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Research Article

Fast Purification of Recombinant Monomeric Amyloid-β from *E. coli* and Amyloid-β-mCherry Aggregates from Mammalian Cells

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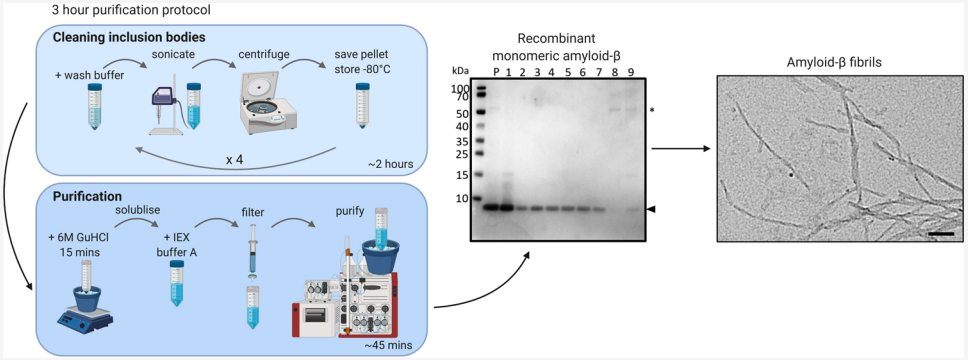

ABSTRACT: The Alzheimer's disease related peptide, Amyloid-beta (Aβ)1−40 and 1−42, has proven difficult to be purified as a recombinant monomeric protein due its expression in *E. coli* leading to the formation of insoluble inclusion bodies and its tendency to quickly form insoluble aggregates. A vast array of methods have been used so far, yet many have pitfalls, such as the use of tags for ease of Aβ isolation, the formation of Aβ multimers within the time frame of extraction, or the need to reconstitute Aβ from a freeze−dried state. Here, we present a rapid protocol to produce highly pure and monomeric recombinant Aβ using a one-step ion exchange purification method and to label the peptide using a maleimide dye. The washing, solubilization, and purification steps take only 3 h. We also present a protocol for the isolation of Aβ-mCherry from mammalian cells.

KEYWORDS: *Aβ42, Aβ40, E22G, arctic mutant, fluorescence, amyloid, ion exchange chromatography, maleimide, dye labeling, inclusion bodies, mCherry*

INTRODUCTION

The presence of Amyloid-beta (Aβ) plaques and τ angles in neurons are hallmarks of Alzheimer's disease, therefore great research effort is put toward understanding how these initially soluble proteins misfold and contribute to pathology. To study protein misfolding, large quantities of protein are required, and while purification protocols for τ proteins are fairly well established, those for Aβ, in its isoforms of 1-39/43 and its mutant variants, are very heterogeneous and lead to variable products.[1]

Many studies investigating aggregation rates and toxicity of Aβ currently use synthetic Aβ due to the ease of purchase and little handling required to obtain monomeric Aβ. However, Aβ can be expensive to purchase and needs to be reconstituted to remove oligomers which can lead to loss of protein in the form of insoluble structures, protein adhering to centrifuge tubes, or buffer exchange steps. There is often variation in the resulting sample due the use of different solvents for reconstitution, which can influence the initial structure.[2] Differences in disaggregation protocols can also introduce heterogeneous structures in the starting peptide, such as the use of hexafluoroisopropanol (HFIP) vs ammonium hydroxide. HFIP is widely used to disaggregate Aβ, yet it can also lead to formation of oligomeric structures, as the peptide is brought to neutral pH via its isoelectric point.[3] Ammonium hydroxide disaggregation has been shown to produce fewer oligomers and a more homogeneous solution compared to a HFIP prepared Aβ solution. In comparison, the HFIP prepared Aβ had increased aggregation propensity.[4] Furthermore, synthetic Aβ has been reported to have high batch variability and lower toxicity compared to recombinantly produced Aβ.[5,6]

Purification of tagged-Aβ is a highly popular method as addition of tags can improve solubility and permit the use of affinity capture chromatography, which can yield highly pure recombinant Aβ samples. In a recent review on Aβ purification methods, it was highlighted that 23/30 protocols utilized

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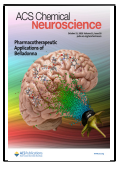

tagged-Aβ for purification.[1] The added benefit of using a recombinant tagged system containing a cleavage site at the Aβ N-terminus is that it can be utilized to release the wild-type Aβ sequence without a methionine (M) start codon. *In vivo*, Aβ is cleaved from the amyloid precursor protein; therefore, the first codon in the sequence is an aspartate, the sequence of which cannot be obtained by expressing Aβ alone, and AβM variants are instead used which have the methionine starting residue before aspartate. However, if tags are not removed prior to further analysis of the peptide, even a small tag, such as a 6xHis-tag, can greatly influence the protein structure and aggregation propensity.[7] Moreover, removal of the tag requires the addition of a cleavage recognition site and additional purification steps which lead to loss of protein, increased time of protein handling, and therefore formation of aggregated species.

The protocol by Walsh *et al.* provided an easy method for purification of AβM variants using urea solubilization to isolate AβM from inclusion bodies, purification by ion exchange chromatography and size exclusion chromatography, centrifugation applying a 30 kDa filter, and lyophilization to store the recombinant protein.[8] However, the DEAE-cellulose chromatography media used in the ion exchange step is no longer commercially available.

Reversed phase (RP) chromatography is another frequently used method to purify Aβ due to the high purity of the resulting recombinant protein. Aβ is eluted from the RP column along a gradient of organic solvent in the presence of an ioniser such as trifluoracetic acid (TFA). The organic solvent is then removed by freeze−drying. The process of freeze−drying induces formation of oligomers, which subsequently can be removed by gel filtration, yet this adds another step to the purification protocol. We have shown for another amyloidogenic protein, α-synuclein, that freeze−drying leads to a compaction of the monomer structure and formation of heterogeneously sized oligomers, even after reconstitution in buffer, compared to samples that were frozen directly after purification.[9] Freeze−drying could also affect the structure of monomeric Aβ, although further studies are needed to confirm this. As with all intrinsically disordered amyloidogenic proteins the structure of the starting material, e.g., the presence of multimers or degraded products, and the surrounding environment heavily influence the aggregation rate, the pathways of aggregation that are taken, and the toxicity of the resulting amyloid.[2]

Here, we provide an improved protocol circumventing some of the outlined issues raised above for the purification of recombinant AβM42 and AβMC40 from *E. coli*. The protocol utilizes a one-step ion exchange chromatography protocol from cleaned and solubilized inclusion bodies, removing the need for affinity tags, and the use of an ion exchange column is cheaper than a gel filtration column. The protocol is rapid, requiring just 45 min to solubilize and purify AβM, reducing researchers' time and reducing the formation of oligomers. There is no requirement for a lyophilization step which can induce oligomer formation. The protocol can be amended to permit the incorporation of a maleimide dye label to the cysteine residue of mutated sequences, such as the AβMC40 sequence. After induction of AβM expression in *E. coli*, the purification protocol takes only around 3 h to obtain highly pure monomeric AβM. We also present a protocol for the isolation of AβM(E22G)-mCherry from HEK293 cells again using one-step ion exchange chromatography.

