Published in final edited form as:

Biochemistry. 2009 September 22; 48(37): 8908–8919. doi:10.1021/bi9006397.

Two disaccharides and trimethylamine N-oxide affect A β aggregation differently, but all attenuate oligomer-induced membrane permeability

Wei Qi¹, Aming Zhang¹, Theresa A. Good², and Erik J. Fernandez¹

¹Department of Chemical Engineering, University of Virginia, Charlottesville, VA 22904

²Department of Chemical and Biochemical Engineering, University of Maryland Baltimore County, Baltimore, MD 21250

Abstract

Interaction between aggregates of amyloid beta protein (A β) and membranes has been hypothesized by many to be a key event in the mechanism of neurotoxicity associated with Alzheimer's disease (AD). Proposed membrane-related mechanisms of neurotoxicity include A β ion channel formation, membrane disruption, changes in membrane capacitance, and lipid membrane oxidation. Recently, osmolytes such as trehalose have been found to delay A β aggregation *in vitro* and reduce neurotoxicity. However, no direct measurements have separated the effects of osmolytes on A β aggregation versus membrane interactions.

In this paper, we tested the influence of trehalose, sucrose and trimethylamine n-oxide (TMAO) on A β aggregation and fluorescent dye leakage induced by A β aggregates from liposomes. In the absence of lipid vesicles, trehalose and sucrose, but not TMAO, were found to delay A β aggregation. In contrast, all of the osmolytes significantly attenuated dye leakage. Dissolution of pre-formed A β aggregates was excluded as possible mechanism of dye leakage attenuation by measurements of Congo red binding as well as hydrogen deuterium exchange detected by mass spectrometry (HX-MS). On the other hand, the accelerated conversion of high order oligomers to fibril caused by vesicles did not take place if any of the three osmolytes presented. Instead, in the case of disaccharide, osmolytes were found to form adducts with A β , and change the dissociation dynamics of soluble oligomeric species. Both effects may have contributed to the observed osmolyte attenuation of dye leakage. These results suggest that disaccharides and TMAO may have very different effects on A β aggregation because of the different tendencies of the osmolytes to interact with the peptide backbone. However, the effects on A β membrane interaction may be due to much more general phenomena associated with osmolyte enhancement of A β oligomer stability and/or direct interaction of osmolyte with the membrane surface.

Keywords

osmolytes; amyloid beta protein; aggregation; membrane interaction

Introduction

Mounting evidence suggests that interaction between aggregated amyloid beta protein (A β) and the neuronal membrane is central to Alzheimer's disease (AD). Aggregated A β has been shown to exhibit ion channel activity (reviewed by (1)), increase membrane permeability and conductance (2–4), and enhance lipid oxidation (reviewed by (5)). Membranes also have the ability to accelerate the aggregation process (6–8). Lipid type (anionic, zwitterionic or cationic), pH and salt concentration, lipid bilayer surface pressure, as well as A β aggregated states have all been shown to affect the A β -lipid interaction (3,7–11). Particularly, the effect of membranes on the oligomer distribution is critical, since neurotoxicity has been found to be a strong function of A β aggregation state, with oligomers being more toxic than monomer or fibrillar species (12–16).

Recently, certain osmolytes, especially trehalose, were found to be effective in slowing the aggregation of several aggregation prone proteins including A β (1–40) (17), insulin(18), W7FW14F(19) and polyglutamine containing protein(20). More importantly, trehalose was able to increase human neuroblastoma cell (SH-SY5Y) viability in presence of A β aggregates and alleviate the polyglutamine induced symptoms in a mouse model of Huntington disease (20). Another osmolyte, Trimethylamine-N-oxide (TMAO), protected MC65 cells under conditional expression of amyloid protein precursor carboxy-terminal fragments(21).

Though encouraging results were reported, the effects of osmolytes on A β aggregation and oligomer-membrane interactions are not well understood. In the above studies, Congo red, Thioflavin T, and circular dichroism were used to assess formation of amyloid or β sheet structure, but these methods could not reveal the distribution of oligomers within a mixture of aggregates. Knowledge of the effects of osmolytes and membranes on the oligomer distribution is important since slowed aggregation could cause accumulation of soluble oligomers, which might be the most neurotoxic oligomeric state (12–17,19,22). Detailed measurements of oligomer distributions would help to clarify whether particular osmolytes affect aggregation, oligomer-membrane interactions, or both.

To explain the beneficial effects of osmolytes on neurotoxicity, mechanisms such as dissociating preformed A β aggregates (17) or increased protein stability (20,21) have been proposed. Unfortunately, these results were obtained from different aggregating peptides, osmolytes, and aggregation conditions, making it hard to draw general conclusions about the property of beneficial osmolytes and dominant mechanisms. Consequently, additional studies are needed of different osmolytes under common aggregation conditions, including effects on membrane interactions. Considering the different preferences of TMAO and carbohydrates to interact with peptide backbones (23,24), it is interesting and worthwhile to compare their effects on A β -membrane interaction with a single model system.

In this work, we have directly compared the effects of two kinds of osmolytes, TMAO and two disaccharides, on A β -liposome interactions. Because of the importance of A β conformation and aggregation state on such interactions, we prepared different kinds of aggregate distributions prior to liposome exposure, as opposed to the simultaneous aggregation liposome interaction studies which have been carried out previously (10,25). To define composition of the aggregates in the samples, we have exploited a methodology based on hydrogen exchange detected by mass spectrometry (HX-MS) (26). Control studies in the absence of liposomes were also performed to distinguish the effects of osmolytes on A β aggregation vs. A β -liposome interactions.

Based on the results, trehalose and sucrose were found to delay A β aggregation significantly in the absence of liposomes, whereas TMAO did not. In contrast, all three osmolytes abolished the acceleration of A β aggregation caused by liposomes and also reduced dye leakage from

liposomes. Thus, while the osmolytes had different effects on aggregation in the absence of liposomes, all three osmolytes increased membrane integrity and delayed membrane-induced fibril formation. Sugar-A β complexes detected in mass spectra suggested that direct A β -carbohydrate interactions could play a role in their different effects on aggregation.

Materials and Methods

Materials

A β (1–40) was purchased from Anaspec, Inc. (San Jose, CA) as 1.0 mg lyophilized aliquots. POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) and P O P G (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac*-1-glycerol [sodium salt]) were obtained from Avanti (Alabaster, AL) and used without further purification. Trehalose and sucrose were from Fisher (Fair Lawn, NJ). 5(6)-carboxyfluorescein and trimethylamine-N-oxide were from Acros Organics (Fair Lawn, NJ). One lot of A β was used for all studies. Others materials were obtained from Sigma unless otherwise specified.

Sample preparation

Stock solutions of A β were prepared by dissolving an aliquot of A β in 100 μ L 0.1% trifluoroacetic acid (TFA, Fluka, Buchs, Switzerland) to a concentration of 10 mg/mL at 25 $\pm$ 0.5 $^{\circ}$ C. After 45 min of dissolution in TFA, “fresh samples” were made by diluting the stock to a final concentration at 100 μ M in PBS (10 mM NaH₂PO₄, 150 mM NaCl, pH 7.4). Aged samples were prepared by diluting TFA stock in PBS as above, and then quiescently incubated at 37 $^{\circ}$ C for 4 hr, 10 hr, 24 hr or 72 hr before labeling. For the samples incubated with osmolytes, osmolytes were added at the same time as PBS was added to TFA stocks, to ensure no aggregation took place prior to osmolyte addition. Osmolyte concentrations were 250 mM unless otherwise specified. This concentration was used to keep the A β :osmolyte molar ratio consistent with the one previously reported to delay A β aggregation as detected by Thioflavin T (ThT) fluorescence assay (17).

