3 ± 0.9 | 10 | 400 ± 70 | 3.8 ± 1.0 | 3 |
| (+/-)BAM1-EG$_6$ High Salt | 80 ± 15 | 1.7 ± 0.3 | 2 | 1,400 ± 60 | 2.2 ± 0.1 | 0.8 |

Data reported as mean values ± SD, n ≥ 3, NA = not applicable

With the charged BAM1-EG$_6$ derivatives in hand, we compared the binding of pure BAM1-EG$_6$ to the binding of a 1:1 mixture of (+)BAM1-EG$_6$:(-)BAM1-EG$_6$ [referred to as (+/-)BAM1-EG$_6$ for short] to aggregated Aβ peptides utilizing a previously reported centrifugation binding assay.[12,22] This intrinsic fluorescence assay was done under equilibrium binding conditions (SI Figure S1). Here, we found a significantly stronger binding interaction between aggregated Aβ and the (+/-)BAM1-EG$_6$ mixture ($K_d$ = 20 nM) compared to the binding of the

compare the strategies for improving the binding of small molecules to amyloid targets using multivalent interactions versus non-covalent, intermolecular interactions (Figure 1), we calculated the enhancement factor, β, proposed by Whitesides and co-workers [β = $K_a$(multi)/$K_a$(mono)][16,17] for both cases, which provides an estimate of the benefit of multimeric binding interactions in systems with unknown number of total binding sites (N) (as is the case with a heterogeneous mixture of amyloid aggregates). Here, we calculated an enhancement factor, β, of 10 for binding



**Figure 2.** Comparison of the normalized binding saturation plots of the covalently linked BAM1 dimer **2** and non-covalent mixture of (+/-)BAM1-EG$_6$ aggregated with β-amyloid (1-42) peptides. Fluorescence intensities were compared for each respective binding curve to estimate the fractional saturation of amyloid aggregates bound by the compounds (Figure 2, also see SI Figures S2 and S3).[23] The analysis revealed that the binding of dimer **2** to Aβ aggregates was slightly negatively cooperative with a hill coefficient of 0.8 (h = 0.005 to h = 1). This binding curve is consistent with the observed negative cooperativity typically found in most reported natural and unnatural multivalent binding systems due to various factors such as unfavorable

uncharged BAM1-EG$_6$, ($K_d$ = 170 nM) (Table 1, SI Figure S2). In order to

geometric strain or entropic cost due to the structural constraint of linkers used to covalently join ligands and receptors.[16] In contrast to the results with BTA dimer **2**, we found that the (+/-)BAM1-EG$_6$ mixture exhibited positive cooperativity upon binding to Aβ aggregates, with a Hill coefficient of h = 3.3; this results was significantly different from the observed Hill coefficient for both the dimer **2** (*p* = 0.001) and neutral parent compound, BAM1-EG$_6$ (*p* = 0.02), as determined by unpaired t-test (Table 1). In order to provide a qualitative visual representation for these differences in cooperativity, Figure 2 shows an overlay of the normalized binding curves of (+/-)BAM1-EG$_6$ and dimer **2** to aggregated Aβ peptides, where the increasing steepness of the sigmoidal curve reflects the larger Hill coefficient for the binding of the mixture of charged BTA molecules compared to dimer **2**.

As a control, we also assessed the binding of pure (+) or (-)BAM1-EG$_6$ to Aβ aggregates. Since the pI of Aβ has been reported to be ~5.5[24], we performed all binding studies at pH 5.5 to minimize electrostatic interactions between the charged BAM agents and aggregated Aβ (Figure S4). Surprisingly, under these conditions we found that pure (+)BAM1-EG$_6$ exhibited a $K_d$ = 290 nM to Aβ aggregates, whereas pure (-)BAM1-EG$_6$ exhibited a $K_d$ = 40 nM to Aβ aggregates (Figure S4) (compared to a $K_d$ = 170 nM for uncharged BAM1-EG$_6$), suggesting that some electrostatic interactions between the charged small molecules and the (apparently positively charged) amyloid surface could not be completely avoided at pH 5.5. However, the low enhancement factor (β = 0.6 and β = 4), the lack of significant positive cooperativity (h = 0.7 and 1.6) found for binding of pure (+) or (-)BAM1-EG$_6$, respectively, as well as the tighter apparent binding of the (+/-)BAM1-EG$_6$ mixture (20 nM) to Aβ aggregates compared to ether pure charged BAM derivative (40 nM or 290 nM) suggests that the intermolecular non-covalent interactions between oppositely charged BAM agents in the 1:1 mixture of (+/-)BAM1-EG$_6$ play an important role in the observed increased apparent binding of this mixture to the amyloid surface.