RESULTS

Thorough Washing of Inclusion Bodies Yields Pure Recombinant AβM. Aβ42 and Aβ40 are the most abundant Alzheimer's disease-associated variants and will be used here to demonstrate the purification method.[10] The Aβ40 variant used in this protocol contains an additional N-terminus cysteine residue, AβMC40, which can be dye-labeled with thiol reactive dyes. AβM42 and AβMC40 were expressed from pET3a plasmids in *E. coli* BL21 (DE3) pLysS strain with 1 mM IPTG for 4 h. The *E. coli* cultures were centrifuged in 50 mL falcon tubes and the pellet stored at −80 °C until needed (Figure 1, light blue box). During expression, AβM peptides form into cytoplasmic insoluble inclusion bodies, which can be beneficial for the purification process as inclusion bodies can contain high quantities and purity of the protein of interest.[11] The inclusion bodies were thoroughly washed to obtain very pure inclusion bodies (Figure 1, medium blue box). The frozen

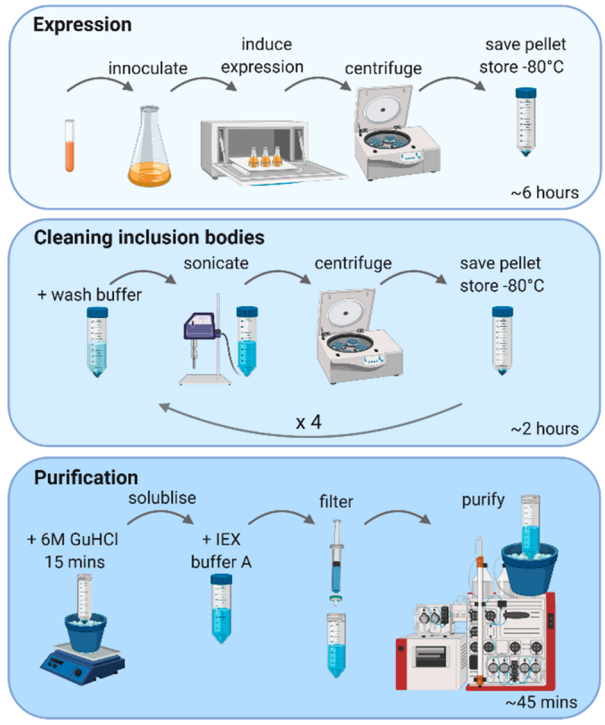

Figure 1. Schematic figure of the expression, isolation, and purification of AβM42 and AβMC40 from *E. coli*. Light blue box (top) − expression: An overnight culture was inoculated in lysogeny broth (LB) medium and then grown at 37 °C until reaching an OD_{600}~0.6−0.8. Expression of AβM was induced upon addition of 1 mM IPTG, 4 h, after which the culture was centrifuged in 50 mL falcon tubes and the pellet stored at −80 °C until use. Medium blue box (center) − cleaning inclusion bodies: AβM is retained in insoluble inclusion bodies. The *E. coli* pellet was resuspended in wash buffer 1 (Table 1) and the inclusion bodies were isolated from the cells by sonication and centrifuged at 10,000*g*. The inclusion bodies were then washed three times with different buffers (Table 1) to remove unwanted proteins and lipids. The final inclusion body pellet was kept at −80 °C until use. Dark blue box (bottom) − purification: The pellet of inclusion bodies was solubilized with 200 μL of 6 M GuHCl per 50 mL of culture for 15 min on ice on a magnetic stirrer before dilution with 15 mL IEX buffer A. The protein solution was filtered through a 0.22 μm membrane and purified using an ÄKTA FPLC with a HiTrap Q HP ion exchange column. Created with BioRender.com.

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pellet from 50 mL of *E. coli* culture was resuspended in wash buffer 1 (Table 1) which contained protease inhibitors to reduce proteolytic cleavage of AβM during cell lysis, 1 M guanidine hydrochloride (GuHCl) to increase washing efficiency by aiding the removal of other proteins, and 1% Triton X-100 to remove lipids bound to the inclusion bodies. The *E. coli* cells were sonicated 5 × 30 s on ice and were centrifuged at 10,000*g* to remove cell debris. This was repeated three times with different additives in the wash buffer (Table 1). Although there was a loss of AβM during the wash steps, as observed in the supernatant of the washes in Supporting Information Figure 1, the cleaner the inclusion bodies then the higher the purity of the purified AβM. By the end of wash 4, the pellet contained highly pure inclusion bodies which were white in color, and which were subsequently stored at −80 °C until solubilization and purification.

Table 1. Wash Buffer and Additives Used to Clean Aβ Containing Inclusion Bodies

10 mM Tris 1 mM EGTA pH 9			
Wash 1	Wash 2	Wash 3	Wash 4
protease inhibitors	protease inhibitors		
1 M GuHCl	1 M GuHCl		
1% Triton X-100	1% Triton X-100	1% Triton X-100	No additives

One-Step Ion Exchange Chromatography Yields Highly Pure Monomeric AβM. The AβM was solubilized from the inclusion bodies before purification. 200 μL of 6 M GuHCl was added to the inclusion body pellet from 50 mL of *E. coli* culture on ice. A small stir bar was added and the pellet left on a magnetic stirrer for 15 min to solubilize the inclusion bodies. GuHCl was chosen as a solubilizing agent instead of urea, as urea decomposition leads to isocyanic acid formation which can cause carbamylation of the N-terminus.[12] After 15 min, 15 mL of ice-cold ion exchange chromatography (IEX) buffer A (10 mM Tris, 1 mM EGTA pH 9) was added to the solution to dilute the GuHCl, reducing the ionic strength of the buffer to permit the protein to bind to the ion exchange column (Figure 1, dark blue box). The protein solution was then filtered through a 0.22 μm filter to remove any precipitate before IEX. At this point, the AβM was already ∼90% pure due to thorough washing of the inclusion bodies (Supporting Information Table 1). The fast protein liquid chromatography (FPLC) machine was not kept in a cold room; therefore, all buffers were kept on ice and an ice bag placed around the column to keep the system as cold as possible to reduce AβM aggregation. A HiTrap Q HP column (GE Healthcare) was used to purify the AβM monomer. To keep purification time to a minimum, the column was equilibrated in IEX buffer A prior to sample preparation. AβM was eluted over seven column volumes with a 0−100% gradient against IEX buffer B (10 mM Tris, 1 mM EGTA, 0.75 M NaCl, pH 9) followed by two column volumes at 100% buffer B. Absorption at 280 nm was used to monitor protein elution from the column (Figure 2a). Analysis of the eluted fractions by SDS-PAGE on a Coomassie blue stained gel showed pure monomeric AβM eluted at ∼30% IEX buffer B (Figure 2, Supporting Information Figure 2). The concentration of AβM42 from each 1 mL fraction ranged 8−12.75 μM, determined by absorption at 280 nm and calculated using the extinction coefficient 1490 M^{-1} cm^{-1} (Supporting Information Table 1). Purification of the AβMC40 variant is shown in Supporting Information Figure 3.

