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Acetylation of A β_{40} Alters Aggregation in the Presence and Absence of Lipid Membranes

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Abstract

A hallmark of Alzheimer's disease (AD) is the formation of senile plaques comprised of the β -amyloid (A β) peptide. A β fibrillization is a complex nucleation-dependent process involving a variety of metastable intermediate aggregates and features the formation of inter- and intramolecular salt bridges involving lysine residues, K16 and K28. Cationic lysine residues also mediate protein–lipid interactions via association with anionic lipid headgroups. As several toxic mechanisms attributed to A β involve membrane interactions, the impact of acetylation on A β_{40} aggregation in the presence and absence of membranes was determined. Using chemical acetylation, varying mixtures of acetylated and nonacetylated A β_{40} were produced. With increasing acetylation, fibril and oligomer formation decreased, eventually completely arresting fibrillization. In the presence of total brain lipid extract (TBLE) vesicles, acetylation reduced the interaction of A β_{40} with membranes; however, fibrils still formed at near complete levels of acetylation. Additionally, the combination of TBLE and acetylated A β promoted annular aggregates. Finally, toxicity associated with A β_{40} was reduced with increasing acetylation in a cell culture assay. These results suggest that in the absence of membranes that the cationic character of

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Author Contributions

A.W.P. and J.L. conceived and designed the study. A.W.P. and M.N. performed ThT assays, AFM experiments, and PDA/lipid binding assays. A.W.P. and J.L. analyzed AFM data. S.J.V. performed MS experiments. J.S. and D.M.S. performed Caspase 3/7 toxicity assays. A.W.P. and J.L. wrote the initial draft. All authors have given approval of the final version of the manuscript.

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acchemneuro.9b00483>.

Supplemental Figure S1, demonstrating reproducibility of acetylation reaction for K16 and K28, and Supplemental Figure S2, demonstrating colocalization of fibrillar and annular aggregates (PDF)

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lysine plays a major role in fibril formation. However, acetylation promotes unique aggregation pathways in the presence of lipid membranes.

Graphical Abstract

Keywords

Amyloid; oligomers; annular aggregates; lysine; lipid bilayers; Alzheimer's disease

INTRODUCTION

Two prominent pathological features associated with Alzheimer's disease (AD), a fatal neurodegenerative disorder, are cerebrovascular, diffuse, and neuritic plaques composed predominantly of the amyloidogenic peptide amyloid- β ($A\beta$) and neurofibrillary tangles comprised of the protein tau. These proteinaceous deposits of tau and $A\beta$ are predominantly comprised of β -sheet-rich fibrous aggregates termed amyloid fibrils. $A\beta$ aggregation can be a multifaceted and complex process that involves a variety of metastable, intermediate species that may or may not be directly on-pathway to fibril formation.¹ The aggregate species that can be formed include a variety of soluble $A\beta$ oligomers ($A\beta$ Os), insoluble protofibrils, and annular aggregates.^{1–3} Beyond the heterogeneity of smaller intermediate aggregate structures, $A\beta$ fibrils can form a variety of distinct structures and morphologies, often termed polymorphs, as a result of preparatory and/or environmental conditions during the aggregation process.^{4–6}

Specific domains or amino acids within the $A\beta$ peptide appear to play a prominent role in dictating the rate of aggregation and the resulting aggregate morphologies.⁷ For example, specific regions within the $A\beta$ peptide have been implicated as being particularly amyloidogenic.^{7–10} Using site-directed spin labeling electron paramagnetic resonance, two β -strand forming domains in $A\beta$ were identified (residues 11–21 and 29–39, respectively) that were separated by a β -turn.¹¹ NMR studies further support the two β -strand regions separated by a β -turn motif in a number of fibril structures.^{12–14} Various fragments of $A\beta$ display different propensities to aggregate.⁷ The first β -strand forming stretch of $A\beta$ also contains a hydrophobic core (residues 16–21) with enhanced amyloidogenic properties.¹⁵ Furthermore, a number of point mutations associated with familial forms of AD clearly alter aggregation rates and aggregate morphologies.¹⁶

Lysine is of particular interest as it contains a positive charge at the end of an aliphatic chain. There are two lysine residues in A β : lysine-16 (K16) and lysine-28 (K28). Electrostatic interactions facilitated by these lysine residues in A β play an essential role in A β folding, nucleation, oligomerization, fibril formation, and toxicity.^{17,18} Specifically, a salt bridge forms between D23 and K28 that stabilizes a β -turn in a variety of fibril structures.^{18–20} K16 is the first amino acid of the previously mentioned hydrophobic core. Additionally, K16 can be involved in intermolecular salt bridge in some fibril structures of A β ;^{18,21} however, K16 is solvent-exposed in other fibril structures.²² Beyond fibril formation, lysine residues are involved in oligomer formation, as substituting alanine for each lysine altered A β assembly and highlighted a role of K16 in A β toxicity.²³

In addition to playing a role in A β aggregation, lysine can play a prominent role in the interaction of A β with lipid membranes. In general, lysine facilitates a variety of peptide-membrane interactions, as it contains the ability to electrostatically interact with charged lipid headgroups while the alkyl chain allows for hydrophobic interactions within the lipid tails.²⁴ Interaction with cellular membranes, comprised predominately of lipids, can profoundly influence A β aggregation,²⁵ promote unique aggregation pathways,^{26–28} nucleate aggregation,^{29–34} and/or stabilize specific A β aggregate species.^{26,32,35,36} It is even suggested that the binding of A β to the plasma membrane is a key step in A β toxicity.^{37,38} A variety of model lipid membranes alter A β conformation and exert enormous influence on the aggregation state, but the exact impact of membranes on A β aggregation is specific to lipid composition.³⁹ In addition to influencing A β aggregation,^{40,41} membranes can be directly targeted by A β , resulting in membrane damage and dysfunction through altered mechanical properties⁴² and/or the formation of discrete pores.^{32,35,43} While lipid composition and biophysical properties of membranes play a role in A β /lipid interactions, physicochemical properties of the residues within A β also influence surface interactions.^{16,44} For example, disease-related point mutations within A β alter the aggregate species observed on model lipid membranes, demonstrating the importance of hydrophilicity and electrostatic properties of specific amino acids within A β .¹⁶

Here, our aim was to understand how acetylating lysine alters A β_{40} aggregation in the presence and absence of lipid membranes. A β_{40} was acetylated by exposure to sulfo-*N*-hydroxysulfosuccinimide acetate (NHSA) producing acetylated K16 (AcK16) and K28 (AcK28). The lipid system used was total brain lipid extract (TBLE), which contain a physiologically relevant mix of lipid components. A β does not appear to be extensively acetylated *in vivo*. Rather, this study was motivated by the dual role lysine plays in both A β aggregation and membrane binding. In addition, this study demonstrates that simple, targeted chemical changes in A β profoundly impact aggregation, membrane interaction, and ultimately toxicity. In the absence of lipid vesicles, acetylation of A β_{40} reduced both oligomerization and fibrillization, with extensive acetylation resulting in complete inhibition of fibril formation. However, the presence of TBLE lipid vesicles appeared to invoke a distinct aggregation mechanism, resulting in fibril formation even when A β_{40} was extensively acetylated. Furthermore, acetylation reduced A β -related toxicity in cell culture.

RESULTS

Lysine-16 Is Preferentially Acetylated over Lysine-28.

To determine the extent at which K16 and K28 were accessible for acetylation and determine experimental conditions under which the impact of acetylation on aggregation could be studied, $A\beta_{40}$ (20 μ M) was exposed to NHSA, an established agent for acetylating primary amines.^{45,46} $A\beta_{40}$ was treated with varying concentrations of NHSA ranging from 0.02 mM to 2.5 mM (resulting in a range of peptide to NHSA molar ratios of 1:1 to 1:125). As there are two lysine residues in $A\beta_{40}$ that can be acetylated (K16 and K28), $A\beta_{40}$ was digested with pepsin to generate peptide fragments containing only one of the lysine residues so that the extent of acetylation of each discrete lysine could be determined by MS.