Congo red binding assay

Congo red stock solution (120 μ M in PBS) first was passed through a 0.22 μ m filter, and then mixed with a 100 μ M A β sample at a volume ratio 1:9. After 45 min incubation at 25 $^{\circ}$ C, absorbance at 405 nm and 541 nm was measured with a SpectraMax Plus³⁸⁴ Microplate Reader (Molecular Devices, Sunnyvale, CA). For each time point, at least three replicates were measured. The molar concentration of Congo red bound to A β was calculated by the well-established Equation of Klunk et al. (27),

$$[CR - A\beta] = ({}^{541}A_t / 47800) - ({}^{403}A_t / 68300) - ({}^{403}A_{CR} / 86200)$$

Here, ${}^{541}A_t$ and ${}^{403}A_t$ are the total absorbance of the Congo red-A β mixtures at 541 nm and 403 nm, respectively, and ${}^{403}A_{CR}$ is the absorbance of Congo red alone in phosphate buffer with and without osmolytes. In the microplate reader, absorbance at 405 nm and 540 nm were assumed to be same as those at 403 nm and 541 nm (15).

Hydrogen exchange mass spectrometry (HX-MS)

HX-MS analysis of A β oligomer distributions was carried out as previously reported (26). Briefly, 5 μ L sample was diluted into 45 μ L of D₂O (Cambridge Isotope Laboratories, D 99.9 %) in a 1.5 mL Eppendorf centrifuge tube. The molar D% in the solvent was 90%, and the pH (as read) was 7.0. Labeling was carried out at ambient temperature for the desired labeling time. Subsequently, a mixture of 150 μ L of dimethylsulfoxide (DMSO) and dichloroacetic

acid (DCA, Fluka, 95/5 vol/vol) were used to rapidly dissolve aggregates back to monomer state and quench the labeling reaction at pH 3.5 (26,28). Finally the sample was sent through a C8 desalting column (Micro Trap™ 1 mm ID × 8 mm, Michrom Bioresources, Inc., Auburn, CA). A gradient from 30% to 80% acetonitrile was used to elute the peptide from the C8 desalting column directly into the mass spectrometer at 50 µL/min. Data were collected in positive ion, zoom scan, and profile mode on a Thermo Finnigan LTQ linear quadrupole ion trap mass spectrometer (San Jose, CA) with a standard ESI source. ESI voltage was 4.5 kV, capillary temperature 275 °C, sheath gas flow rate 20 units, and tube lens voltage 135 V. All mass spectra presented were averages of approximately 400 scans. The most abundant +4 state was used for HX analysis, and other charge states (+3, +5 and +6) were also observed.

Liposome preparation

Either POPC or POPG was prepared in PBS buffer to give multilamellar lipid dispersions. To prepare large unilamellar vesicles (LUVs), the dispersion was subjected to five freeze-thaw cycles in liquid nitrogen and in a 37 °C water bath, followed by extrusion (at least 15 times) through polycarbonate membranes with a 100 nm pore size (Avestin, Ottawa, Canada). To prepare the 5(6)-carboxyfluorescein (CF) loaded liposomes, lipids were dissolved in approximate 90 mM CF in water, and went through the same procedure as above. After the extrusion step, the free carboxyfluorescein was removed by overnight dialysis at 4 °C against PBS buffer with balanced osmosis pressure inside and outside the lipid vesicles in Slide-A-Lyzer Dialysis cassette from Thermo (Rockford, IL).

Dye-leakage assay measure by fluorescence

The release of CF dye from POPG LUVs was measured with a Fluorolog-3 spectrofluorometer (Jobin-Yvon, Edison, NJ) at excitation and emission wavelengths of 480 nm and 516 nm, respectively, through a 10 mm cuvette. Pre-aged fresh, 4 hr and 72 hr Aβ samples (100 µM) in PBS were then added to POPG liposome in PBS, trehalose, sucrose and TMAO solutions. In the final mixture, the molar ratio of lipid to peptide was 20:1 and osmolytes concentrations were approximately 250 mM. The solution was carefully mixed and further incubated for 1 hr at 37 °C to allow the leakage to reach maximum. Previously, others have shown 10 min was sufficient for Aβ (1–42) to induce significant leakage from POPC giant unilamellar vesicles (29), and for Aβ (1–40), almost 100% leakage was observed for aged samples within approximately 8 min for PG vesicles (9). Relative leakage was expressed as a percentage relative to the total amount of dye release obtained by adding 1% Triton X-100 to the CF loaded vesicles (29,30). CF loaded LUVs were taken as 0% leakage, which showed only slight leakage (less than 3%) within 1 hr measuring time. Each sample was measured in triplicate.

Results

Osmolytes' effects on Aβ aggregation in the absence of liposomes

Congo red assay—Congo red binding was used to assess the effects of osmolytes on Aβ aggregation under our conditions. Because Congo red binds to extended β-sheet structure, and not specifically to any particular aggregates or fibril (31), the results were used qualitatively. For PBS controls (Figure 1, filled bar), Congo red intensity quickly reached maximum after only 4 hr incubation. Even in the freshly prepared Aβ sample, Congo red showed some binding, suggesting the existence of some aggregation intermediate with extended β-sheet structure, possibly and oligomer or protofibril. This rapid assembly is consistent with our previous results from the same lot of Aβ under the same conditions (26,32). For Aβ samples incubated with osmolytes, the Congo red binding time course was not dramatically different. However, the time at which the maximum Congo red binding observed was later for the osmolyte containing samples (4 hr for PBS, 10 hr for TMAO; 72 hr for sucrose and trehalose). These results suggest

that osmolytes may delay the aggregation to a certain extent. However, the relative amounts of oligomers vs. fibrils present could not be assessed from this data.

HX-MS measurements of aggregate structure distributions—HX-MS was used to provide complementary information about the distribution of aggregated A β structures. We have shown previously that HX-MS can distinguish A β in monomeric, low molecular weight (LMW), high molecular weight (HMW), and fibrillar forms (26). In the same work, we carefully deconvoluted spectra by typical Gaussian peak fitting and assigned peaks to different aggregated structures based on their degree of deuterium exchange and complementary measurements (15,26). It should be mentioned that HMW and LMW are both distributions with centroids representing the average mass. Thus, their distributions might be partially overlapped.

Figure 2 compares A β structural distributions incubated in the absence and presence of the osmolytes. The A β concentration was fixed at 100 μ M and osmolyte concentrations were set at 250 mM. At this molar ratio, the inhibition of A β aggregation by trehalose has been shown to be efficient (17). A β samples in PBS without osmolytes that served as controls in this study showed the same trends in distributions as we observed previously (26). The top row of Figure 2 shows the fresh sample contained fully labeled monomer (M) and some partially protected LMW oligomers (L). Samples incubated for longer periods prior to deuterium labeling (Figure 2, 4 hr through 72 hr) were all mixtures, with a major peak of partially protected HMW oligomers (H) and a growing fibril peak (F) at long times. In the second row of Figure 2, samples of A β aggregated with TMAO showed a similar labeling pattern as PBS controls at every incubation time point. Markedly different labeling distributions were generally observed for A β samples incubated in the presence of trehalose and sucrose. The fresh sample was similar to PBS controls in terms of both initial distribution and dynamics. However, for 4 hr and 10 hr aging times, solvent exposed monomer made up to more than 50% of the distributions for samples incubated with carbohydrate, while the HMW species was the major component in PBS and TMAO-containing samples. After 24 hr incubation with carbohydrate, monomer still persisted with an abundance of approximately 30%. After 72 hr of incubation with carbohydrate, the fibril peak was almost completely absent, and the predominant species was HMW, analogous to PBS control samples observed at 4 to 10 hours aggregation. The rate of aggregation was significantly diminished in the presence of trehalose and sucrose.

For each incubation time, experiments at different subsequent labeling times were used to assess the dissociation rates of the aggregated species formed during the incubation time. Our previous study showed that for PBS controls, LMW oligomers dissociated to monomer within 10 min, while HMW oligomers and fibrils did not dissociate significantly within a 1 hr labeling time (26). A β incubated with TMAO showed similar labeling behavior as PBS controls for samples at all the incubation time points (data not shown), suggesting the dynamics of A β aggregate dissociation were not disturbed by TMAO. However, samples incubated 24 hr with trehalose and sucrose showed very different subsequent dynamic labeling behavior. The spectra of fresh A β prepared with trehalose were similar to that of fresh PBS controls (Figure 2, left column). With increasing incubation time prior to labeling (4 hr, 10 hr and 24 hr), more LMW were formed, and the time constant for converting LMW to fully labeled peak was increased. It should be noted that the dissociation rate was measured in labeling buffer prepared without trehalose, and hence it is a characterization of the aggregate product rather than dissociation during the aggregation with trehalose.

