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Exploration of Non-Specific Peptide Inhibitors of Amylin Aggregation in Type-2 Diabetes

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EXPLORATION OF NON-SPECIFIC PEPTIDE INHIBITORS OF AMYLIN AGGREGATION IN TYPE-2 DIABETES

By

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Abstract

Polypeptide aggregation is seen in a variety of diseases (termed amyloidoses) but remains poorly understood. This study uses the polypeptide amylin to investigate how non-specific interactions between peptides can inhibit aggregation. A set of 5 heptapeptides was assessed for its ability to impact amylin-induced membrane damage and aggregation. Solvent effects precluded accurate assessment of the impact on membrane damage; however a method of subtracting out the solvent effect is offered. The two most hydrophobic compounds showed the ability to substantially slow amylin aggregation, while those compounds carrying charged side chains tended to increase the rate of aggregation. Only one compound showed a concentration-dependent effect, which is offered as evidence for specific interactions. This study demonstrates that even random peptide sequences can have a significant impact on the behavior of an aggregating species, via specific and nonspecific associations.

Introduction

Protein aggregation is a widespread but poorly understood phenomenon that occurs in diseases ranging from Alzheimer's, Mad Cow, and Parkinson's to Type-2 Diabetes [for general reviews on amyloid formation, see ref. 1-3]. In each of these diseases, a unique polypeptide chain exists which undergoes a transition from a soluble state—usually considered devoid of well-defined secondary structure—to a β-sheet rich conformer capable of self-assembling into aggregates. Interestingly, there is little or no sequence homology among these aggregate-competent polypeptides, despite the structural similarities of the mature fibrils they form [2]. The relationship between the aggregation of the polypeptide and the pathogenesis of the disease is unclear, and may be different for different conditions. However, the presence and deposition of these structurally similar aggregates, termed amyloid plaques, over the course of the development of these diseases has led researchers to call these conditions amyloidoses.

In the case of Type-2 Diabetes, the aggregating polypeptide is known as amylin or human islet amyloid polypeptide (hIAPP). Amylin is normally stored alongside and secreted with insulin from pancreatic β-cells, and is thought to be involved in blood glucose homeostasis [4]. The polypeptide is a 37-residue chain, which contains three distinct, but somewhat overlapping regions capable of forming amyloid fibrils (8-20, 20-29, and 30-37) [5-9]. *In vitro*, full-length amylin forms fibrils at concentrations as low as 5 μM [4,10,11], whereas *in vivo* the polypeptide remains soluble despite being stored at concentrations estimated at 800 μM [11,12], suggesting that the body has a successful method by which it prevents amylin aggregation.

In addition to aggregating, amylin has also been shown to possess cytotoxic properties [2,13-15], thus providing a direct conceptual link explaining how it may participate in the development of Type-2 Diabetes. Counter-intuitively, however, it is not the mature aggregates of amylin which damage cells, but rather the oligomer states. This observation, along with other research, has led many to suggest that it is the aggregation process itself that damages cells, not the mature fibrils [13-16]. A possible mechanism for the observed cytotoxicity is that amylin may form pores or channels in cell membranes [14].

One model being developed suggests that amylin monomers form an α-helical intermediate that inserts into lipid bilayers prior to aggregation [17,18]. These intermediates may then cluster together within the membrane prior to transitioning into a β-sheet rich structure, which dissociates from the membrane to form the mature aggregate. In this model, while inserted within the membrane the amylin molecules form pore structures that allow cell contents to leak out. In fact, it is well established that amylin aggregation is catalyzed in the presence of lipids [19], which is consistent with a model in which a membrane-bound intermediate is present on the pathway to aggregation.

One natural question arising from research on amylin is how the bodies of healthy individuals prevent amylin aggregation and cell damage. Insulin is the most likely molecular mechanism by which an organism may regulate amylin behavior [4,10,20], though larger scale metabolic regulation is also a potential candidate—and, in fact, the two are not mutually exclusive ideas. Research has shown that the 10-19 region of the insulin B-chain binds amylin most strongly [20,21]. Because of this, our lab previously

synthesized different length fragments of this region of the B-chain in order to determine the shortest sequence still capable of inhibiting amylin aggregation and membrane damage [22]. While peptide-based drugs are difficult to deliver to patients, understanding the underlying molecular mechanisms by which insulin inhibits amylin aggregation is a first step toward the rational design of a potential treatment for Type-2 Diabetes.

Fragments of the 10-19 region of the insulin B-chain were, indeed, capable of slowing aggregation and decreasing membrane damage at sufficiently high concentrations; however none of our compounds showed the full effect of insulin, and at lower concentrations the compounds demonstrated various effects, including increasing the rate of aggregation in some cases [22]. Consistent with the model in which the oligomerization of amylin is responsible for membrane damage in cells, those compounds we synthesized which slowed aggregation to the greatest extent simultaneously showed the greatest amount of membrane damage. It is worth noting that insulin itself does not show this effect, and is capable of slowing aggregation without damaging the membrane. How this is accomplished remains unclear.

Because of the variety of effects witnessed with the insulin fragments we synthesized, a question of specificity was raised: to what extent will the presence of any compound in solution with an aggregating species impact aggregation kinetics and membrane damage? In the field of chemical biology, which is responsible for much of the current drug design and research in modern medicine, non-specific effects of small molecules are well established. Research by McGovern and others has shown that high-throughput screening (HTS) produces a multitude of false-positives from compounds that show non-specific reactions with numerous drug target candidates [23-26]. Interestingly, these small-

molecules may produce false-positives by forming small aggregates capable of trapping or non-specifically binding to drug target candidates [23-26].

Our results with different length fragments of the insulin B-chain, as well as knowledge from the field of chemical biology, led to the design of the project presented herein, whose aim is to explore whether or not amylin aggregation and the associated membrane damage can be impacted non-specifically by the presence of other peptide compounds in solution. To begin addressing this, we have designed and synthesized a series of five peptide compounds, called the NS series (Non-Specific series), which explore a small region of sequence space. These compounds are assessed here for their impact on amylin aggregation and amylin-induced membrane damage.

Table 1. NS peptide compounds assayed for their effect on amylin aggregation and membrane damage.

Peptide	*Sequence*	*Molecular Formula*	*Molar Mass (g/mol)*
NSx5	YAFDVVG	$C_{37}H_{52}N_8O_{10}$	768.87
NSx6	YFSPSFY	$C_{47}H_{56}N_8O_{11}$	909.01
NSx7	YFKPKFY	$C_{53}H_{70}N_{10}O_9$	991.20
NSx8	YAYPYAY	$C_{47}H_{56}N_8O_{11}$	909.01
NSx9	YFEPEFY	$C_{51}H_{60}N_8O_{13}$	993.08

Table 1 shows the sequence of each compound in the NS series. NSx5 is a modified version of the neuropeptide Deltorphin-1, which is unrelated to the other sequences and has no known involvement with amylin in the human body—thus, this peptide may be thought of as a 'random' sequence. The remaining compounds, NSx6- NSx9, are symmetrical heptapeptides of increasing hydrophobicity. The molecules are symmetrical about a proline residue, and this residue serves to prevent aggregation by opposing formation of β-sheets [27], and we predict that the symmetry and restricted conformational freedom should minimize the nature and type of interactions possible

between amylin and each compound*. Constructing a non-specific inhibitor of amylin aggregation and membrane damage can provide a control compound by which the efficacy of future potential inhibitors may be assessed.

