

Research Article

Cell Surface Binding and Internalization of $A\beta$ Modulated by Degree of Aggregation

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The amyloid peptides, $A\beta_{40}$ and $A\beta_{42}$, are generated through endoproteolytic cleavage of the amyloid precursor protein. Here we have developed a model to investigate the interaction of living cells with various forms of aggregated $A\beta_{40/42}$. After incubation at endosomal pH 6, we observed a variety of $A\beta$ conformations after 3 ($A\beta^3$), 24 ($A\beta^{24}$), and 90 hours ($A\beta^{90}$). Both $A\beta_{42}^{24}$ and $A\beta_{40}^{24}$ were observed to rapidly bind and internalize into differentiated PC12 cells, leading to accumulation in the lysosome. In contrast, $A\beta_{40/42}^{90}$ were both found to only weakly associate with cells, but were observed as the most aggregated using dynamic light scattering and thioflavin-T. Internalization of $A\beta_{40/42}^{24}$ was inhibited with treatment of monodansylcadaverine, an endocytosis inhibitor. These studies indicate that the ability of $A\beta_{40/42}$ to bind and internalize into living cells increases with degree of aggregation until it reaches a maximum beyond which its ability to interact with cells diminishes drastically.

1. Introduction

Alzheimer's disease (AD) is a progressive neurological disorder resulting from the deposition of Alzheimer β -Amyloid peptide ($A\beta$) as senile plaques, the appearance of neurofibrillary tangles, and selective neuronal loss. The most abundant forms of $A\beta$ are 40 and 42 amino acid residues long and referred to as $A\beta_{40}$ and $A\beta_{42}$, respectively [1].

The endocytic pathway has been implicated in the extracellular secretion of $A\beta_{40}$ and $A\beta_{42}$ [2, 3]. These peptides are derived from the endoproteolytic cleavage of the Amyloid precursor protein (APP) with β -secretase, followed by γ -secretase. β -secretase cleavage occurs in the acidic late endosomes [4, 5]. After γ -secretase cleavage, $A\beta_{40}$ or $A\beta_{42}$ is free in the endosomal lumen [6]. The endosomal contents can be either secreted from the cell [7–9] or transferred to the lysosome [10].

The endosome has been found to be quite acidic (pH 6) with the recycled endosome slightly less acidic (pH 6.5) [11, 12]. Exposure of $A\beta$ to endosomal pH conditions has been found to induce various conformational and oligomeric states [13–15]. Many oligomeric forms of $A\beta$ have been proposed and characterized as intermediates in the pathway to forming the Amyloid fibre. Some of these structures include trimers, pentamers, high molecular weight $A\beta$ -derived diffusible ligands (ADDLs), protofibrils, and fibrils [16–21].

Here we present a method for generating a mixed population of $A\beta$ conformations using model endocytic conditions. Using this method, we demonstrate that when $A\beta$ is exposed to endosomal conditions for an extended period of time, the ability of the peptide to bind and internalize into living rat adrenal pheochromocytoma (PC12) cells increases with time until it reaches a maximum beyond which its ability to interact with PC12 cells diminishes drastically.

2. Materials and Methods

2.1. Peptide Synthesis and Purification. A β 40 and A β 42 were synthesized and purified as described previously [22]. Before cleavage from the resin, the fluorophore, N α -(9-Fluorenylmethoxycarbonyl)-N ϵ -tetramethylrhodamine-(5-carbonyl)-L-lysine (Molecular Probes, Eugene, OR) (abbreviated TMR), was coupled to the N-terminus via a glycine linker. The crude peptides were purified by HPLC using a Superdex Tricorn 10/300 GL Peptide column (Amersham Biosciences, Piscataway, NJ) with 30 mM NH₄OH running buffer. To maintain stock peptide solutions free from fibril seeds, solutions were stored at pH 10 and 4°C immediately after chromatographic separation of monomeric peptides. A β preparations were never lyophilized, as this process may allow for seeds to form. These solution conditions have been previously shown to maintain the monomeric state [16, 23, 24]. Peptide purity and identity was confirmed using both MALDI mass spectrometry and amino acid analysis. Concentrations of stock peptide solutions were determined using amino acid analysis and confirmed by either tyrosine absorbance (275 nm, $\epsilon = 1390 \text{ cm}^{-1} \text{ M}^{-1}$) or TMR absorbance for labelled peptides (550 nm, $\epsilon = 92000 \text{ cm}^{-1} \text{ M}^{-1}$). At least three separate synthesized lots of A β were used in this study and each displayed identical cell association rates when compared for quality assurance. Oligomeric samples were prepared by diluting stock A β samples to 30 μM with 30 mM NH₄OH and reducing to pH 6 with 0.2 M HCl and incubating for zero (A β^0), 3 (A β^3), 24 (A β^{24}), or 90 hours (A β^{90}) in the dark at 20°C.

2.2. Dynamic Light Scattering (DLS). Hydrodynamic radius (Rh) measurements were made at 20°C with a DynaPro DLS instrument (Protein Solutions Inc., Piscataway, NJ). Peptide samples (30 μM) were reduced to pH 6 using 0.2 mM HCl, centrifuged at 12000 $\times g$ for 3 minutes and then rapidly added to a 1 cm path length cuvette and left in the instrument. DLS data was collected at various time points over 90 hours. Particle translational diffusion coefficients were calculated from decay curves of autocorrelation of light scattering data and converted to hydrodynamic radius (Rh) with the Stokes-Einstein equation. Histograms of intensity versus Rh were calculated using Dynamics data analysis software (Protein Solutions Inc., Piscataway, NJ).

2.3. Filter Assay. A β samples (30 μM) were either not filtered or spin-filtered for 30 minutes at 14,000 $\times g$ using 10, 30, 100 kDa (Amicon ultra cellulose KMWO), or 0.1 μm (Amicon PVDF) spin filters. Absorbance at 550 nm was collected on a Molecular Devices SpectraMax M5 (Molecular Devices Corp., Sunnyvale, CA) and graphs were created normalizing the absorbance signal from each filtered sample to the corresponding unfiltered sample.

2.4. Thioflavin-T Assay. Fluorescence measurements were obtained using 200 μL of 30 μM A β samples, within a 96-well plate, after addition of 5-fold molar excess of thioflavin-T and incubation at room temperature for 30 minutes.

Emission at 485 nm was collected using 440 nm excitation on a Molecular Devices SpectraMax M5 (Molecular Devices Corp., Sunnyvale, CA).

2.5. Cell Culture. PC12 cells were maintained in DMEM/F12 containing 10% fetal bovine serum (HyClone, Logan, UT) with 100 units/mL penicillin and 100 $\mu\text{g}/\text{mL}$ of streptomycin. To induce differentiation of PC12 cells and for cell imaging, they were plated at 2.2×10^4 cells/cm² in Lab-tech chambered cover glass chambers and suspended in phenol red free DMEM/F12 containing N2 supplement and 10 ng/mL NGF. Cells were differentiated for 72 hours before media was replaced and peptide treatments (final concentration 1.5 μM) were performed. Cells were maintained at 37°C in a humidified incubator with 5% carbon dioxide.