Spectra shown in Figure 3 as are representative examples of the labeling dynamics observed after A β was incubated more than 24 hr with and without trehalose. For PBS controls (Figure 3, left), HMW oligomer was the predominant species in the sample, with ~ 20% fibril. Only subtle changes in the spectra occur with increased labeling time with small increases in the

fraction of monomer and a slight reduction in fibril at longer labeling times, indicating that little dissociation occurred. In contrast, samples incubated in trehalose buffer yielded subsequent labeling spectra markedly different in three respects (Figure 3, right). First, no fibril peak was present. Second, the monomer peak was prominent in the spectrum of the sample labeled for 10 s. The less labeled peak in the spectrum of the 10 s labeled sample was ~4.0 Da heavier in mass than the HMW peak in the PBS control spectrum, suggesting the species were more solvent accessible and/or smaller than the HMW oligomer. The centroid mass for the protected species in the trehalose spectrum (4345.9 ± 1.0 Da) was close to the mass previously reported for LMW for freshly dissolved A β (4346.0 ± 0.9 Da) (26). Finally, with increased labeling time, the intensity of the more protected oligomer peak decreased while that of the monomer peak increased accordingly in presence of trehalose. This indicated that the protected species was dissociating to a fully solvent exposed, presumably monomeric state. This is in contrast to the lack of dissociation for A β samples incubated for 24 hr in absence of trehalose. Notably, fresh samples in PBS showed a similar labeling pattern indicating dissociation, albeit with shorter time constant (26).

Nonspecific A β -sugar complex formation—The above HX-MS data clearly showed that A β aggregation could be delayed by trehalose or sucrose but not by the addition of TMAO. Similar observations of delayed A β aggregation have been previously reported for carbohydrates (17,19,22). However, the mechanism by which disaccharides influence the aggregation remains unknown. Furthermore, direct measurements of the oligomer-oligomer interactions and/or A β -carbohydrate interactions are not available.

One possible line of evidence of saccharide-peptide interactions from this work is the formation of adducts arising from noncovalent association during the electrospray process. Figure 4 shows evidence for such noncovalent association between A β and the carbohydrate osmolytes, but not with TMAO. A β in PBS buffer (Figure 4, upper left) showed isolated +3, +4, +5 and +6 charge state peaks with centroid masses 1444.18, 1083.45, 867.00 and 722.73, respectively. The +4 charge state was the most abundant signal under electronic spray conditions used in this study, and for clarity subsequent discussion focuses on this peak. For A β in both trehalose and sucrose solutions, the +4 charge state (m/z 1083.45) was still the strongest peak in the spectra, but it was followed by a series of adduct peaks separated by an average mass of 342.0 ± 0.8 Da (85.5 ± 0.2 m/z at +4 charge state, and molecular weight of trehalose and sucrose is 342.3). Adduct peak intensity decreased with increased number of sugar molecules associated with the peptide. In contrast, A β samples in TMAO yielded spectra almost identical to that of the PBS sample. No adducts were observed with the mass matching TMAO, even though experiments were conducted under same experimental procedures and conditions as for the carbohydrate osmolytes.

Sugar and protein adduct formation during the electrospray ionization (ESI) process have been previously taken to reflect non-covalent interactions between the molecules, probably involving hydrogen bonding (33). The abundant -OH groups in sugar molecules and peptide backbones are potential hydrogen bonding candidates. In contrast to the carbohydrates, TMAO is not believed to interact preferentially with the peptide backbone, based on simulation (34) and experimental investigations (reviewed in (23)). Further, in a recent theoretical transfer free energy model analysis, urea and TMAO represented the two extremes in osmolytes preferentially accumulated or excluded from the protein backbone, respectively. Sugars like trehalose and sucrose fell in between these two extremes (23,24). Therefore, the trend in adduct formation observed here was consistent with previous estimates of osmolyte association with the peptide backbone. However, it should be noted that the N-O bond in TMAO has a large dipole; this might allow TMAO to decompose during MS analysis into a positive ion that would be much less likely to form an adduct with the positively charged protein during the electrospray process. This may also contribute to the reduced adduct formation for TMAO.

To test whether the carbohydrate-A β interaction is noncovalent, MS experiments were repeated with A β concentration fixed at 100 μ M and trehalose concentration adjusted from 100 μ M up to 250 mM. As shown in Figure 5, both the intensity and number of adducts increased at the higher trehalose concentrations. This concentration-dependent behavior is typical of the non-covalent complex formation between carbonic anhydrase and a number of sugar molecules (33). In addition, the hydrogen exchange behavior was observed to be the same for the main peak and all adducts (see Supplementary Information), indicating that the association with trehalose and sucrose was not sufficiently strong and/or long-lived to exclude solvent access to the backbone. Indeed, simulation studies suggest that such lifetime of the disaccharide-peptide interaction might only be on the scale of $\sim$ ns (35). This would be an insufficient period to exclude D₂O molecules capable of labeling polypeptides in a small fraction of our 10-second labeling period (26).

Osmolyte Effects on the A β -liposome interaction

Carboxyfluorescein leakage from liposomes—CF loaded POPG liposomes were used here as an *in vitro* model to detect the effect of pre-aged A β samples on membrane permeability as well as osmolyte influences A β -induced membrane permeability (9,29). Figure 6 (black bars) shows the impact of pre-aged A β aggregates on induced membrane permeability in the absence of osmolytes. Fresh and 4 hr samples led to the marked CF leakage of approximately 70% and 80%, respectively. In contrast, the 72 hr sample only resulted in approximately 30% leakage. The combined fraction of HMW and LMW oligomers in each sample prior to mixing with the liposomes was previously reported as 50%, 60% and 35 % for fresh, 4 hr and 72 hr sample respectively (26). Therefore, the A β induced leakage appeared to be roughly correlated with the abundance of HMW and LMW oligomers in each sample.

Figure 6 also shows the effect of osmolytes on permeability. For fresh and 4 hr samples, A β -induced leakage was reduced to $\sim$ 10% and $\sim$ 30% regardless of which osmolyte was used. For the 72 hr sample, the osmolytes had no significant effect on membrane leakage. It is interesting to note that the osmolytes had very similar effects on permeability, despite their very different effects on aggregation in the absence of liposomes. This might be because osmolytes alter preformed oligomer distributions, or because they modulate the A β -membrane interaction.

Osmolyte effects on pre-formed aggregates—Dissociation of the preformed aggregates was the proposed mechanism for the reduction of A β -induced neurotoxicity brought about by trehalose (17). To test this hypothesis under our conditions, each of the three osmolytes was added to parallel 4 hr and 72 hr samples to a final concentration of 250 mM. The mixture was then incubated at 37 °C for another hour and aggregation was assessed by Congo red binding assay.

Although Congo red binding can not be used to distinguish between the presence of oligomers and fibril in solution, Congo red does not bind to A β monomer, while it does bind to aggregates, making it useful in differentiating between these two species in solution. Therefore, if aggregates were dissolved back to non-Congo red binding species, the intensity should be significantly reduced. In Figure 7, for both 4 hr and 72 hr samples, Congo red intensities for A β controls and samples treated with all three osmolytes were not statistically different. This indicated an insignificant amount of aggregates were dissolved back to monomer within the tested time period. To further support above results, HX-MS was also used to detect the effect of trehalose on 4 hr and 72 hr samples. HX labeling distributions were similar at same labeling time for both samples with or without trehalose, and no increase in fully labeled peak (monomer) was observed in the trehalose treated samples (see Supplementary Information).