Results & Discussion

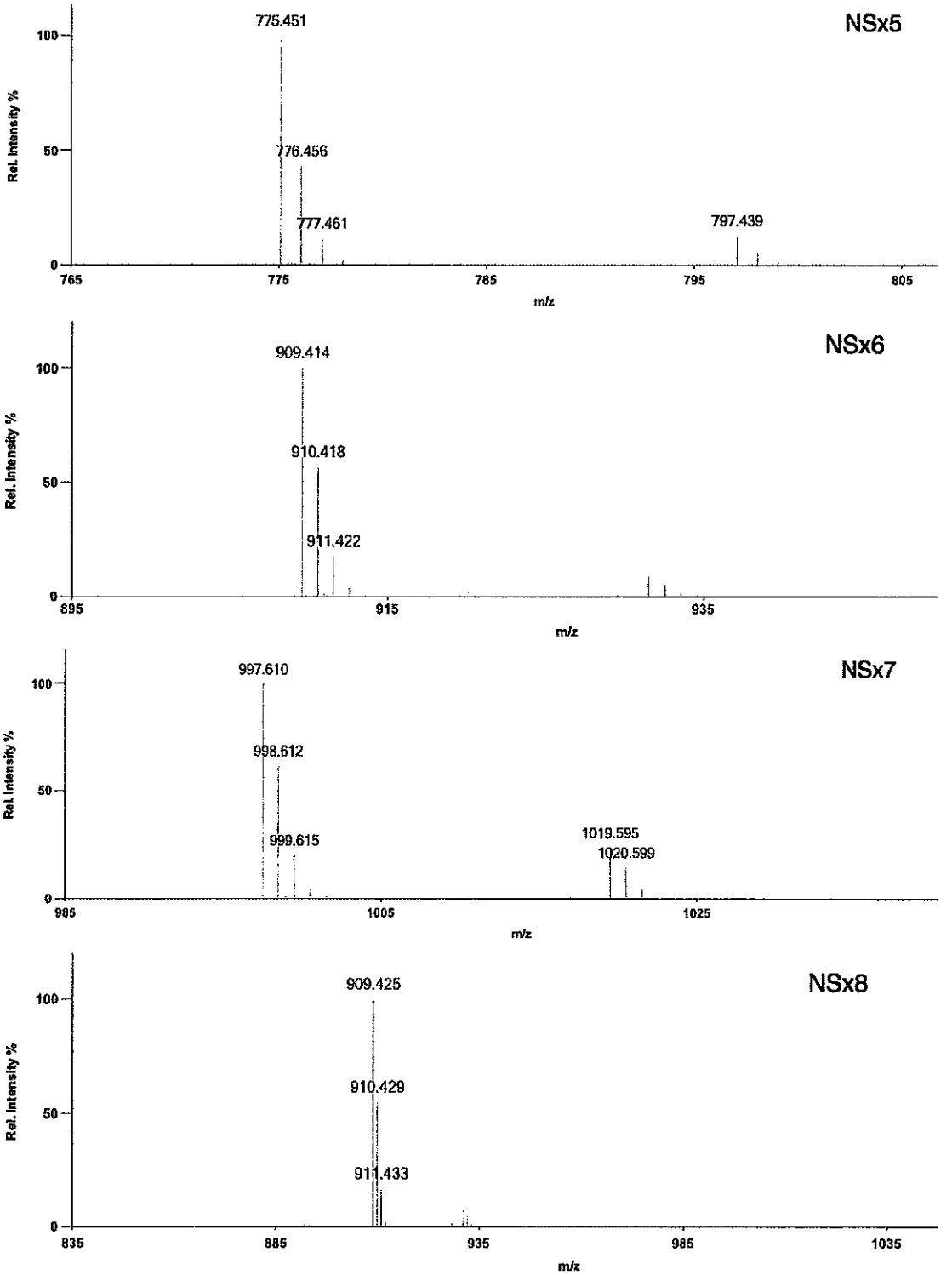
Electrospray Ionization-Mass Spectrometry Experiment

Figure 1 below shows the results of an electrospray ionization mass spectrometry experiment conducted on the NS series of peptides assayed here, using acidic methanol as solvent. Refer to Table 1 in the introduction for the predicted molecular weights and sequences of each compound. Three of the peptides synthesized, NSx6, -8, and -9, have spectra which match the predicted mass. The other two compounds, NSx5 and -7 show spectra with an M+1 peak that is 6 mass units higher than expected. It is difficult to find a justification for this discrepancy. The most likely cause is a miscoupling during synthesis; however none of the amino acids common between NSx5 and NSx7 are not also present in the other peptides, and none of the amino acids within the sequences differ by 6 mass units.

Oligomerization of peptides during MS experiments has been observed [28]. A trimer of peptides surrounding a water molecule would produce a peak that is 6 units higher than predicted, however this scenario is admittedly far-fetched, and the separation of isotope peaks by 1 unit suggests that the peaks are, in fact, M+1. The combination of a sodium adduct followed by loss of an OH- group would also produce peaks that are 6 units larger than anticipated. Another avenue worth investigating is whether or not

* Symmetry here refers only to symmetry in the primary sequence. The chirality of each residue, the 'bend' of the proline side chain, and the directionality of the peptide bond generally preclude the synthesis of a truly symmetrical peptide.

incomplete cleavage of one of the protecting groups on the side chains of either peptide during synthesis would explain the ESI results.



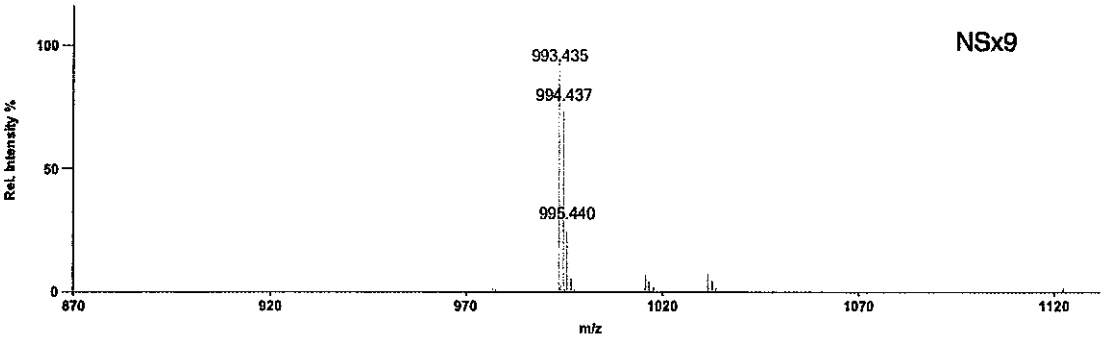


Figure 1. Results of ESI-MS of each NS peptide, using acidic methanol as solvent. Compare MW values to the predicted values given in Table 1 in the introduction. Compounds NSx6, -8, and -9 show the predicted mass. Compounds NSx5, and -7 show an M+1 peak that is 6 mass units higher than predicted. The explanation for this is unclear, however likely possibilities include a miscoupling during synthesis, or failure of a protecting group to be cleaved during purification. Alternatively, oligomer states could explain the observed peak, but these are difficult to justify. Since the spectra are extremely clean (i.e. there is no sign of significant impurity) the compounds were carried through the assay process with the assumption that the modification responsible for the deviation from the predicted molecular mass would not be significant. This study attempts to explore whether or not amylin aggregation can be impacted non-specifically by other peptide molecules in solution, and so the exact structure of the compounds can be somewhat ambiguous without hindering the integrity of the results. Further experiments will be conducted, regardless, to attempt to figure out the cause of the discrepancy between the observed and calculated masses.

It is worth noting that all of the spectra are extremely 'clean,' in other words there is no suggestion of any impurity in the samples. This shows that in each case some peptide was successfully synthesized and purified, and the spectrometry results are not likely the result of an impurity or side reaction during the synthetic process. Since the study presented here attempts to examine whether or not amylin aggregation can be influenced non-specifically by the presence of other peptide molecules in solution, even slight alterations in the predicted structure of NSx5 and NSx7 do not impact the integrity of the results since they can simply serve to sample a larger portion of sequence space. Therefore, all of the NS compounds were carried through the entire assay process despite the discrepancies observed in the ESI experiments. We are continuing to explore avenues

by which we may resolve the disagreement in molecular weights, including NMR and proteolytic experiments.