2.6. Analytical Ultracentrifugation. TMR-labelled A β samples (6 μM) were prepared in phenol red free DMEM/F12 media containing N2 supplement and 10 ng/mL NGF. Either fresh media or cultured supernatants, obtained from cell culture after 72 hours, were used. To avoid interference from cell culture components, molecular weights of TMR-labelled A β were obtained by selective monitoring of TMR absorbance at 550 nm. Sedimentation experiments were performed at 20°C on a Beckman XLI analytical ultracentrifuge using an AN50-Ti rotor. Molecular weights were calculated using Beckman XLI data analysis software in which absorbance versus radial position data were fitted to the sedimentation equilibrium equation using nonlinear least-squares fitting.

2.7. Confocal Microscopy. Three-dimensional stacks of fluorescence micrographs were taken at 20°C with a confocal laser-scanning system consisting of an LSM 510 Zeiss META NLO confocal microscope with a C-APO 40X water immersion objective (numerical aperture 1.2) and HeNe laser with a 543 nm laser line. The displayed images were captured using Zeiss LSM Image version 4 and prepared using ImageJ version 1.37v and represent a single cross-section through the cells.

2.8. Inhibition of Endocytosis. Differentiated PC12 cells were treated with 50 μM monodansylcadaverine (MDC) for 20 minutes at 37°C with 5% carbon dioxide. After inhibitor treatment, A β^{24} (final concentration 1.5 μM) was added to the media containing MDC and imaged after 4 hours at 37°C. A β^{24} (final concentration 1.5 μM) was also added to differentiated PC12 cells media for 4 hours at either 37°C or 4°C prior to imaging.

2.9. Intracellular Localization. Differentiated PC12 cells were first treated with A β^{24} (final concentration 1.5 μM) for 6 hours at 37°C followed by treatment with 40 nM Mito-Tracker Deep Red and 50 nM LysoTracker Green DND-26 for 20 minutes. After treatment, the media was exchanged with fresh phenol red free DMEM/F12 media containing N2 supplement and 10 ng/mL NGF with 1.5 μM DAPI for nuclear staining. Cells were imaged using Argon laser with 488 nm laser line for LysoTracker, HeNe laser with 633 nm

laser line for MitoTracker, HeNe Laser with 543 nm laser for TMR-labelled $A\beta^{24}$, and a tunable Chameleon laser at 730 nm for two-photon excitation of DAPI. 2D histograms and correlation coefficients were determined using Image J version 1.42q with colocalisation threshold plugin [25].

2.10. Toxicity. Differentiated PC12 cell media was replaced with media containing 0.6 to 20 μM $A\beta^0$, $A\beta^{24}$, $A\beta^{90}$, or 10 μM Melitin as a positive control for 48 hours. Cell survival was quantified using the Sulforhodamine B assay [26], and absorbance was measured at 560 nm using Molecular Devices SpectraMax M5 (Molecular Devices Corp., Sunnyvale, CA). LC50 values were determined as the concentration of $A\beta$ required to kill fifty percent of the cells from an absorbance versus $A\beta$ concentration plot.

3. Results

3.1. $A\beta$ Aggregation Is Mediated through Cell Interaction. To study the interaction between $A\beta$ and live cells, we synthesized and fluorescently labelled $A\beta_{40/42}$. The synthesized $A\beta$ was maintained in solution from purification to storage and was never lyophilized, as these solution conditions are known to significantly reduce the formation of $A\beta$ aggregation seeds [16, 23, 24]. We covalently attached tetramethylrhodamine (TMR) to the N-terminus of $A\beta$ via a flexible glycine linker to generate TMR- $A\beta$. The N-terminus of $A\beta$ is highly accessible even in the fibril state [21, 27, 28] and attaching a fluorescent label to this site has been shown to neither alter its amyloidogenic properties [16, 29, 30], nor its solubility behaviour [31, 32].

We have previously shown that treating cultured cells with 1.5 μM monomeric TMR- $A\beta_{42}$ leads to the formation of visible aggregates on the surface of PC12 cells within one hour of treatment [29]. We initiated our current study by investigating whether $A\beta$ aggregation could occur in cell culture media alone. Using analytical ultracentrifugation, we measured the molecular weights of $A\beta_{40}$ and $A\beta_{42}$ present in phenol red free cell culture medium, both freshly prepared and conditioned media taken from differentiated PC12 cells 3 days postdifferentiation. Following the addition of 6 μM $A\beta$ to each medium and subsequent 24-hour incubation at room temperature, both media preparations were centrifuged to equilibrium at room temperature in an analytical ultracentrifuge in order to determine the molecular weight of $A\beta$ conformations. The molecular weights of $A\beta_{40}$ and $A\beta_{42}$ from both media preparations were measured to be approximately 4103 Da and 4425 Da, respectively. These values both correspond to the expected monomeric molecular weights of $A\beta$, falling within the 95% confidence intervals of 3650–4570 Da for $A\beta_{40}$ and 3650–4770 Da for $A\beta_{42}$. Thus, the aggregation of $A\beta$ seen by confocal microscopy apparently occurs only after interaction with the differentiated PC12 cells and not with cultured supernatants. It should be noted that the concentration previously used to treat cells (1.5 μM) and the concentration used for ultracentrifugation (6 μM) are considerably lower than the reported 20 to 50 μM range required for *in vitro* aggregation [33–35].

3.2. $A\beta$ Oligomerizes at Endosomal pH. We have previously shown that $A\beta_{40}$ and $A\beta_{42}$ aggregate significantly at pH 6 [29]. Since $A\beta_{40}$ and $A\beta_{42}$ are generated through endoproteolytic cleavage [7–9], and the pH of the endosome and recycled cellular vesicles is equivalent to pH 6 [12], we characterized the $A\beta$ conformations formed under endosome conditions. $A\beta$ (30 μM) was reduced to pH 6, and hydrodynamic radius calculations were collected after zero ($A\beta^0$), 3 ($A\beta^3$), 24 ($A\beta^{24}$), and 90 ($A\beta^{90}$) hours using dynamic light scattering (Figure 1). The average hydrodynamic radius of $A\beta_{40}$ was found to increase from 2.0 nm at time zero ($A\beta_{40}^0$) to 216 nm after 90 hours at pH 6 ($A\beta_{40}^{90}$). A more striking increase was found with $A\beta_{42}$, beginning with 1.7 nm at time zero ($A\beta_{42}^0$) to 451 nm after 90 hours at pH 6 ($A\beta_{42}^{90}$). For each of these samples, the development of increasingly higher ordered aggregates was observed over time and the samples that were treated for 90 hours contained particles over 1000 nm in radii.

