A β 40 has a subtle effect on A β 42 protofibril formation, but to a lesser degree than A β 42 concentration, in A β 42/A β 40 mixtures

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¹Abbreviations used – AD, Alzheimer's disease, A β , amyloid- β protein, aCSF, artificial cerebrospinal fluid, DLS, dynamic light scattering, HFIP, hexafluoroisopropanol, R_H , hydrodynamic radius, R_g , root mean square radius, MALS, multi-angle light scattering, SEC, size exclusion chromatography, ThT, thioflavin T.

ABSTRACT: Recent findings suggest that the senile plaques in Alzheimer's disease may contain soluble amyloid- β peptide ($A\beta$) fibril precursors along with insoluble fibrils.. These soluble $A\beta$ species, including oligomers and protofibrils, have been well-studied *in vitro* and are formed via non-covalent self-assembly of $A\beta$ monomers. While both 40- and 42-residue forms of $A\beta$ are observed in the human body, the majority of the $A\beta$ aggregation work has been conducted on $A\beta_{42}$ or $A\beta_{40}$ separately, with relatively few investigations of mixtures. In order to study the effect of different combinations of $A\beta_{40}$ and $A\beta_{42}$ on protofibril formation, mixtures of either dry solid peptide, or purified $A\beta_{40}$ and $A\beta_{42}$ monomer solutions were mixed together and protofibril/monomer distributions were quantified. Increases in the $A\beta_{42}/A\beta_{40}$ ratio increased protofibril formation but the presence of $A\beta_{40}$ in the mixed $A\beta$ solutions had a significant negative impact on protofibril formation compared to equivalent solutions of pure $A\beta_{42}$. Protofibril size was less affected, but β -sheet structure increased with protofibrils formed from higher $A\beta_{42}/A\beta_{40}$ ratio solutions. Direct measurement of $A\beta_{42}/A\beta_{40}$ ratios by C-terminal-selective ELISA found very little $A\beta_{40}$ incorporated into protofibrils. The cumulative data emphasizes the critical importance of $A\beta_{42}$, yet establishes $A\beta_{40}$ as a regulator of $A\beta_{42}$ aggregation.

1. Introduction

Alzheimer's disease (AD) is the most prevalent type of dementia in older adults and is caused by progressive neurodegeneration in the hippocampus and the amygdala in affected patients [1]. As of 2013, AD was the sixth leading cause of death in the United States. It has been estimated that 5.3 million Americans are currently living with AD and in the coming years the number will increase significantly due to the aging baby boom generation [2, 3]. Furthermore, it is estimated that the number of people currently living with dementia worldwide (46.8 million) is expected to double in 20 years [4],

Two pathological hallmarks, neurofibrillary tangles (NFTs) and senile (amyloid) plaques, still dominate the biochemistry of AD [5]. NFTs are intraneuronal deposits of insoluble filamentous aggregates of tau, a microtubule-associated protein. Senile plaques are extracellular deposits in the brain parenchyma of insoluble amyloid- β protein (A β) fibrils. Clinicopathologic studies have shown that the clinical progression of AD correlates closely with the density and distribution of abnormally-folded tau NFTs in the brain; while the initial cause of the disease appears to be linked to A β aggregation and accumulation [6].

A β is a collection of secreted peptide fragments produced by β - and γ -secretase-catalyzed proteolytic cleavage of the amyloid precursor protein (APP). The cleavage yields A β fragments 37-43 amino acids in length (reviewed in [7-10]). The two most commonly-studied fragments are A β 40 and A β 42. Both peptides are found circulating normally in the blood and cerebrospinal fluid with A β 40 at much higher levels than A β 42 [11, 12]. Even though the two isoforms differ by only two amino acid residues, their biophysical properties are very different. It is widely recognized that A β 42 is more

fibrillogenic than A β 40 [13] and this property has many ramifications on the disease process. In early-onset familial AD, mutations in the APP gene surrounding the γ -secretase cleavage site alter A β metabolism by increasing the total A β amount and the A β 42/A β 40 ratio [14-16]. While the circulating levels of A β are dominated by A β 40, senile plaques consist overwhelmingly of A β 42 [17]. A β deposits in the leptomeningeal vessels also have a greater percentage of A β 42 [18]. Furthermore, studies in mouse models, human presenilin-expressing mouse fibroblast lines, and human plasma indicate that the total A β concentration may not be as important as the A β 42/A β 40 ratio for plaque formation [14, 19-21]. These observations, along with other findings, support the critical, and detrimental, role of A β 42 in AD pathogenesis [22]. In fact, the A β 42 level in cerebrospinal fluid (CSF) is one component of an important biomarker test for AD patients that also includes CSF tau and amyloid imaging [23]. The reduction in circulating plasma and CSF A β 42 is believed to be due to aggregation and deposition of A β 42 in the brain prior to onset of AD symptoms [24, 25].

Much of the detailed knowledge about A β aggregation kinetics and mechanisms has been acquired through *in vitro* studies using synthetic peptides. These studies have been invaluable for understanding the process by which A β undergoes non-covalent self-assembly in solution and identifying the conditions that modulate this process. There is general agreement that unstructured monomeric A β 42 and A β 40, at sufficient concentration, will nucleate and begin to form soluble oligomers [26, 27] and protofibrils with significant β -sheet content [28-30]. The initial nucleation step, conformational pathway, and subsequent oligomerization can be influenced by pH, ionic strength, temperature, and agitation [28, 31-33]. Many of the soluble A β species ultimately

progress to insoluble fibrils, which are indistinguishable from those isolated from brain tissue [34]. Although fibril-bearing senile plaques are the most visible A β pathological feature, the failure of insoluble A β load to correlate strongly with memory loss [35] significantly increased the interest in soluble A β aggregates for their role in neurodegeneration [36, 37].