Dye Leakage Assay

Because amylin is known to compromise cell membranes such that cell contents leak out, we can use lipid vesicles (liposomes) containing dye to measure the extent of membrane damage [16-18]. As amylin molecules form pores in the liposomes, the dye encapsulated within is allowed to escape, which we can monitor as an increase in fluorescence compared to control reactions without amylin. In this study, we use detergent-treated vesicles as a positive, 100% control showing maximal fluorescence and vesicles in buffer as a measure of the baseline (0%) fluorescence in the absence of amylin. The extent of amylin-induced membrane damage is calculated as the percent emission above baseline, compared to the detergent-treated control. To test what effect the NS peptides have on membrane damage, the amylin is fixed at 10 μM and assayed against a range of NS peptide concentrations from 2 to 100 μM.

Figure 2 shows the percent change in dye leakage compared to an amylin sample run concurrently for each of the NS compounds synthesized. Negative values indicate reduced membrane damage while positive values mean greater dye leakage was observed. All assays were performed in triplicate, and average values are reported. The variation in fluorescence readings inherent to the instrument is small (2 fluorescence units or less for all trials); the variation among individual samples within a single assay is ~10% in all cases (error bars).

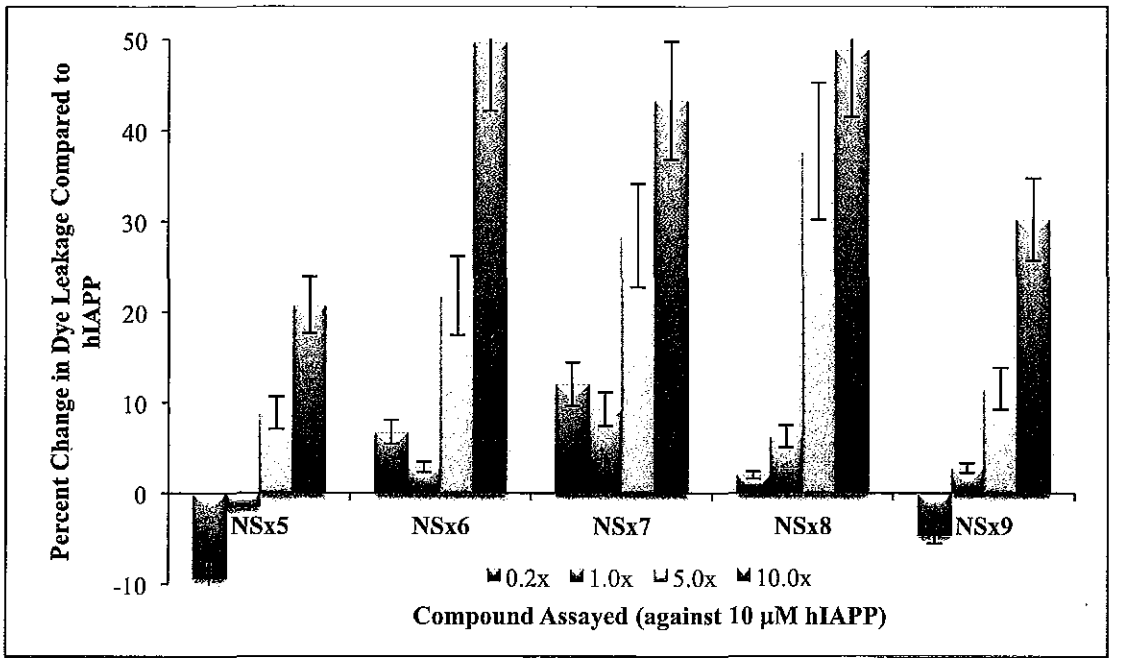


Figure 2. Dye leakage assay results for each of the NS compounds. In this assay, membrane damage is measured as the increase in fluorescence (compared to a baseline control containing only vesicles and buffer) caused by leakage of carboxyfluorescein dye from model liposomes in the presence of amylin. A detergent-treated buffered vesicle solution serves as a 100% leakage control (maximal fluorescence). The average value of triplicate runs are reported as percent changes compared to the amount of dye leakage observed with a 10 μM sample of amylin run concurrently. The amount of NS peptide added was varied from 2 to 100 μM (0.2x amylin concentration up to 10.0x amylin concentration). All effects observed are concentration-dependent (Figure 3). Negative values indicate a decrease in membrane damage, while positive values show an increase. In all cases, the amount of membrane damage was observed to increase significantly at higher concentrations. This startling effect prompted further investigation, which has suggested the results of this assay are due to solvent effects as discussed in the text. Error bars represent an ~10% variation in reactions in all cases.

The results of our dye leakage assay are surprising. In all cases, a significant increase in membrane damage was observed at higher concentrations of NS peptide (above equimolar for all compounds). This implies that all of the peptides synthesized here either enhance the effect of amylin or are themselves capable of damaging membranes. Figure 3 shows a plot of the percent change in dye leakage as a function of NS concentration. Reasonable linear fits are obtained, suggesting that the effects are concentration-dependent. Additional dye leakage assays with each NS peptide showed that membrane

damage was, in fact, observed with the NS series alone—i.e. even in the absence of amylin [data not shown].

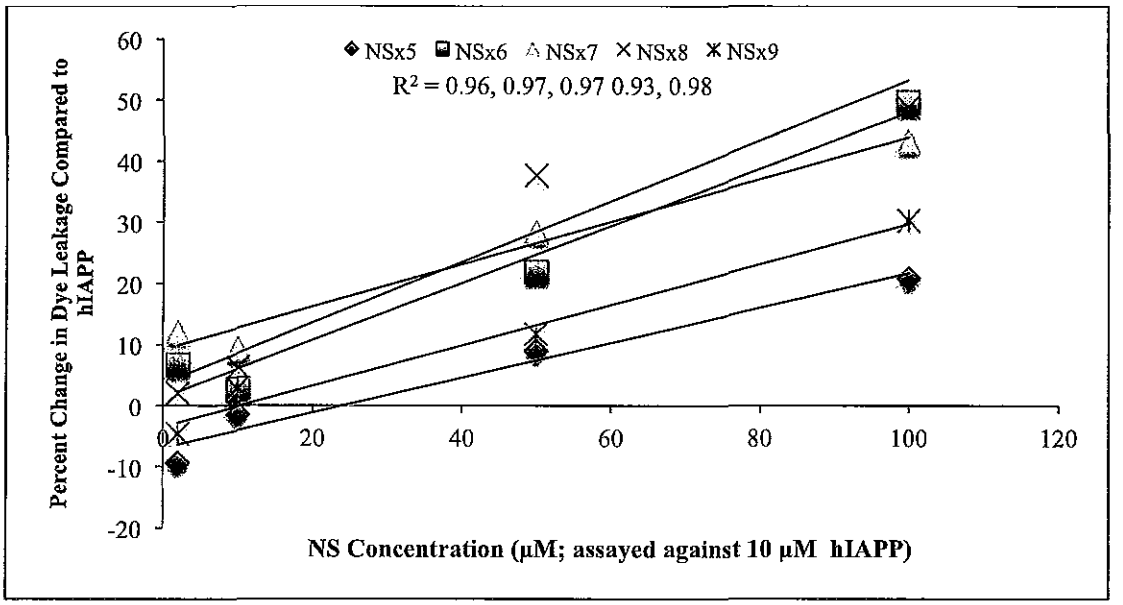


Figure 3. Concentration-dependence of the dye leakage assay results, presented in Figure 2. The significant increase in dye leakage observed in all cases at higher concentrations, coupled with the near-perfect linearity of the effect prompted further investigation which suggests that the results seen here are due primarily to solvent effects, as discussed in the text.