HX-MS analysis—HX-MS was used to determine whether the A β oligomer distributions in the presence of lipids could be affected by osmolytes. A β in fresh, 4 hr and 72 hr samples were first pre-aged, then further incubated with POPG or POPC liposomes without CF dye, and finally labeled as described in *Methods* for A β samples in PBS only. In Figure 8, the mass spectra for samples in PBS buffer only (first row, PBS) are shown. In the second and third rows (POPC and POPG) mass spectra of pre-aged A β samples after incubation with liposomes without osmolytes are displayed. For both kinds of liposomes, fresh A β showed a similar HX pattern, indicating either no A β -lipid interaction or no effects of the interaction on the initial structural distribution (Figure 8, left column). However, for 4 hr incubated A β (Figure 8, middle column), a highly protected peak appeared, producing a spectrum similar to that of the 72 hr sample in the absence of lipid. For 72 hr incubated A β , only a fibril peak was observed (Figure 8, right column). Thus, liposomes clearly accelerated A β aggregation under our conditions. This was supported by the observation of visible precipitation when A β aged for 4 hr (see Supplementary Information) or 72 hr (data not shown) was added into the liposome solution.

Figure 8 also shows how the above acceleration A β aggregation is affected by the presence of osmolytes. Surprisingly, HX labeling distributions in presence of both osmolytes and liposomes for all incubation times (fresh, 4 hr and 72 hr) (4th and 5th rows) were very similar to samples in PBS without liposomes (1st row). Thus, HX-MS shows that both osmolytes abolish the acceleration effect of liposomes on aggregation. This could indicate that they both block the interaction between A β and liposomes. Alternatively, since disaccharides in the absence of liposomes appeared to slow down the rate of A β aggregation (Figure 2), it is possible that the liposomes and osmolytes have opposing effects on A β aggregate distribution, that at least some fraction of the A β present interacts with osmolytes and with liposomes. Thus, the results we observed might be a combination of those two effects.

Discussion

Osmolytes are small organic molecules, which in nature stabilize intracellular proteins against the environment stress, such as temperature extremes, dehydration and high extracellular osmotic pressure (23). Recently, certain osmolytes have been shown to increase cell viability in presence of aggregated A β (17,21), as well as other disease-associated aggregation prone proteins such as α -synuclein and polyglutamine (20,36). However, the protective effects of osmolytes are still not well understood because of varied conditions between studies as well as limited investigations distinguishing their effects on protein aggregation versus peptide membrane interactions. In this study, we evaluated the effects of selected osmolytes on A β -induced membrane permeability, as an *in vitro* surrogate of neurotoxicity. In addition, HX-MS was used to monitor aggregate population distributions in the presence and absence of both liposomes and osmolytes. The overall goal was to determine whether and if possible how each osmolyte affected A β -membrane interactions, A β aggregation in the absence of liposomes, or both.

The first portion of this work is concerned with examining the effects of osmolytes on A β aggregation in the absence of liposomes, because A β biological activity has been widely assumed to be aggregation state dependent. Two classes of osmolytes, the carbohydrates (sucrose and trehalose) and TMAO had different effects on A β aggregation. Disaccharides increased the typical time HMW appeared from 4 hr (Figure 2, 1st row) to 72 hr (Figure 2, 3rd and 4th row). However, fibrils were the majority of the sample after 6 days (data not shown) with or without carbohydrates. Hence, disaccharides appear to affect mainly the formation rate of HMW. Further, after 24 hr, samples incubated with disaccharides dissociated much more rapidly than controls in PBS (Figure 3). In contrast, TMAO showed little effect on A β aggregation. All HX-MS spectra from samples with added TMAO were almost identical to PBS-only controls at same time points (Figure 2, 1st and 2nd rows).

The observed reduction in aggregation rate in the presence of disaccharide osmolytes is generally consistent with previous literature reports. Trehalose delayed the aggregation of A β (1–40), A β (1–42), W7FW14F and insulin (17,19). Trehalose and sucrose also attenuated the aggregation of myoglobin containing a 35-glutamine repeat (20) and reduced aggregates formation of rhu-MAb HER2 when the sugar/peptide ratio exceeds 500 (37). However, under certain conditions, such as low A β concentration (10 μ M) and different sugar:peptide ratios, sucrose has been found to increase the number of A β (1–40) seeds, small aggregates, protofibrils, and fibrils (22).

Fewer studies are available on the effects of TMAO on protein aggregation. It has been found to accelerate the A β conformation transition from random coil to β -sheet (38), β -synuclein aggregation (39) and the formation of Tau protein fibrils (40). The merely neutral effect of TMAO on A β aggregation here is probably attributable to the early appearance of β -sheet structure in our A β samples. At the relative high concentration (100 μ M) used here, freshly prepared A β contained a significant fraction of protected species termed LMW (Figure 2, 1st row). Furthermore, CD indicated fresh sample conformation was already β -sheet instead of random coil (15,41 and Supplementary Information). Under similar conditions, dimer was observed in equilibrium with monomer (42). Thus, it is perhaps not surprising that in this case, TMAO effect on β -sheet formation was not significant.

We then examined the effects of osmolytes on aggregated A β induced dye leakage from liposomes and accelerated fibril formation. It should be emphasized that in many studies of A β cell and/or liposome interaction, monomeric A β was incubated with liposomes or cells, and then viability or/and membrane-induced aggregation was monitored (8,10,22,25,43). However, in our study, fresh and pre-aged A β samples (4 hr and 72 hr), with well-characterized differences in solvent accessibility and dynamics, were mixed with liposomes in absence or presence of the tested osmolytes. Since osmolyte effects on A β aggregation in the absence of liposomes were specifically studied in early portion of this paper, above approach allowed us to separate osmolyte effects on aggregation and A β aggregates-liposome interactions.

In the absence of osmolytes, we observed that pre-formed A β aggregates induced substantial increases in membrane permeability (Figure 6), with the samples causing the highest CF leakage (fresh and 4 hr, Figure 6) having the highest combined fraction of LMW and HMW as measured by HX-MS (Figure 2). This trend is in good agreement with previous reports that A β oligomers are particularly effective at increasing vesicle or/and membrane permeability (2,4,9) and causing cell toxicity (3,12,14,15,44). At the same time, we also found that the liposome interaction clearly accelerated conversion of HMW to fibrils (Figure 8) as well as visible precipitation under some conditions (see Supplementary Information). Such membrane accelerated aggregation was also observed previously in studies carried out without osmolytes (6,10,45).

In the presence of all osmolytes examined, the marked A β -induced leakage observed for fresh and 4 hr samples was reduced substantially (Figure 6). In addition, the accelerated fibril formation induced by liposomes was abolished by osmolytes (Figure 8) as well as visible precipitation (see Supplementary Information). Increased osmotic pressure has been shown previously to reduce leakage from liposomes under different experimental conditions (46). While the effect on leakage was much less than that observed in Figure 6, it is possible that osmotic effect is one means by which osmolytes are stabilizing the liposomes.

Effects of trehalose and TMAO on cell toxicity in the presence of the A β aggregates have been investigated. At a similar osmolyte:peptide molar ratio, trehalose was found to increase viability of SY5Y cells (17). Likewise, TMAO significantly improved MC56 cell viability under conditional expression of amyloid precursor protein fragments (21). On the other hand,

osmolyte effects of other protein-liposome interaction have been examined. Trehalose decreased the liposome aggregation induced by HSA-liposome interactions (47). It reduced lipid vesicle adsorption to polystyrene with only transient intravesicular solute leakage (48). It can also block the aggregation of human antibody light chain induced by lipid-derived aldehydes (49), which presented in plasma of AD patients at elevated concentration (50) and may also accelerate A β aggregation and protofibril formation (51). The correlation between our measured trends in dye leakage and the documented benefits to cell viability suggest that membrane permeability effects of osmolytes may play a role in their beneficial effects on cell viability.