Recombinant AβM42 Forms Long Fibril-like Structures over Time. The recombinant AβM42 was analyzed by liquid chromatography mass spectrometry (LC-MS) to ensure a pure protein of the correct mass/charge had been purified. A deconvoluted mass of 4645 Da was obtained, which corresponded to the predicted mass of AβM42 (Figure 3a, m/z data presented in Supporting Information Figure 4.). To compare whether there were differences in the AβM42 product from different purification batches, such as the presence of degradation products or contaminants which may influence cell responses, we treated the neuronal cell line SHSY-5Y with AβM42 at 1−2 μM and investigated their vitality via metabolic activity using an MTT assay. We observed a slight, but not significant, increase in cell vitality upon the addition of AβM42 compared to cells without AβM42 treatment, which has previously been observed.[13] No significant difference in cell vitality after treatment with AβM42 from batch 1 or 2 was

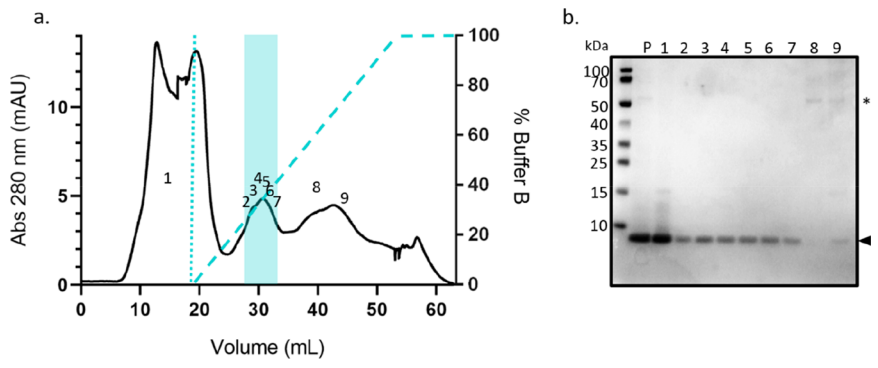

Figure 2. Highly pure monomeric AβM42 is purified by ion exchange chromatography from GuHCl solubilized inclusion bodies. AβM42 was solubilized in 6 M GuHCl and diluted in IEX buffer A before being applied to the HiTrap Q HP ion exchange column (shown up to the first dotted line in a). (a) Chromatograph of the absorption at 280 nm shows the elution of protein from the HiTrap Q HP column over a gradient of 0−100% buffer B containing 0.75 M NaCl over seven column volumes, followed by two column volumes of 100% buffer B (dashed line showing gradient in a). (b) In order to determine when AβM42 eluted from the column the fractions were collected and analyzed using SDS-PAGE on a 4−12% bis-tris gel with Coomassie blue staining. The numbers on chromatograph (a) correspond to the lane on the gel in (b). The AβM42 sample prior to IEX (P) was highly pure. Protein bands correlating to ∼4.5 kDa (shown by the arrow next to b) show monomeric AβM42 in fractions 2−7 which are highlighted in blue in the chromatograph (a). AβM42 eluted at ∼30% buffer B. Higher-molecular-weight species (indicated by a star in b) elute later in the buffer B gradient in fractions 8 and 9.

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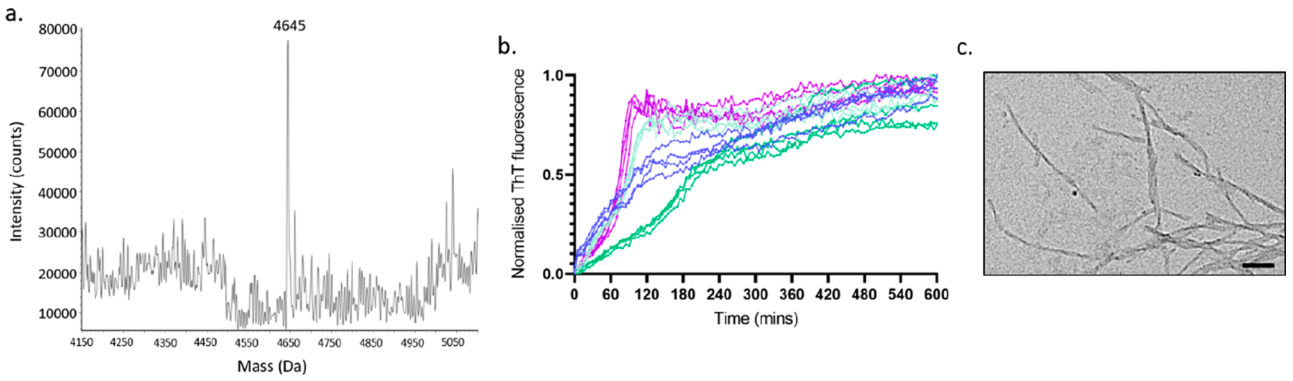

Figure 3. Pure recombinant AβM42 forms long fibrillar structures. Recombinant AβM42 was analyzed by mass spectrometry and the (a) deconvoluted spectrum shows the expected MW of 4645 Da (see Supporting Information Figure 4 for m/z spectrum). (b.) Normalized ThT-based aggregation assays show slight batch variation (each color represents a batch), but good reproducibility within the batches (individual lines represent individual wells, four wells per batch). 5 μM of AβM42 in 100 μM Tris 200 mM NaCl, pH 7, with 20 μM of ThT was incubated in a half-area 96 well plate at 37 °C with double orbital agitation at 300 rpm for 1 min before each read every 5 min for 600 min. (c) TEM image of fibrils formed during incubation of 5 μM of AβM42 with constant rotation at 20 rpm at 37 °C for 2 days. Scale bar = 100 nm.