The fact that each compound shows such a striking increase in membrane damage at higher concentrations, and yet shows varying effects at equimolar and substoichiometric concentrations prompted further investigation of the dye leakage assay results. Coupled with the near-perfect linearity of the effects (see Figure 3), it was suggested that the solvent used to dissolve the peptides for the dye leakage assay (DMSO) might be responsible for the membrane damage. In order to test this directly, a control dye leakage assay was performed on DMSO and methanol, each in the presence and absence of dye-containing liposomes. Figure 4 shows the results of this control run. Volumes of DMSO and methanol were chosen based on the volumes of NS peptide stock solution that had been used for the dye leakage assays (NS peptides were originally dissolved in pure

DMSO, see Materials and Methods section). Unlike Figure 2, the values reported are raw emission values (528 nm), not percent changes, in order to better illustrate the effect. In the absence of vesicles, neither DMSO nor methanol shows any fluorescence. However, once vesicles are added, a large increase in fluorescence is observed, indicating that both organic solvents may be capable of damaging lipid membranes. This further suggests that the results depicted in Figure 2 with each of the NS compounds may actually be due to solvent effects, and not a result of interactions between the peptides and amylin.

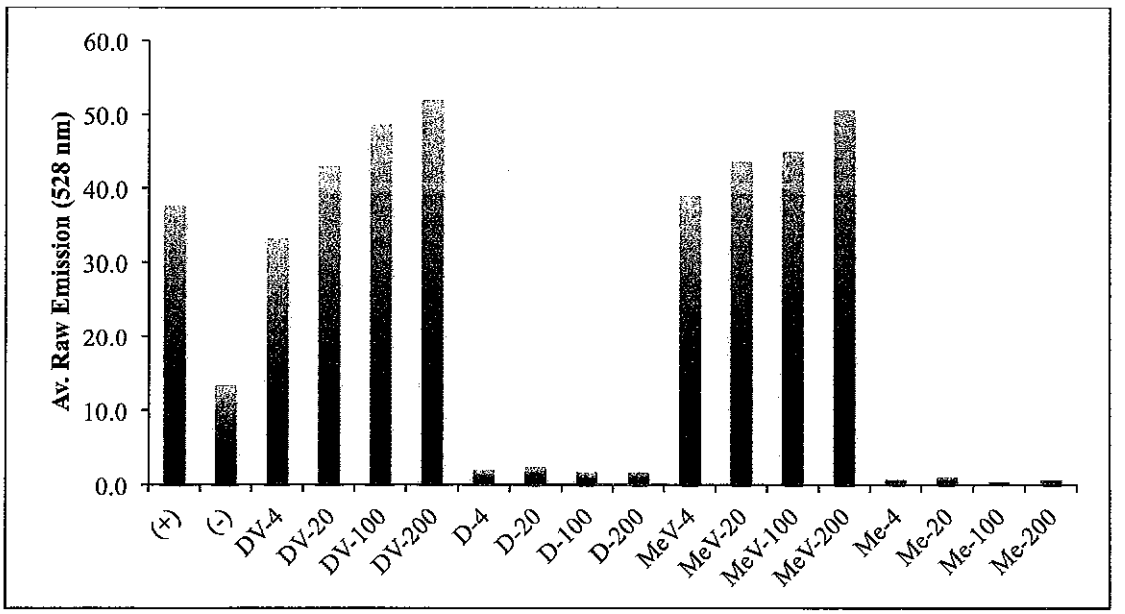


Figure 4. Control dye leakage assay to determine if organic solvents DMSO or methanol (MeOH) can incur membrane damage. Positive control (+) is a detergent-treated solution with dye-containing vesicles, while the negative (-) control is dye-containing vesicles in buffer. The volume (in μL) of solvent assayed is indicated by a numerical designation, and the volumes were chosen to reflect the volume of NS peptide solution assayed in the trial dye leakage assays reported in Figure 2. In the absence of vesicles, neither DMSO nor MeOH show any significant fluorescence (D-# and Me-# columns). Once vesicles are added, however, a striking increase in fluorescence is seen (DV-#, MeV-# columns). This effect is concentration-dependent (data not shown), and suggests that the results of our dye leakage assays on the NS compounds may be due to solvent effects.

In order to determine whether or not the DMSO solvent was in fact responsible for the observed increases in dye leakage, and whether or not this effect could be accounted for, we assayed the NSx5 compound in a different manner. The results are given in Figure 5.

One aliquot of NSx5 was dissolved in pure DMSO, while another was dissolved in 50 μL of DMSO and then diluted with sodium phosphate buffer up to 2620 μL total volume (the same volume as that of the sample dissolved in pure DMSO, see Materials and Methods section). These two solutions of NSx5 were assayed alone as well as against 10 μM amylin.

Concurrently, a series of DMSO solutions was assayed in order to build a 'standard curve' to measure the increase in emission over baseline as a function of the volume of DMSO added. This standard curve provided only a general estimation of the exact volume dependence of the emission change, however, and is not shown here. From the curve, we subtracted out the *estimated* effect of the DMSO solvent for both the NSx5 solution dissolved in pure DMSO as well as the solution diluted with buffer.

Looking at Figure 5, it can be seen that simply diluting the peptide with buffer is sufficient to remove the enhanced dye leakage effect seen in the pure DMSO solution. Subtracting out the effect of DMSO from both solutions removes virtually any observed increase in dye leakage, which is consistent with the hypothesis that the results depicted in Figure 2 are due solely to solvent effects. Additionally, it is worth noting that diluting the peptide solution with buffer or subtracting out the effect of DMSO abolished any concentration-dependence [data not shown]. It also appears that NSx5 may actually decrease the amount of membrane damage seen to a small extent. At this point, we cannot explicitly generalize these findings to all of the compounds in the NS series until we have finished re-assaying them in dilute buffer or a different solvent system. However, it seems likely that all of the compounds will display significantly different

effects on amylin-induced membrane damage once the effect of DMSO has been accounted for.

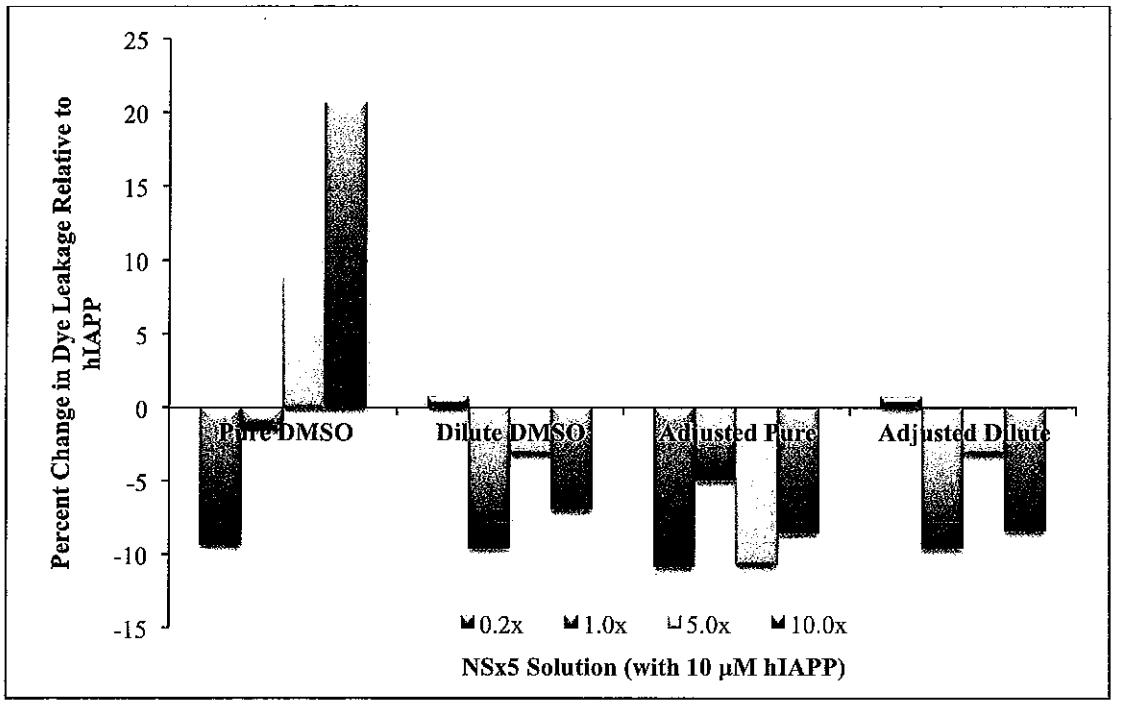


Figure 5. An attempt to account for the observed effect of DMSO on the dye leakage assay using the NSx5 compound. Two solutions of the NSx5 peptide were prepared, one in pure DMSO, the other in dilute DMSO. This alone resulted in a significant change in the observed effect on dye leakage; in fact, the NSx5 peptide does not seem to increase membrane damage at all when in dilute DMSO. Assayed concurrent with these two solutions was a series of DMSO controls with dye-containing vesicles in order to build a standard curve for the increase in fluorescence over baseline caused by DMSO. Based on the values obtained, the results of the Pure DMSO and Dilute DMSO percentages were adjusted to subtract out the effect of solvent. Performing this operation results in both NSx5 solutions showing little if any increase in dye leakage, consistent with the idea that the original results were due only to solvent effects.