The percent of acetylated K16 (AcK16) and K28 (AcK28) was determined by comparing the abundance of the peptic fragments of different charge states containing just one lysine that was acetylated or not (Figure 1). When no NHSA was added to $A\beta_{40}$, no acetylation of lysine was observed. At lower concentrations of NHSA, ranging from 0.02 mM to 0.16 mM, K16 is preferentially acetylated over K28 with K16 acetylation being ~2–3 $\times$ more abundant than K28 (Figure 1B). With the 0.16 mM treatment of NHSA, ~47% of K16 was acetylated, but only ~17% of K28 had been modified. Effectively, these lower concentration treatments of NHSA resulted in conditions at which K16 was the predominately acetylated lysine; however, most of the available lysine residues (both K16 and K28) were not acetylated. At NHSA concentrations of 0.50 mM, ~78% of K16 was acetylated, while ~48% of K28 was acetylated, resulting in a condition in which the vast majority of $A\beta$ was acetylated with AcK16 being the most abundant form (~1.6 $\times$). Further increasing the concentration of the acetylating agent to 1.0 mM and 1.5 mM produced an increase in the acetylation of K28 to about ~70–78% and reduced the ratio of AcK16:AcK28 to about 1:1. While AcK16 and AcK28 were observable with all treatments of NHSA, the NHSA concentrations of 0.02 mM to 0.50 mM represent a regime in which K16 is predominantly more acetylated than K28; whereas, for concentrations of 1.0 mM and above of NHSA both K16 and K28 are acetylated to similar and significant extents. Further supporting the notion that K16 was more accessible for acetylation compared to K28 was the reproducibility of the NHSA reaction from experiment to experiment (Figure S1). While the abundance of AcK16 and AcK28 always increased with larger NHSA concentration and was relatively reproducible, the extent of K28 acetylation varied to a greater extent.

Acetylation of $A\beta_{40}$ Extends the Lag Phase and Extent of Aggregation.

Having established the extent of acetylation of K16 and K28 exposed to varying amounts of NHSA, we next determined the impact of AcK16 and AcK28 on the aggregation of $A\beta_{40}$. ThT assays were performed with 20 μ M $A\beta_{40}$ that had been exposed to increasing concentrations of NHSA (Figure 2). In general, increasing acetylation of $A\beta_{40}$ inhibited aggregation. That is, as the concentration of NHSA increased, the lag phase was extended and the total amount of β -sheet content decreased. The addition of 0.16 mM and 0.32 mM NHSA did not significantly change the lag phase associated with $A\beta_{40}$ compared to control ($A\beta_{40}$ that was not exposed to NHSA). $A\beta_{40}$ in the absence of an NHSA treatment, with 0.16 mM, or 0.32 mM treatment of NHSA exhibited similar lag phases of 6.8 ± 0.16 h, $6.4 \pm$

0.22 h, and 7.0 ± 0.17 h, respectively (Figure 2B), and the relative slope of the growth phase, R_{slope} (h^{-1}), was also not significantly changed ($0.40 \pm 0.058 \text{ h}^{-1}$, $0.33 \pm 0.037 \text{ h}^{-1}$, and $0.27 \pm 0.046 \text{ h}^{-1}$ respectively, Figure 2C). Treatment with 0.16 mM, 0.32 mM, and 0.50 mM NHSA reduced the total extent of fibrillization as determined by the relative fluorescent signal of the steady-state plateau region (RF_{ss}) of the ThT curve (reduced by 23%, 45%, and 53% respectively, Figure 2D). Increasing the concentrations of NHSA to 0.50 mM resulted in a statistically significant extension of the lag phase, 7.7 ± 0.13 h. In addition, the slope of the growth phase at 0.50 mM NHSA significantly deviates from the control, $0.15 \pm 0.040 \text{ h}^{-1}$ (Figure 2C), and the total extent of fibril formation (measured as RF_{ss}) was reduced by 58%. Finally, treatment with 1.0 mM NHSA and 1.5 mM NHSA completely suppressed fibril formation over the 20 h ThT assay, although there is a hint of a potential fibril nucleation event after ~18 h. As such, accurate lag phase and growth phases could not be determined; however, the relative fluorescence at the end of the experiment is less than 20% of the control for both conditions.

Acetylation of $\text{A}\beta_{40}$ Inhibits Fibril Formation and Oligomerization.

While the ThT assays demonstrate that acetylation of lysine via exposure to NHSA reduces fibrillization of $\text{A}\beta_{40}$, these assays do not provide information concerning the potential of acetylation to promote polymorphic fibril structure or $\text{A}\beta\text{O}$ formation. In light of previous observations that substituting lysine in $\text{A}\beta$ with alanine results in altered fibril morphology,²³ we incubated $\text{A}\beta_{40}$ under the exact same conditions as used in the ThT assays and sampled these solutions at multiple time points for AFM analysis. Beyond looking at fibril morphology, this has the added benefit of allowing for the assessment of $\text{A}\beta\text{O}$ formation. To analyze the population and dimensions of $\text{A}\beta$ fibrils and $\text{A}\beta\text{Os}$, criteria based on aggregate morphologies were used for automated image analysis.⁴⁷ $\text{A}\beta\text{Os}$ were defined in AFM images as being larger than 1 nm in height, having an aspect ratio of between 1.0 and 2.5 (indicating a nonfibrillar morphology), and occupying a surface area less than $9,500 \text{ nm}^2$. The surface area criteria were based on our observation that the smallest fibrils occupied a surface area of $\sim 10,000 \text{ nm}^2$ and that oligomers occupied an area typically 5 times smaller than this. Fibrils were identified as aggregates that were at least 1 nm in height with an aspect ratio greater than 2.5 and occupying a surface area of at least $10,000 \text{ nm}^2$. Upon solubilization, an initial aliquot of $\text{A}\beta_{40}$ was sampled for all conditions (effectively time point 0 h), and no aggregates were observed.

Based on AFM images and consistent with the ThT assays, increasing acetylation of $\text{A}\beta_{40}$ impaired aggregation (Figure 3). $\text{A}\beta\text{O}$ growth was quantified by counting the number of discrete oligomers per unit area observed on mica (Figure 4A); fibril growth was quantified by the percent of the mica surface covered by fibrils (Figure 4B). This approximation was used to quantify the extent of fibril formation due to fibril bundling and heterogeneity in length, making it difficult to manually, or with an algorithm, determine the exact numbers of fibrils. When nonacetylated $\text{A}\beta$ was incubated, a large population of oligomers was observed within 3 h with a peak population appearing between 5 and 7 h, as measured by the number of $\text{A}\beta\text{Os}$ per unit area (Figure 4A). Fibrils appeared after 7 h of incubation, and the fibril density on the mica surface steadily increased, as measured by the % area of the

surface occupied by fibrils (Figure 4B). The appearance of fibrils coincided with a steady decline in the $A\beta$ O population.