In order to provide additional experimental evidence that electrostatic interactions play a role in the binding of (+/-)BAM1-EG$_6$ to Aβ aggregates, we examined the binding of (+/-)BAM1-EG$_6$ in pH 5.5 water containing high salt concentrations (i.e. 500 mM NaCl), which we expect would diminish electrostatic interactions.[25] As anticipated, under high salt conditions we observed a significantly lower apparent binding affinity for (+/-)BAM1-EG$_6$ to Aβ aggregates compared to when no NaCl was added (Table 1). Additionally, the positive cooperativity that was observed for the (+/-)BAM1-EG$_6$ mixture in pure water was eliminated in the presence of high salt, exhibiting a Hill coefficient that was similar (i.e., not significant by unpaired t-test) to neutrally-charged BAM1-EG$_6$ bound to aggregated Aβ (Table 1). Taken together, the observed decrease in apparent affinity and cooperativity for binding of (+/-)BAM1-EG$_6$ to Aβ aggregates under high salt conditions suggests that electrostatic interactions play a dominant role for the improved binding of (+/-)BAM1-EG$_6$ to Aβ compared to BAM1-EG$_6$ in pure water.

To examine whether the use of non-covalent, intermolecular interactions could improve the binding of BTA derivatives to amyloids derived from peptides or proteins other than Aβ, we next tested the binding of (+/-)BAM1-EG$_6$ to a preparation of aggregated α-Synuclein (αS) at pH 5 (the pI of αS has been reported to be ~4.7[26]), a protein associated with Lewy Bodies found in patients with Parkinson's disease. As was the case with Aβ aggregates, we found that (+/-)BAM1-EG$_6$ bound stronger ($K_d$ = 400 nM) to aggregated αS compared to the uncharged parent compound, BAM1-EG$_6$ ($K_d$ = 1100 nM), with an estimated enhancement factor β of 3 (Table 1, Figure S5). Under high salt conditions, this enhancement of apparent binding of (+/-)BAM1-EG$_6$ to aggregated αS was abolished and the apparent binding was comparable of BAM1-EG$_6$. Furthermore, a Hill coefficient of h = 3.8 (with no added salt) suggested that (+/-)BAM1-EG$_6$ exhibited positive cooperativity for binding to aggregated αS (Table 1, *p* = 0.006 compared to binding of neutral BAM1-EG$_6$). These binding results with αS further support that non-covalent,

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electrostatic interactions between oppositely charged BTA derivatives can improve the apparent binding of small molecules to multiple (here, two) amyloidogenic protein aggregates.

In conclusion, while binding to amyloids remains a complex system, we have demonstrated that non-covalent interactions between synthetic molecules can be used to improve their binding to amyloid targets. We show that a 1:1 mixture of negatively charged (-)BAM1-$EG_6$ and positively charged (+)BAM1-$EG_6$ exhibited a 10-fold enhancement of binding to aggregated Aβ peptides compared to the neutrally charged parent compound, BAM1-$EG_6$, and a 3-fold enhancement for binding to aggregated αS proteins. The observed enhancement in binding was substantially reduced in the presence of high salt, supporting the significant role of electrostatic interactions in the binding of (+/-)BAM1-$EG_6$ to the amyloid surface. We hypothesize that differences in average binding site densities for small molecules on different amyloid compositions[15,27] could play a role in influencing the extent of improved binding between molecules bound to nearby sites on an amyloid surface (Figure S6). While it remains to be seen, such differences in average binding site densities may potentially be further exploited to improve the targeting of one amyloid composition over another using a similar approach as described here. Furthermore, we show that using non-covalent interactions between monomeric amyloid-binding molecules led to the same degree of enhancement in binding to aggregated Aβ as a covalently attached dimeric molecule **2**, and demonstrated the possibility of using non-covalent interactions to impart positive cooperativity into the amyloid-binding properties of small molecules. Given that the affinity of BTA analogs for binding to amyloids was directly correlated with potency for reducing seminal amyloid-medicated HIV infection,[12] the work presented here could lead to improved design of microbicide supplements for reducing transmission of certain sexually transmitted diseases. Current efforts are focused on exploring other non-covalent or reversible interactions (such as metal chelation) in the design of high affinity amyloid-binding small molecules to improve biocompatibility and targeting of amyloids associated with Alzheimer's and other amyloid-related diseases.