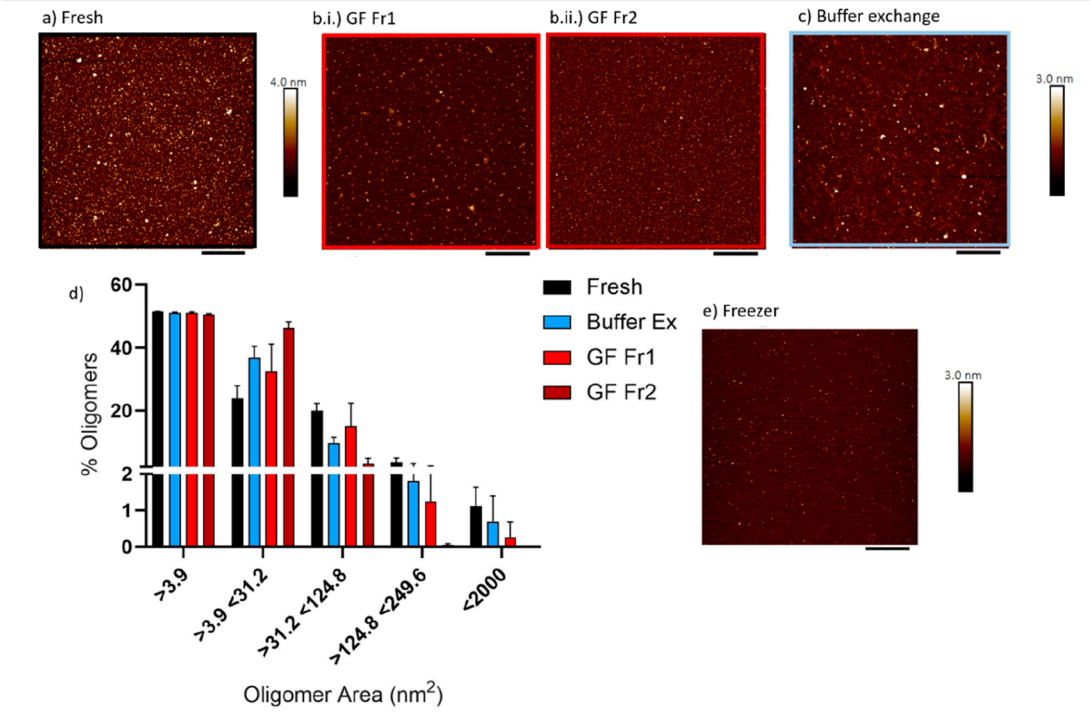

Figure 4. Atomic force microscopy shows that small multimeric AβM42 structures are present on mica, but most abundant after buffer exchange. 10 μL of AβM42 was deposited on freshly cleaved mica (a) directly after purification by IEX (6.7 μM), (b) after GF into 100 mM Na_2HPO_4, pH 7, where AβM42 eluted between (i) 17.5 min (8 μM) and (ii) 18 min (7 μM), (c) after buffer exchange in cold 100 mM Na_2HPO_4, pH 7, using PD MiniTrap G-10 columns (5 μM). (d) Size of the oligomers was extracted from each image and presented as a percentage of the total for each image and averaged for 6 images per condition. 3.9 nm^2 = 1 pixel; 31.2 nm^2 = 8 pixels; 124.8 nm^2 = 32 pixels; 249.6 nm^2 = 64 pixels. (e) AβM42 after storage at −80 for six months in IEX buffer A, pH 9, (8 μM) did not show large oligomeric structures. Black scale bar = 400 nm. The height profile bar shown at the right of the figure shows a height of up to 3−4 nm for multimeric species.

observed showing the purification protocol to reproduce recombinant AβM42 to a similar quality (Supporting Information Figure 5). A thioflavin-T (ThT) based aggregation assay was then used to investigate the aggregation properties of the purified AβM42 between batches. The ThT molecule fluoresces when it intercalates into the backbone of a fibril containing β-sheet structure, leading to a sigmoidal curve over time as the protein aggregates and the ThT fluorescence intensity increases.[14] To investigate the aggregation propensity, AβM42 was diluted to 5 μM in 100 mM Tris, 200 mM NaCl, pH 7, with 20 μM ThT and aggregated for 600 min with 1 min shaking at 300 rpm before every reading, every 5 min (Figure 3b). AβM42 aggregation was fairly rapid under the conditions used due to the presence of salt,[15] although not all replicates show a clear lag phase, a a sigmoidal curve of the increase of ThT fluorescence, as expected for a nucleation-dependent protein aggregation assay.[16] Shown are AβM42 fibrils formed and imaged by transmission electron microscopy (TEM) (Figure 3c, Supporting Information Figure 6). Finally, we also show that during the solubilization step (Figure 1, medium

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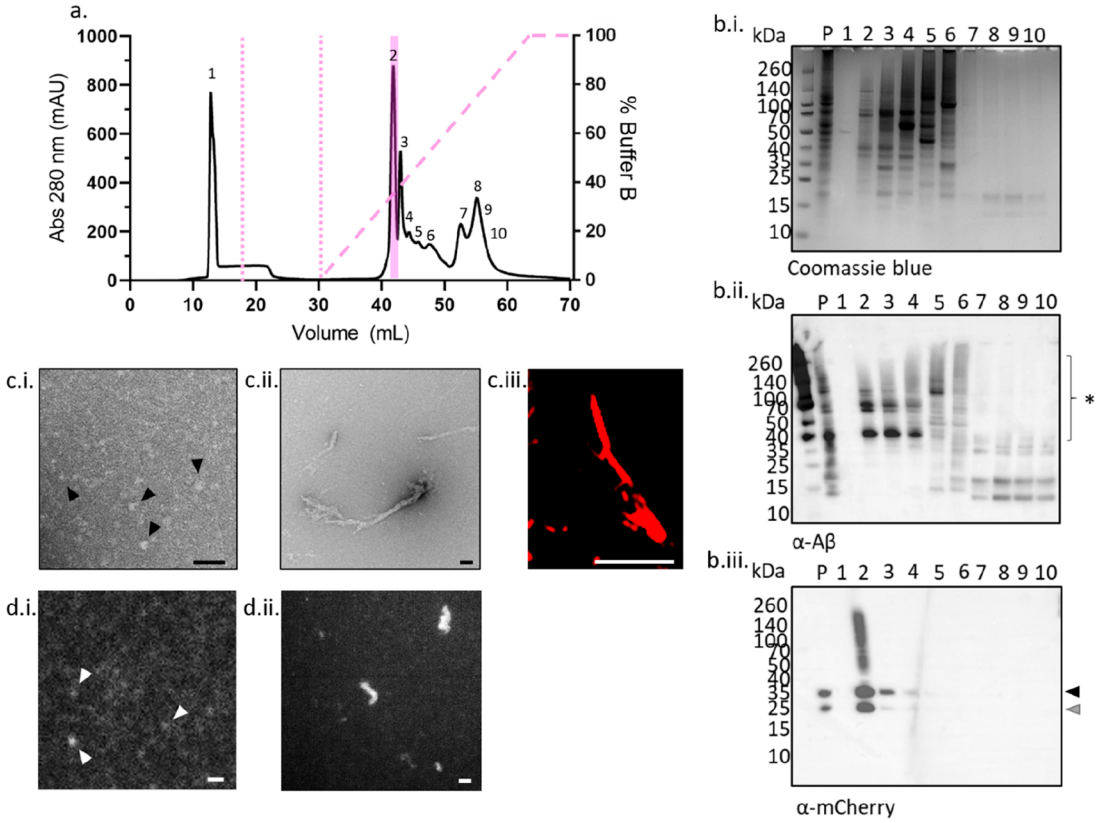