When treated with NHSA concentrations of 0.16 mM, 0.32 mM, and 0.5 mM, $A\beta$ O also appeared after 3 h of incubation; however, the $A\beta$ O population was significantly reduced in an acetylation-dependent manner (Figure 3 and Figure 4A). With all three of these NHSA treatments, fibrils were still observed at later time points. For both 0.16 mM and 0.32 mM concentrations of NHSA, fibrils could be observed after 7 h of incubation, but fibrils were not observed by AFM for $A\beta_{40}$ treated with 0.5 mM NHSA until after 9 h of incubation (Figure 3 and Figure 4B). The density of fibrils observed on mica was also decreased as a function of acetylation (Figure 4B). Consistent with the ThT assays, treatment with 1.0 mM and 1.5 mM NHSA resulted in no observable fibrils over the 11 h incubation time. In addition, the number of $A\beta$ O observed at any given time point was significantly reduced (~ 20 times), suggesting that extensive acetylation arrests $A\beta_{40}$ aggregation at the oligomeric phase. It should be noted that the detection limit of $A\beta$ O associated with this AFM analysis is likely on the order of octamers. That is, smaller $A\beta$ O may still have formed but would be difficult to observe with this assay. Hexameric and smaller $A\beta$ O have been observed for $A\beta_{40}$ in which the lysine residues have been substituted for with alanines.²³ With that being said, size analysis demonstrates that reaching a condition in which $A\beta_{40}$ is significantly acetylated results in smaller and fewer $A\beta$ O. Specifically, $A\beta$ O formed without NHSA treatment or with NHSA treatments of 0.32 mM or less resulted in oligomers with a broad height distribution with a mode of ~ 1.5 – 3.5 nm (Figure 4C). NHSA treatments of 0.5 mM or greater resulted in oligomers that had a mode height of ~ 1 – 1.5 nm (Figure 4C). As the size of $A\beta$ O observed under a specific condition did not change as a function of time, and so few oligomers were observed at extensive NHSA treatments, the height histograms presented were compiled over all time points.

To determine if acetylation promoted unique fibril polymorphs, morphological features of fibrils grown under the different treatments of NHSA were determined by AFM image analysis (Figure 5). Height profiles were taken perpendicular to the axis of each fibril for $A\beta_{40}$ fibrils formed at NHSA concentrations of 0.0 mM, 0.16 mM, 0.32 mM, and 0.50 mM (Figure 5A). The 1.0 mM and 1.5 mM NHSA condition were not analyzed as fibrils were not observed. Profiles were taken in a manner to avoid fibril crossings in order to represent the height scale of single fibril species better. The fibril height profiles of $A\beta_{40}$ at 0.0 mM, 0.16 mM, 0.32 mM, and 0.50 mM NHSA concentrations all had a mode fibril height of ~ 2.5 nm (Figure 5A), suggesting that the fibrils formed under these conditions were not polymorphic. As fibril height can vary along the contour of the fibrils, the average height along fibril contour of every individual fibril was determined. As fibril heights did not appear to change as a function of time, data time points of 7 h and beyond were compiled for each variable to produce histograms of the average fibril contour height (Figure 5B). The median fibril contour height for NHSA concentrations of 0.0 mM, 0.16 mM, 0.32 mM, and 0.50 mM was 2.4, 2.2, 3.1, and 2.5 nm, respectively, with an interquartile range of 1.0, 0.8, 0.4, and 0.6 nm, respectively (Figure 5B), confirming the absence of any polymorphic fibril structures for $A\beta_{40}$ under these acetylating concentrations.

Exposure to TBLE Membranes Promotes Fibrillization of Acetylated A β ₄₀.

As the presence of lipid membranes has been implicated as a modifier of A β aggregation^{39,48} and lysine plays a prominent role in peptide/lipid interactions,^{49–51} we next wanted to determine how the presence of lipid membranes modifies the aggregation of A β ₄₀ under the various acetylating conditions. ThT assays were performed in the presence of large unilamellar vesicles (LUV) of TBLE. TBLE was chosen as a model system due to its physiologically relevant mixture of membrane lipids and its extensive use in studying A β membrane interactions.^{7,16,52,53} In short, A β ₄₀ (20 μ M) was incubated in the presence of TBLE LUVs at a lipid to peptide ratio of 30:1 under the same experimental and instrumental conditions as the assays performed in the absence of lipids.

There are examples in the literature of the presence of lipids both accelerating^{54–56} or slowing^{39,57} A β aggregation. While the lipid composition of the membrane plays a role,^{39,58,59} the preparatory history of A β may also influence the A β /lipid interaction.⁵³ With the A β preparation protocol used here, TBLE membranes slowed aggregation compared to aggregation in the absence of lipids (compare the ThT assay curves of A β ₄₀ without NHSA treatment in Figure 2 and Figure 6).⁶⁰ For example, the presence of TBLE extended the lag phase of A β ₄₀ aggregation ~5 h. While acetylation associated with NHSA treatment of 1.0 mM or greater completely prevented fibrillization in the absence of TBLE vesicles, A β ₄₀ formed fibrils under all acetylating conditions based on the ThT assay curves (Figure 6), and this was later confirmed by AFM images of these solutions at the end of the ThT assay (Figure 7). In the presence of TBLE, the lag phase decreased with increasing acetylation, with the following lag-phase times exhibited: A β ₄₀, 9.8 $\pm$ 0.67 h; 0.16 mM NHSA, 7.8 $\pm$ 0.40 h; 0.32 mM NHSA, 7.2 $\pm$ 0.31 h; 0.50 mM NHSA, 6.8 $\pm$ 0.34 h; 1.0 mM NHSA, 7.1 $\pm$ 0.36 h; and 1.5 mM NHSA, 6.8 $\pm$ 0.99 h (Figure 6B). This trend suggests that A β acetylation promotes the formation of the critical nucleus associated with fibril initiation. This is in stark contrast to the impact of acetylation in the absence of TBLE, in which the lag phase was extended. In addition to the shortened lag phase in the presence of TBLE vesicles, acetylating conditions of 0.5 mM, 1.0 mM, and 1.5 mM NHSA all exhibited a statistically significant decrease in the slope of the growth phase in the presence of TBLE compared to control (A β ₄₀, 0.43 $\pm$ 0.042 h⁻¹; 0.5 mM NHSA, 0.26 $\pm$ 0.026 h⁻¹; 1.0 mM NHSA, 0.17 $\pm$ 0.027 h⁻¹; 1.5 mM NHSA, 0.18 $\pm$ 0.031 h⁻¹). Concurrent with this shortened lag phase, the overall β -sheet load, as measured by relative fluorescence at steady state, systematically decreased with increasing acetylated lysine content. The reduction in β -sheet formation started with ~20–35% reduction at 0.16 mM NHSA and 0.32 mM NHSA concentrations to ~60% reduction for 1.0 mM NHSA and 1.5 mM NHSA (Figure 6D).

TBLE Promotes Unique Aggregate Morphologies of Acetylated A β ₄₀.

While not resulting in unique fibril morphologies in the absence of lipids, point mutations in A β associated with familial forms of AD aggregate into unique polymorphic structures in the presence of TBLE bilayers.¹⁶ This suggests that acetylation could promote polymorphic fibrils in the presence of TBLE, despite not producing polymorphs in the absence of lipids. To determine if acetylated forms of A β ₄₀ produced polymorphic fibrils, A β ₄₀ (20 μ M) was incubated in the presence of TBLE LUVs at a lipid to peptide ratio of 30:1. After 20 h samples were deposited on a mica substrate, and AFM image analysis was performed for all

conditions. Height profiles of fibrils demonstrated a fibril height of 2.5–3.0 nm (Figure 7A), like that observed in the absence of TBLE (Figure 5A). However, there are several regions along the contour of the fibrils formed in the presence of TBLE that appeared thicker. That is, the morphology along the contour of the fibril deviated to a greater extent. As a result, the height along fibril contours had a much broader distribution when compared to fibrils in the absence of lipids. Median heights of fibrils in the presence of TBLE were 3.6 nm (0.0 mM NHSA), 3.3 nm (0.16 mM NHSA), 3.3 nm (0.32 mM NHSA), 3.9 nm (0.50 mM NHSA), 2.8 nm (1.0 mM NHSA), and 3.3 nm (1.5 mM NHSA), and the distribution of heights defined by the interquartile range were 2.6 nm (0.0 mM NHSA), 2.0 nm (0.16 mM NHSA), 1.9 nm (0.32 mM NHSA), 2.0 nm (0.50 mM NHSA), 2.1 nm (1.0 mM NHSA), and 2.1 nm (1.5 mM NHSA) (Figure 7B). An example of this broadening can be observed for A β ₄₀ and 0.50 mM NHSA in Figure 8B. Collectively, it appears that TBLE does not promote a fibril polymorph in acetylated A β ₄₀; however, the increased variation in morphology along the fibril contour may indicate that lipids are being incorporated into or decorating the observed fibrils. This does not appear to be dependent on acetylation. Rather, it appears to be a consequence of fibril formation in the presence of TBLE vesicles.