To further investigate the relative levels of peptide aggregation at endocytic pH, we filtered the $A\beta_{40}$ and $A\beta_{42}$ samples through various molecular weight cutoff (MWCO) spin filters (Figure 2). Approximately 67% of $A\beta_{40}^3$, 60% of $A\beta_{40}^{24}$, and 55% of $A\beta_{40}^{90}$ were recovered through the 10 kDa MWCO spin filter and approximately 80% were recovered through the 100 kDa MWCO filter, except for $A\beta_{40}^{90}$ with only 60% recovered. In contrast, only 55% of $A\beta_{42}^3$, $A\beta_{42}^{24}$, and $A\beta_{42}^{90}$ were recovered through the 10 kDa filter and approximately 60% were recovered through the 100 nm filter. These results indicate that the majority of peptide conformations present under these conditions were able to pass through a 10 kDa molecular weight filter, but that just over 40% could not be recovered through the 100 nm filter for the $A\beta_{40}^{90}$, $A\beta_{42}^3$, $A\beta_{42}^{24}$, and $A\beta_{42}^{90}$ samples.

We also used thioflavin-T to assess the time-dependence of the extent of Amyloid fibril formation at endocytic pH. Thioflavin-T is a dye known to shift its fluorescence from 430 nm to 490 nm upon binding specifically to the cross- β -structure of Amyloids but not to monomeric or small oligomeric complexes [36, 37]. We observed enhanced thioflavin-T fluorescence at all time points (Figure 3); however, Thioflavin-T bound most strongly to $A\beta_{40}^{90}$, $A\beta_{42}^{24}$, and $A\beta_{42}^{90}$. The high thioflavin-T binding to $A\beta^{90}$ samples suggests that these late stage $A\beta$ conformations are the most aggregated.

3.3. Endocytic $A\beta$ Undergoes Rapid Cellular Interaction. Using confocal microscopy, cell surface association and internalization of peptides can be monitored. We have previously shown that monomeric $A\beta_{42}$ associates with cells more rapidly than $A\beta_{40}$, with significant staining observable after six hours of treatment [29]. To determine whether the aggregation state of $A\beta$ affects cell association, we exposed each of the $A\beta_{40}$ and $A\beta_{42}$ samples to differentiated PC12 cells and monitored the kinetics of association by confocal microscopy. Upon treating cells with $A\beta_{40}^{24}$, the cell surface association was observed within one hour of treatment (Figure 4). Moreover, $A\beta_{40}^{24}$ was observed to significantly internalize into these cells after only 6 hours, whereas significant internalization of $A\beta_{40}^0$ was only visualized around

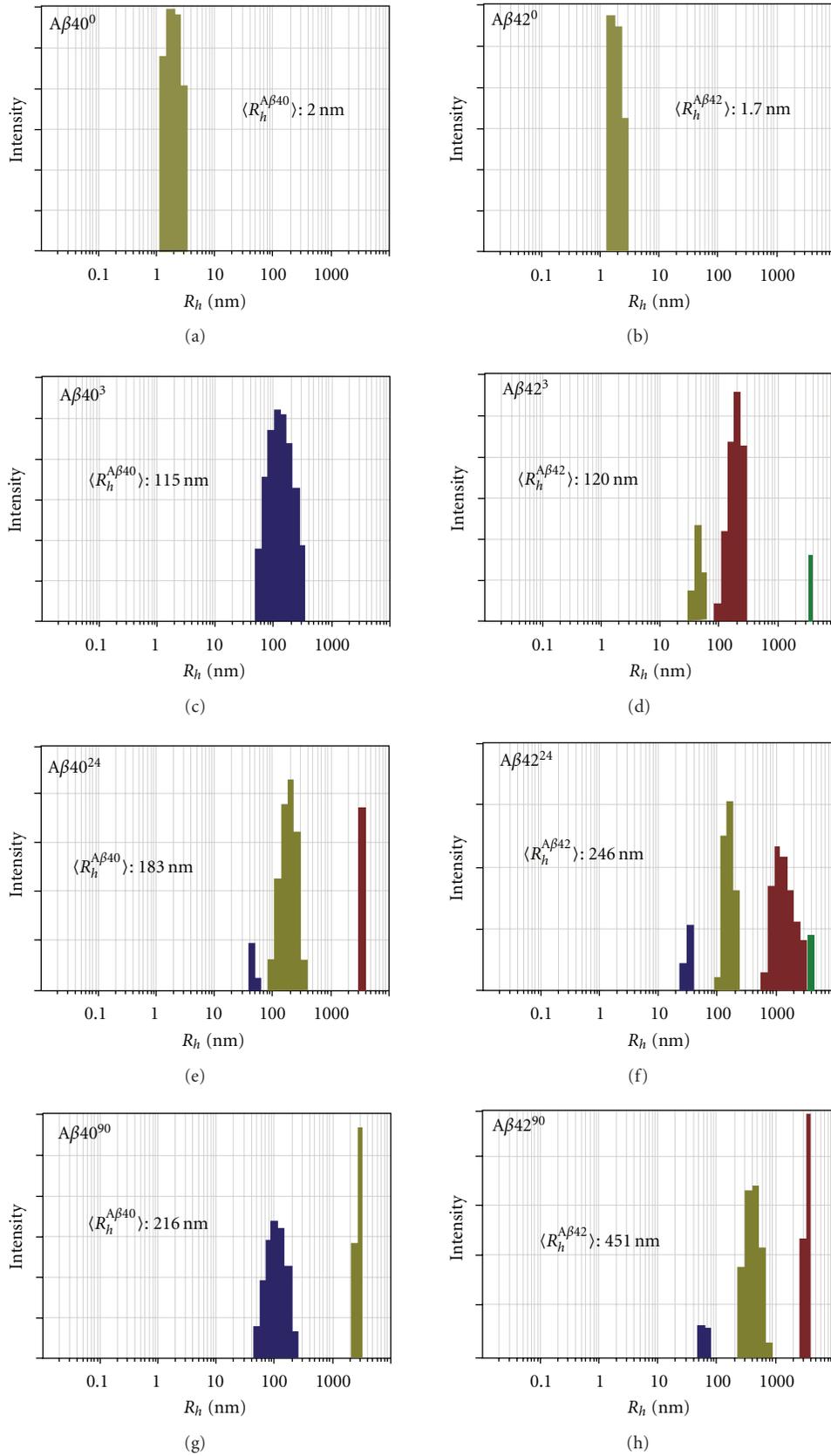


FIGURE 1: Dynamic light scattering of endocytic Aβ. A time-dependent increase in the average hydrodynamic radius (R_h) was observed with incubation of Aβ under endocytic conditions. At each time point, a variety of aggregates are present.