## METHODS

**Measurement of the binding affinity to aggregated Aβ (1-42) or α-synuclein (αS).** Binding of compounds to aggregated Aβ (1-42) and αS was measured according to a previously described assay.[8] Briefly, 200 μL of various concentrations of BTA/BAM compounds in DI $H_2O$ or 500 mM NaCl/ DI $H_2O$ (high salt conditions) were incubated in the absence or presence of 10 μg of pre-aggregated amyloid (total volume 220 μL). Solutions were allowed to equilibrate overnight at room temperature. Samples were then centrifuged at 16,000 x g for 20 min at 4°C. The supernatants were removed and the pellet was re-suspended in 220 μL of fresh DI $H_2O$ or 500 mM NaCl. Fluorescence of the bound molecule was determined using a spectrofluorometer (Photon Technology International, Inc., Birmingham, NJ). Each experiment was repeated at least three times and error bars denote standard deviation from the mean. Graphs shown in Figures S1-S5 were fit using the following one-site specific binding algorithm with Hill slope to determine $K_d$: $Y = B_{max} \times X^h/ (K_d^h + X^h)$, where X is the concentration of small molecule, Y is the specific binding intensity, $B_{max}$ is the apparent maximal observable fluorescence upon binding to Aβ/αS and h is the hill slope. Data was processed using Origin 7.0 (MicroCal Software, Inc., Northampton, MA) and GraphPad Prism 6. For statistics comparing Hill coefficients, the unpaired t test with Welch's correction was used and calculated from GraphPad Prism 6 (GraphPad Software, Inc. La Jolla, CA).

## ASSOCIATED CONTENT

**Supporting Information**. Additional details for the synthesis and characterization of negatively charged (-)BAM1-$EG_6$ and positively charged (+)BAM1-$EG_6$, for preparation and characterization of amyloid aggregates, and for methods used to quantify additional binding characteristics can be found in the supplemental information. Additional raw

binding data can also be found in the supplementary information. This information is available free of charge via the Internet at http://pubs.acs.org.

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### Author Contributions

J.L.C. and J.Y. designed the research and analyzed the data. J.L.C. and C.C.C. executed the experiments. J.L.C. and J.Y. wrote the manuscript.

### Notes

The authors declare no competing financial interests
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## REFERENCES