While the morphology of fibrils formed in the presence of TBLE did not appear to be altered by acetylation, increased acetylation promoted the formation of annular aggregates in the presence of TBLE (Figure 7A and Figure 8). For conditions of 1.0 and 1.5 mM NHSA, a large population of annular aggregates formed (2.6 ± 0.4 to 6.4 ± 0.6 per $25 \mu\text{m}^2$, respectively) that were not readily observed in the absence of lipid or with lower levels of acetylation. These annular aggregates were considerably thicker than fibrils with heights ranging from ~5–10 nm (Figure 8B). The inner diameter of these annular aggregates ranged from ~60–200 nm with a median size of ~114 nm (Figure S9B). Many annular aggregates appeared to be strongly associated with fibril aggregates as they were colocalized on the mica surface, with some fibrils appearing to protrude directly from annular aggregates (Figure S9A).

Acetylation Reduces the Affinity of A β ₄₀ for TBLE Membranes.

As the presence of TBLE vesicles clearly influenced the aggregation of acetylated A β ₄₀, a polydiacetylene (PDA) lipid-binding assay was performed to measure the total interaction between A β ₄₀ and TBLE membranes (Figure 9). Polydiacetylene is a lipid moiety that is easily incorporated into vesicles and photo-cross-linked, resulting in hybrid vesicles with enhanced fluorescence in response to mechanical stress associated with peptide binding and aggregation. By monitoring the fluorescence of these vesicles as a function of time, the total interaction of A β with the TBLE/PDA vesicles can be monitored. Control experiments were performed with the various doses of NHSA, and a fluorescent response was not invoked in these control experiments. Upon exposure of TBLE/PDA vesicles to A β ₄₀, the fluorescent signal steadily increases as the peptide binds and aggregates on the vesicles. As the extent of A β ₄₀ acetylation was increased, the total interaction between the peptide and vesicles was gradually reduced. As binding to and aggregation on the vesicle can both induce PDA response, it is important to interpret this assay with the results of the ThT assay in mind. As the shortest lag phase for any condition was ~7 h as determined by the ThT assay, the PDA response for the first 7 h is dominated by the initial binding of A β to the membrane. It

appears that acetylation reduces the overall affinity of $A\beta_{40}$ for TBLE vesicles. As the steady-state phase of fibril formation is reached for all conditions by 11 h of incubation (as determined by the ThT assay), the total fibril load has its largest contribution to the total PDA signal. In this regard, the magnitude of the PDA decreases with increasing acetylation in a similar fashion in comparison with the ThT assay. Lastly, at ~17 h the PDA/TBLE fluorescence signal associated with exposure to β that had not been treated with NHSA or treated with 0.16 mM NHSA began to decrease instead of maintaining a plateaued maximum response. This drop-in signal eventually reaches that of the maximum response of other experimental conditions. Extensive fibrillization on lipid vesicles can lead to PDA vesicle instability that results in flocculation, and these two conditions were associated with the most substantial fibril load. As a result, this likely reflects a loss in signal to the instability of the PDA/TBLE vesicles upon prolonged exposure to late-stage aggregates that alter the colloidal suspension.

$A\beta_{40}$ -Induced Cytotoxicity Is Reduced by Acetylation in a Dose Dependent Manner.

To determine the impact of acetylation on the $A\beta$ -induced cytotoxicity, mouse neuroblastoma X Rat neuron hybrid, ND7/23, cells were exposed to 5 μ M $A\beta_{40}$ that had been treated with varying doses of NHSA (0.0, 0.16, 0.32, 0.50, 1.00, and 1.50 mM). Cell death was determined with a Caspase 3/7 toxicity assay 24 h after exposure to the different $A\beta$ preparations (Figure 10). Unmodified $A\beta_{40}$ (0 mM treatment of NHSA) resulted in $21.7 \pm 0.8\%$ toxicity. The percent toxicity decreased in a dose dependent manner with increasing acetylation. The smallest treatment with NHSA (0.16 mM), a condition for which a third of available lysine residues were acetylated, significantly reduced $A\beta$ toxicity ($15.5 \pm 1.0\%$) compared to control. Under conditions when approximately two-thirds of available lysine residues are acetylated (0.5 mM NHSA treatment), $A\beta$ -induced toxicity dropped below 10%, and when 85–90% of the available lysine residues were acetylated (1.5 mM NHSA treatment), the percent toxicity was only $4.3 \pm 1.4\%$.

DISCUSSION

Understanding how individual amino acids of $A\beta$ affect aggregation and peptide-membrane interactions is significant as it provides the possibility for focused, therapeutic targeting and may provide more effective treatment strategies. In this work, we explored the role of lysine residues in the aggregation of $A\beta_{40}$ in the presence and absence of membranes through removal of the positive charge of lysine side chains via acetylation. In summary (Figure 11), we demonstrate that 1) acetylation reduces the aggregation propensity of $A\beta_{40}$, eventually abolishing fibril formation; 2) acetylation impedes the formation of $A\beta$ Os; 3) under our experimental conditions, TBLE membranes slow $A\beta_{40}$ aggregation; however, acetylation of $A\beta_{40}$ promotes aggregation in the presence of TBLE compared to control; 4) acetylation of $A\beta_{40}$ reduces the total $A\beta$ /membrane interaction; 5) the combination of acetylation and the presence of TBLE can promote the formation of annular aggregates of $A\beta_{40}$. This work suggests that the $A\beta$ aggregation process (in both the presence and absence of lipid bilayers) is highly dependent on the charge state of lysine residues and that the $A\beta$ aggregation mechanism is unique on TBLE bilayers.

Through increasing the concentration of NHSA, both K16 and K28 could be acetylated, but K16 was more readily acetylated. The preferential acetylation of K16 is consistent with structural studies of monomeric A β demonstrating that K16 is solvent-exposed while K28 is partially protected.^{22,61} The experimental conditions created two regimes of A β acetylation: one in which AcK16 predominated (0.16 mM and 0.32 mM NHSA) and the other in which the abundance of AcK16 to AcK28 was similar (1.0 mM and 1.5 mM NHSA). As the complete absence of fibrils was observed only when K28 was appreciably acetylated, it is tempting to associate K28 acetylation with this observation. However, as substitution of either K16 or K28 with alanine impacted A β aggregation,²³ it is likely that acetylation of both lysine residues plays a role in the observed inhibition of aggregation. There are plausible mechanisms associated with acetylation of either lysine. K16 is associated with an amyloidogenic core sequence in A β that plays a crucial role in fibril formation,^{9,10} and as a result, altering this region is likely to impact aggregation. There is a precedent for such a notion as the Flemish mutation in A β , which changes an alanine to glycine and is at the end of this amyloidogenic core sequence, significantly reduces the fate of A β fibrillization.^{62–64} Indeed, low concentration treatments of NHSA, when K28 is not appreciably acetylated, exhibited significant (although not absolute) reduction in fibril and oligomer formation. A potential mechanism for AcK16 inhibition of aggregation is provided by computational studies of A β ₄₂[K16A] demonstrating that the substitution of lysine results in a loss of intrapeptide contacts between the hydrophobic core and C-terminus of A β , therefore, reducing the propensity of β -sheet formation required for aggregation.⁶⁵ With regard to K28, acetylation would disrupt its ability to participate in a salt bridge with D23 or E22. This salt bridge is associated with the turn region of a β -hairpin observed in an A β fibril structure.¹⁷ The removal of K28's positive charge through acetylation would, therefore, destabilize A β fibrils. Support for this mechanism can be seen in the success of a variety of A β fibril inhibitors directly targeting this salt bridge.^{66–70} Furthermore, artificially creating the salt bridge between D23 and K28 via the addition of a lactam linker increases the rate of fibril formation by removing the entropic barrier imposed by the establishment of the salt bridge and effectively removing the lag phase.⁷¹ Changes in the net charge of A β ₄₀ from –3 to –4 or –5 may also play a role in impeding fibril formation as increases in the solvation energy would reduce the strength of the hydrophobic collapse of A β that is associated with the initial stages of aggregation.^{72,73}