As discussed above, numerous *in vivo* investigations have established that the A β 42/A β 40 ratio is an important factor in AD pathology. Despite this information, most studies utilize only one form of A β . Although fewer in number, there have been several reports on A β aggregation kinetics and mechanisms in A β 42/A β 40 mixtures with most suggesting that A β 40 inhibits A β 42 aggregation. For example, the inclusion of monomeric A β 40 suppressed both A β 42 monomer and protofibril conversion to fibrils and failed to promote A β 42 fibril elongation [38]. Other reports have demonstrated shorter lag times and/or faster aggregation rates as the A β 42/A β 40 solution ratio increased [39, 40]. It has been postulated that A β 40 inhibits A β 42 aggregation by preferentially binding to A β 42 aggregates thereby preventing further polymerization [39]. A contrasting theory maintains that A β 40 potentially delays A β 42 aggregation through “non-productive” interactions [41]. The findings that a minor increase in the solution A β 42/A β 40 ratio increases neurotoxicity [40, 42] emphasizes the importance of understanding the aggregation dynamics of A β 42/A β 40 mixtures and the impact on A β structure and biological activity.

The current study examined the effect of varying A β 42/A β 40 ratios on protofibril formation. Protofibrils are a well-characterized member of an ever-broadening class of soluble A β aggregates. Their formation *in vivo* may play a role in AD pathogenesis

making them an important potential therapeutic target [43]. The investigation revealed marked changes in both the extent of protofibril formation and the character of the protofibrils as the A β 42/A β 40 ratio changed. The findings demonstrated profound effects by A β 42 on the initial nucleation phase, which is closely coupled to rapid protofibril formation.

2. Materials and methods

2.1. A β preparation. A β 42 and A β 40 peptides were obtained from W.M. Keck Biotechnology Resource Laboratory (Yale School of Medicine, New Haven, CT) in lyophilized form, individually dissolved in 100% hexafluoroisopropanol (HFIP) (Sigma-Aldrich, St. Louis) at 1 mM, separated into aliquots in sterile microcentrifuge tubes, and evaporated uncovered at room temperature overnight in a fume hood. The following day, the aliquots were vacuum-centrifuged to remove any residual HFIP and stored in dessicant at -20°C . Preparation of A β 42/A β 40 dry peptide mixtures was accomplished by resuspending A β 42 and A β 40 aliquots separately in 100% HFIP and mixing the two solutions in the desired amounts to form either pure A β 42 or A β 40 or molar A β 42/A β 40 ratios of 4:1, 1:1, and 1:4. The volumes used to prepare the planned dry peptide ratios were calculated to yield a total A β concentration of 200 μM upon reconstitution. Briefly, A β 42 and A β 40 (1 mg) were resuspended in 100% HFIP to a concentration of 1 mM. Ratios of 4:1, 1:1, and 1:4 A β 42/A β 40 were prepared by mixing selected volumes of each A β /HFIP into three separate tubes. The volumes used were 160 μL A β 42 (0.72 mg) and 40 μL A β 40 (0.17 mg) for 4:1; 100 μL A β 42 (0.45 mg) and 100 μL A β 40 (0.43 mg) for 1:1; and 40 μL of A β 42 (0.18 mg) and 160 μL A β 40 (0.69 mg) for 1:4. The A β 42/A β 40 HFIP mixtures were evaporated overnight in a fume hood uncovered. Vacuum centrifugation was used to remove any remaining HFIP and samples were stored in a desiccant at -20°C .

2.2. Size exclusion chromatography. Dry A β 42/A β 40 peptide mixtures were dissolved in 50 mM NaOH to yield a 2 mM solution followed by dilution to 200 μM A β in prefiltered

artificial cerebrospinal fluid (aCSF, 15 mM NaHCO₃, 1 mM Na₂HPO₄, 130 mM NaCl, 3 mM KCl, pH 7.8) and incubation for 30 min at 25 °C to allow protofibril formation. Protofibrils and monomers were separated as previously described [44]. The A β solutions were centrifuged at 18,000g for 10 min and the supernatant was fractionated using size exclusion chromatography (SEC) on a Tricorn Superdex 75 10/300GL column (fractionation range 3-70 kD, GE Healthcare) attached to an AKTA FPLC system (GE Healthcare). Bovine serum albumin (Sigma) was routinely run each day to prevent non-specific binding of A β to the column matrix. A β was eluted at 0.5 mL min⁻¹ in aCSF and 0.5 mL fractions were collected and immediately placed on ice. A β concentrations were determined in-line by UV absorbance using an extinction coefficient for A β of 1450 cm⁻¹ M⁻¹ at 280 nm. For aggregation of A β ₄₂/A β ₄₀ monomer mixtures directly isolated by SEC, solutions were diluted with aCSF to 40 μ M total A β , supplemented with 0.05% sodium azide (NaN₃) to prevent contamination, and quiescently incubated at 25 °C for 25 days with numerous measurements during the incubation. For aggregation of A β ₄₂/A β ₄₀ monomer mixtures prepared from separately SEC-purified A β ₄₂ and A β ₄₀ monomers, solutions were mixed together at different ratios to a total A β concentration of 40 μ M in aCSF buffer and incubated at 37 °C without disturbance for 24 h. Following an 18,000g spin, supernatants were further separated on SEC in-line with light scattering.

2.3. Dynamic light scattering. Hydrodynamic radius (R_H) measurements were made as previously described [44]. Measurements were taken at room temperature with a DynaPro Titan instrument (Wyatt Technology, Santa Barbara, CA). Samples (30 μ l)

were placed into a quartz cuvette and light scattering intensity was collected at a 90° angle using a 5-second acquisition time. Particle diffusion coefficients were calculated from auto-correlated light intensity data and converted to R_H with the Stokes-Einstein equation using Dynamics software (version 6.12.0.3). Histograms of percent intensity vs. R_H were generated by data regularization and intensity-weighted mean R_H values were derived from the regularized histograms.

2.4. Thioflavin T fluorescence assay. A β samples were assessed by ThT fluorescence as previously described [45]. Briefly A β samples were diluted to 5 μ M (unless otherwise noted) in aCSF pH 7.8 containing 5 μ M ThT. Fluorescence emission scans (460-520 nm) were acquired on a Cary Eclipse fluorescence spectrophotometer using an excitation wavelength of 450 nm and integrated from 470-500 nm to obtain ThT relative fluorescence values. Excitation and emission slit widths and power settings were the same for all fluorescence measurements. Buffer controls did not show any significant ThT fluorescence in the absence of A β . All ThT fluorescence numbers are reported in relative fluorescence units (RFU). Extended aggregation assays of SEC-purified A β ₄₂/A β ₄₀ monomer mixtures were monitored by ThT fluorescence similarly.