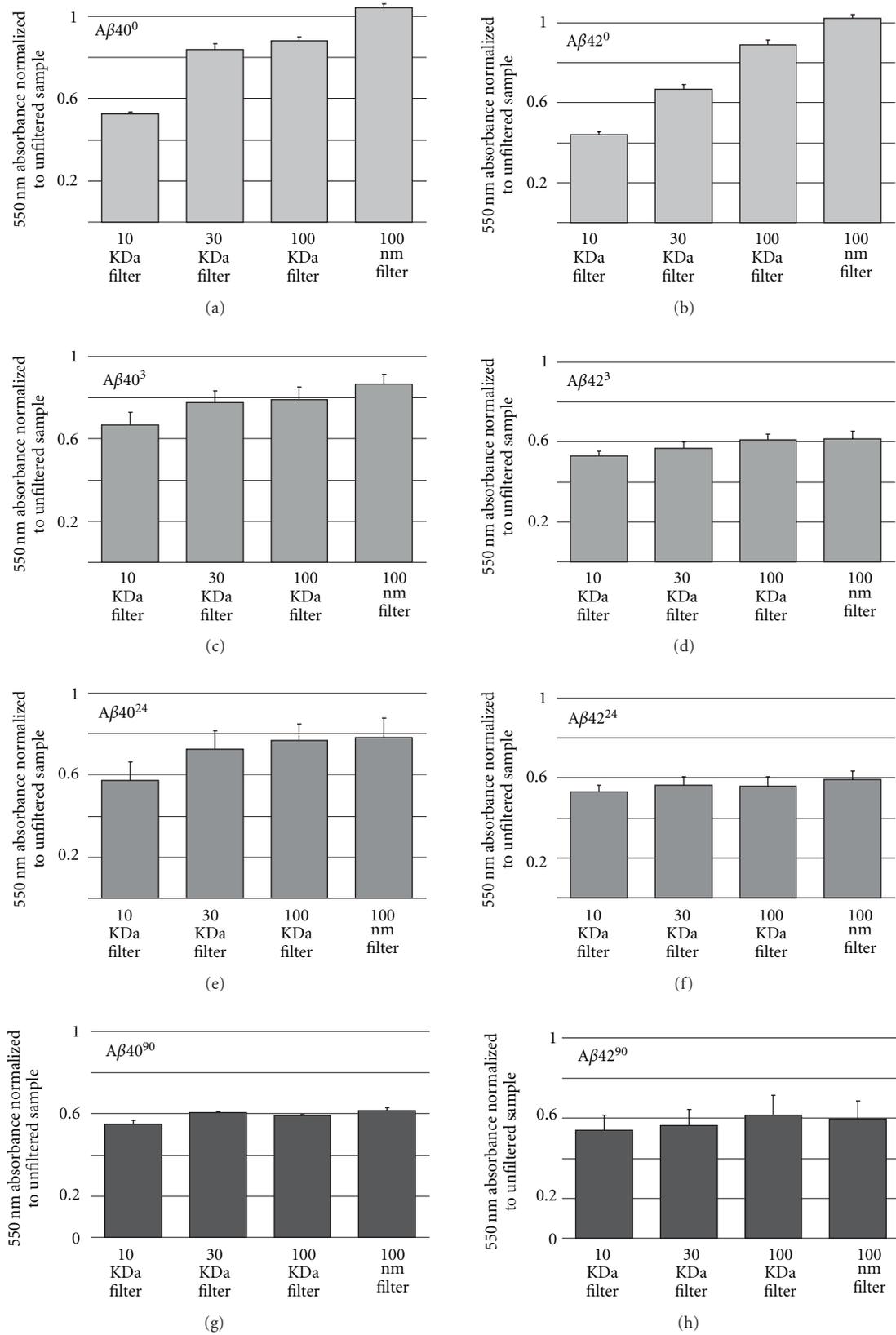


FIGURE 2: Separation of Aβ by ultrafiltration. Graphs show the TMR absorbance of the filtrates for each Aβ sample divided by the absorbance of the unfiltered sample at 550 nm. Error bars represent the range from two independent experiments.

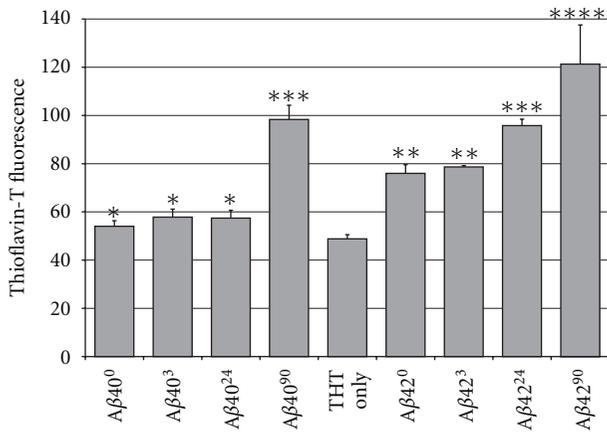


FIGURE 3: Thioflavin-T fluorescence of A β exposed to endocytic conditions. Significant thioflavin-T fluorescence for the A β 40⁹⁰ and A β 42⁹⁰ samples indicates the highest level of aggregation present. Error bars represent the range of two independent experiments. Statistical significance is indicated (*) $P < .05$.

24 hours (Figure 4). Similarly, A β 42²⁴ internalized into differentiated PC12 cells after only one hour of treatment, whereas A β 42⁰ treatment only became observable at 24 hours (Figure 4). Interestingly, when differentiated PC12 cells were treated with late-stage A β 40⁹⁰ or A β 42⁹⁰, very few cells underwent internalization or even exhibited cell surface interaction with these aggregated peptide forms (Figure 4). To illustrate the contrast between these treatments, we collected the three-dimensional image slices through a cell from the base to the apex for the 6-hour treatment with A β 42⁰, A β 42²⁴, and A β 42⁹⁰ (Figure 5). A β 42⁰ was only observed around the periphery of the cell, whereas some A β 42²⁴ was located inside the cell. By contrast, A β 42⁹⁰ treatment seemed to localize to extracellular regions and did not produce the punctate pattern as observed with the A β 42⁰ sample.

To quantify the frequency of cells that exhibited peptide internalization, we randomly selected five fields of view from at least three separate 6-hour treatment experiments and plotted the percentage of cells having internalized A β (Figure 6). Approximately 25% of cells internalized A β 40/42³, whereas more than 90% of cells internalized A β 40/42²⁴. In contrast, very few cells were found to internalize A β 40/42⁹⁰. Since late A β 40/42⁹⁰ was found to have a large number of aggregates with hydrodynamic radius over 1000 nm (Figure 1) and were found to significantly bind thioflavin-T (Figure 3), then these aggregates may favour self-association over cell association.