Beyond inhibiting fibril formation, acetylation of A β reduces oligomerization. This finding appears to be in contradiction to the oligomerization of A β ₄₀[K16A] and A β ₄₀[K28A] which displayed similar oligomerization distributions to wild-type A β ₄₀.²³ However, the detection limitations of the AFM analysis used here would not allow for the detection of the small oligomers (hexamers or smaller) observed by Sinha et al. With regard to the oligomers observable by AFM, these tend to be classified as high molecular weight (HMW) oligomers, and a number of toxic HMW A β Os have been reported.^{74–77} The smaller, low molecular weight (LMW) A β Os tend to be less toxic or even benign compared with their HMW counter-parts.^{74,76–78} The analysis suggests that even if acetylation does not alter the formation of LMW A β Os, the more toxically relevant HMW oligomers are reduced by acetylation. With regards to potential mechanisms for this inhibition of A β O formation, molecular dynamics suggests that A β oligomerization is governed through electrostatic

interactions⁴⁰ and that reducing electrostatic attraction promotes smaller ordered oligomers.⁴¹ Such a scenario is consistent with our observation that high levels of acetylation resulted in fewer and smaller A β O_s, as acetylation would reduce the electrostatic attraction between A β peptides.

The impact of acetylation on A β aggregation and toxicity suggests that targeting K16 and K28 can be an effective way to manipulate aggregation for potential therapeutic purposes. Indeed, there are several indications that such a strategy may be effective. A molecular tweezer, CRL01, that selectively binds to lysine side chains inhibits the formation of A β O_s and fibrils.⁷⁹ Aspirin, which can act as an acetylating agent,^{80–82} can reduce the risk of AD⁸³ and protects HEK239 cells against cytotoxicity associated with exposure to A β fibrils.⁸⁴ More broadly, aspirin reduces protein aggregation in several amyloid-based, neurodegenerative diseases,^{85,86} and this protection was directly linked to its ability to acetylate proteins in *C. elegans* models of Huntington's disease and AD.^{85,87} However, aspirin's ability to acetylate proteins is not lysine-specific.⁸⁸

Lipid membrane environments have been shown to influence the aggregation of a number of amyloid-forming proteins^{29,58,89–94} and can even induce unique aggregation pathways.^{26,91} The impact of membranes on A β aggregation is sensitive to several biochemical and biophysical factors.³⁷ For example, membrane thickness regulates A β aggregation, as POPC and DOPC catalyzed fibrillization at a wide range of lipid to peptide ratios but DLPC stabilized cytotoxic oligomers at stoichiometric or higher lipid to peptides ratios.⁹⁵ Interestingly, A β adopts a partially folded α -helical structure spanning from K16-E22 when binding zwitterionic lipids.⁹⁶ The inclusion of a charged lysine (K16) in this α -helix forming stretch associated with lipid binding implies potential importance of the positive charge associated with this residue in interacting with membranes and that removing the positive charge, e.g., by acetylation, should profoundly impact A β 's interaction on membranes. In a similar fashion, mutations, Arctic (E22G) and Italian (E22K) of the negatively charged glutamic acid, at the end of this sequence promote morphologically distinct fibrils on supported TBLE bilayers.¹⁶ Ganglioside clusters within lipid membranes facilitate the A β /membrane interaction and promote fibrils formation;⁹⁷ however, these fibrils differ than those formed in aqueous solution in morphology, secondary structure, and cytotoxic properties.³⁹ Importantly, ganglioside clusters appear to trap K28.⁹⁸ Beyond a role in fibril formation, specific lipid interactions may stabilize A β O intermediates, as has been demonstrated with polymethacrylate copolymer encased lipid-nanodiscs.^{99,100}

A unique, lipid-induced aggregation pathway would be consistent with our results with regard to A β aggregation in the presence and absence of TBLE vesicles. While acetylation extended the lag-phase A β aggregation in the absence of TBLE vesicles, the opposite was observed in the presence of such vesicles, and A β was able to form fibrils even after extensive acetylation associated with high concentration treatments of NHSA. Despite the shortened lag phase associated with acetylation of A β in the presence of TBLE vesicles, the total fibril load was reduced, and polymorphic annular aggregates were observed. Collectively, this suggests that, in the presence of lipids, acetylation enhances the formation of the critical nucleus associated with initiating fibrillization but promotes secondary aggregation pathways. The enhanced nucleation could be related to enhanced diffusion

along the membrane surface. That is, once bound to the lipid membrane, the removal of the cationic nature of lysine via acetylation could reduce the interaction with anionic head-groups, which may allow for freer diffusion of A β peptides along the membrane surface and increase the ability of peptides colliding and coalescing into a critical nucleus. A plausible scenario for the observed decrease in the slope of the growth phase, associated with the reduction in fibril elongation and β -sheet load, is that the inability to form a salt bridge between K28 and other residues increases the dissociation rate of monomers from the growing fibril ends relative to the association rate, resulting in an overall slower elongation.

As mentioned earlier, the preparatory history of A β influences A β aggregation in the presence and absence of lipids interaction.^{53,101–103} The impact of preparatory history on aggregation suggests that environmental differences strongly impact aggregation, and lipids can be considered an environmental factor. Under our experimental conditions, extensive acetylation (>85% of available lysine residues acetylated) completely arrested A β ₄₀ fibrillization in the absence of lipid; however, fibrils still formed with extensive acetylation when incubated with TBLE. This demonstrates that complete inhibition of fibril formation due to acetylation may not be universal across preparation protocols and environmental conditions. With that being said, both K16 and K28 participate in intermolecular and intramolecular electrostatic interactions that stabilize a variety of fibril structures,^{17,18} and it is likely that acetylation would universally retard fibrillization, albeit to varying extents. While K28 typically forms salt bridges with D23,^{18–20} acetylating K28 may have a more pronounced impact of aggregation compared to K16, which appears to be solvent exposed in fibrils under some experimental conditions.²² While the formation of the salt bridge between K18 and D23 may facilitate and stabilize fibrils, it does not appear to be required. For example, the Iowa mutation (D23N) in A β , which removes a negative charge involved in salt bridge formation with K28, slows aggregation but does not completely suppress fibril formation.⁴⁴ However, the Iowa mutation does promote a distinct polymorphic fibril structure when aggregating on lipid membranes.¹⁶

While A β does not appear to be extensively acetylated *in vivo*, several other amyloid-forming proteins, including tau, α -synuclein (α -syn), and huntingtin (htt), are acetylated. Acetylation of tau is elevated in human tauopathy brains with acetylation of various lysine residues impacting different aspects of tau biology, i.e., turnover, aggregation, and interactions with microtubules.^{104–107} Importantly, acetylation modifies tau-associated toxicity,^{105,106,108} and tau acetylation alters susceptibility to A β -associated toxicity.¹⁰⁷ Enzymes that regulate tau acetylation can be targeted to reduce Tau toxicity.^{105,109,110} N-Terminal acetylation occurs in α -syn *in vivo*,^{111–113} which enhances its propensity to form α -helical structures when binding lipids and reduces aggregation.^{114,115} Acetylation of lysine residues within the first 17 amino acids of htt have been identified by mass spectroscopy,¹¹⁶ and acetylation of some of these residues reduces htt's ability to bind lipid membranes and reduces toxicity in cell cultures.¹¹⁷ However, there are conflicting reports with regards to the impact of acetylation of htt on aggregation, as chemical acetylation reduced aggregation in htt-exon1 fusion protein¹¹⁷ but synthetic htt peptides with selected acetylated lysine residues demonstrated a minimal impact on aggregation.¹¹⁸ The discrepancy may lie in the extent of acetylation, as the chemical acetylation was nonselective compared to the precise acetylation of specific residues in the synthetic peptide.