2.5. Multi-angle light scattering (MALS). MALS data was obtained in-line with SEC using a DAWN DSP instrument (Wyatt Technology, Santa Barbara, CA). Samples were analyzed under continuous flow through a K12 quartz cell and were illuminated with a helium-neon laser. Light scattering data were obtained every 0.167 mL at fixed-angle

detectors 4-16 and plotted using the Debye formalism of the Rayleigh-Debye-Gans approximation [46, 47] for large anisotropic particles (eq 1).

$$R_{\theta} / Kc = M_w P(\theta) \quad (\text{eq 1})$$

This equation relates the Rayleigh ratio (scattering intensity) to molecular weight (M_w) multiplied by a form factor, $P(\theta)$, where c is solute concentration (g/mL), θ is the scattering angle, K is an optical constant equal to $4 \pi^2 (dn/dc)^2 n_o^{-2} \lambda_o^{-4} N_A^{-1}$, n is the refractive index of the solution, n_o is the refractive index of the solvent, λ_o is the wavelength of incident light in a vacuum (632.8 nm), and N_A is Avagadro's number. $P(\theta)$, which takes into account the destructive interference of scattering by large particles, is the scattering intensity for a large particle divided by the scattering intensity without interference and can be expanded in a power series. The limiting form of the form factor equation as θ approaches zero is shown in eq 2 where R_g^2 is the mean square radius, and λ is λ_o / n_o .

$$P(\theta) = 1 - (16 \pi^2 / 3\lambda^2) R_g^2 \sin^2(\theta/2) \quad (\text{eq 2})$$

Substitution of eq 2 into eq 1 and plotting of R_{θ} / Kc versus $\sin^2(\theta/2)$ yields M_w as the y-intercept and a limiting slope equal to $M_w (16 \pi^2 / 3\lambda^2) R_g^2$. Debye plots were fit with a linear regression using dn/dc values for A β and BSA of 0.186 mL/g and 0.190 mL/g respectively.

2.6. Circular dichroism. Samples (minimum 0.2 ml) were placed into a Jasco J0556 quartz rectangular quartz cuvette with a 0.1 cm pathlength, or smaller volumes (15 μ L) were measured via quartz-windowed microsampling disc (Jasco MSD-462) with 0.1 cm pathlength. Spectra were obtained with a Jasco J-1500 circular dichroism spectrometer by wavelength scan from 260 to 190 nm using scan speeds, data integration times, and response factors as provided in the Figure legend. Five successive wavelength scans were averaged for each A β sample. Buffer control spectra were averaged and subtracted from A β sample spectra, and each resulting point ($[\theta]_{\text{obs}}$, deg) was converted to mean residue ellipticity ($[\theta]$, deg cm² dmol⁻¹) with the equation $[\theta] = [\theta]_{\text{obs}} \times (\text{MRW}/10lc)$, where MRW is the mean residue molecular weight of A β (1-42) (4514.1 g/mol divided by 42 residues), l is the optical path length (cm), and c is the concentration (g/cm³).

2.7. A β C-terminal-selective ELISA. A β antibodies were obtained as a generous gift from Mayo Clinic Jacksonville Department of Neuroscience. C-terminal selective antibodies Ab2.1.3 (A β 42 specific) and Ab13.1.1 (A β 40 specific) were employed as capture antibodies. Antibody Ab5, which recognizes both A β peptides, was used as the detection antibody. Ab5 was conjugated to HRP using a previously described protocol [48]. Briefly, 2 mg HRP (Sigma) was dissolved in 400 μ L water and incubated at room temperature for 20 min with 100 μ L of 0.1 M sodium periodate on an orbital shaker protected from light. The enzyme was then dialyzed at 4°C against 1 mM sodium acetate buffer (pH 4.4) overnight. The dialyzed HRP solution (190 μ L) was supplemented with 10 μ L of 200 mM sodium carbonate buffer (pH 9.5) and mixed with

200 μ L of Ab5 (1.77 mg/mL) and 300 μ L of 10 mM sodium carbonate buffer (pH 9.5). The mixture was incubated for 2 h at room temperature on an orbital shaker followed by the addition of 50 μ L of freshly prepared sodium borohydride solution (4 mg/mL) and further mixing at 4°C for 2 h. The Ab5-HRP conjugate was purified by SEC as described above. The absorbance of each fraction was assessed at 280 nm (protein) and 403 nm (HRP heme groups) for determination of Ab5 concentration.

Measurement of A β 42 and A β 40 in SEC-isolated A β 42/A β 40 mixtures by ELISA with all steps carried out at room temperature. Briefly, a 96-well plate was coated overnight with 100 μ L of 5 μ g/mL Ab2.1.3 or Ab13.1.1 capture antibodies. The wells were washed with PBS containing 0.05% Tween 20, blocked for 1 hr with PBS containing 1.0% BSA, 5.0% sucrose and 0.05% NaN₃, and washed again. Successive treatments with washing in between were done with samples or standards (2 h), Ab5-HRP detection antibody (2 h), and equal volumes of HRP substrates TMB and hydrogen peroxide (0.5 h). The reaction was stopped by the addition of 1% H₂SO₄ solution and the optical density for each sample was determined at 450 nm with a reference reading at 630 nm. SEC-purified A β 42 and A β 40 monomers were used to construct separate standard curves from 1,000-10,000 pM and were done for each ELISA. Standards and samples were diluted to 1 μ M in 7.2 M GuHCl for 30 min and then further diluted to fall within the standard curve. The denaturant step enhanced antibody recognition for both monomeric and oligomeric A β . Additional ELISA development included testing of A β 40 standard curves with Ab2.1.3 (A β 42 specific) and A β 42 with Ab13.1.1 (A β 40 specific). No cross-reactivity was observed.