HX-MS measurements of the aggregated subpopulation distribution before and after exposure to liposomes were a valuable complement to permeability measurements, since they could reveal effects of membrane interactions on the aggregates (8,52,53). The marked reduction in changes in the aggregate distribution upon addition of osmolytes (Figure 8) suggests that reduction of membrane permeability and/or reduced membrane interactions may be at least one mechanism by which osmolytes reduce A β neurotoxicity.

It is important to consider that different aggregated states are thought to differ in their membrane interactions and effects on neurotoxicity (2,4,12,14,15,44,54). For example, aggregated A β was shown to have greater affinity for the membrane than monomeric A β (52,55). More specifically, only oligomeric intermediates could interact with a lipid bilayer and reduce membrane fluidity (54), induce the ion channel activity (4), bind to a specific antibody (14) and change membrane capacitance (2). It was also reported that A β -induced liposome aggregation decreased when A β was aged for longer time (56). Therefore, it was hypothesized that assembly into soluble oligomers generates hydrophobic domains that interact with membranes, and that these hydrophobic domains disappear in fibrils (57). Our HX-MS and CF leakage data support this hypothesis. LMW oligomers present in fresh samples and HMW oligomers predominant at 4 hr were both more solvent exposed (Figure 2) and induced more leakage (Figure 6) than fibrils in the 72 hr samples. Proteolytic HX-MS data suggested the solvent accessibility of residues 1–16 and/or 20–34 are more solvent exposed in protofibril (58) or HMW (41) than fibril.

In addition, HX-MS also showed that for both POPC and POPG, HMW was converted into a more solvent protected species readily (Figure 8). It is possible that the solvent inaccessibility due to peptide insertion into lipids could contribute to reduced HX (59), but the reduction observed is highly consistent with fibril formation, and the conclusion of accelerated aggregation would be consistent with previous studies (6,10,45). In addition, A β aggregates could induce ion channel activity from liposome made of either neutral or negatively charged lipids (25). Thus, accelerated aggregation and ion channel activity with both types of lipids suggest that HMW interacts with either neutral or negatively charged liposomes with similar strength. Measured binding constants for A β with POPC and POPG were not statistically different (52) and this interaction was regarded to be driven by hydrophobic rather than electrostatic interactions (53). CD analysis of pre-aged A β samples upon interaction with POPC liposomes was also carried out (see Supplemental Information). No marked conformation change was observed except a slight intensity increase at 218 nm, consistent with a modest increase in amount of β -sheet structure when fibril formed. Thus, during incubation of pre-aged A β with liposomes, it is possible that little change in secondary structure composition took place other than assembly of units with β -sheet structure (41).

The dissolution of pre-formed aggregates has been proposed as a competing hypothesis for the beneficial effects of trehalose (17). However, under our conditions both Congo red binding (Figure 7) and HX-MS (see Supplementary Information) showed that once HMW was formed, it could not be dissolved back to monomer by adding concentrated osmolytes within 1 hr testing

time. In addition, fibril still formed even in presence of trehalose or sucrose if the incubation time was longer than 6 days (data not shown). Others have also reported that small osmolytes can not dissolve aggregates substantially. TMAO, PBA (4-phenylbutyric acid) and glycerol were found to protect MC56 cells from A β -induced toxicity, but did not alter triton insolubility of the aggregate band compared to no treatment (21). Thus, under our conditions, the osmolytes preserving effect on dye leakage can not be explained as converting pre-formed A β oligomers to monomer. The fact that we did not observe the dissociation of aggregates reported by others (17) may be related to the higher A β concentration used and faster aggregation observed in our study. Our measurements demonstrated that the osmolytes mainly delayed of the formation of HMW rather than dissolving HMW. Therefore, one possible explanation for the different behavior observed (17) is that because of slower aggregation at 20 μ M, A β samples might still be LMW when trehalose was added after 2 days.

An alternative explanation for the effect of osmolytes on dye leakage from liposomes in the presence of aggregated A β is that the osmolytes either increase the stability of oligomers and/or increase the membrane integrity of the liposomes, or both. As discussed earlier, the solvent-exposed hydrophobic patch presented in oligomers may make them the less stable kinetically in solution and more likely to interact with lipid membranes. Previous results showed that the intermediate-time incubated samples (4 hr and 8 hr) contain oligomers with lowest apparent stability upon guanidine treatment, and also induce the most toxicity in SH-SY5Y cells (15). The direct measurement of stability was not carried out in this work. However, the HX-MS data clearly demonstrated that in presence of the osmolytes, the membrane accelerated transition from HMW to fibril was blocked (Figure 8) and the presence of a more rapidly dissociating LMW species was extended from less than 4 hr up to 24 hr (Figure 2). Given that without osmolytes, samples with the highest content of LMW and HMW induced the most dye leakage (Figure 6), it seems somewhat contradictory that in the presence of osmolytes, the formation and/or stability of LMW and HMW species are enhanced but dye leakage is attenuated. One explanation for this apparent contradiction is that osmolytes both enhance the stability of LMW and HMW oligomeric species and reduce their tendency to interact with the lipid membrane surface. This was also proposed to be possible mechanism of osmolyte action on A β by other investigators (20,21,60).

On the other hand, those osmolytes may also affect the liposome surface to prevent encapsulated dye leakage, protein adsorption and liposome aggregation (47,48,61). Disaccharides have been shown to associate with the lipid membrane (62), form hydrogen bond to the phosphate groups (63) and replace water molecules at the membrane surface (64,65). TMAO could attenuate membrane disruption, even in the presence of denaturants such as urea (66). Considering the excess amount of osmolyte in the solution as compared to A β and lipids, it is possible that osmolytes coat on the liposome surface and hence help maintain the membrane integrity (47).

Notably, while the different osmolytes had similar effects on dye leakage, TMAO showed different effects on A β aggregation in the absence of liposomes. Presumably, the different osmolytes affect the kinetics and/or thermodynamics of steps in the aggregation pathway differently (reviewed by (57)). For example, the preferential exclusion of osmolytes has been proposed to be their stabilizing mechanism for globular proteins, but the extent of osmolytes-peptide interaction varies from one osmolyte to another (23,24,60). TMAO is preferentially excluded from both native and denatured states, and forces proteins to fold (reviewed by (23)). Sucrose and trehalose have both been shown to shift the conformation equilibrium towards the native state and reduce aggregation (35,60,67). Monomer collapse might be another explanation, but it alone is not necessarily sufficient to protect A β from hydrogen exchange (68). Thus, it is possible that, in presence of trehalose and sucrose, the conformational

ensemble A β was shifted to a set of less aggregation prone conformations and/or the opportunity for A β –A β interaction to form large aggregates was reduced.

The detailed mechanism of how each osmolyte exerts their different effects on aggregation kinetics is largely beyond the scope of this paper. However, one aspect of the osmolyte-peptide interaction was revealed by adduct formation during electrospray ionization. A β -osmolyte adduct formation (Figure 4) was correlated with their delaying effect on aggregation. The differences in osmolyte-peptide interactions are also consistent with the results of a recent transfer free energy model analysis. In this model, TMAO exhibited the highest unfavorable interaction with the peptide backbone while trehalose and sucrose interact more strongly (23, 24)

While the effects of osmolytes on oligomer distributions and lipid interactions have potential implications for therapeutic strategies, they are not straightforward. In addition, it should be noted that even effective osmolytes like trehalose, may not completely block the formation of neurotoxic oligomers as seen in our results and the work of others (17,19), nor does it completely attenuate A β induced membrane permeability and dye leakage. Therefore, any strategy for modifying the neurotoxic properties and oligomer distribution by such molecules or derivatives will require careful optimization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations list

AD	Alzheimer's disease
A β	Amyloid beta protein
CF	5(6)-carboxyfluorescein
DCA	dichloroacetic acid
DMSO	dimethylsulfoxide
F	Fibril
HMW	High molecular weight oligomer
HX-MS	Hydrogen deuterium exchange detected by mass spectrometry
LUV	large unilamellar vesicles
LMW	Low molecular weight oligomer
M	Monomer
POPC	palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine
POPG	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phospho- <i>rac</i> -1-glycerol [sodium salt]
TFA	trifluoroacetic acid
TMAO	Trimethylamine N-oxide

Acknowledgments

The author thanks Dr. Luiz C. Salay, Dr. Alex Lai and Professor Lukas Tamm for their kind help with the liposome related studies. This research was funded by NIH 1R01 NS 042686 to TAG and EJJ, and in part by award N° 08-3 from the Commonwealth of Virginia's Alzheimer's and Related Diseases Research Award Fund, administered by Virginia Center on Aging, Virginia Commonwealth University.