Collectively, it appears that the cationic nature of lysine residues in general plays a role in amyloid formation that can be manipulated by acetylation.

Aspirin-induced lysine acetylation impedes the aggregation of SOD1, leading to the hypothesis that targeting the net charge of native or misfolded proteins may represent a viable therapeutic target.¹¹⁹ As A β is not extensively acetylated *in vivo*, targeting enzymes to promote its acetylation or deacetylation is likely not a viable therapeutic strategy. However, targeting the net charge of A β , as suggested by the SOD1 study, to influence its propensity to aggregate and/or bind lipid membranes may be an effective strategy, provided a method to selectively target lysine residues within A β can be developed. In particular, the reduced affinity of acetylated A β for lipid membranes could underlie the observed reduced toxicity as a function of acetylation, as binding to and damaging the plasma membrane plays a role in A β toxicity^{120,121}.

METHODS

Peptide Preparation.

Synthetic A β_{40} (Invitrogen) was equilibrated to room temperature for 30 min. A β_{40} was dissolved in 10% NH₄OH(w/v) to acquire an alkaline stock solution of A β_{40} (0.5 mg/mL). The stock solution was unperturbed for 10 min followed by 5 min of bath sonication. The A β_{40} stock solution was aliquoted and snap-frozen. Aliquots were lyophilized overnight and stored at -80°C until the day of experimentation. For experiments, individual aliquots of A β_{40} were equilibrated to room temperature. An alkaline solution, 60 mM NaOH(aq), was used to solubilize A β_{40} (target concentration of 1.5 mg/mL), creating what is referred to as the A β_{40} -NaOH solution. The A β_{40} -NaOH solution was incubated at room temperature for 10 min, after which it was gently pipet mixed. The actual concentration of the A β_{40} -NaOH solution was measured (NanoDrop, ThermoScientific) via absorbance at 280 nm with an extinction coefficient of $1440\text{ M}^{-1}\text{ cm}^{-1}$. The pH of the A β_{40} -NaOH solution was controlled to be 11.23. For all experiments, the A β_{40} -NaOH solution was diluted into a 20 mM HEPES 150 mM NaCl buffer, pH = 7.23.

Mass Spectrometry.

For the MS experiments, the A β_{40} -NaOH solution was divided into 10 μg aliquots. These aliquots were diluted in the HEPES buffer and treated with NHSA at increasing molar ratios of NHSA:A β_{40} . Samples were allowed to incubate at room temperature for 1 h before the NHSA reaction was quenched with 1 M Trizma (Sigma-Aldrich, St. Louis, MO). All samples were snapfrozen and lyophilized overnight. The following day, samples were dissolved in 1 M acetic acid containing 0.5 mg/mL of pepsin (10:1 pepsin:A β_{40}). Samples were left overnight protected from light and incubated at room temperature. On the third day samples were analyzed via liquid chromatography (LC)-MS (ThermoFisher Scientific Q Exactive Mass Spectrometer, an LC-MS/MS with orbitrap). To remove residual salts, chromatography was performed utilizing a C18 column (ZORBAX RRHD Eclipse XDB 80 Å C18, $2.1 \times 100\text{ mm}$, $1.8\text{ }\mu\text{m}$; Agilent, Santa Clara, CA) and a mobile phase of 0.1% formic/water (v/v) (A) and 0.1% formic acid/acetonitrile (v/v) (B) with a gradient of 5%B for 0–2 min, 40%B for 5 min, 50%B for 6 min, 95%B for 7–8 min, and 5% B for 8.5–10

min with a flow rate of 300 $\mu\text{L}/\text{min}$. A full MS scan was performed for the mass-to-charge range (m/z) of 200–2000 with a resolution of 70,000 and a resolution of 17,500 for the Top 5 tandem MS (MS/MS) method that was used. Mass spectral analysis was performed using BioPharma Finder to determine the percent abundance of AcK16 and AcK28. This was carried out by comparing lysine-specific fragments of K16 (YEVHHQKLVF) and K28 (FAEDVGSNKGAIIGL) of $A\beta_{40}$ to the same peptide fragments containing a 43.05 Da mass shift, for the +1, +2, and +3 charge states. LC-MS/MS analyses for several notable reagent:analyte molar ratios were performed in duplicate (see the Supporting Information) to demonstrate the reproducibility in % K acetylation assignments.

Thioflavin-T Fluorescence Assay (ThT).

Monitoring fibril formation was achieved with thioflavin T (ThT; Sigma-Aldrich' St. Louis, MO) assays. Assays were carried out in a Costar 96-well black clear flat bottom plate. The $A\beta_{40}$ -NaOH solution was added to 20 mM HEPES 150 mM NaCl solutions containing 3 mol equiv of ThT to $A\beta_{40}$ and varied molar equivalents of NHSA to $A\beta_{40}$ at a pH of 7.23. The resulting solutions contained 20 μM $A\beta_{40}$ and the addition of 0.16, 0.32, 0.50, 1.00, and 1.50 mM NHSA. Additionally, a 3 mm borosilicate bead was introduced to facilitate homogeneous mixing across wells.¹²² The assay plate was sealed with plate tape and incubated at 37 °C for 24 h on a SpectraMax M2Multi-Mode plate reader (Molecular Devices, Sunnyvale, CA). Fluorescence intensities were measured in 5 min intervals at excitation and emission wavelengths of 440 and 484 nm, respectively, with shaking in between reads. Assays performed in the presence of TBLE were performed in the same manner with the addition of TBLE vesicles prepared as described below.

Data analysis was performed utilizing GraphPad Prism 6 to average replicates and perform baseline corrections and error analysis reported as the standard error mean (SEM). Additionally, through the use of a Boltzmann sigmodal function (eq 1) kinetic parameters such as the nucleation time, commonly referred to here as lag phase (t_{lag}), was obtained by fitting the individual traces of ThT curves using the following equation:^{123,124}

$$Y = (y_i + m_i x) + \frac{y_f + m_f x}{1 + e^{-\left(\frac{x - x_a}{\tau}\right)}} \quad (1)$$

Utilizing GraphPad Prism 6 the initial lag phase and plateau regions of the curves are defined by linear fits where the initial fluorescence and slope are defined by y_i and m_i and the final fluorescence and slope are defined as y_f and m_f respectively. The time to reach the half-maximum of the curve is defined by x_o , and the elongation time constant of the growth phase is defined as τ . As a nonideal sigmoidal function, no constraints were applied. A least-squares fitting operation was then performed for a maximum iteration limit of 1000. The lag-phase time was calculated from the kinetic parameters, $t_{\text{lag}} = x_o - 2\tau$. The slope of the growth phase is associated with the growth of fibrils. The slope was calculated by normalizing the ThT traces to the mean maximum response of the control group (no NHSA added) within each independent experiment for comparison across experiments. To obtain the relative slope of the growth phase, R_{slope} (h^{-1}) the linear fits of the growth phase were

performed by defining the boundaries of the fit to only include the linear region between the lag phase and the plateau region as visually determined for each ThT curve. The maximum signal of fluorescence at steady state was calculated averaging the signal over the plateau region of the sigmoidal curve. Initial starting points were visually determined to be near the inflection point where the sigmoidal curve enters the plateau region. The fits were performed from these starting points to the end of the data set. Once the boundaries were defined, the average fluorescence signal within this plateau region was determined. For comparison between trials, these were converted to the relative fluorescence at steady state (RF_{ss}) by dividing each value by the control experiment (no NHSA for that experiment). All kinetic parameters were then averaged from triplicates of independent experiments to determine the mean $\pm$ SEM. Significance was reported as greater than 95% confidence calculated using a standard Student's *t* test to determine variations in parameters across varied NHSA concentrations.