3. Results

3.1. Protofibril formation increases as the A β 42/A β 40 ratio increases in mixed solutions.

We have previously detailed the preparation and characterization of the biophysical and proinflammatory properties of protofibrils rapidly formed by A β 42 and to a slower extent by A β 40 [44, 45, 49]. A variety of solution and microscopic techniques indicated that A β 42 protofibrils were short, curvilinear species less than 100 nm in length, rich in β -sheet structure, yet relatively small and quite soluble (hydrodynamic radius, R_H 20-25 nm and molecular weight, M_w , 200-2600 kDa). Those findings are consistent with previous reports [29, 30, 50] and expand the current analysis of these soluble precursors to fibrils. A β protofibrils may be an important therapeutic target in AD therefore a better understanding of their properties will enhance this effort. Furthermore, the observation that circulating A β in plasma and CSF contains both A β 42 and A β 40 underscores the importance of understanding the effect of varying ratios of the two isoforms on protofibril formation. Since protofibril formation occurs rapidly upon reconstitution, different mixtures of dry A β 42 and A β 40 peptide were prepared to ensure that the correct mixture would be present upon reconstitution. This procedure is described in the Methods and was done by mixing 100% HFIP solutions of A β 42 and A β 40 together at predetermined volumes to obtain the correct molar ratios and then removing the HFIP under vacuum centrifugation. Upon reconstitution in the appropriate volume of modified artificial cerebrospinal fluid (aCSF) [45], the desired final individual solution concentrations of A β 42 and A β 40 were achieved while maintaining the total A β concentration at 200 μ M (depicted in Figure 1A). The extent of rapid protofibril formation in the A β 42/A β 40 mixtures after a 30 min incubation at 25 °C was assessed by SEC-

separation and spectroscopic quantitation. A solution of A β 42 alone yielded a typical 2-peak elution profile on Superdex 75 (fractionation range 3-70 kD) with roughly equivalent amounts of protofibrils and monomers (Figure 1A). Our previous work showed that protofibrils were larger than 70 kD [49], so protofibrils eluted in the void volume. The calculated distribution was 56% protofibrils and 44% monomers (Figure 1B). This finding is consistent with our previous reports on A β 42 protofibril formation [44, 45]. However, a decrease in the A β 42 concentration and an increase in the A β 40 concentration resulted in a decrease in protofibril formation. Accordingly, the decrease in protofibril formation corresponded with an increase in residual monomers (Figure 1). The quantitation of this effect is depicted in Figure 1B. These findings demonstrate the effect that the A β 42/A β 40 ratio and A β 42 concentration have on A β nucleation and protofibril formation.

3.2. Protofibril size range slightly increases as the A β 42/A β 40 ratio increases in mixed solutions.

Dynamic light scattering (DLS) analysis was conducted on the protofibril pools in Figure 1A obtained following SEC-separation of the selected A β 42/A β 40 mixtures. Diffusion coefficients were measured and converted to hydrodynamic radius (R_H) in Dynamics software 6.12.0.3 using the Stokes-Einstein equation. Regularization of the light scattering data separated the light scattering of the protofibrils from the buffer contribution and any trace presence of large particles with R_H values greater than 1000 nm. Regularized intensity-weighted histograms revealed a subtle change in protofibril size for the different A β 42/A β 40 ratios (Figure 2). Determination of the mean R_H from

the histograms of each sample yielded values of 21 nm for 200/0, 16 nm for 160/40, 16 nm for 100/100, 14 nm for 40/160, and 13 nm for 0/200 (data not shown). The decrease in the A β 42/A β 40 peptide ratio appeared to alter protofibril amount more dramatically than protofibril size.

3.3. Protofibril thioflavin T fluorescence increases as the A β 42/A β 40 ratio increases in mixed solutions.

Thioflavin-T (ThT) is a benzothiazole dye, which shows increased fluorescence when bound to amyloid fibrils [51]. The β -sheet architecture of aggregated A β in particular creates a ThT binding site [52]. ThT binding and fluorescence has previously been used to characterize soluble A β protofibril species [30, 44]. Here we used ThT to analyze the SEC-isolated protofibril pools obtained from each of the different A β 42/A β 40 mixtures in Figure 1A. ThT fluorescence measurements were conducted at a final A β concentration of 5 μ M for each of the protofibril samples to ensure that a change in fluorescence was not due to lesser or greater numbers of protofibrils in the sample. ThT analysis revealed that the extent of ThT binding and fluorescence decreased as the A β 42/A β 40 ratio decreased (Figure 3). The ThT data suggested that protofibrils formed from different A β 42/A β 40 mixtures differed in their conformation or the ThT binding and/or ThT molecular rotation was altered in protofibrils formed from solutions containing a lower A β 42/A β 40 ratio. The small peak of material eluting in the void volume (protofibril peak) for the 40 μ M A β 42/160 μ M A β 40 and the 200 μ M A β 40 solutions exhibited minimal β -sheet character based on ThT fluorescence levels very close to the background levels.

3.4. Mixed monomer fractions after SEC-isolation display same A β 42/A β 40 ratio-dependence on nucleation.

A second set of dry A β 42/A β 40 peptide mixtures were prepared and separated by SEC as in Figure 1. The same trend of protofibril and residual monomer elution was observed for the same A β 42/A β 40 mixtures. The aggregation kinetics of the SEC-eluted monomers (containing mixtures of A β 42 and A β 40) was conducted at a lower concentration than the protofibril formation described earlier at 200 μ M A β . In contrast to rapid protofibril formation, this process permitted observation of aggregation lag times. The SEC-purified A β 42/A β 40 monomer mixtures were each brought to 40 μ M total A β in a solution of aCSF containing 0.05% NaN₃ and incubated at room temperature. ThT fluorescence measurements were taken at numerous time points to assess the aggregation of each A β 42/A β 40 monomer mixture over time. The SEC-purified all-A β 42 (40 μ M) and the 4:1 A β 42/A β 40 ($\approx$ 32 μ M/8 μ M) monomer exhibited a very rapid initial aggregation with significant ThT fluorescence by 21 hrs (Figure 4). The 1:1 A β 42/A β 40 monomer solution ($\approx$ 20 μ M/20 μ M) showed an extended aggregation lag phase with little ThT fluorescence at 21 hrs, but significant levels at 47 hrs (Figure 4). All five solutions, once nucleated, reached roughly the same ThT fluorescence levels. The 1:4 A β 42/A β 40 ($\approx$ 8 μ M/32 μ M) and all-A β 40 (40 μ M) monomer solutions aggregated much slower with lag times of 3 days, decreased polymerization rates, and lower overall maximum ThT fluorescence levels (Figure 4). Similar to rapid protofibril formation, this data shows that as A β 42 increases and A β 40 decreases within a monomeric A β solution, lag times decrease and the overall ThT binding/fluorescence increases.