Figure 5. Varying aggregate sizes of AβM(E22G)-mCherry isolated by ion exchange chromatography exhibit weak fluorescence. AβM(E22G)-mCherry was lysed from HEK293 cells by sonication. (a) The soluble fraction was applied to the column (shown up to the first dotted line in (a), and the unbound protein was washed from the column (shown up to the second dotted line in (a)). The chromatograph of absorption at 280 nm shows protein elution from the HiScreen Capto Q ImpRes column eluted over a gradient of 0−100% of buffer B containing 1 M NaCl over seven column volumes, followed by two column volumes of 100% buffer B (dashed line showing gradient in (a). (b) In order to determine when AβM(E22G)-mCherry eluted off the column, the fractions were collected and analyzed using SDS-PAGE on a 4−12% bis-tris gel and (b.i) Coomassie blue staining, or transferred to a membrane for Western blot using antibodies against (b.ii) Aβ and (b.iii) mCherry. The sample prior to IEX (P) contained many proteins, including aggregated Aβ and mCherry. Protein bands correlating to ∼32.3 kDa (shown by the black arrow in b.iii) show the predicted MW for monomeric AβM(E22G)-mCherry. Fraction 2 contained the highest content of AβM(E22G)-mCherry, although the presence of degraded mCherry (b.iii, gray arrow) and aggregated Aβ (b.ii, star) was also apparent. The morphology of the purified AβM(E22G)-mCherry was determined by TEM, and both (c.i) oligomers and (c.ii, Supporting Information Figure 8) larger aggregates were present. (c.iii) SIM image of a section view of an AβM(E22G)-mCherry aggregate inside a cell prior to purification reveals a similar structure to those identified by TEM after purification. These purified aggregates were also analyzed to determine whether they were still fluorescent using wide-field imaging with a 561 nm laser, both (d.i) small oligomers and (d.ii) large aggregates were weakly fluorescent (also see Supporting Information Figure 9). Black scale bar = 100 nm; white scale bar = 2 μm.

blue box), it is also possible to produce fluorescently labeled Aβ via a dye-labeled cysteine modified Aβ, where the thiol-reactive dye reacts with the monomeric Aβ as it becomes solubilized. 1 mM tris(2-carboxyethyl)phosphine (TCEP) was added to all buffers to keep the cysteine residue in a reduced form to allow the maleimide dye reaction to occur (Supporting Information Figure 7). The concentration of protein and degree of labeling (DOL) was calculated, taking into account the absorption of the dye at 490 nm and the influence of the dye on absorption at 280 nm (Supporting Information Table 2).

Buffer Exchange by Gel Filtration Is Preferable to Reduce AβM42 Oligomers Compared to Buffer Exchange Columns. Aβ42 is the most aggregation-prone isoform of Aβ;[17] it is therefore very difficult to maintain in a monomeric form during storage. Ideally, AβM42 would be purified and used straight away to prevent formation of multimeric or oligomeric structures; however, the purification buffer may not be ideal for many assays at pH 9. Two buffer exchange methods were compared to determine the extent of oligomerization of the same aliquot of AβM42 when exchanged into 100 mM Na_2HPO_4, pH 7, using PD MiniTrap G-10 buffer exchange columns (GE Healthcare) and a gel filtration column (GF) (Superdex 75 10/300 GL (GE Healthcare)) (Supporting Information Figure 8). Atomic force microscopy (AFM) was used to investigate presence of multimeric species (Figure 4 and Supporting Information Figure 9). The radius of gyration of AβM42 is ∼0.9 nm[18] and the resolution of the AFM images is 3.9 nm per pixel; therefore, multimers of AβM42 up to four are defined as one pixel, and surface-induced aggregation on the mica may increase the percentage of aggregates in comparison to that in solution.[19] In comparison to freshly prepared AβM42 in pH 9 buffer and AβM42 buffer exchanged using G-10 buffer exchange columns with a gravity elution protocol , the AβM42 after GF had the least amount of oligomeric species, particularly the second fraction (dark red) collected which had a lower concentration (Figure 4a−d) (Figure 4c). Dependent on the downstream assays, the AβM42 is required for the G-10 columns provide a quick method for buffer exchange, but

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ACS Chem. Neurosci. 2020, 11, 3204−3213

AβM42 has begun to aggregate; yet, the use of a GF column takes longer, but yields a more monomeric protein. AβM42 which had been kept at −80 °C in pH 9 buffer had minimal aggregation (Figure 4e), yet prolonged storage at high pH may lead to chemical alteration of some residues.

AβM(E22G)-mCherry Purified from HEK293 Cells Remains Fluorescent. While purification of Aβ from *E. coli* is useful, as it produces a high quantity and purity, it is still unclear whether the monomeric and/or aggregate structures of Aβ formed *in vitro* are representative of those that form *in vivo*. Here, we present a protocol to isolate AβM(E22G)-mCherry from HEK293 cells. The AβE22G Alzheimer's disease associated mutant is highly aggregation prone.[20] Although the protein is tagged to a large fluorescent protein, mCherry, which may influence folding and structure, the fluorescent protein permits the study of Aβ aggregation in cells.[21] In this cell line, we have previously identified five categories of intracellular AβM(E22G)-mCherry aggregates—oligomers, single fibrils, fibril bundles, clusters, and aggresomes. These stages underline the heterogeneity of Aβ42 aggregates and represent the progression of Aβ42 aggregation within the cell.[21] Isolation of endogenous AβM(E22G)-mCherry may allow further insight into morphology or seeding ability of endogenously structured Aβ by microscopy.

Expression of AβM(E22G)-mCherry was induced by 1 μg/mL tetracycline for 7 days. The cells were centrifuged at 4000*g* for 15 min and the pellet was frozen until use. The cells were lysed by sonication in 50 mM Tris, pH 8, with protease inhibitors before centrifuging to separate the soluble and insoluble fractions. Western blot analysis, probing both Aβ and mCherry, showed that the soluble fraction contained more AβM(E22G)-mCherry than the insoluble fraction (Supporting Information Figure 10). The soluble fraction was purified using a HiScreen Capto Q ImpRes ion exchange column and eluted on a linear gradient against IEX buffer B (50 mM Tris, 1 M NaCl, pH 8) over seven column volumes, followed by two column volumes of 100% buffer B (Figure 5a). The eluted fractions were analyzed by SDS-PAGE separation and the gels were either stained with Coomassie blue (Figure 5b.i) or transferred onto a membrane and probed by Western blot for Aβ (Figure 5b.ii) and mCherry (Figure 5b.iii). The pre-IEX sample (P) contained many proteins (Figure 5b.i), yet a lot appeared to be aggregates of AβM(E22G) (Figure 5b.ii). Unbound proteins (Figure 5a, peak 1; b.i, lane 1) eluted from the column during the sample application and column wash. Peak 2, corresponding to lane 2 in Figure 5b contained the most abundant AβM(E22G) and mCherry by Western blot. The expected MW for monomeric AβM(E22G)-mCherry was 32.3 kDa (Figure 5b.iii, black arrow); therefore both degraded products (Figure 5b.iii, light gray arrow) and aggregated products were present (Figure 5b.ii, star). The presence of Aβ aggregates was to be expected as the E22G mutant is a highly aggregation prone mutant,[20] which was expressed in HEK cells for 7 days, and no denaturing step was employed during the purification protocol. It appears that mCherry is not always present in Aβ aggregates, as the Western blot displaying Aβ bound antibodies is more highly populated than the mCherry Western blot. It possible that the AβM(E22G)-mCherry has become degraded in the cell; the expected molecular weight of mCherry is 26.7 kDa; therefore, mCherry fragments may be identified in the Western blot Figure 5b.iii. Another explanation for the discrepancy between the Aβ and mCherry probed blots may be due to steric hindrance preventing the antibody binding to mCherry in an aggregated form. The concentration of fraction 2 was 77 μM, as determined by absorption at 280 nm and calculated using the extinction coefficient of 35 870 M^{-1} cm^{-1}.