Lipid Vesicle Preparation.

A 5 mg amount of TBLE (Avanti Polar Lipids, Alabaster, AL) was hydrated with a 20 mM HEPES 150 mM NaCl buffer, pH = 7.23, and allowed to incubate for 30 min at 60 °C and 1400 rpm in a Thermomixer. Lipid samples were vigorously pipetted and scraped to remove all traces of TBLE from the walls of the centrifuge tube; after which, the samples were put through 7 freeze–thaw cycles and 30 min of bath sonication at 37 °C. Lipid stocks were again heated to 60 °C before being extruded through a 100 nm polycarbonate membrane filter. Resulting vesicle solutions were kept at 37 °C before being diluted into a HEPES solution at a final concentration of 0.432 mg/mL within a 96-well plate for a protein to lipid ratio of 30:1.

Ex Situ Tapping Mode Atomic Force Microscopy (TMAFM).

Incubations of $A\beta_{40}$ (20 μ M) were performed in a similar manner to previous ThT assays with the same concentrations and ratios of $A\beta_{40}$ to NHSA. Solutions were incubated at 37 °C and shaken and read at the same intervals as previous ThT assays; however, at the time points of 0, 1, 3, 5, 7, 8, 9, 11, and 24 h the 96-well plate was removed from the plate reader, and two 2 μ L samples were taken from each solution. The experiment was done in this manner in order to track the progress of $A\beta$ aggregate formation. Once samples were taken, they were allowed to incubate for 1 min before being washed with 100 μ L of 18 ultrapure water. Samples were dried with compressed air immediately after the wash. After sampling, the plate was returned and incubated until the next time interval. Samples were imaged via atomic force microscopy (AFM) under tapping mode on a Nanoscope V Multimode (Veeco, Santa Barbara, CA) with a closed-loop vertical engage J-scanner. Cantilevers used for images were a diving-board-shaped silicon oxide cantilever with a resonance frequency of ~300 kHz and a spring constant of 40 N/m (Budget Sensors).

AFM images were processed utilizing Matlab equipped with the image processing toolbox (MathWorks, Natick, MA) as described previously.⁴⁷ To correct for background curvature, height images were flattened using a second polynomial flattening algorithm. To aid in automatically identifying individual aggregates, AFM images were converted into binary maps after establishing a height threshold of 1.0 nm. Individual aggregates were determined

from the binary maps using a recognition algorithm. Physical features of individual aggregates (e.g., height, volume, diameter, shape factor, length, aspect ratio, length, and area covered) were measured. Average heights of fibrils were determined by measuring the fibrils along their contour length while excluding any cross over regions of overlapping fibrils. The range of fibril contour length, or the spread of fibril heights, was calculated as the interquartile range which involves taking the median of the lower and upper of a given data set. Populations of oligomers and fibrils, along with the percent area covered by fibrils, were plotted using GraphPad Prism 6 to average across replicates and perform error analysis reported as a standard error mean (SEM).

Preparation of TBLE/Polydiacetylene Vesicles (PDA).

Separately, 8.4 mg of 10,12-tricosadiynoic acid (PDA) (GFS Chemicals, Columbus, OH) and 5.0 mg of TBLE were dissolved in a 1:1 chloroform:ethanol solution. The two were combined in a glass vial at a 60:40 mass ratio of PDA to TBLE, and solvents were removed under vacuum to produce a TBLE/PDA film. This film was hydrated with 8 mL of 20 mM HEPES 150 mM NaCl buffer (pH = 7.23) at 75 °C. The solution was probe sonicated at 180 W continuously for 10 min. The resulting solution was stored protected from light in a 4 °C fridge overnight. The following day the TBLE/PDA solution was allowed to reach ambient temperature before transferring it to a 100 mL beaker. The solution was stirred at 300 rpm for 10 min under cover of a black box adapted with a TLC lamp that irradiated the solution at 254 nm for the full length of time. PDA assays were performed in a Costar 96 black clear flat bottom well plate with the addition of 3 mm borosilicate glass beads. Peptide-lipid interactions were observed fluorescently at room temperature with excitation and emission wavelengths of 485 and 560 nm utilizing a SpectraMax M2Multi-Mode plate reader (Molecular Devices, Sunnyvale, CA). Data analysis was performed by averaging triplicates and performing baseline corrections with GraphPad Prism 6.

Caspase 3/7 Toxicity Assay.

For cell based assays, synthetic A β ₄₀ was initially prepared as previously described above. Disaggregated A β ₄₀ was added to a 20 mM HEPES 150 mM NaCl buffer, pH = 7.23 along with NHSA. Acetylated forms of A β ₄₀ were obtained by incubating 20 μ M A β ₄₀ in a 20 mM HEPES 150 mM NaCl buffer, pH = 7.23, along with the simultaneous addition of NHSA at 0.0, 0.16, 0.32, 0.50, 1.00, or 1.50 mM NHSA. The subsequent solutions were kept at room temperature for 1 h without agitation. The NHSA reaction was quenched with a lysine solution for a final concentration of 2.7 mM. Dialysis was performed against H₂O at 4 °C in 2 h intervals for a total of 4 h (Slide-A-Lyzer MINI Dialysis Devices, MWCO 3.5K, Thermo fisher Scientific). Samples of A β ₄₀ were alkalified with 10% NH₄OH (w/v) and lyophilized to produce monomeric acetylated forms of A β ₄₀.

Mouse neuroblastoma X Rat neuron hybrid, ND7/23 (Sigma-Aldrich, St. Louis, MO), was grown in a Dulbecco's Modified Eagle Medium augmented with 4.5 g/L glucose, 110 mg/L sodium pyruvate, 1% penicillin-streptomycin (stabilized with 10,000 units penicillin and 10 mg streptomycin/mL), 2 g/L sodium bicarbonate, 584 mg/L L-glutamine (Sigma-Aldrich, St. Louis, MO), 5 mM HEPES (Fisher BioReagents, Waltham, MA), and 10% HyClone fetal bovine serum (Thermo Scientific, Waltham, MA) at 37 °C and 5% CO₂. Cells were seeded

at 5×10^3 cells/well and grown for 24 h. $A\beta_{40}$ previously acetylated with NHSA treatments of 0.0, 0.16, 0.32, 0.50, 1.00, or 1.50 mM was added to cells for a final concentration of 5 μ M $A\beta_{40}$. After 24 h of incubation the Caspase 3 reagent (Anaspec, Sensolyte AMC Caspase 3/7 assay kit) was added and allowed to shake for 45 min while protected from light at room temperature. Fluorescence intensities were measured at 45 min, 1, 3, and 4 h at Ex/Em = 354 nm/442 nm on a Synergy H1 (BioTek, Winooski, VT) plate reader. Data analysis was performed by subtracting the fluorescence readings of untreated cells from $A\beta_{40}$ exposed cells and then dividing by the difference of the positive control, the proteasome inhibitor MG132, and untreated cells to produce a measure of relative Caspase 3/7 activity. Relative Caspase 3/7 activities were compared using a two-way ANOVA, followed by a Dunnett's multiple comparisons test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AD	Alzheimer's disease
$A\beta$	β -amyloid peptide
$A\beta_{40}$	β -amyloid peptide (1-40)
$A\beta$Os	$A\beta$ oligomers
K16	lysine-16
K28	lysine-28
NHSA	sulfo- <i>N</i> -hydroxysulfosuccinimide acetate
AcK16	acetylated K16
AcK28	acetylated K28
TBLE	total brain lipid extract
AFM	atomic force microscopy
ThT	thioflavin T
t_{lag}	lag-phase time

R_{slope}	relative slope of the growth phase
RF_{ss}	relative fluorescent signal of steady-state
LUV	large unilamellar vesicle
LMW	low molecular weight
HMW	high molecular weight

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Figure 1.