3.5. A β 40 has an impact on A β 42 aggregate yields.

The findings heretofore indicate that at high A β concentrations, particularly A β 42, nucleation is rapid and protofibrils are formed but with a significant dependence on the ratio between the two peptides. However, working with stored dry A β 42, and to a lesser extent A β 40, peptide samples without further purification increases the potential for pre-existing structures that may influence the ensuing aggregation process. In order to preclude this possibility, studies were done in solution mixtures of A β 42 and A β 40 using separately SEC-purified monomers of each. For these studies, both A β 42 and A β 40 were reconstituted in 6 M GuHCl containing 10 mM NH₄OH to suppress rapid protofibril formation and increase monomer yields. Purified monomer fractions were obtained from the included volume of a Superdex 75 column, immediately placed on ice, and promptly used for aggregation studies. A total A β (A β 42 + A β 40) concentration of 40 μ M was selected so that 0.5 mL (90 μ g A β) could be loaded for in-line SEC-MALS analysis and post-elution evaluation of fractions. This amount was the realistic minimum for sample detection and analysis [49]. A β 42 and A β 40 monomers were isolated in aCSF pH 7.8 containing 30 mM NaCl concentration, rather than 130 mM to encourage soluble aggregate formation and discourage insoluble fibril formation [49]. Using this paradigm previously with A β 42, we have observed the formation of both protofibrils and protofilaments [49]. Furthermore, in order to better elucidate whether A β 40 directly impacts A β 42 aggregation, or is merely a bystander during A β 42 nucleation, additional and separate control experiments were conducted with A β 42 alone.

A β 42 and A β 40 mixtures prepared from SEC-purified monomers were incubated for 24 h at 37 °C and then monitored for ThT fluorescence. Increased A β 42/A β 40 ratio correlated with higher ThT fluorescence after 24 h (Figure 5A). A solution of purely A β 40 did not display any aggregation at all while purely A β 42 displayed the greatest aggregation. The presence of A β 40 at a small ratio (4:1 A β 42:A β 40) slowed the overall aggregation based on ThT fluorescence (Figure 5A). A separate experiment was conducted using identical conditions and the same four A β 42 concentrations (40, 32, 20, and 8 μ M) but without A β 40 in the solutions. The decrease in ThT fluorescence as A β 42 concentration decreased (Figure 5B, 40 μ M to 32 μ M) was not nearly as dramatic as with the inclusion of A β 40 (e.g. 32 μ M A β 42/8 μ M A β 40). The aforementioned A β 42/A β 40 solution mixtures were centrifuged at 18,000g for 10 min and the supernatants were re-purified on SEC. The pure A β 40 solution did not yield any soluble protofibrils in the Superdex 75 void volume (Figure 6A), but increasing A β 42/A β 40 ratio significantly increased soluble protofibril levels (Figure 6A). A plot of the protofibril/monomer distribution (Figure 6B) highlighted the significant impact of A β 42 on protofibril formation and the difference in aggregation propensity between A β 42 and A β 40. The ThT fluorescence in Figure 5A and elution chromatograms in Figure 6A indicated that more protofibrils were being formed as the A β 42/A β 40 ratio increased. Further inspection of the data also indicates that the presence of A β 40 significantly decreased protofibril formation by A β 42 (Figure 6A). This observation is more clearly evident when comparing the protofibril/monomer chromatogram distributions in A β 42/A β 40 solution mixtures (Figure 6A, B) with the control solutions containing A β 42 alone (Figure 6C, D). The effect of A β 42 concentration on protofibril formation was not

as dramatic as with the inclusion of A β 40. Certainly, the increased concentration of A β 40 monomers, which are slower to aggregate, in the aggregation solutions will skew the protofibril/monomer distribution. However, careful inspection of the protofibril peaks formed by A β 42 alone and those including A β 40 demonstrates an apparent subtle inhibitory effect of A β 40 on A β 42 protofibril formation (Figure 6A and 6C).

3.6. The A β 40/A β 42 ratio has a negligible effect on protofibril molecular weight.

In order to establish if the A β 42/A β 40 ratio affected the protofibril size, we took advantage of multi-angle light scattering in-line with SEC (SEC-MALS) to obtain molecular weight (M_w) values for each solution. The three solutions that yielded sufficient aggregates for evaluation were 40 μ M A β 42/0 μ M A β 40 (40/0), 32 μ M A β 42/8 μ M A β 40 (32/8), and 20 μ M A β 42/20 μ M A β 40 (20/20) (Figure 7A). M_w values were obtained every 0.167 mL and remained fairly constant across the Superdex 75 void peaks for all three solutions (Figure 7A), with little difference between varying A β ratios. Weight-averaging of the “slice” data through the protofibril concentration peaks (full width half max) for each solution gave M_w values of 2.95×10^6 Da, 3.13×10^6 Da, and 2.92×10^6 Da for 40/0, 32/8, and 20/20 solutions respectively. The M_w determinations were representative of approximately 650-700 monomer units within the A β aggregates, which is on the higher end of protofibril M_w and nearing protofilaments. Another size parameter, root mean square radius (R_g), was also obtained from the MALS data and confirmed there was little change in size between aggregates formed from the three solutions. Z-average R_g values were of 57 nm, 57 nm, and 58 nm for 40/0, 32/8, and 20/20 solutions respectively (data not shown). The control solutions containing A β 42

alone at different concentrations were also evaluated with SEC-MALS. For this series, M_w values were found to be larger than those obtained for the series of A β 42/A β 40 mixtures, but did not vary much between the different A β 42 concentrations (40 μ M, 4.21×10^6 Da; 32 μ M, 4.64×10^6 Da; and 20 μ M, 5.25×10^6 Da) (data not shown). SEC-MALS-determined R_g values for the pure A β 42 solutions were similar to the initial set of A β 42/A β 40 mixture with values of 55 nm, 57 nm, and 56 nm for 40, 32, and 20 μ M respectively (data not shown). Overall, the findings revealed that the presence of A β 40 in A β 42/A β 40 mixtures lowered the number of soluble protofibrils formed. However, the protofibrils that were formed had similar M_w and size to solutions of A β 42 alone. This suppressive effect was greater than simply lowering the A β 42 concentration in solutions of A β 42 alone.