References

1. Arispe N, Diaz JC, Simakova O. A[β] ion channels. Prospects for treating Alzheimer's disease with A[β] channel blockers. *Biochimica et Biophysica Acta (BBA) - Biomembranes Amyloidogenic Protein-Membrane Interaction* 2007;1768:1952–1965.
2. Sokolov Y, Kozak JA, Kayed R, Chanturiya A, Glabe C, Hall JE. Soluble Amyloid Oligomers Increase Bilayer Conductance by Altering Dielectric Structure. *J. Gen. Physiol* 2006;128:637–647. [PubMed: 17101816]
3. Kaye R, Sokolov Y, Edmonds B, McIntire TM, Milton SC, Hall JE, Glabe CG. Permeabilization of Lipid Bilayers Is a Common Conformation-dependent Activity of Soluble Amyloid Oligomers in Protein Misfolding Diseases. *J. Biol. Chem* 2004;279:46363–46366. [PubMed: 15385542]
4. Demuro A, Mina E, Kaye R, Milton SC, Parker I, Glabe CG. Calcium Dysregulation and Membrane Disruption as a Ubiquitous Neurotoxic Mechanism of Soluble Amyloid Oligomers. *J. Biol. Chem* 2005;280:17294–17300. [PubMed: 15722360]
5. Komatsu H, Liu L, Murray IVJ, Axelsen PH. A mechanistic link between oxidative stress and membrane mediated amyloidogenesis revealed by infrared spectroscopy. *Biochimica et Biophysica Acta (BBA) - Biomembranes Amyloidogenic Protein-Membrane Interaction* 2007;1768:1913–1922.
6. Yip CM, McLaurin J. Amyloid- β Peptide Assembly: A Critical Step in Fibrillogenesis and Membrane Disruption. *Biophys. J* 2001;80:1359–1371. [PubMed: 11222297]
7. Terzi E, Holzemann G, Seelig J. Self-association of [β]-Amyloid Peptide (1–40) in Solution and Binding to Lipid Membranes. *Journal of Molecular Biology* 1995;252:633–642. [PubMed: 7563079]
8. Terzi E, Holzemann G, Seelig J. Interaction of Alzheimer beta-Amyloid Peptide(1–40) with Lipid Membranes. *Biochemistry* 1997;36:14845–14852. [PubMed: 9398206]
9. McLaurin J, Chakrabarty A. Membrane Disruption by Alzheimer beta -Amyloid Peptides Mediated through Specific Binding to Either Phospholipids or Gangliosides. IMPLICATIONS FOR NEUROTOXICITY. *J. Biol. Chem* 1996;271:26482–26489. [PubMed: 8900116]
10. Bokvist M, Lindstrom F, Watts A, Grobner G. Two Types of Alzheimer's [β]-Amyloid (1–40) Peptide Membrane Interactions: Aggregation Preventing Transmembrane Anchoring Versus Accelerated Surface Fibril Formation. *J. Mol. Bio* 2004;335:1039–1049. [PubMed: 14698298]
11. Ege C, Lee KYC. Insertion of Alzheimer's A{ β }40 Peptide into Lipid Monolayers. *Biophys. J* 2004;87:1732–1740. [PubMed: 15345552]
12. Bucciantini M, Giannoni E, Chiti F, Baroni F, Formigli L, Zurdo J, Taddei N, Ramponi G, Dobson CM, Stefani M. Inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases. *Nature* 2002;416:507–511. [PubMed: 11932737]
13. Dahlgren KN, Manelli AM, Stine WB Jr, Baker LK, Krafft GA, LaDu MJ. Oligomeric and Fibrillar Species of Amyloid- β Peptides Differentially Affect Neuronal Viability. *J. Biol. Chem* 2002;277:32046–32053. [PubMed: 12058030]
14. Kaye R, Head E, Thompson JL, McIntire TM, Milton SC, Cotman CW, Glabe CG. Common Structure of Soluble Amyloid Oligomers Implies Common Mechanism of Pathogenesis. *Science* 2003;300:486–489. [PubMed: 12702875]
15. Lee S, Fernandez EJ, Good TA. Role of aggregation conditions in structure, stability, and toxicity of intermediates in the A β fibril formation pathway. *Protein Science* 2007;16:723–732. [PubMed: 17327396]
16. Kirkitadze MD, Bitan G, Teplow DB. Paradigm shifts in Alzheimer's disease and other neurodegenerative disorders: The emerging role of oligomeric assemblies. *Journal of Neuroscience Research* 2002;69:567–577. [PubMed: 12210822]
17. Liu R, Barkhordarian H, Emadi S, Park CB, Sierks MR. Trehalose differentially inhibits aggregation and neurotoxicity of beta-amyloid 40 and 42. *Neurobiology of Disease* 2005;20:74–81. [PubMed: 16137568]
18. Arora A, Ha C, Park CB. Inhibition of insulin amyloid formation by small stress molecules. *FEBS Letters* 2004;564:121–125. [PubMed: 15094052]
19. Vilasi S, Iannuzzi C, Portaccio M, Irace G, Sirangelo I. Effect of Trehalose on W7FW14F Apomyoglobin and Insulin Fibrillization: New Insight into Inhibition Activity. *Biochemistry* 2008;47:1789–1796. [PubMed: 18205397]