The morphology and fluorescence of the purified AβM-(E22G)-mCherry from peak 2 were analyzed by TEM and fluorescence microscopy. Both small oligomeric aggregates (Figure 5c.i, indicated by black arrows) and larger aggregates (Figure 5c.ii, Supporting Information Figure 11) were present. The large aggregates with fibrillar morphology were very similar to those identified within the cells using SIM imaging prior to purification (Figure 5c.iii).[21] The aggregates also emitted weak fluorescence when excited with a 561 nm laser (Figure 5d.i., oligomers indicated with white arrows; and Figure 5d.ii, shows larger fluorescent aggregates, see also Supporting Information Figure 12). Mass spectrometry analysis of AβM(E22G)-mCherry showed the expected weight of the monomeric AβM(E22G)-mCherry, 32.3 kDa, was not highly abundant, but that a smaller degraded product of 25.2 kDa and a larger product of 36.3 kDa were the dominant species, among many other species of differing molecular weights (Supporting Information Figure 13). In cells, Aβ is commonly degraded or altered by post-translational modification; therefore, the presence of species of differing molecular weights of AβM(E22G)-mCherry may be reflective of different truncations and modifications that occur within a cellular environment.[22]

CONCLUSION

We present a fast method for the purification of the Alzheimer's disease related peptide AβM. The benefits of this protocol include the use of commercially available reagents, a rapid one-step chromatography step using an ion exchange column and the lack of freeze-drying step which induces oligomerization and the need for further gel filtration steps requiring pricey columns. The purification protocol provided requires only 45 min to solubilize and purify, if the protein and buffers are kept ice-cold, highly pure monomeric recombinant AβM can be obtained. The protocol can be stopped and the products frozen at two key points, after centrifugation of *E. coli* expressing AβM, or after cleaning of the inclusion bodies prior to solubilization and purification. Ideally, AβM would be purified and used straight away to prevent freezing of monomeric peptide which can induce dimer and oligomer formation. Furthermore, we provide a protocol to isolate fluorescent AβM(E22G)-mCherry structures from mammalian cell lines which can be used to track seeding of Aβ in cells with fluorescent endogenously structured protein.

METHODS AND MATERIALS

Expression of Recombinant AβM Variants in *E. coli*. The plasmid pET3a containing human AβM42 and AβMC40 cDNA was transformed into *Escherichia coli* (*E. coli*) One Shot BL21 (DE3) pLysS (Thermo Fisher Scientific, USA). The plasmids were a kind gift from Prof. Sara Linse. The protein sequences encoded in the pET3a plasmids are AβM42: MDAEFRHDSGYEVHHQKLVFFAEDVGSN-KGAIIGLMVGGVVIA and AβMC40: MCDAEFRHDSGYEVHH-QKLVFFAEDVGSNKGAIIGLMVGGVV. One liter cultures of *E. coli* in Lysogeny Broth (LB) containing carbenicillin (100 μg/mL) were grown at 37 °C with constant shaking at 250 rpm and induced for expression of AβM when the OD_{600} reached 0.6−0.8 with addition of 1 mM isopropyl-β-thiogalactopyranoside (IPTG). After 4 h of AβM expression, the cells were pelleted by centrifugation in 50 mL falcon

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ACS Chem. Neurosci. 2020, 11, 3204−3213

tubes at 4000 × g for 15 min. The supernatant is discarded, and at this point, the pellets can be frozen until further use.

Cleaning of AβM Containing Inclusion Bodies. 50 mL of culture from a 4 h induction (Table 1) was found to be a suitable amount for purification of monomeric AβM, as increasing the concentration can lead to aggregation during purification. At this point, multiple 50 mL pellets can be cleaned at the same time and frozen prior to solubilization and purification. The pellet was resuspended in 30 mL wash buffer 1 containing 10 mM Tris, 1 mM EDTA, protease inhibitor tablets (cOmplete EDTA-free cocktail, Roche), 1 M GuHCl, 1% Triton-X100, pH 9, and was sonicated on ice for 30 s on, 30 s off, five times using an XL-2020 sonicator (Heat Systems, USA). The suspension was centrifuged at 4 °C at 10,000 × g for 15 min. The supernatant was discarded and the pellet was resuspended in 30 mL wash buffer 2 (Table 1) before sonicating it on ice for 30 s on, 30 s off, three times. The suspension was centrifuged at 4 °C at 10,000 × g for 15 min. The supernatant was discarded and the pellet was resuspended in 30 mL wash buffer 3 (Table 1) before being sonicated on ice for 30 s on, 30 s off, three times. The suspension was centrifuged at 4 °C at 10,000 × g for 15 min. The supernatant was discarded and the pellet was resuspended in 30 mL wash buffer 4 (Table 1) before being sonicated on ice for 30 s on, 30 s off, three times. The suspension was centrifuged at 4 °C at 10,000 × g for 15 min. The washing steps are important to remove impurities from the inclusion bodies and to obtain a high purity of the final recombinant AβM. At this point, the pellet should be white and can be frozen until use or solubilized before chromatography.

Solubilizing and Ion Exchange Chromatography of AβM. The washed inclusion body pellet from 50 mL of culture was placed on ice with a small magnetic stir bar on a magnetic stirrer. 200 μL of 6 M GuHCl was added to the pellet and stirred vigorously for 15 min to solubilize the AβM containing inclusion bodies. 15 mL of ice-cold IEX buffer A (10 mM Tris, 1 mM EDTA, pH 9) was added slowly to the solubilized pellet to dilute the 6 M GuHCl and to permit binding of AβM to the ion exchange column. The solubilized AβM was filtered through a 0.22 μm filter before being placed on ice prior to chromatography. If the chromatography system is not kept in a cold room, then all buffers must be kept on ice and the column wrapped in an ice bag to keep the chromatography process as cold as possible to reduce aggregation of AβM. The ion exchange column must be equilibrated prior to the AβM sample being ready for purification to reduce the amount of time for AβM handling. AβM was loaded onto a HiTrap Q HP column (GE, Healthcare) and eluted against a linear gradient of IEX buffer B (10 mM Tris, 1 mM EDTA, 0.75 M NaCl, pH 9) over seven column volumes followed by two column volumes of 100% buffer B. Purification was performed on an ÄKTA Pure FPLC and monitored by absorption at 280 nm (GE Healthcare). AβM eluted at ∼30% buffer B and must be immediately placed in aliquots for storage at −80 °C. The concentration of AβM was determined by absorption at 280 nm on a NanoVue spectrometer using the extinction coefficient of 1490 M^{-1} cm^{-1} for both AβM variants. AβM42 mass/charge was determined using ESI-MS at the Department of Chemistry, University of Cambridge. To note, for buffer exchange into required buffers for different assays, the fastest method is to use desalting columns and centrifugation. The PD MiniTrap G-10 columns (GE, Healthcare) are suitable for use with small peptides down to 700 Da.