Thioflavin T Aggregation Assay

The commercially available dye, thioflavin T, preferentially binds to the β-sheet structure of amyloid fibrils. When it does so, it shows enhanced fluorescence with excitation and emission maxima at around 450 and 480 nm, respectively [29]. The binding mode of the dye to amyloid fibrils is not well characterized. However, as fibrils form, more and more dye binds to the growing aggregates, producing an increase in

fluorescence that can be monitored over time. In the case of amylin aggregation, a typical thioflavin T assay will show a lag phase, in which aggregation nucleation centers are forming, followed by a sharp increase in fluorescence as fibers elongate. This phase is short because of the cooperative nature of the aggregation process. After this, once the maximum number of fibers has formed, a plateau phase occurs at some maximal fluorescence value. The overall shape of the graph should be sigmoidal, which is typical of cooperative processes. Figure 6 shows a representative plot of a typical amylin thioflavin T assay under our experimental conditions. The reader should note that the analysis performed on the graph is similar to that used for enzyme kinetics.

Typically, the value of primary interest is the time at which 50% of the amylin is in the aggregated form ($t_{1/2}$). This is conceptually similar to the Michaelis-Menton constant, or the K_i of inhibitor studies. In the presence of an inhibitor of aggregation, the $t_{1/2}$ should increase and the graph should shift to the right (since a greater amount of time will be needed for half of the amylin to aggregate). The converse is also true, and is often seen: if a compound accelerates aggregation, a shift to the left will be seen and the $t_{1/2}$ will decrease. In this study, we have scaled all plots as a percent of maximal fluorescence, which allows one to read the $t_{1/2}$ value directly from the graph by simply scanning along the line, y= 50%. It is worth noting that this assay is performed in the presence of model liposomes (without dye) in order to catalyze the aggregation process as well as to mimic the *in vivo* environment in which amylin aggregates in Type-2 Diabetes.

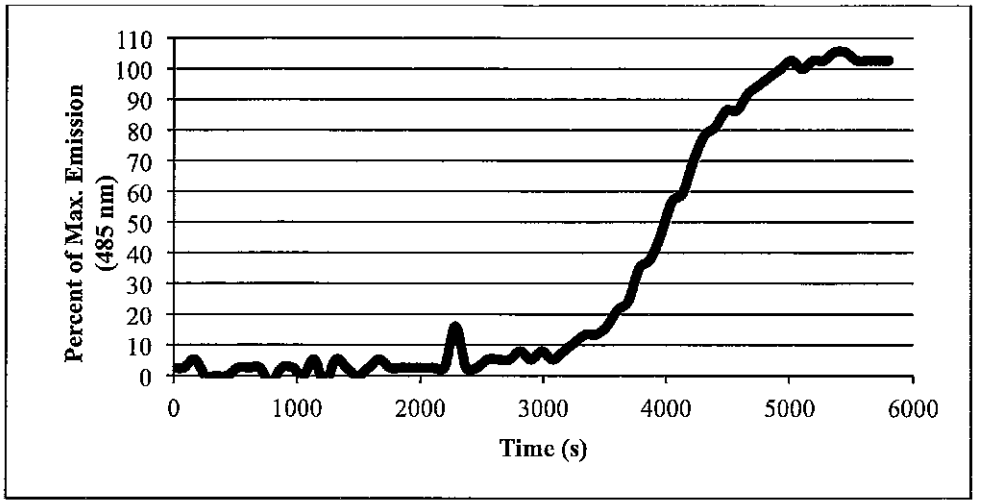
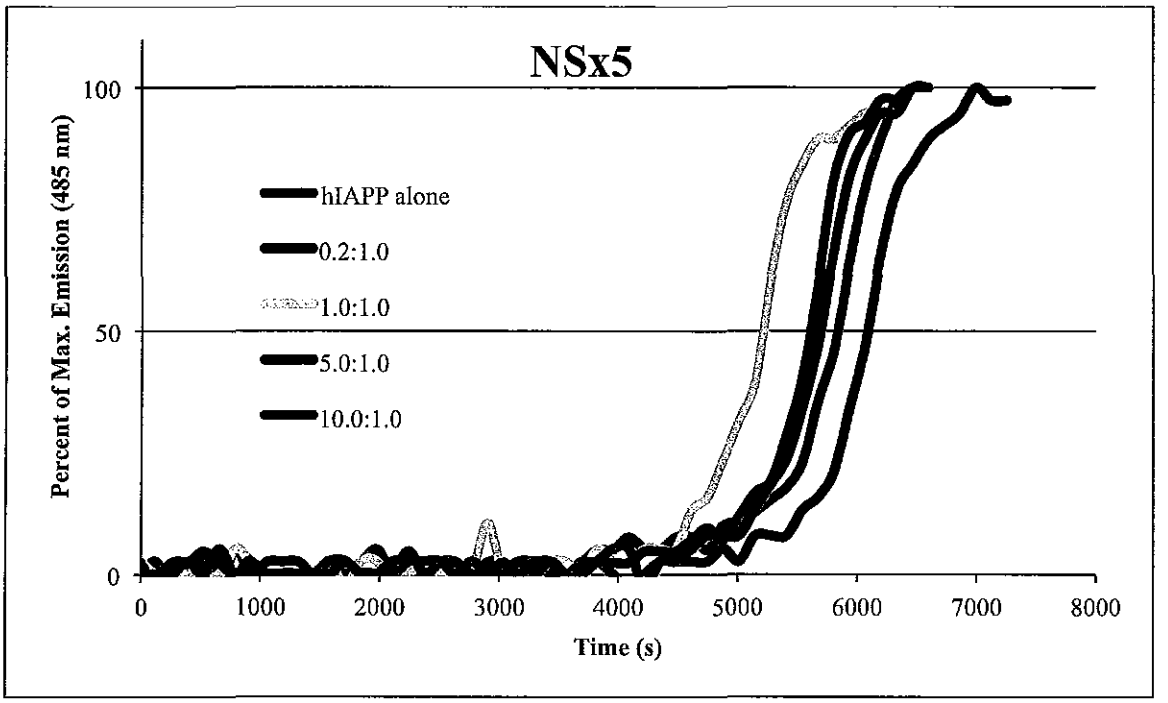
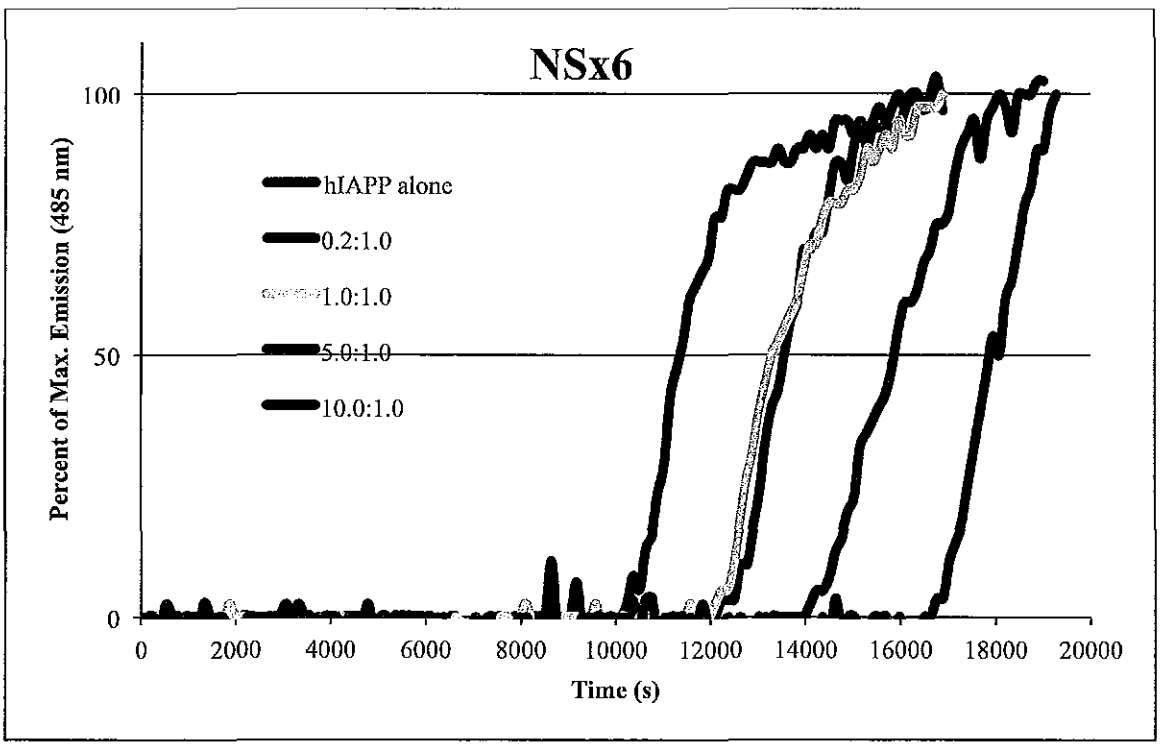
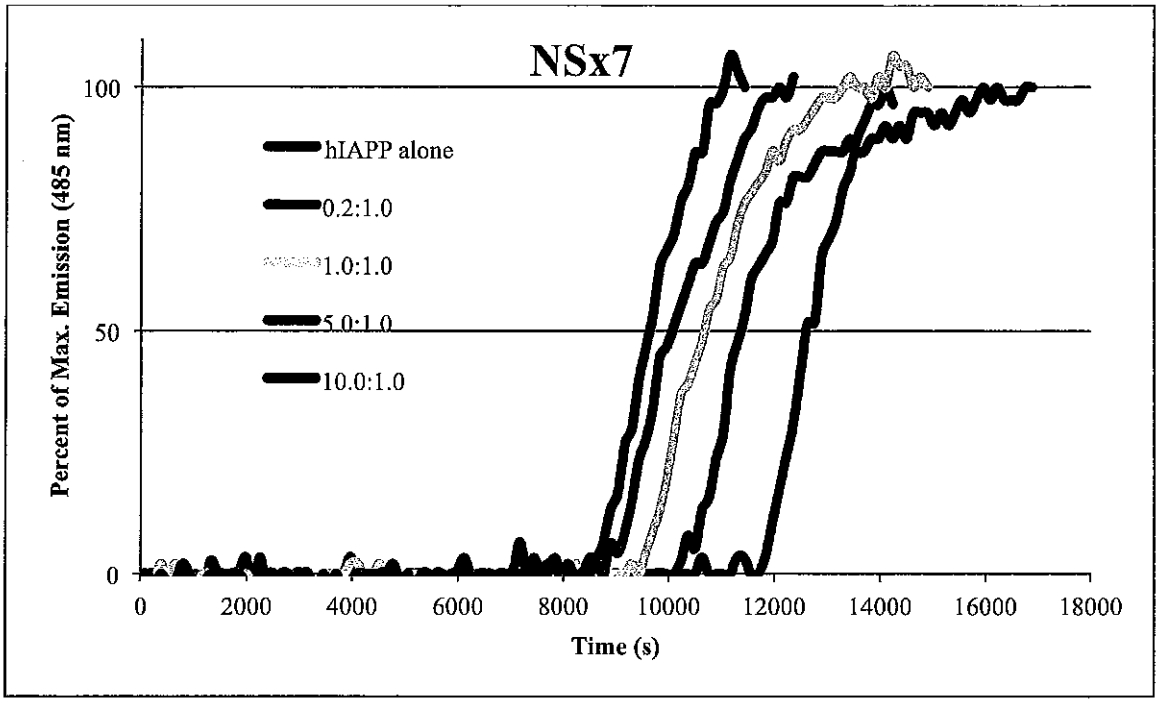