(1) Eisenberg, D., and Jucker, M. (2012) The amyloid state of proteins in human diseases. *Cell 148*, 1188–203.
(2) Knowles, T. P. J., Vendruscolo, M., and Dobson, C. M. (2014) The amyloid state and its association with protein misfolding diseases. *Nat. Rev. Mol. Cell Biol. 15*, 384–96.
(3) Hardy, J., and Selkoe, D. J. (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science 297*, 353–6.
(4) Moore, D. J., West, A. B., Dawson, V. L., and Dawson, T. M. (2005) Molecular Pathophysiology of Parkinson's Disease. *Annu. Rev. Neurosci. 28*, 57–87.
(5) Hull, R. L., Westermark, G. T., Westermark, P., and Kahn, S. E. (2004) Islet amyloid: A critical entity in the pathogenesis of type 2 diabetes. *J. Clin. Endocrinol. Metab. 89*, 3629–3643.
(6) Münch, J., Rücker, E., Ständker, L., Adermann, K., Goffinet, C., Schindler, M., Wildum, S., Chinnadurai, R., Rajan, D., Specht, A., Giménez-Gallego, G., Sánchez, P. C., Fowler, D. M., Koulov, A., Kelly, J. W., Mothes, W., Grivel, J. C., Margolis, L., Keppler, O. T., Forssmann, W. G., and Kirchhoff, F. (2007) Semen-Derived Amyloid Fibrils Drastically Enhance HIV Infection. *Cell 131*, 1059–1071.
(7) Chiti, F., and Dobson, C. M. (2006) Protein misfolding, functional amyloid, and human disease. *Annu. Rev. Biochem. 75*, 333–66.
(8) Olsen, J. S., Brown, C., Capule, C. C., Rubinshtein, M., Doran, T. M., Srivastava, R. K., Feng, C., Nilsson, B. L., Yang, J., and Dewhurst, S. (2010) Amyloid-binding small molecules efficiently block SEVI (semen-derived enhancer of virus infection)- and semen-mediated enhancement of HIV-1 infection. *J. Biol. Chem. 285*, 35488–96.
(9) Inbar, P., Li, C. Q., Takayama, S. A., Bautista, M. R., and Yang, J. (2006) Oligo(ethylene glycol) derivatives of thioflavin T as inhibitors of protein-amyloid interactions. *Chembiochem 7*, 1563–6.
(10) Cifelli, J. L., Chung, T. S., Liu, H., Prangkio, P., Mayer, M., and Yang, J. (2016) Benzothiazole Amphiphiles Ameliorate Amyloid β-Related Cell Toxicity and Oxidative Stress. *ACS Chem. Neurosci. 7*, 682–8.
(11) Habib, L. K., Lee, M. T. C., and Yang, J. (2010) Inhibitors of catalase-amyloid interactions protect cells from beta-amyloid-induced oxidative stress and toxicity. *J. Biol. Chem. 285*, 38933–43.
(12) Capule, C. C., Brown, C., Olsen, J. S., Dewhurst, S., and Yang, J. (2012) Oligovalent amyloid-binding agents reduce SEVI-mediated enhancement of HIV-1 infection. *J. Am. Chem. Soc. 134*, 905–8.
(13) Sheik, D. A., Brooks, L., Frantzen, K., Dewhurst, S., and Yang, J. (2015) Inhibition of the enhancement of infection of human immunodeficiency virus by semen-derived enhancer of virus infection using amyloid-targeting polymeric nanoparticles. *ACS Nano 9*, 1829–36.
(14) Qin, L., Vastl, J., and Gao, J. (2010) Highly sensitive amyloid detection enabled by thioflavin T dimers. *Mol. Biosyst. 6*, 1791–5.
(15) Lockhart, A., Ye, L., Judd, D. B., Merritt, A. T., Lowe, P. N., Morgenstern, J. L., Hong, G., Gee, A. D., and Brown, J. (2005) Evidence for the presence of three distinct binding sites for the thioflavin T class of Alzheimer's disease PET imaging agents on beta-amyloid peptide fibrils. *J. Biol. Chem. 280*, 7677–84.
(16) Krishnamurthy, V. M., Estroff, L. A., and Whitesides, G. M. (2006) Multivalency in Ligand Design, in *Fragment-based Approaches in Drug Discovery* (Jahnke, W., and Erlanson, D. A., Eds.). Wiley-VCH Verlag GmbH & Co., Weinheim.
(17) Mammen, M., Choi, S.-K., and Whitesides, G. M. (1998) Polyvalent Interactions in Biological Systems: Implications for Design and Use of Multivalent Ligands and Inhibitors. *Angew. Chemie Int. Ed. 37*, 2754–2794.
(18) Savjani, K. T., Gajjar, A. K., and Savjani, J. K. (2012) Drug solubility: importance and enhancement techniques. *ISRN Pharm. 2012*, 195727.
(19) Pajouhesh, H., and Lenz, G. R. (2005) Medicinal chemical properties of successful central nervous system drugs. *NeuroRx 2*, 541–53.
(20) Cifelli, J. L., Dozier, L., Chung, T. S., Patrick, G. N., and Yang, J. (2016) Benzothiazole Amphiphiles Promote the Formation of Dendritic Spines in Primary Hippocampal Neurons. *J. Biol. Chem. 291*, 11981–11992.
(21) Rong Liu (Ed.). (2008) Water-Insoluble Drug Formulation Second Edition. CRC Press, Boca Raton.
(22) LeVine, H. (2005) Multiple ligand binding sites on Abeta(1-40) fibrils. *Amyloid 12*, 5–14.
(23) Weiss, J. (1997) The Hill equation revisited: uses and misuses. *FASEB J. 11*, 835–841.
(24) Rauk, A. (2009) The chemistry of Alzheimer's disease. *Chem. Soc. Rev. 38*, 2698.
(25) Capone, R., Blake, S., Restrepo, M. R., Yang, J., and Mayer, M. (2007) Designing Nanosensors Based on Charged Derivatives of Gramicidin A. *J. Am. Chem. Soc. 129*, 9737–9745.
(26) Vigneswara, V., Cass, S., Wayne, D., Bolt, E. L., Ray, D. E., and Carter, W. G. (2013) Molecular Ageing of Alpha- and Beta-Synucleins: Protein Damage and

Repair Mechanisms. *PLoS One 8*, e61442.

(27) Ye, L., Velasco, A., Fraser, G., Beach, T. G., Sue, L., Osredkar, T., Libri, V., Spillantini, M. G., Goedert, M., and Lockhart, A. (2008) In vitro high affinity alpha-synuclein binding sites for the amyloid imaging agent PIB are not matched by binding to Lewy bodies in postmortem human brain. *J. Neurochem. 105*, 1428–37.

**Non-covalent, Intermolecular Interactions Enhance Amyloid Binding**