Expression of Recombinant AβM(E22G)-mCherry in HEK293 Cells. The plasmid pcDNA5/FRT/TO:Aβ(E22G)-mCherry encodes the arctic mutant of Aβ42 sequence (E22G) and a C-terminus encoded linker sequence GSAGSAAGSGESH followed by the mCherry fluorescent protein sequence. This plasmid was subsequently transfected along with the pOG44 plasmid encoding the FLp recombinase into the Flp-In T-REx-293 cell line (#R78007, Thermo Fisher Scientific). The gene of interest, the coding sequence of AβM(E22G)-mCherry, was integrated into the genome to generate an inducible, stable, and single-copy cell line expressing the Arctic mutant (E22G) of Aβ42 fused to mCherry.[21] Complete media (DMEM (high glucose), 10% FBS and 2 mM L-glutamine) was during cell line construction. Stable transfectants were selected using complete media with the addition of the antibiotic hygromycin B for 6−12 weeks. After selection, single cell clones were collected to generate a homogeneous cell line and the expression level was characterized by flow cytometry in our previous paper.[21]

To induce AβM(E22G)-mCherry expression, we administered complete media with addition of 1 μg/mL tetracycline to the cells. To harvest enough protein for purification, six T75 tissue flasks were seeded and cells induced for protein expression for 7 days. Approximately 12 million cells were collected and centrifuged at 4000 × g for 15 min and directly frozen in −80 °C.

Purification of AβM(E22G)-mCherry. The HEK293 cell pellet was resuspended in 25 mL IEX buffer A (50 mM Tris, pH 8) with protease inhibitors (cOmplete, EDTA-free cocktail, Roche) and sonicated 20 s on, 30 s off, four times using an XL-2020 sonicator (Heat Systems). The suspension was centrifuged at 800 × g for 5 min at 4 °C to remove unbroken cells. The supernatant was removed and centrifuged for a further 15 min at 21,000 × g at 4 °C. The supernatant was saved as the soluble fraction, and the insoluble fraction was resuspended in IEX buffer A. To determine which fraction contained the most AβM(E22G)-mCherry the fractions were analyzed by SDS-PAGE on a 4−12% bis-tris gel and subjected to Western blot analysis to probe for the presence of Aβ and mCherry. The membrane was probed with an anti-Aβ antibody targeted to residues 1−16 (1:1000, #E 610, Biolegend) and a secondary anti-mouse IgG HRP-conjugated antibody (1:1000, #NA931, GE Healthcare). The membrane was dried and reprobed with an anti-mCherry antibody (1:1000, #125096, abcam) and a secondary anti-mouse IgG HRP-conjugated antibody (1:1000, #NA931, GE Healthcare). The soluble fraction was found to contain more AβM(E22G)-mCherry than the insoluble fraction (Supporting Information Figure 10) and was therefore used for further purification. The soluble fraction was filtered through a 0.22 μm membrane before being loaded on a HiScreen Capto Q ImpRes ion exchange column (GE Healthcare). The AβM(E22G)-mCherry was eluted from the column over seven column volumes on a linear gradient against IEX buffer B (50 mM Tris, 1 M NaCl, pH 8) followed by two column volumes of 100% buffer B. Western blot analysis, using the same antibodies as described above, was used to confirm in which eluted fractions the AβM(E22G)-mCherry resided. Monomeric AβM(E22G)-mCherry has a MW of 32.3 kDa, the Western blots show the presence of both degraded and aggregated AβM(E22G)-mCherry from purification. The AβM(E22G)-mCherry positive fraction was slightly pink in the column, but it is noted that the HEK293 cell medium is also pink and residues of the latter can be present in other eluted fractions; therefore, Western blot analysis is required to confirm the presence of AβM(E22G)-mCherry rather than just relying on color of eluted fractions. The concentration of the eluted fraction containing AβM(E22G)-mCherry was calculated from absorption at 280 nm on a NanoVue spectrometer using the extinction coefficient of 35,870 M^{-1} cm^{-1}. AβM(E22G)-mCherry mass/charge was determined using ESI-MS at the Department of Chemistry, University of Cambridge.

SDS-PAGE and Western Blot. To determine in which fractions AβM eluted from the ion exchange columns, SDS-PAGE was ran. 20 μL of protein solution was incubated with 4 μL of LDS sample buffer and incubated at 100 °C for 5 min before 10 μL was loaded on a 4−12% bis-tris gel (NuPAGE, Thermo Fisher Scientific). The gel was either stained with Coomassie blue or transferred to a 0.22 μm polyvinylidene fluoride (PVDF) membrane and probed for Aβ. The membrane was first blocked with 5% BSA in PBS with 0.05% Tween-20 for 30 min before incubation with the primary antibody against residues 1−16 of Aβ (1:1000, #E610, biolegend) for 1 h. After washing for 2 min three times, the membrane was incubated with the secondary antibody, anti-mouse IgG linked to HRP (1:1000, #NA931, GE Healthcare) for 1 h. The membrane was washed for 2 min five times and incubated with chemiluminescent subtrate (SuperSignal WEST pico PLUS, Thermo Fisher Scientific) and imaged using a G:Box (Syngene). The membrane was dried and subsequently reprobed using similar conditions, by blocking with 5% BSA, incubating with the primary antibody against mCherry (1:1000,

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ACS Chem. Neurosci. 2020, 11, 3204−3213

#[1C51] ab125096, abcam) followed by washing and incubation with the secondary antibody anti-mouse IgG linked to HRP (1:1000, #NA931, GE Healthcare). To determine purity of the AβM fractions, during purification the gel images were analyzed using ImageJ software[23] to determine the percentage of AβM present. Regions of interest were selected with a rectangle and a histogram of the intensity of dyed protein within the area displayed using the measure function. From the peaks displayed in the histogram, correlating to protein bands, the percentage purity of AβM could be calculated from the peak of AβM as a percentage of the total area of peaks.

Cell Vitality Assays. Human neuroblastoma cells (SH-SY5Y) were obtained from the European Collection of Cell Cultures (ECACC, Sigma-Aldrich, Dorset, United Kingdom) and grown in a 1:1 minimal essential medium (MEM) (Sigma-Aldrich) and nutrient mixture Ham's F-12 (Sigma-Aldrich) supplemented with 15% FBS, 1% nonessential amino acids, 2 mM GlutaMAX, and 1% antibiotic−anti-mycotic (all Thermo Fisher Scientific, Epsom, United Kingdom). The vitality of neuroblastoma SH-SY5Y cells in the presence of AβM42 was determined using the MTT (3 [4,5-dimethylthiazole 2]-2,5-diphenyltetrazolium bromide) kit from Promega (Madison, Wisconsin, US). Briefly, 3×10^4 cells were seeded into each well of 96-well culture plates overnight followed by incubation of AβM42 from two pufication batches at a final concentration of 1 and 2 μM for 24 h. MTT was added to the cells for 4 h and the absorption was measured with a microplate reader at 590 nm (Envision, PerkinElmer, Waltham, Massachusetts, US). The absorbance is proportional to the number of viable cells. Each experiment was performed three times in duplicate for each purification batch. Data was normalized to the cell only control in each experiment.