20. Tanaka M, Machida Y, Niu S, Ikeda T, Jana NR, Doi H, Kurosawa M, Nekooki M, Nukina N. Trehalose alleviates polyglutamine-mediated pathology in a mouse model of Huntington disease. *Nature Medicine* 2004;10:148–154.
21. Woltjer RL, McMahan W, Milatovic D, Kjerulf JD, Shie F-S, Rung LG, Montine KS, Montine TJ. Effects of chemical chaperones on oxidative stress and detergent-insoluble species formation following conditional expression of amyloid precursor protein carboxy-terminal fragment. *Neurobiology of Disease* 2007;25:427–437. [PubMed: 17141508]
22. Fung J, Darabie AA, McLaurin J. Contribution of simple saccharides to the stabilization of amyloid structure. *Biochemical and Biophysical Research Communications* 2005;328:1067–1072. [PubMed: 15707986]
23. Bolen DW. Effects of naturally occurring osmolytes on protein stability and solubility: issues important in protein crystallization. *Methods Macromolecular Crystallization* 2004;34:312–322.
24. Street TO, Bolen DW, Rose GD. A molecular mechanism for osmolyte-induced protein stability. *Proceedings of the National Academy of Sciences* 2006;103:13997–14002.
25. de Planque MRR, Raussens V, Contera SA, Rijkers DTS, Liskamp RMJ, Ruyschaert J-M, Ryan JF, Separovic F, Watts A. [beta]-Sheet Structured [beta]-Amyloid(1–40) Perturbs Phosphatidylcholine Model Membranes. *Journal of Molecular Biology* 2007;368:982–997. [PubMed: 17382345]
26. Qi W, Zhang A, Patel D, Lee S, Harrington JL, Zhao L, Schaefer D, Good TA, Fernandez EJ. Simultaneous monitoring of peptide aggregate distributions, structure, and kinetics using amide hydrogen exchange: Application to Abeta(1–40) fibrillogenesis. *Biotechnology and Bioengineering* 2008;100:1214–1227. [PubMed: 18351682]
27. Klunk WE, Jacob RF, Mason RP. Quantifying Amyloid [beta]-Peptide (A[beta]) Aggregation Using the Congo Red-A[beta] (CR-A[beta]) Spectrophotometric Assay. *Analytical Biochemistry* 1999;266:66–76. [PubMed: 9887214]
28. Whittemore NA, Mishra R, Kheterpal I, Williams AD, Wetzel R, Serspersu EH. Hydrogen-Deuterium (H/D) Exchange Mapping of Aβ Amyloid Fibril Secondary Structure Using Nuclear Magnetic Resonance Spectroscopy. *Biochemistry* 2005;44:4434–4441. [PubMed: 15766273]
29. Ambroggio EE, Kim DH, Separovic F, Barrow CJ, Barnham KJ, Bagatolli LA, Fidelio GD. Surface Behavior and Lipid Interaction of Alzheimer {beta}-Amyloid Peptide 1–42: A Membrane-Disrupting Peptide. *Biophys. J* 2005;88:2706–2713. [PubMed: 15681641]
30. Weinstein JN, Klausner R, Innerarity T, Ralston E, Blumenthal R. Phase transition release, a new approach to the interaction of proteins with lipid vesicles. Application to lipoproteins. *Biochim. Biophys. Acta* 1981;647:270–284. [PubMed: 6895324]
31. Walsh DM, Hartley DM, Kusumoto Y, Fezoui Y, Condron MM, Lomakin A, Benedek GB, Selkoe DJ, Teplow DB. Amyloid beta -Protein Fibrillogenesis. STRUCTURE AND BIOLOGICAL ACTIVITY OF PROTOFIBRILLAR INTERMEDIATES. *J. Biol. Chem* 1999;274:25945–25952. [PubMed: 10464339]
32. Lee, S. Chemical & Biochemical Engineering. Baltimore County, Baltimore: University of Maryland; 2006. Role of Aggregation Conditions and Presence of Small Heat Shock Proteins on Aβ Structure, Stability and Toxicity.
33. Wang W, Kitova EN, Klassen JS. Nonspecific Protein-Carbohydrate Complexes Produced by Nanoelectrospray Ionization. Factors Influencing Their Formation and Stability. *Analytical Chemistry* 2005;77:3060–3071. [PubMed: 15889894]
34. Zou Q, Bennion BJ, Daggett V, Murphy KP. The Molecular Mechanism of Stabilization of Proteins by TMAO and Its Ability to Counteract the Effects of Urea. *J. Am. Chem. Soc* 2002;124:1192–1202. [PubMed: 11841287]
35. Lins RD, Pereira CS, Hunenberger PH. Trehalose-Protein Interaction in Aqueous Solution. *PROTEINS: Structure, Function, and Bioinformatics* 2004;55:177–186.
36. Sarkar S, Davies JE, Huang Z, Tunnacliffe A, Rubinsztein DC. Trehalose, a Novel mTOR-independent Autophagy Enhancer, Accelerates the Clearance of Mutant Huntingtin and {alpha}-Synuclein. *J. Biol. Chem* 2007;282:5641–5652. [PubMed: 17182613]
37. Cleland JL, Lam X, J BK, Yang a, Tzung-horng, David Y, Overcashier, Brooks D, Hsu C, Carpenter JF. A specific molar ratio of stabilizer to protein is required for storage stability of a lyophilized monoclonal antibody. *Journal of Pharmaceutical Sciences* 2001;90:310–321. [PubMed: 11170024]

38. Yang D-S, Yip CM, Huang THJ, Chakrabartty A, Fraser PE. Manipulating the Amyloid-beta Aggregation Pathway with Chemical Chaperones. *J. Biol. Chem* 1999;274:32970–32974. [PubMed: 10551864]
39. Uversky VN, Li J, Fink AL. Trimethylamine-N-oxide-induced folding of [alpha]-synuclein. *FEBS Letters* 2001;509:31–35. [PubMed: 11734201]
40. Scaramozzino F, Peterson DW, Farmer P, Gerig JT, Graves DJ, Lew J. TMAO Promotes Fibrillization and Microtubule Assembly Activity in the C-Terminal Repeat Region of Tau. *Biochemistry* 2006;45:3684–3691. [PubMed: 16533051]
41. Zhang A, Qi W, Good TA, Fernandez EJ. Structural Differences between A β (1–40) Intermediate Oligomers and Fibrils Elucidated by Proteolytic Fragmentation and Hydrogen/Deuterium Exchange. *Biophys. J* 2009;96:1091–1104. [PubMed: 19186145]
42. Pallitto MM, Murphy RM. A Mathematical Model of the Kinetics of β -Amyloid Fibril Growth from the Denatured State. *Biophys. J* 2001;81:1805–1822. [PubMed: 11509390]
43. Kanapathipillai M, Lentzen G, Sierks M, Park CB. Ectoine and hydroxyectoine inhibit aggregation and neurotoxicity of Alzheimer's [beta]-amyloid. *FEBS Letters* 2005;579:4775–4780. [PubMed: 16098972]
44. Kayed R, Pensalfini A, Margol L, Sokolov Y, Sarsoza F, Head E, Hall J, Glabe C. Annular Protofibrils Are a Structurally and Functionally Distinct Type of Amyloid Oligomer. *J. Biol. Chem* 2009;284:4230–4237. [PubMed: 19098006]
45. Koppaka V, Axelsen PH. Accelerated Accumulation of Amyloid β Proteins on Oxidatively Damaged Lipid Membranes. *Biochemistry* 2000;39:10011–10016. [PubMed: 10933822]
46. Niven RW, Carvajal TM, Schreier H. NEBULIZATION OF LIPOSOMES .3. THE EFFECTS OF OPERATING-CONDITIONS AND LOCAL ENVIRONMENT. *Pharm. Res* 1992;9:515–520. [PubMed: 1495897]
47. Bados-Nagy I, Galantai R, Laberge M, Fidy J. Effect of Trehalose on the Nonbond Associative Interactions between Small Unilamellar Vesicles and Human Serum Albumin and on the Aging Process. *Langmuir* 2003;19:146–153.
48. Adams DR, Toner M, Langer R. Role of Trehalose in Prevention of Giant Vesicle Adsorption and Encapsulated Solute Leakage in Anhydrobiotic Preservation. *Langmuir* 2007;23:13013–13023. [PubMed: 17983248]
49. Nieva J, Shafton A, Altobelli LJ III, Tripuraneni S, Rogel JK, Wentworth AD, Lerner RA, Paul WJ. Lipid-Derived Aldehydes Accelerate Light Chain Amyloid and Amorphous Aggregation. *Biochemistry* 2008;47:7695–7705. [PubMed: 18578541]
50. McGrath LT, McGleenon BM, Brennan S, McColl D, McIlroy S, Passmore AP. Increased oxidative stress in Alzheimer's disease as assessed with 4-hydroxynonenal but not malondialdehyde. *QJM* 2001;94:485–490. [PubMed: 11528012]
51. Chen K, Kazachkov M, Yu PH. Effect of aldehydes derived from oxidative deamination and oxidative stress on β -amyloid aggregation; pathological implications to Alzheimer's disease. *Journal of Neural Transmission* 2007;114:835–839. [PubMed: 17401529]
52. Kremer JJ, Murphy RM. Kinetics of adsorption of [beta]-amyloid peptide A[beta](1–40) to lipid bilayers. *Journal of Biochemical and Biophysical Methods* 2003;57:159–169. [PubMed: 12915007]
53. Lin M-S, Chiu H-M, Fan F-J, Tsai H-T, Wang SS-S, Chang Y, Chen W-Y. Kinetics and enthalpy measurements of interaction between [beta]-amyloid and liposomes by surface plasmon resonance and isothermal titration microcalorimetry. *Colloids and Surfaces B: Biointerfaces* 2007;58:231–236.
54. Kremer JJ, Pallitto MM, Sklansky DJ, Murphy RM. Correlation of β -Amyloid Aggregate Size and Hydrophobicity with Decreased Bilayer Fluidity of Model Membranes. *Biochemistry* 2000;39:10309–10318. [PubMed: 10956020]
55. Good TA, Murphy RM. Aggregation state-dependent binding of β -amyloid peptide to protein and lipid components of rat cortical homogenates. *Biochemical and Biophysical Research Communications* 1995;207:209–215. [PubMed: 7857267]
56. Kurganov B, Doh M, Arispe N. Aggregation of liposomes induced by the toxic peptides Alzheimer's A[beta]s, human amylin and prion (106–126): facilitation by membrane-bound GM1 ganglioside. *Peptides* 2004;25:217–232. [PubMed: 15063003]