3.4. Internalization of A β Is Mediated through Cellular Import Mechanisms. To determine if the internalization of endocytic A β is mediated through cellular processes, such as receptor-mediated endocytosis or through direct peptide-mediated processes like membrane pore formation, we monitored the effects of temperature and monodansylcadaverine (MDC)

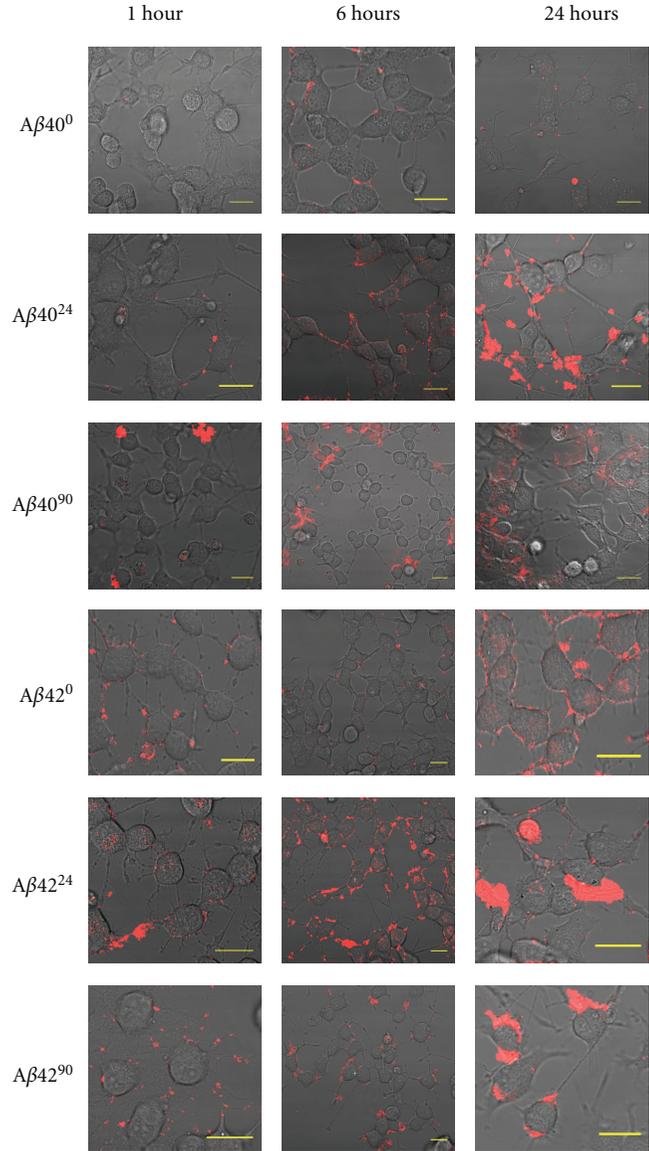


FIGURE 4: Confocal Microscopy images showing A β time course of cell entry. Confocal microscopy images of differentiated PC12 cells treated with various A β samples exposed to endosomal conditions after 1, 6, and 24 hours. All scale bars are 20 μ m in length. A β 40²⁴ associates rapidly to differentiated PC12 compared to A β 40⁰, whereas A β 40⁹⁰ displays weak cell association over its treatment course. A β 42²⁴ displays rapid internalization compared to A β 42⁰. A β 42⁹⁰ displays reduced cell interaction compared to A β 42⁰ and A β 42²⁴.

on internalization. MDC is a known inhibitor of receptor-mediated clathrin-dependent endocytosis [38, 39]. At physiological temperature (37°C), after a 4-hour treatment of cells with A β 40²⁴ and A β 42²⁴, internalization was observed (Figure 7). At 4°C membrane vesicle formation is inhibited, preventing endocytosis of extracellular and cell surface components [40]. When A β 40²⁴ or A β 42²⁴ association with differentiated PC12 cells was monitored at 4°C, none of the cells were found to have internalized these peptides

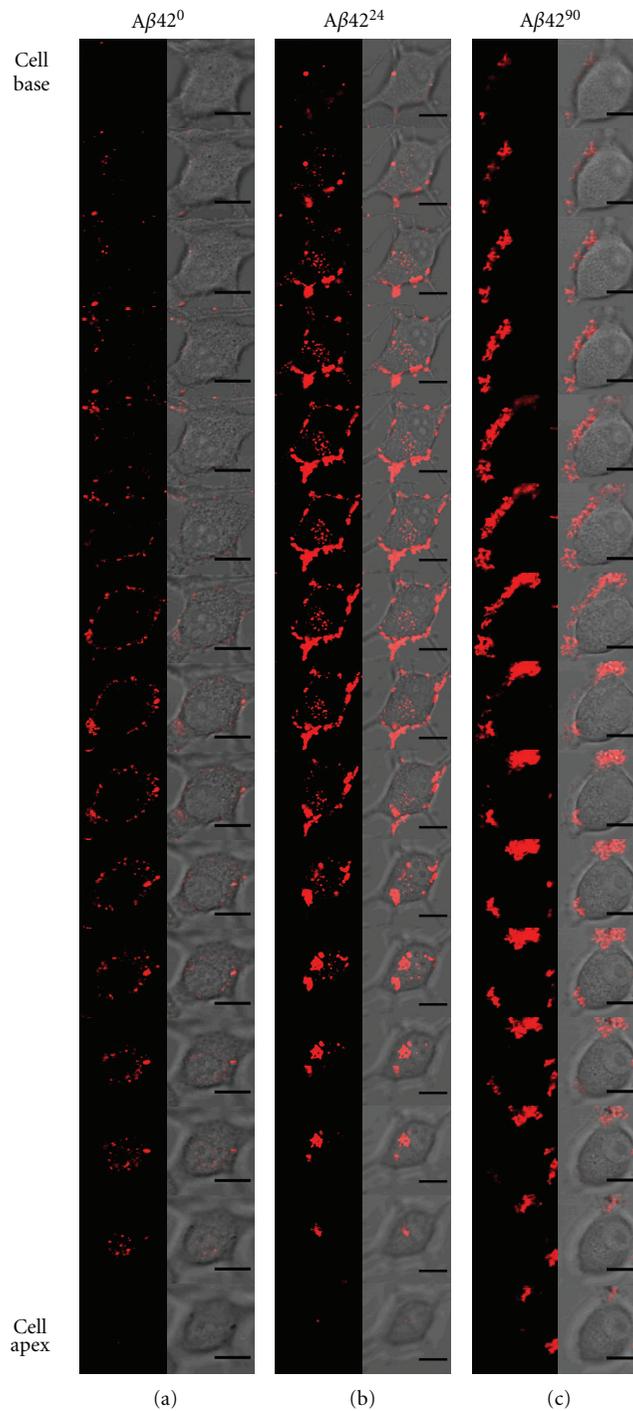


FIGURE 5: Image planes through a single cell for each $A\beta_{42}$ treatment. Confocal microscopy images from differentiated PC12 cell base to cell apex for $A\beta_{42}^0$, $A\beta_{42}^{24}$, and $A\beta_{42}^{90}$, with the TMR signal only (a) and TMR cell merged image (c). All scale bars are $10\ \mu\text{m}$ in length.

(Figure 7). Similarly, when differentiated PC12 cells were treated with MDC, neither $A\beta_{40}^{24}$ nor $A\beta_{42}^{24}$ were observed within the cells. These observations indicated that $A\beta^{24}$ was internalized through a cell-directed import mechanism, rather than an independent penetration route through the cell membrane.