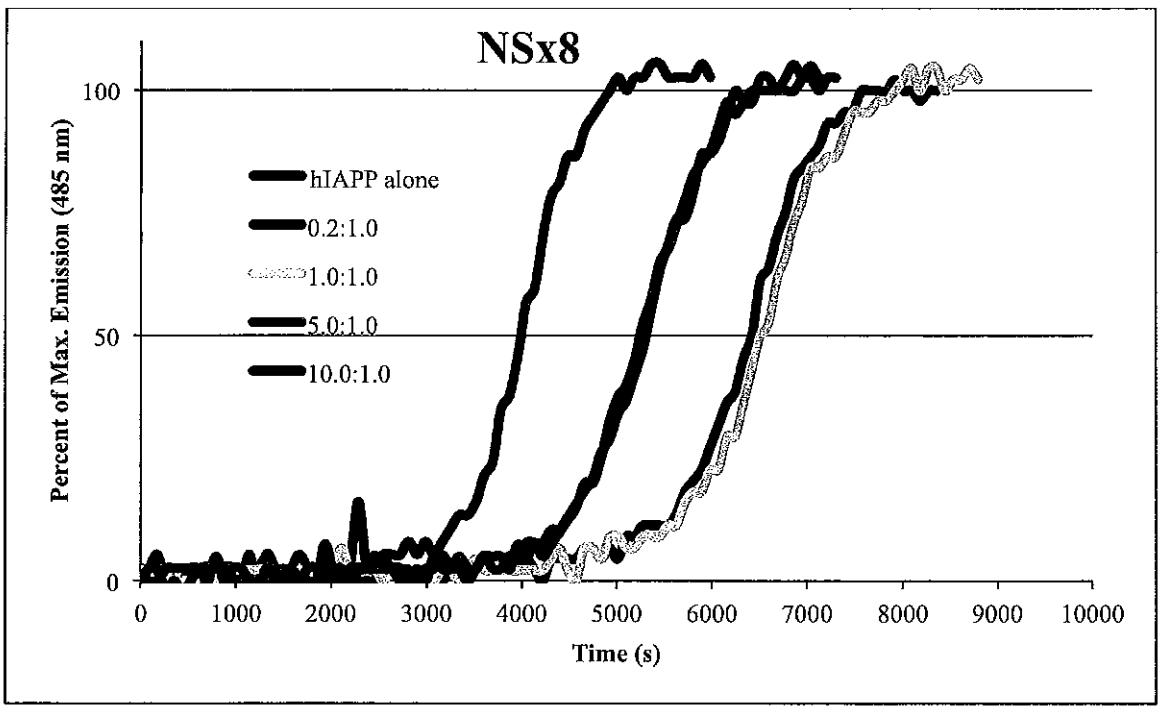
Figure 6. Representative thioflavin T profile for amylin alone under the assay conditions used in this study. A sigmoidal curve is indicative of cooperative amyloid formation. A clear lag time is visible, during which aggregate nucleation centers are forming. This is followed by a sharp increase in fluorescence as fibers elongate cooperatively. Finally, the fluorescence levels off once the maximum amount of fiber has formed. It is practical to scale the plot such that the maximum fluorescence is 100%. This allows one to read the $t_{1/2}$ directly from the graph (in this case, just under 4000 sec). This value represents the time at which 50% of the amylin has formed fibers, and is the value typically used to assess changes in aggregation kinetics in the presence of potential inhibitors.