57. Murphy RM. Kinetics of amyloid formation and membrane interaction with amyloidogenic proteins. *Biochimica et Biophysica Acta (BBA) - Biomembranes* Amyloidogenic Protein-Membrane Interaction 2007;1768:1923–1934.
58. Kheterpal I, Chen M, Cook KD, Wetzel R. Structural Differences in A β Amyloid Protofibrils and Fibrils Mapped by Hydrogen Exchange - Mass Spectrometry with On-line Proteolytic Fragmentation. *J. Mol. Bio* 2006;361:785–795. [PubMed: 16875699]
59. Demmers JAA, Haverkamp J, Heck AJR, Koeppe RE, Killian JA. Electrospray ionization mass spectrometry as a tool to analyze hydrogen/deuterium exchange kinetics of transmembrane peptides in lipid bilayers. *Proceedings of the National Academy of Sciences of the United States of America* 2000;97:3189–3194. [PubMed: 10725361]
60. Kim Y-S, Jones LS, Dong A, Kendrick BS, Chang BS, Manning MC, Randolph TW, Carpenter JF. Effects of sucrose on conformational equilibria and fluctuations within the native-state ensemble of proteins. *Protein Science* 2003;12:1252–1261. [PubMed: 12761396]
61. Wendorf JR, Radke CJ, Blanch HW. Reduced protein adsorption at solid interfaces by sugar excipients. *Biotechnology and Bioengineering* 2004;87:565–573. [PubMed: 15352054]
62. Zhang P, Klymachyov AN, Brown S, Ellington JG, Grandinetti PJ. Solid-state NMR investigations of the glycosidic linkage in [alpha]-[alpha] ' trehalose. *Solid State Nuclear Magnetic Resonance* 1998;12:221–225. [PubMed: 9800267]
63. Crowe LM. Lessons from nature: the role of sugars in anhydrobiosis. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology* 2002;131:505–513.
64. Crowe LM, Mouradian R, Crowe JH, Jackson SA, Womersley C. Effects of carbohydrates on membrane stability at low water activities. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1984;769:141–150.
65. Luzardo, MdC; Amalfa, F.; Nunez, AM.; Diaz, S.; Biondi de Lopez, AC.; Disalvo, EA. Effect of Trehalose and Sucrose on the Hydration and Dipole Potential of Lipid Bilayers. *Biophysical Journal* 2000;78:2452–2458. [PubMed: 10777741]
66. Barton KN, Buhr MM, Ballantyne JS. Effects of urea and trimethylamine N-oxide on fluidity of liposomes and membranes of an elasmobranch. *Am J Physiol Regul Integr Comp Physiol* 1999;276:R397–R406.
67. Kaushik JK, Bhat R. Why is trehalose an exceptional protein stabilizer?: An analysis of the thermal stability of proteins in the presence of compatible osmolyte trehalose. *J. Biol. Chem* 2003;278:26458–26465. [PubMed: 12702728]
68. Zhang S, Iwata K, Lachenmann MJ, Peng JW, Li S, Stimson ER, Lu Ya, Felix AM, Maggio JE, Lee JP. The Alzheimer's Peptide A[beta] Adopts a Collapsed Coil Structure in Water. *Journal of Structural Biology* 2000;130:130–141. [PubMed: 10940221]

Figure 1.

Congo red assay for the amount of bound Congo red [CR-Aβ] (see Methods). Aβ samples were incubated at 37 °C quiescently in PBS (filled bar), trehalose (slashed bar), sucrose (grid bar) and TMAO (open bar). Incubation times are shown as x-axis labels. Aβ concentration was 100 μM, and each osmolyte concentration was 250 mM. Asterisks indicate conditions significantly different from PBS controls (ANOVA, $p < 0.01$).

Figure 2.

Representative hydrogen exchange mass spectra (+4 charge state) for Aβ samples in absence and presence of TMAO, trehalose (Tre) and sucrose (Suc) at different times of Aβ aggregation. Incubation time points were selected as fresh (0 hr), 4 hr, 10 hr, 24 hr and 72 hr. The Aβ concentration was 100 μM and each osmolyte concentration was 250 mM. Labeling time was 10 s. Vertical lines in the columns indicated the centroid masses of completely (gray line) unlabeled Aβ and (dark line) fully deuterated Aβ controls. M, L, H, F indicate the peak arising from monomer, LMW, HMW and fibril, respectively. Each spectrum was normalized to its own maximum intensity.

Figure 3. Representative hydrogen exchange mass spectra (+4 charge state) for samples incubated for 24 hr in PBS (left column) and in trehalose buffer (right column), labeled subsequently for the times indicated. The A β concentration during incubation was 100 μ M, and the trehalose concentration was 250 mM. Each spectrum was normalized to its own maximum intensity.

Figure 4.

Representative mass spectra in full range scan for A β samples in PBS, TMAO, trehalose (Tre) and sucrose (Suc) solution. A β concentration was 100 μ M, and each osmolyte concentration was 250 mM. Samples were treated using the same procedure as for labeling experiments excluding the D₂O dilution step. Each spectrum is normalized to its own maximum intensity. For A β in PBS buffer only, +3, +4, +5 and +6 charge states correspond to observed m/z values of 1444.18, 1083.45, 867.00 and 722.73 respectively. In trehalose and sucrose spectra, the arrows point to the adduct peaks formed with +4 charge state which are separated by an average mass 342.0 ± 0.8 Da (the approximate molecular weight of both sucrose and trehalose). Adducts were also observed for +3 and +5 charge states.

Figure 5.

Representative mass spectra in full range scan for A β samples in different concentrations of trehalose. A β concentration was 100 μ M and trehalose concentration was 0.1 mM (A), 10 mM (B), 50mM (C), 100 mM (D) and 250 mM (E). Samples were treated using the same procedure as labeling experiments excluding the D₂O dilution step. q=0 to 9 was used to indicate the number of the trehalose adducted to A β at +4 charge state. Each spectrum is normalized to its own maximum intensity.

Figure 6.

Relative percent leakage of CF from POPG LUVs upon incubation with pre-aged Aβ samples in PBS (filled bar), trehalose (slashed bar), sucrose (grid bar) and TMAO (open bar) solutions. Osmolyte concentration was 250 mM, and lipid/Aβ ratio was 20:1. Once Aβ was added to the liposome solution in the absence or presence of the osmolytes, the mixture was incubated quiescently for another 1 hr at 37 °C.

Figure 7.

Congo red assay of dissolution of pre-aged A β samples by trehalose (slashed bar), sucrose (grid bar) and TMAO (open bar). A β samples were incubated for 4 hr and 72 hr in PBS (filled bar), respectively. After A β sample aggregation, concentrated osmolyte solutions were added to a final concentration of 250 mM, after which osmolytes and aggregated A β were allowed to interact for an additional 1 hr at 37 C quiescently. Samples treated with osmolytes are not statistically different from PBS controls (ANOVA, $p > 0.05$).

Figure 8.

Representative hydrogen exchange mass spectra for A β samples upon interaction with POPC and POPG LUVs, as well as POPG LUVs in absence and presence of the osmolytes. Labeling time was 10 s for all the spectra. Pre-aged A β samples in PBS, fresh, 4 hr and 72 hr, were shown in the first row. POPC or POPG liposomes were added to pre-aged samples with lipid/A β molar ratio 10:1 and additional 1 hr incubation at 37 °C. For the fourth and fifth rows, osmolytes and POPG were added to pre-aged A β solution in turn and also incubated for another 1 hr 37 °C. Osmolyte concentration was 250 mM for both trehalose and TMAO.