3.5. Internalized $A\beta$ Is Targeted to the Lysosome. The location of deposited intracellular $A\beta_{40/42}^{24}$ was examined using intracellular organelle markers in differentiated PC12 cells. Cells treated with $A\beta_{40}^{24}$ and $A\beta_{42}^{24}$ for 6 hours were visualized using LysoTracker Green DND-26 for the lysosome, MitoTracker Deep Red for the mitochondria, and DAPI for

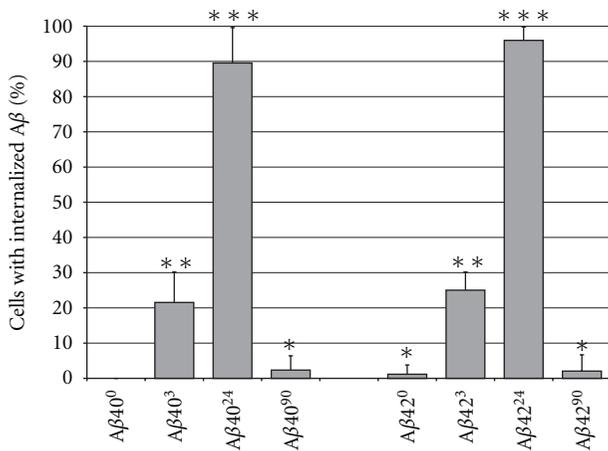


FIGURE 6: Percentage of PC12 cells with internalized A β . A β 40²⁴ and A β 42²⁴ were more significantly internalized into differentiated PC12 cells than the other forms of A β . A β 40⁹⁰ and A β 42⁹⁰ display a striking drop in the amount of internalization. Error bars represent the standard deviation from at least three individual experiments with $n > 200$ cells per condition. Statistical significance is indicated (*) $P < .005$.

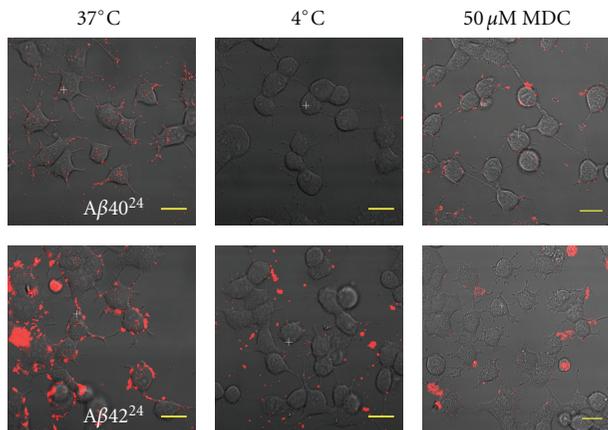


FIGURE 7: Inhibition of A β internalization. Confocal microscopy images of differentiated PC12 cells treated with A β 40/42²⁴ for 4 hours. All scale bars are 20 μ m in length. Internalization was only observed at 37°C. Membrane integrity was maintained throughout all treatments, indicating that internalization is mediated through a cellular process such as clathrin-dependent endocytosis.

nucleus staining (Figure 8). The staining pattern of each cellular organelle marker in the same image plane as the A β 40²⁴ and A β 42²⁴ treatment was determined (Figure 8) and quantified (Figure 9). Only the signal from the lysosome marker costained with both internalized A β 40²⁴ and A β 42²⁴. The red signal from the peptides costained well with green signal from the lysosomes, resulting in the yellow signal in the merged image. When quantified, 71% of the lysosome signal intensity colocalised with the A β 40 channel and 80% with the A β 42 channel (Figure 9). Whereas only 9% of the mitochondria signal intensity colocalised with A β 40 channel and less than 1% with the A β 42 channel

(Figure 9). These findings suggest that the internalized vesicles containing A β 40/42²⁴ are directed to the lysosome. The intensity of both A β 40²⁴ and A β 42²⁴ fluorescence signals was found to increase in the lysosomes over time, which may reflect the accumulation of A β 40/42²⁴ in the lysosome.

To determine the relative toxicity of these A β conformations, a cytotoxicity assay was performed where differentiated PC12 cells were treated with various peptide concentrations for 48 hours (Figure 10). We calculated the lethal concentration at which 50% of cells were killed (LC50), for each of the A β conformations. We found that A β 40²⁴ was moderately more toxic than A β 40⁰, with a lower LC50 value (Figure 10). Surprisingly, A β 40⁹⁰ was also found to have a lower LC50 value compared to A β 40⁰. A β 42²⁴ was also found to be moderately more toxic than A β 42⁰, whereas A β 42⁹⁰ had a much higher LC50 value compared to A β 42⁰ (Figure 10).

4. Discussion

We have developed a method to produce a collection of A β conformations that differ in their extent of aggregation and investigated the interaction between these states of A β 40 and A β 42 and differentiated PC12 cells. Others have described the methods to isolate individual soluble oligomeric forms of A β , using various chemical reagents and protocols [41–44]. In addition, purified oligomeric A β molecules from cell culture [45, 46] or transgenic mice [47] have also been monitored. These purified sources of oligomeric A β offer great potential in understanding the progression of A β aggregation; however, they cannot be directly visualized over the time course of their effects on cells during maturation from earlier to later conformations. Since it is well established that A β monomers can oligomerize under the physiological pH of the endosome [13–15], and A β is formed through endoproteolytic cleavage, we have utilized this condition to determine which conformations are present and how these mixed conformations interact with differentiated PC12 cells. It is known that the extent of oligomerization/fibrillation is very dependent on experimental conditions. Necula et al. [48] have indicated that A β can be induced to form fibrils via dilution from 100 mM NaOH to neutral pH in the presence of 10 mM HEPES/100 mM NaCl buffer, while dilution into phosphate buffered saline results in oligomer formation. In our experiments, the peptide was diluted from 30 mM ammonium hydroxide (pH 10) to pH 6 with final condition of 1 mM ammonium chloride as the only additional chemical. We do note that the normal human physiological concentration of ammonium chloride in blood and cerebrospinal fluid is approximately 20 to 50 μ M [49, 50], and that hyperammonemia has been linking to Alzheimer type II astrocytosis [51, 52].