ThT fluorescence in the SEC-eluted protofibril pools was similar between A β 42/A β 40 solution mixtures after normalization for concentration (data not shown). The comparable fluorescence per micromolar A β for the protofibrils was consistent with the similarity in M_w values between protofibrils formed by A β 42 and A β 42/A β 40 mixtures. Despite these similarities, circular dichroism (CD) measurements uncovered subtle structural differences in protofibrils formed by A β 42 and mixtures of A β 42 and A β 40 (Figure 8A). Protofibrils formed from a pure A β 42 solution (40/0) contained significant β -sheet structure, while protofibrils isolated from the 32/8 and 20/20 A β mixtures exhibited less β -structure and some elements of α -helical structure. Interestingly, this same lessening of β -structure was observed in pure A β 42 solutions when the concentration was lowered. Again, protofibrils formed in the 40 μ M A β 42 solution were mostly β -sheet, while the lower two concentrations (32 μ M and 20 μ M

A β 42) had elements of α -helix (Figure 8B). Cumulatively, a decrease in solution A β 42 concentration effected aggregation rate, and the yield and secondary structure of protofibrils. However, the presence of A β 40 enhances these effects.

3.7. There is little to no incorporation of A β 40 into protofibrils formed from A β 40/A β 42 solution mixtures.

The extent of protofibril formation was governed by A β 42/A β 40 ratio in solution mixtures. However, it was not known what the relative amounts of each peptide that were incorporated into SEC-isolated protofibril pools. A C-terminal-selective A β ELISA was developed in order to distinguish and quantitate differences in A β 42 and A β 40 concentrations. The ELISA was utilized to determine the A β 42/A β 40 ratio in the solution mixtures in Figure 5A and the peak protofibril fractions from the SEC elutions in Figure 6A. The ELISA-determined ratios are presented in Table 1 and underscored the difference between the initially prepared A β 42/A β 40 aggregation solution mixtures and which peptide ends up in the protofibril fraction. The initial aggregation solutions and protofibrils that contained only A β 42 (40/0) were only detected by the A β 42 capture antibody (Ab 2.1.3) and the samples that contained only A β 40 (0/40) were only detected by the A β 40 capture antibody (Ab 13.1.1). These findings confirmed the C-terminal selectivity of the ELISA (Table 1). Ratios of >10 or <0.01 were presented in Table 1 for clarity but the footnote indicates that only one peptide was detected in each pure solution.

The ELISA data indicated that the initial aggregation solution mixtures had A β 42/A β 40 ratios close to the expected values to the original dry peptide mixtures

(Table 1). However, the predominant A β peptide found in the protofibrils formed from A β 42/A β 40 solution mixtures was A β 42. Particularly emphasizing this point was the 20 μ M A β 42/20 μ M A β 40 (20/20) mixture, wherein SEC-isolated protofibrils contained virtually all A β 42 (Table 1). C-terminal-selective ELISA analysis of the initial 20/20 mixture was close to an even mix between the two peptides. Our findings indicate that that much of the protofibril formation in A β 42/A β 40 mixtures occurred by A β 42 with very little A β 40 incorporation. While A β 40 may impact A β 42 aggregation and possibly alter the aggregate structure, it does not extensively incorporate within the oligomeric assembly.

3.8. A β 40 has a lesser effect on protofibril formation when A β 42 concentration remains fixed.

In order to directly assess the impact of A β 40 on A β 42 protofibril formation, solution mixtures of newly SEC-purified A β 42 and A β 40 monomers were prepared. A β 42 concentration was maintained at 20 μ M, while A β 40 concentration varied. In this paradigm, A β 40 had only a small effect on A β 42 protofibril formation. ThT fluorescence was moderately reduced in the solution mixtures containing A β 40 after a 24 h incubation (data not shown), but only small changes were observed in the protofibril peak after SEC separation (Figure 9A). Accordingly, monomer levels increased in the Superdex 75 chromatograms as the A β 40 concentration increased in the solution aggregation mixtures (Figure 9B). While the protofibril / monomer ratio decreased as the A β 40 concentration increased (Figure 9C), a closer look at the SEC protofibril peak integration (nmol) indicated that the effect of A β 40 on the amounts of A β 42 protofibrils

was slight (Figure 9D). However, there was an observable decrease in the ThT fluorescence in the collected SEC peak protofibril fractions from aggregation solutions that contained A β 42 plus A β 40 (Figure 9E). Further analysis with CD of the same peak SEC protofibril fractions suggested that the decrease in ThT binding/fluorescence of A β 42 protofibrils formed in the presence of A β 40 may reflect a change in the β -sheet character of the A β 42 protofibrils (Figure 9F). Additional elements of secondary structure beyond β -sheet were noted in the spectra when the A β 40 concentration increased. This was particularly evident after SEC-isolation and analysis of the 20 μ M A β 42 / 56 μ M A β 40 aggregation solution (Figure 9F). The overall findings demonstrate that decreased A β 42 concentration and the presence of A β 40 may work together to prevent protofibril formation, but A β 42 concentration has a much more significant impact at the ratios examined in this study.