Thioflavin-T (ThT) Based Kinetic Aggregation Assays. 20 μM freshly made ThT (Abcam, Cambridge, UK) was added to 50 μL of 5 μM AβM42 after buffer exchange into 100 mM Tris, 100 mM NaCl pH 7 using PD MiniTrap G-10 columns (GE, Healthcare). All samples were loaded onto nonbinding, clear-bottom, 96-well half area plates (Greiner Bio-One GmbH, Germany). The plates were sealed with a SILVERseal aluminum microplate sealer (Grenier Bio-One GmbH). Fluorescence measurements were taken with a FLUOstar Omega plate reader (BMG LABTECH GmbH, Ortenbery, Germany). The plates were incubated at 37 °C with double orbital shaking at 300 rpm for 1 min before each was read every 5 min for 600 min. Excitation was set at 440 nm with 20 flashes and the ThT fluorescence intensity measured at 480 nm emission with a 1300 gain setting. Two ThT assays were run using four fractions of AβM42 from two purification runs with four wells per fraction. Data were normalized to the sample with the maximum fluorescence intensity for each plate.

Buffer Exchange. 300 μL of AβM42 at 25.5 μM was buffer exchanged into 100 mM Na_2HPO_4 pH 7 using PD MiniTrap G-10 columns (GE Healthcare) following the gravity exchange protocol provided. The buffers were kept ice-cold to reduce oligomerization. 500 μL of the same aliquot of AβM42 at 25.5 μM was also injected into a Superdex 75 10/300 GL gel filtration column. The 100 mM Na_2HPO_4 pH 7 buffer was kept on ice and the column wrapped in an ice bag to keep the protein as cold as possible. The AβM42 was eluted isocratically at 0.8 mL/min and eluted between 17.5 and 18 min, as expected for a protein of 4.5 kDa.

Atomic Force Microscopy. 10 μL of AβM42 were incubated on freshly cleaved mica for 10 min while at 4 °C. The mica was washed five times with dH_2O and dried gently under a flow of compressed air. Images were acquired in Peak Force Quantitative Nanomechanical Property mode using ScanAsyst Air probes on a BioScope Resolve (Bruker AXS GmbH). Each field of view was 2 μm × 2 μm acquired with 512 lines at 0.966 Hz. Images were flattened using NanoScope Analysis software, ver. 1.8 and exported. The pixel area of the oligomers was extracted from the AFM images using the ICY imaging software (http://icy.bioimageanalysis.org/) and were gray rendered before analysis. The area of oligomers was then calculated using the "Connected Components" plugin. The gray-scale 8-bit images were given a threshold of 80/256 to remove background and a group of connected pixels was detected as a cluster which was converted to a size where one pixel = 3.9 nm^2.

Transmission Electron Microscopy of AβM42 and AβM-(E22G)-mCherry Aggregates. 5 μM of AβM42 was incubated in 100 mM Tris 200 μM NaCl, pH 7 for 2 days with constant rotation at 20 rpm on a rotator (SB2, Stuart Scientific) at 37 °C. AβM(E22G)-mCherry was used at a concentration of 77 μM. 10 μL of each sample was deposited on a carbon 400 mesh grid for 1 min. The grid was washed twice for 15 s in dH_2O before incubating in 2% uranyl acetate for 30 s to negatively stain the sample. The grid was imaged using a Tecnai G2 80−200kv TEM at the Cambridge Advanced Imaging Centre.

Fluorescence Imaging of AβMC40-AF488 and AβM(E22G)-mCherry. A glass coverslip was cleaned with 1 M KOH for 15 min and washed extensively with dH_2O and dried. 5 μM of AβMC40-AF488 sample was incubated at 37 °C for 2 days with rotation at 20 rpm. The solution was centrifuged at 21,000 × g for 5 min and 10 μL deposited on the glass and incubated in the dark for 15 min. 10 μL of AβM(E22G)-mCherry at 77 μM was deposited on the glass and incubated in the dark for 15 min. Both samples were washed three times to remove unbound protein with dH_2O and imaged. Images of the samples were collected using a custom-built three-color structured illumination microscopy (SIM) setup which we have previously described.[24] A 60×/1.2NA water immersion lens (UPLSAPO 60XW, Olympus) and a sCMOS camera (C11440, Hamamatsu) were used. The laser excitation wavelengths used were 488 nm (iBEAM-SMART-488, Toptica) for imaging AβMC40-AF488 and a 561 nm laser for AβM(E22G)-mCherry (OBIS 561, Coherent). The samples were also imaged in the other laser channel and in the 640 nm laser (MLD 640, Cobolt) channel to check for cross talk or nonspecific fluorescence contaminants, no/little fluorescence was observed in other channels. The laser intensity used was between 10 and 20 W/cm^2 with an exposure time of 150 ms. Although a SIM setup was used, the intensity of the signal was too low in the samples to use artifact-free SIM reconstruction, so widefield reconstruction was used and the average intensity from nine SIM images is presented. The same setup was used to image AβM(E22G)-mCherry aggregates in cells, but the signal was strong enough for SIM reconstruction, which was performed with LAG SIM, a custom plugin for Fiji/ImageJ available in the Fiji Updater. LAG SIM provides an interface to the Java 691 functions provided by fairSIM.[25]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschemneuro.0c00300.

Coomassie stained gel of AβM42 expression and cleaning of inclusion bodies in preparation for purification by chromatography; Ion exchange chromatography; Purity determined by densitometry and concentrations of fractions; Liquid chromatography and mass/charge spectrum; Viability of SHSY 5Y cells; Fibrillar structures formed after incubation of AβM42; Formation of fluorescent fibrils and oligomers and purification by ion exchange chromatography; Calculation of the concentration and degree of labeling for eluted fractions containing AβMC40-AF488; Atomic Force Microscopy; TEM showing purified fractions; Mass spectra; Raw images of Coomassie blue stained SDS PAGE gels and Western blots (PDF)

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Author Contributions

A.D.S designed experiments and performed purification, kinetic assays, AFM, TEM, and widefield microscopy imaging. M.L. constructed the AβM(E22G)-mCherry Flp-In T-REx-293 cell line and performed cell culture, performed SIM imaging, reconstruction of images of AβM(E22G)-mCherry in cells and helped with widefield microscopy imaging. A.F.-V. maintained SHSY-5Y cells and performed MTT assays. A.D.S. made the figures. A.D.S. and G.S.K.S. wrote the manuscript. All authors contributed to the manuscript and gave their final approval.

Notes

The authors declare no competing financial interest.

Raw data files are available at the Cambridge University Repository at https://doi.org/10.17863/CAM.57720.

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