Figure 7 shows the results of the thioflavin T assay performed with 10 μM amylin in the presence of varied concentration of each NS compound (range: 2 to 100 μM, the same concentrations used for the dye leakage assay). NS and amylin stock solutions were prepared using the minimal amount of DMSO needed to dissolve the peptide and diluted with buffer. Control runs with each NS compound at all concentrations, DMSO in the presence and absence of vesicles, and buffered dye alone showed that no assay component other than amylin produces any fluorescence above baseline (0-2 fluorescence units). Within each assay, a variation of ~3% was seen for all reactions in all cases (error bars in Figure 8).


NSx5
Percent of Max. Emission (485 nm)
100
50
0
hIAPP alone
0.2:1.0
1.0:1.0
5.0:1.0
10.0:1.0
0
1000
2000
3000
4000
5000
6000
7000
8000
Time (s)


NSx6
Percent of Max. Emission (485 nm)
100
50
0
hIAPP alone
0.2:1.0
1.0:1.0
5.0:1.0
10.0:1.0
0
2000
4000
6000
8000
10000
12000
14000
16000
18000
20000
Time (s)


NSx7
Percent of Max. Emission (485 nm)
100
50
0
hIAPP alone
0.2:1.0
1.0:1.0
5.0:1.0
10.0:1.0
0
2000
4000
6000
8000
10000
12000
14000
16000
18000
Time (s)


NSx8
Percent of Max. Emission (485 nm)
100
50
0
hIAPP alone
0.2:1.0
1.0:1.0
5.0:1.0
10.0:1.0
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
Time (s)

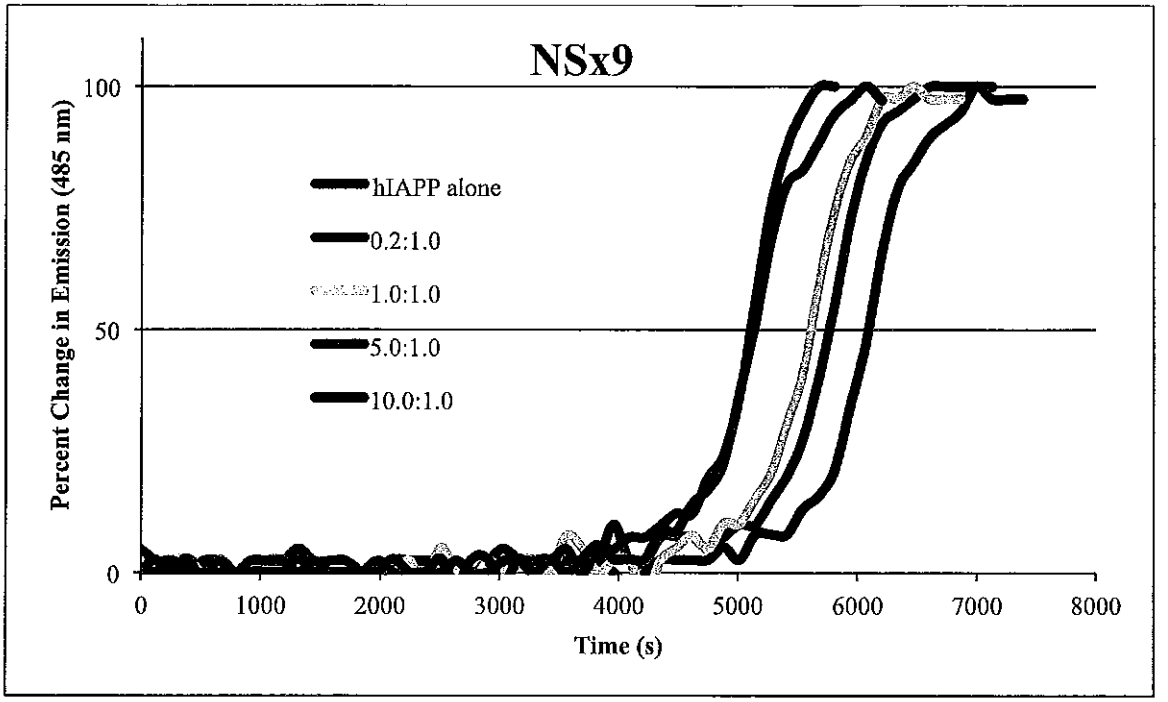


Figure 7. Thioflavin T results for 10 μM amylin assayed against increasing amount of NS compound (range: 2 to 100 μM, legend indicates the ratio of NS peptide: amylin). The color codes across all plots match, and the dark blue line represents amylin alone, without NS peptide. All plots have been scaled as percent of the maximal emission to allow ease of comparison. Compared to amylin by itself, a shift to the right indicates slower fiber formation, while a shift to the left indicates an increased rate of aggregation. A variation of ~3% was seen for all reactions. NSx6 slows aggregation considerably, such that at its maximal concentration a plateau phase was not seen until near the end of the assay. The effect of NSx6 seems concentration dependent (Figure 9). NSx8 also slows amylin aggregation, however the effect does not appear to correlate with concentration or molar ratio, perhaps indicating a non-specific effect. Of the remaining compounds, NSx5 and NSx9 both increase the rate of aggregation. NSx7 shows varied effects, sometimes increasing, sometimes slowing the aggregation rate. These results underscore the fact that even a random peptide in solution with amylin can have a substantial impact on the rate of aggregation observed, and that future studies must take care to account for non-specific effects their peptides might exert.

The results of Figure 7 show that even a 'random' peptide in solution with amylin can have a substantial effect of aggregation kinetics. NSx6 shows a concentration-dependent effect in which amylin aggregation is slowed significantly. At the highest concentration (100 μM NSx6), the lag phase is extended until almost 4 hr 40 min. NSx8 also seems capable of slowing amylin aggregation to a significant extent; however, the effect does not correlate with concentration, which may indicate a non-specific effect. Of the

remaining compounds, both NSx5 and NSx9 increase the rate of fibril formation compared to amylin alone. NSx7 shows varied effects, sometimes increasing and sometimes decreasing the rate of aggregation. These results do not undermine previous studies, but rather serve as an example that peptide compounds synthesized without any particular connection to amylin can still show a profound impact on its behavior. Future studies with amylin should be aware of this, and a strategy designed to deal with possible non-specific effects of any potential peptide inhibitors.

Another way of presenting the data in Figure 7 is to compare the $t_{1/2}$ values for each NS compound. It is important to note that not all compounds were assayed together at the same time. Therefore, it is not appropriate to compare their $t_{1/2}$ values directly. In different assays, amylin may show different lag times and slightly different maximal fluorescence values. This is partly due to the fact that both the thioflavin T dye and the liposome solutions used to catalyze the reaction must be prepared fresh for each assay. Slight differences in dye concentration may impact the intensity of the readings. Using different lipid stock solutions for each assay means that the same vesicle concentration will not be present for each trial, which can impact the rate of amylin aggregation.

Because of these factors, it is necessary to adjust each $t_{1/2}$ value in order to make them comparable across assays. This is most readily accomplished by dividing the NS $t_{1/2}$ value by the $t_{1/2}$ of the amylin sample run at the same time and converting the value to a percent. This allows one to compare the change in the rate of aggregation incurred by each NS compound measured against the rate of amylin aggregation alone. Figure 8 presents the $t_{1/2}$ values for all of the NS compounds adjusted in this way. Bars extending in the positive y-direction indicate a slowing of aggregation, while bars in the negative y-

direction show an increased rate of aggregation. From this plot, it is easy to see the substantial effect that the compounds NSx6 and NSx8 have on amylin behavior, as well as the general trend of the remaining compounds to increase the rate of aggregation.