4. Discussion

A β assembly occurs via a nucleation-dependent polymerization process whereby the rate-limiting nucleation step is preceded by a lag phase and followed by rapid polymerization and ultimately fibril formation [53]. The length of the lag is inversely correlated with concentration and it can be abolished by the introduction of a pre-formed fibril seeds. The lag phase duration is highly variable even in homogeneous aqueous solutions and is sensitive to many conditions [28]. Protofibril formation is closely tied to the nucleation step in the A β aggregation pathway. Our investigation of A β 42/A β 40 mixtures allowed us to follow those early assembly steps and the effect of each peptide on the process.

Measurements of human plasma A β levels have yielded concentration ranges of 30-230 pM for A β 40 and 6-31 pM for A β 42 [11, 24, 54, 55]. The reported A β 42/A β 40 ratio from these studies ranges from 0.06-0.38 for control patients with the most common ratio centering about 0.15. While most techniques do not have the appropriate sensitivity at these low pM concentrations, the current study investigated A β 42/A β 40 ratios that might recapitulate those found in normal or AD physiology. Comparisons were then made to solutions composed of purely A β 42 or A β 40.

As the A β 42/A β 40 ratio was modulated in the prepared mixtures, we observed several phenomena. One, the extent of protofibril formation increased at higher A β 42/A β 40 ratios based on SEC elution profiles. Two, while the size (R_H) and M_w of the SEC-isolated protofibrils from the mixtures increased only modestly or not at all in solutions with different ratios, the samples formed from higher A β 42/A β 40 solutions exhibited greater ThT fluorescence per μ M A β suggesting that they had a more developed or defined β -sheet structure. Three, CD analysis confirmed a more developed β -sheet structure at higher A β 42 concentration in the absence of A β 40. Four, control experiments with pure A β 42 solutions suggested that while A β 42 concentration was a factor in the mixtures, A β 40 appears to act as a modulator of A β 42 aggregation. These findings highlighted the important role of A β 42 concentration in both triggering protofibril formation and defining the structure and properties of the protofibrils.

The most straightforward explanation for A β 40 retarding A β 42 aggregation is a direct association with A β 42 monomers. However, direct measurement of the protofibril fractions by C-terminal-selective ELISA indicated that the protofibrils contained predominately A β 42 with negligible A β 40 incorporation. This argues against an

association-inhibition mechanism for A β 40, at least at the nucleation stage. However, it is possible that trace amounts of A β 40 monomers may have the capacity to physically interact with A β 42 and interfere with aggregation at substoichiometric ratios. The introduction of one monomeric “cap” to a growing A β 42 oligomeric chain could retard the process. There is previous evidence that once A β 42 protofibrils are formed, they may be able to undergo additional growth via addition of A β 40 monomers leading to a mixed A β 42/A β 40 protofibril [38]. A similar type of cross-deposition of A β 42 and A β 40 onto sonicated fibril seeds prepared from either peptide has also been demonstrated [41]. While further studies will be necessary to confirm that a single protofibril can be composed of both A β 42 and A β 40, the earlier published findings indicate that interactions between the two peptides may be more productive once a conformational template is present. Overall, the results demonstrate that A β 42 self-assembly dominates the process at the nucleation stage, but A β 40 may impede early aggregate (protofibril) formation. This report provides evidence of important roles for both A β 42 and A β 40 in nascent soluble A β aggregate formation and reinforces the critical consequence of increasing A β 42/A β 40 ratio.

Conflict of interest statement

The authors declare that there is no conflict of interest in this work.

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Tables

Table 1. Measured A β 42/A β 40 ratios in mixed monomer solutions and in SEC-isolated protofibrils. Initial A β 42/A β 40 solution mixtures and SEC-eluted protofibril peak fractions were collected and analyzed by C-terminal-selective A β ELISA as described in the Methods. Determined A β 42/A β 40 numerical ratios (pM A β 42/pM A β 40) for each sample are presented plus or minus standard error measurement for n= 3 ELISA analyses with n=4 replicates for each sample. In some samples, no A β 42 or A β 40 was detected (ND).

A β 42/A β 40 mixture (μ M)	Aggregation solution	Protofibril peak
40/0	>10 ^a	>10 ^a
32/8	3.0 $\pm$ 0.62	>10 ^b
20/20	1.4 $\pm$ 0.19	>10 ^b
8/32	0.8 $\pm$ 0.06	ND ^{a,c}
0/40	<0.01 ^c	ND ^{a,c}

a - no A β 40 detected; b - trace A β 40 detected; c - no A β 42 detected

Figures

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Figure 8.

Figure 9.

Figure Legends

Figure 1. A change in the A β 42/A β 40 ratio directly impacts the extent of protofibril formation. Panel A. Lyophilized peptide mixtures containing varying ratios of A β 42 and A β 40 were solubilized with NaOH followed by dilution into aCSF at a final total A β concentration of 200 μ M. The ensuing solution was centrifuged and the supernatant eluted on Superdex 75 into 0.5 ml fractions. UV absorbance at 280 nm was monitored during the elutions and the traces are shown above. Solution concentrations for each sample after reconstitution and prior to elution are shown in the legend. A β elution recoveries were 57% (200/0), 64% (160/40), 59% (100/100), 63% (40/160), and 62% (0/200). Panel B. The distribution of SEC-isolated protofibrils and monomers in each A β 42/A β 40 mixture was determined by integration of the two primary UV absorbance peaks observed for each elution in Panel A and dividing each peak by the total A β eluted.

Figure 2. R_H histograms of protofibrils formed from varying A β 42/A β 40 mixtures. The protofibril pools shown in Figure 1A were analyzed immediately after SEC-isolation by DLS and regularized histograms of percent intensity versus R_H are presented for each A β 42/A β 40 mixture. Intensity-weighted mean R_H values were derived from the regularized histograms and are shown in each histogram along with the A β 42/A β 40 mixture concentration (μ M).

Figure 3. ThT fluorescence for protofibrils formed from A β 42/A β 40 mixtures decreases as the A β 42/A β 40 ratio decreases. The protofibril pools shown in Figure 1A were analyzed immediately after SEC-isolation by ThT binding and fluorescence as described in the Methods. A β concentrations were obtained from UV absorbance at 280 nm, and the protofibril samples were diluted to 5 μ M A β in aCSF containing 5 μ M ThT for fluorescence acquisition. Fluorescence emission was quantitated as described in the Methods.