When extracellular monomeric A β associates to the surface of cells, we speculate there are three possible outcomes: (1) it can act as a stable template to allow further A β aggregation; (2) the peptide can penetrate through the cell membrane depositing in the cytoplasm; or (3) the peptide may be internalized into the cell within endocytic vesicles, which would result in a reduction in the surrounding pH. In the third case, the endocytic vesicles containing A β can

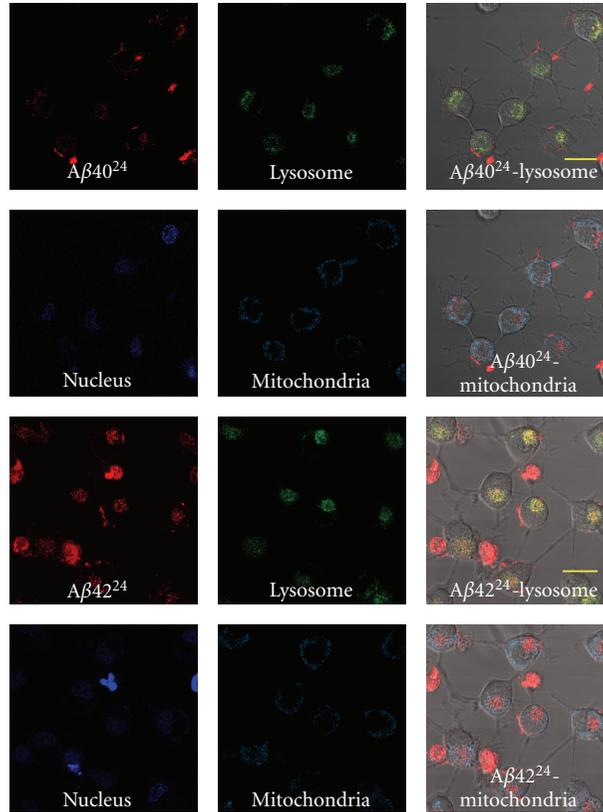


FIGURE 8: Intracellular localization of endocytic Aβ. Confocal microscopy images through a single plane of cells treated with either Aβ40²⁴ or Aβ42²⁴ and intracellular markers. All scale bars are 20 μm in length. Colocalization between the red Aβ40/42²⁴ signal and the green lysosome signal is shown in yellow in the merged image. Colocalization was not observed with either mitochondrial or nuclear stains.

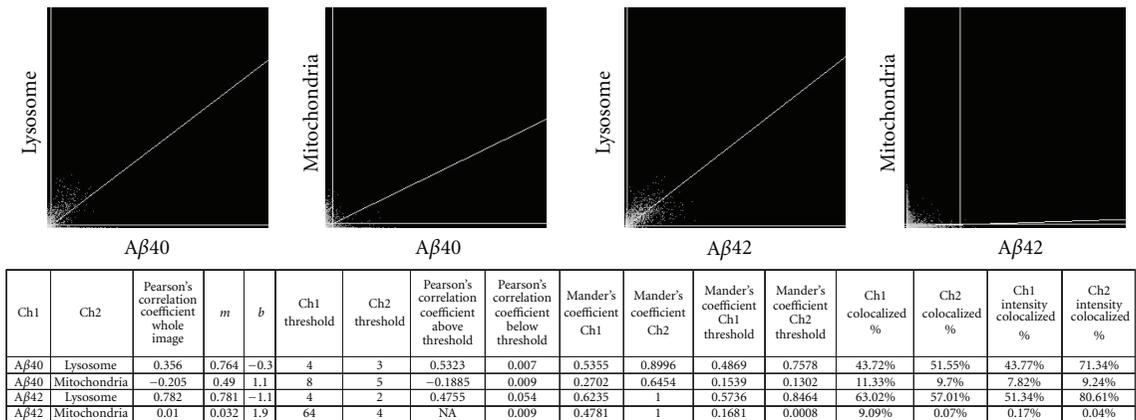


FIGURE 9: Quantitative colocalisation analysis of intracellular Aβ. Quantitative analysis indicates significant colocalisation of Aβ40²⁴ and Aβ42²⁴ with the lysosome marker within live cells.

theoretically be recycled back to the cell surface or directed to the lysosome where a further reduction in pH would occur. We have previously visualized oligomeric Aβ on the surface of neuronal cell lines [29], and here sought to determine first whether this aggregation occurred prior to cell surface deposition in the cell culture media or are aggregation and cell surface binding concomitant and linked processes. Using ultracentrifugation analysis of Aβ preparations either

in freshly prepared cell culture media or in conditioned media removed from cultured cells after 3 days, we did not observe any conformation larger than the monomer. These results indicate that components in our cell media or secreted factors from cultured cells are not responsible for the observed Aβ aggregation present on the surface of neuronal cells. After adding monomeric Aβ to cells, we noted a maturation time for the visual appearance of Aβ on

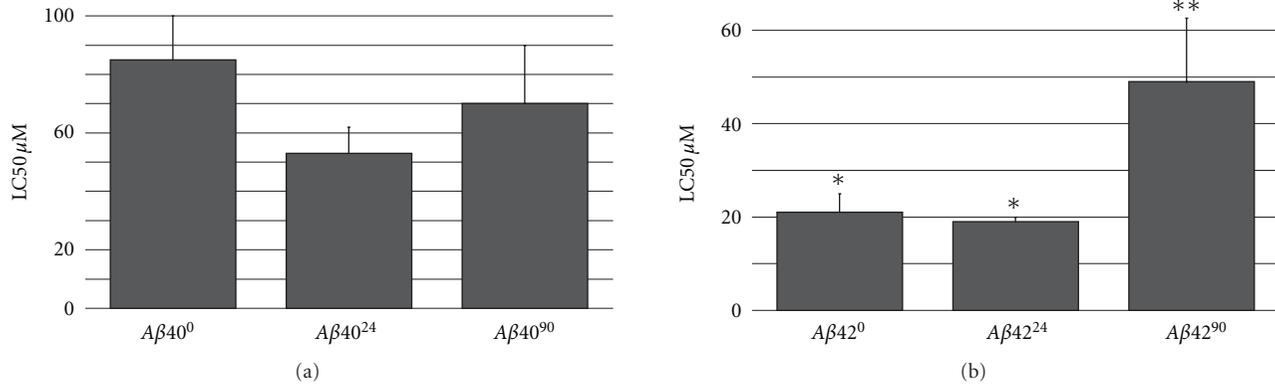


FIGURE 10: Relative toxicity of endocytic Aβ. Lethal concentration of endocytic Aβ required to kill 50% of differentiated PC12 cells at 48 hours is shown. Error bars represent the standard deviation from the mean using three replicates. Statistical significance is indicated (*) $P < .05$.

the cell surface and in the cell interior (Figure 4; [29]). We postulate that the peptides on the cell surface might undergo a series of pH reductions through endocytic recycling, before becoming visible punctae on and inside the cell. Since pH 6 is approximately endocytic pH, we characterized the kinetic effects of this condition on peptide conformations. Exposing Aβ40 for up to 24 hours at pH 6, we observed an increase in the average hydrodynamic radius from 2.0 nm (Aβ40⁰) to 183 nm (Aβ40²⁴) (Figure 1). In addition, approximately 60% of these peptides were recovered through a 10 kDa MWCO filter and 80% were recovered through a 100 kDa MWCO filter (Figure 2), and only minor thioflavin-T binding was found (Figure 3). Following exposure of up to 24 hours at pH 6, Aβ42 had an increase in the average hydrodynamic radius from 1.7 nm (Aβ42⁰) to 246 nm (Aβ42²⁴) (Figure 1), approximately 55% were recovered through 10 kDa MWCO filter and only 60% were recovered through 100 nm filter (Figure 2) and more significant thioflavin-T binding was measured (Figure 3). Both Aβ40 and Aβ42 treated for 90 hours at pH 6 were observed to have large average hydrodynamic radius particles, with 40% of these samples being withheld by a 100 nm filter and both displayed significant thioflavin-T staining. Also, these early- and late-stage Aβ samples were observed to have an ensemble of Aβ conformations (Figure 1).