Determining the extent of lysine acetylation by NHSA. (A) MS analysis of K16 and K28 specific peptide fragments reported as the percent abundance of acetylated to unmodified lysine. Percent abundance reported as average of +3, +2, +1 charge states of K16 and K28 specific peptide fragments. (B) Ratio of AcK16 to AcK28 as a function of increasing concentrations of NHSA.

Figure 2.

ThT assays investigating the impact of acetylation on $A\beta_{40}$ aggregation. (A) Representative ThT curves performed at 37 °C of 20 μ M $A\beta_{40}$ with increasing NHSA treatments of 0.0 mM (green), 0.16 mM (blue), 0.32 mM (purple), 0.50 mM (pink), 1.0 mM (orange), and 1.5 mM (red). Solid lines represent fits calculated with eq 1. (B) Lag-phase time, t_{lag} (h), (C) slope of growth phase, R_{slope} (h^{-1}), and (D) normalized maximum response of the steady state region of ThT curves, RF_{ss} , averaged over three independent experiments are shown as a function

of NHSA treatment. Error bars represent SEM in all bar plots. (*) $P = 0.05$, (**) $P = 0.01$, (***) $P = 0.001$, and (****) $P = 0.0001$.

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Figure 3.

Representative AFM images of Aβ₄₀ aggregates as a function of NHSA treatment and time. *Ex situ* AFM images sampled at multiple time points over 20 h of Aβ₄₀ (20 μM) that had been treated with various concentrations of NHSA: 0.0 mM, 0.16 mM, 0.32 mM, 0.50 mM, 1.0 mM, and 1.5 mM. The scale bar is applicable to all images, and the same color scale is used in all images.

Figure 4. AFM image analysis of the impact of acetylation on $A\beta_{40}$ aggregate populations and morphologies. (A) Oligomers per μm^2 and (B) percent surface area occupied by fibrils are presented as a function of incubation time. Error bars represent the SEM. (C) Histogram of $A\beta O$ height compiled over the entire time course of incubation for $A\beta_{40}$ treated with NHSA concentrations of 0.0 mM, 0.16 mM, 0.32 mM, 0.50 mM, 1.0 mM, and 1.5 mM.

Figure 5.

Comparison of fibril morphologies of $A\beta_{40}$ as a function of acetylation. (A) Height profiles of representative *ex situ* AFM images of $A\beta_{40}$ (20 μM) treated with 0.0 mM, 0.16 mM, 0.32 mM, and 0.50 mM NHSA. Higher concentration treatments of NHSA are not shown as fibrils were not observed under these conditions. Green and blue lines in each image indicate the location corresponding to height profiles provided on the right. (B) Histogram of the average fibril height measured along the contour of each fibril. (A, B) Red dashed line represents height of 2.5 nm.

Figure 6.

ThT assays investigating the impact of acetylation on $A\beta_{40}$ aggregation in the presence of TBLE LUVs. Representative ThT curves performed at 37 °C of 20 μ M $A\beta_{40}$ with increasing NHSA treatments of 0.0 mM (green), 0.50 mM (pink), 1.0 mM (orange), and 1.5 mM (red) in the presence of TBLE LUVs at a lipid to peptide ratio of 30:1. (B) Lag-phase time, t_{lag} (h), (C) slope of growth phase, R_{slope} (h^{-1}), and (D) normalized maximum response of the steady state region of ThT curves, RF_{ss} , averaged over three independent experiments are

shown as a function of NHSA treatment. Error bars represent SEM in all bar plots. (*) P 0.05, (**) P 0.01, (***) P 0.001, and (****) P 0.0001.

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Figure 7.

Comparison of fibril morphologies of $A\beta_{40}$ formed in the presence of TBLE as a function of acetylation. (A) Height profiles of representative *ex situ* AFM images, taken after 20 h of incubation, of $A\beta_{40}$ (20 μM) treated with 0.0 mM, 0.16 mM, 0.32 mM, 0.50 mM, 1.0 mM, or 1.5 mM NHSA. Green and blue lines in each image indicate the location corresponding to height profiles provided directly below each image. (B) Histogram of the average fibril height measured along the contour of each fibril. (A, B) Red dashed line represents height of 2.5 nm.

Figure 8.

Acetylation of $A\beta_{40}$ in the presence of TBLE produces annular aggregates. (A) *Ex situ* AFM images of $A\beta_{40}$ (20 μM) treated with 1.0 mM and 1.5 mM NHSA in the presence of TBLE. Boxes indicate regions presented as zoomed in images in (B). (B) Zoomed in $0.5 \times 0.5 \mu\text{m}$ images of annular aggregates for 1.0 mM and 1.5 mM NHSA. Green and blue lines correspond to height profiles directly below each image. The red dashed line in height profiles indicates 8.0 nm height. Color scale is consistent in all images presented.

Figure 9. Polydiacetylene binding assay of $A\beta_{40}$ at increasing states of acetylation. PDA/TBLE vesicle fluorescence response as a function of time upon exposure to unmodified $A\beta_{40}$ (0.0 mM NHSA, green) and $A\beta_{40}$ treated with 0.16 mM NHSA (blue), 0.32 mM NHSA (purple), 0.50 mM NHSA (pink), 1.0 mM NHSA (orange), or 1.5 mM NHSA (red).

Figure 10.

Caspase 3/7 activity of ND7/23 neuroblastoma upon exposure to acetylated $A\beta_{40}$.

Standardized Caspase 3/7 activity of ND7/23 neuroblastoma after 24 h of treatment with (5 μ M) pretreated with 0.0 mM (green), 0.16 mM (blue), 0.32 mM (purple), 0.50 mM (pink), 1.0 mM (orange), and 1.5 mM (red) NHSA concentrations. Data shown as mean $\pm$ SEM, $n = 12$, of the standardized fluorescence signals. (*) $P = 0.05$, (**) $P = 0.01$, (***) $P = 0.001$, and (****) $P = 0.0001$.

Figure 11.

The impact of acetylation of lysine residues on Aβ₄₀ aggregation in the presence and absence of lipid membranes. (A) Unmodified Aβ₄₀ readily forms fibrils via a classic aggregation pathway that includes LMW AβOs, HMW AβOs, and protofibrils. (B) With extensive acetylation of K16 and K28, aggregation appears to be arrested with a very small population of LMW AβOs. (C) While there are changes in rate of aggregation and small differences in aggregate morphology, unmodified Aβ₄₀ readily forms a variety of AβOs, protofibrils, and fibrils in the presence of lipid membranes. (D) In the presence of lipid membranes, acetylated Aβ₄₀ aggregation is reduced compared to unmodified Aβ. However, extensive acetylation does not completely arrest fibril formation and begins to promote the formation of large annular aggregates.