Figure 4. The A β 42/A β 40 ratio modulates the nucleation phase prior to aggregation. SEC-purified monomer pools from a second series of A β 42/A β 40 reconstituted dry peptide mixtures were prepared as described in the Methods and Figure 1. The A β 42/A β 40 monomer samples were diluted to 40 μ M total A β in aCSF with the inclusion of 0.05% NaN₃ and were incubated at room temperature. Aggregation was monitored by ThT fluorescence as described in the Methods. Fluorescence data points are the average $\pm$ SEM for n=3 separate aggregation solutions for each A β 42/A β 40 ratio. A β 42/A β 40 ratios were determined by C-terminal-selective A β ELISA and were (3.2 ± 0.3) for the 4:1 sample, (0.6 ± 0.2) for the 1:1 sample, and (0.2 ± 0.05) for the 1:4 sample. No A β 40 or A β 42 was detected in the “all A β 42” or “all A β 40” monomer samples, respectively. Determined A β 42/A β 40 numerical ratios (pM A β 42/pM A β 40) within each mixed monomer sample represent 4 replicate measurements of each sample with each C-terminal-selective antibody plus or minus the absolute standard deviations determined by propagation of uncertainties from two means (A β 42 and A β 40).

Figure 5. Increasing A β 42/A β 40 ratio and A β 42 concentration accelerates monomer aggregation. Solution mixtures of SEC-purified A β 42 and A β 40 monomers (total A β 40 μ M) in aCSF buffer were prepared and incubated at 37 °C for 24 h. Panel A. Following incubation, solution mixtures were measured for ThT fluorescence as described using a 1/10th dilution of each sample. Panel B. Solutions of SEC-purified A β 42 monomers were prepared at different concentrations in aCSF buffer and incubated at 37 °C for 24 h. Following incubation, solutions were measured for ThT fluorescence as in Panel A.

Figure 6. Increasing A β 42/A β 40 ratio increases the yields of soluble protofibrils but A β 40 retards the process. Solution mixtures of SEC-purified A β 42 and A β 40 monomers (total A β 40 μ M) were prepared and incubated at 37 °C for 24 h. Panel A. Samples were centrifuged at 18,000g for 10 min and supernatants were separated on SEC. Elution chromatograms monitored by A_{280nm} are shown in the graph and each trace is labeled as described in the legend. A β elution recoveries were 72% (40/0), 76% (32/8), 78% (20/20), 81% (8/32), and 90% (0/40). Panel B. The percent distribution between protofibrils and monomers was calculated directly from the absorbance traces in A. Panel C, D. Solutions of SEC-purified A β 42 monomers were prepared at different concentrations in aCSF buffer and incubated at 37 °C for 24 h, centrifuged at 18,000g for 10 min, and supernatants were separated on SEC. Elution chromatograms were acquired and quantitated as in Panel A and B. A β 42 elution recoveries were 45% (40 μ M), 57% (32 μ M), 74% (20 μ M), and 78% (8 μ M).

Figure 7. Increasing A β 42/A β 40 ratio increases protofibril yields but does not significantly protofibril molecular weight. Panel A. Molecular weights of the measureable protofibril peaks were obtained using SEC-MALS during the elution of the A β 42/A β 40 mixtures in Figure 6A. The absorbance (concentration) traces (continuous lines, right y-axis) are shown along with the measured M_w values (left y-axis) obtained every 0.167 mL through the protofibril peak. Panel B. Molecular weights of the protofibril peaks were obtained using SEC-MALS during the elution of the different A β 42 concentration solutions in Figure 6C and depicted as described for Panel A.

Figure 8. Increasing A β 42/A β 40 ratio enhances protofibril β -structure. Peak protofibril fractions from the SEC-purified A β 42 and A β 40 solution mixtures in Figure 6A or different A β 42 concentrations solutions in Figure 6C were analyzed by CD. Spectra were obtained as described in the Methods for the post-SEC peak protofibril fractions of the three solution A β 42/A β 40 mixtures and different A β 42 concentrations solutions with measureable peaks. All spectra are presented as mean residue ellipticity. Panel A. Protofibril fractions from the SEC-purified A β 42 and A β 40 solution mixtures in Figure 6A. Panel B. Protofibril fractions from the SEC-purified pure A β 42 solutions in Figure 6C. Panel A spectra were obtained using a scan speed of 100 nm/min and a 4 sec data integration time. Panel B spectra were obtained using a scan speed of 50 nm/min and an 8 sec data integration time. A response factor of 200 mdeg/1.0 OD was used in both panels.

Figure 9. Protofibril formation at fixed A β 42 concentration is not dramatically effected by inclusion of increasing A β 40 concentration in the mixture. Solution mixtures containing SEC-purified A β 42 monomers (20 μ M) and increasing A β 40 monomers (10, 20, 40 and 56 μ M) were prepared and incubated at 37 °C for 24 h. Panel A, B. Samples were centrifuged at 18,000g for 10 min and supernatants were separated on SEC. Elution chromatograms monitored by A_{280nm} are shown in the graph and each trace is labeled as described in the legend. The elution traces are split between protofibrils and monomers in Panel A and Panel B respectively in order to display the peaks on different y-axes. A β elution recoveries were 73% (20/0), 85% (20/10), 85% (20/20), 89% (20/40), and 91% (20/56). Panel C. Percent distribution between protofibrils and monomers calculated directly from the absorbance traces. Panel D. The protofibril elution peaks in Panel A were integrated in nmol between 6 and 10 mL. Panel E. The peak fraction (0.5 mL) from each of the SEC-eluted protofibril peaks in Panel A were measured for ThT fluorescence. The values represent relative fluorescence units based on integrated emission curves as described in the Methods. Panel F. The same peak protofibril fractions depicted in Panel E were analyzed by CD. Spectra (presented as mean residue ellipticity) were obtained and averaged over 20 accumulations using a scan speed of 100 nm/min, 4 sec data integration time, and a response factor of 200 mdeg/1.0 OD.