The interactions of these early- and late-stage Aβ conformations were monitored with differentiated PC12 cells. Whereas late stage Aβ40⁹⁰ and Aβ42⁹⁰ exhibited very few interactions with cells (Figures 4, 5) and internalized at a very low frequency (Figure 6), early stage Aβ40²⁴ and Aβ42²⁴ interacted with cells rapidly (Figure 4) and internalized into the majority of the cells present (Figure 6). Interestingly, Aβ40²⁴ showed a more rapid cellular association and internalization compared to Aβ40⁰ (Figure 4). Similarly, Aβ42²⁴ was found to internalize more rapidly than Aβ42⁰, indicating that a specific conformation may play a role in cell binding and internalization. Furthermore, our model of endocytic Aβ toxicity (Figure 10) is in agreement with previous findings utilizing chemically-produced oligomers of Aβ which showed that Aβ fibrils were less toxic than

soluble oligomers [41, 53]. Our results with Aβ⁹⁰ are also in agreement with studies that have indicated that regions with large Amyloid plaques do not correlate directly with regions of significant neuronal loss [54, 55].

Many studies have reported that certain oligopeptides are freely imported into cells. For example, the peptide sequences corresponding to Tat (48–60), penetratin, and oligoarginine are known to internalize into live cells. Cellular import of Tat (48–60) and penetratin was shown to be temperature dependent, indicating the possible role of endocytosis for internalization [56, 57]. In contrast, oligoarginine containing C-terminal tryptophan was shown to follow a nonendocytic pathway, independent of energy requirements and temperature [57]. Another type of internalization process has been studied in the bacterium *Clostridium septicum*, whose alpha toxin contains functional domains responsible for oligomerization and cellular pore formation. Using specific domains that bind cell surface receptors, alpha toxin monomers oligomerize to form pores in human cells and thus impose direct entry [58]. By exposing Aβ to endosomal conditions, we were able to observe internalization of Aβ40/42²⁴, which was inhibited at 4°C or with the endocytosis inhibitor MDC (Figure 7). Throughout the course of our confocal experiments, we did not observe any disruption of the cell membrane, which would have been expected if membrane channels or pores were being formed by Aβ. Thus, the structural conformation of Aβ that was observed to internalize into cells seems to follow a cell-mediated import mechanism.

Our results with the various Aβ conformations correlate directly with previous reports that indicate the deposition of Aβ in the lysosome [15, 59, 60] and with the previous study that internalized Aβ can persist undegraded for days [61]. From our findings taken together with previous studies, we present the following model for the interaction of Aβ with neuronal cells (Figure 11). As monomeric Aβ binds the cell surface, this event could lead to a conformational change allowing for the further catalytic aggregation of Aβ. Recently, it has been shown that oligomeric Aβ binds to GM1 ganglioside and alters physical properties of the plasma

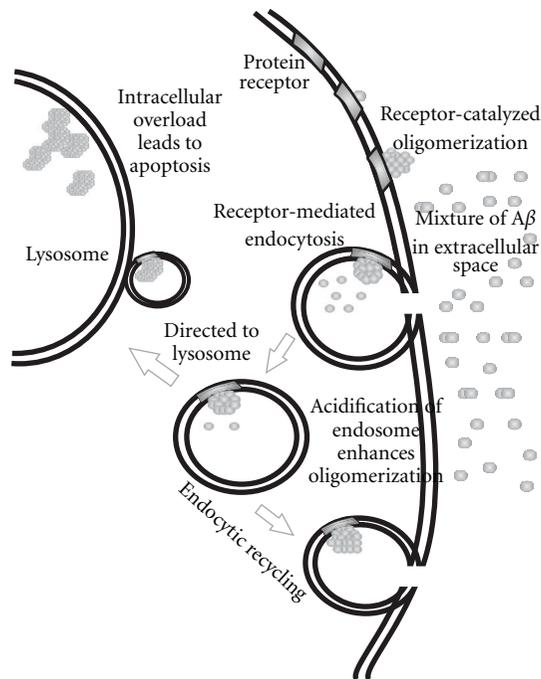


FIGURE 11: A plausible mechanism of $A\beta$ neurotoxicity.

membrane which stimulates the amyloidogenic processing pathway of APP [62, 63]. $A\beta$ that is bound to the cell surface may become internalized and recycled, allowing for acidification and further aggregation, which in turn may stimulate additional $A\beta$ generation [62]. Once the surface $A\beta$ reach some specific structural form, they may be internalized and directed to the lysosome for potential degradation. However, the reduced pH of the lysosome may instead cause further aggregation and persistence of $A\beta$, resulting in lysosomal overload and cell death. The recently reported high turnover rate of monomeric $A\beta$ [64, 65] and the formation of large aggregate pools of $A\beta$ may indicate that the human body uses these mechanisms to sequester and remove the toxic $A\beta$ oligomers from neuronal cells. With age, the turnover rate for monomeric $A\beta$ may slow down, which could then result in the initiation of our proposed mechanism and may contribute to the late onset of Alzheimer's disease. It is also possible that endocytosis of $A\beta$ and lysosomal targeting are mechanisms by which the cell clears $A\beta$ aggregates from the cell surface. Also, certain large aggregates of $A\beta$ may just be too large for the cell to internalize, and they may represent the precursor for extracellular Amyloid formation.

5. Conclusion

Our study shows that using a simple method to generate various $A\beta$ conformations, the rates of cellular interaction and targeting can be followed with live cell cultures. Using this model, we found that early endocytic conformations, rather than highly aggregated late forms, serve as the major contributors to rapid cell internalization. The mechanism

of internalization likely involves a cell surface receptor-mediated process instead of peptide-mediated direct entry, resulting in accumulation in the lysosome. This method allows for conformation-specific therapeutics and conditions to be screened with live cells, circumventing the need to purify specific $A\beta$ conformations.

Abbreviations

AD:	Alzheimer's disease
$A\beta$:	Amyloid β
ADDLs:	$A\beta$ -derived diffusible ligands
APP:	Amyloid precursor protein
DAPI:	4',6-diamidino-2-phenylindole
DMEM/F12:	Dulbecco's modified eagle's medium: nutrient mixture F-12 1 : 1 mixture
D-PBS:	Dulbecco's phosphate-buffered saline
MDC:	Monodansylcadaverine
MWCO:	Molecular weight cut-off
NGF:	Nerve growth factor
PC12:	Rat adrenal pheochromocytoma
TMR:	Tetramethylrhodamine.

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