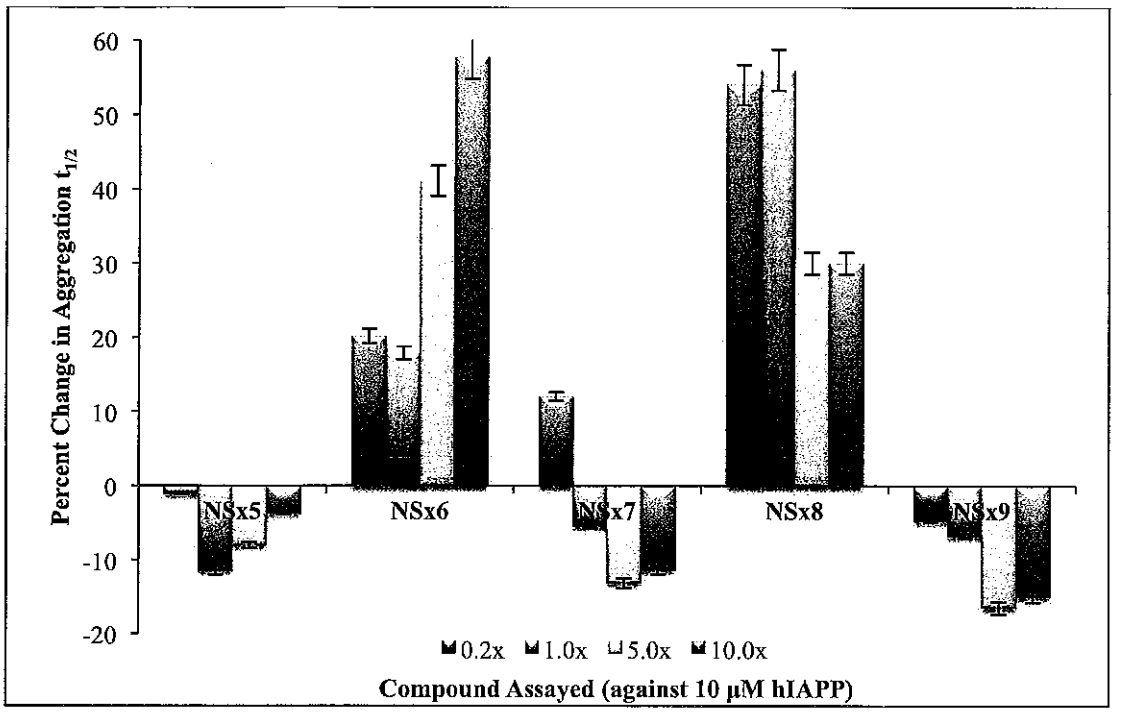


Figure 8. Comparison of adjusted $t_{1/2}$ values for each NS compound. The $t_{1/2}$ is obtained from the graphs shown in Figure 7. By dividing these values by the $t_{1/2}$ of the amylin sample run at the same time and converting to a percent, one obtains a dimensionless value, which can be compared with other assay results not run at the same time. Positive values indicate a decrease in the rate of aggregation, while negative values show an increase in aggregation kinetics. Both NSx6 and NSx8 substantially decrease the rate of aggregation. Only the effect of NSx6 seems concentration-dependent. NSx5 and 9 both increase the rate of aggregation. NSx7 increases the rate of aggregation at all but the lowest molar ratio; the effect does not appear concentration-dependent. Error bars represent an ~3% variation in all reactions.

Finally, we checked for concentration dependence with respect to the thioflavin T results (Figure 9). Only NSx6 seems to exert a concentration-dependent effect. NSx9 may show a weakly concentration-dependent effect if the regression analysis is nonlinear (data not shown, $R^2 = 0.8$). The effect of all other compounds is not concentration-dependent.

We suggest that a concentration *independent* effect may be an indicator of non-specific effects.

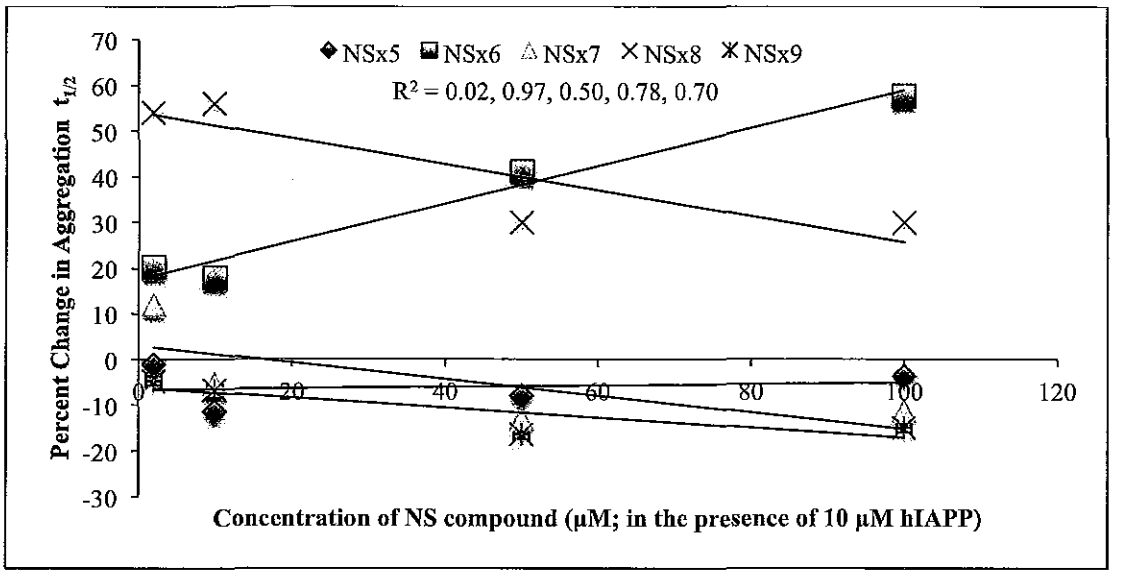


Figure 9. Concentration-dependence of the results of the thioflavin T assays. Only the effect of NSx6, which was to significantly slow the rate of amylin aggregation, appears concentration dependent (R^2= 0.97). All other compounds do not seems to exert concentration-dependent effects, perhaps indicating that their interactions with amylin are non-specific.

Conclusions/ Future Prospects

This study has shown that even a random peptide can have a significant effect on the behavior of an aggregating species. This underscores the need for understanding the molecular mechanisms behind protein aggregation as well as taking care to rule out non-specific effects in future studies dealing with amylin aggregation. It seems likely that the concentration-dependence seen in the way that NSx6 slows amylin aggregation may indicate a specific effect. Because of the high sequence similarity between NSx6-9, it is reasonable to speculate as to why the compounds show such disparate effects. Of all the compounds assayed, only NSx8 and NSx6 do not carry charged side chains (see Table 1, NSx7 carries two lysines, NSx5 and NSx9 carry a single aspartic acid and two glutamic

acid residues, respectively), and both of these compounds demonstrated an ability to slow amylin aggregation.

Hydrophobicity is therefore one logical explanation for the observed effects, since the symmetric nature of each compound as well as the intervening proline restricts the conformational freedom of each NS peptide. However, our previous research showed a correlation between the ability to form salt bridge interactions with amylin and slowing of its aggregation [22]. It was observed that a glutamic acid residue on potential inhibitors was able to bind to an arginine residue on amylin. Modeling studies indicated that the strength of this salt bridge interaction was strongly related to the ability of the compounds to slow aggregation. We would therefore expect that NSx9 and NSx5 might be able to slow aggregation by forming an analogous salt bridge association; this does not appear to be the case, however. Conformational factors may be responsible, but further experiments are needed to confirm this.

Our lab has also previously correlated increased membrane damage with slowing the rate of amylin aggregation [22]. As such, it would be desirable to be able to plot the change in aggregation kinetics of each of the NS compounds against the change in dye leakage to see whether or not this trend holds for the series investigated here. However, due to the solvent effects of the DMSO, we cannot do this until all of the dye leakage assays have been repeated under different solvent conditions. It is worth noting, however, that when the effect of DMSO is subtracted from the dye leakage assay performed on NSx5, a decrease in membrane damage is seen at all concentrations (compare Figures 2 and 5 with respect to NSx5). Since NSx5 also shows an increase in the rate of

aggregation at all concentrations (Figures 7 and 8), this suggests that the two observable behaviors may be correlated in this series of peptides.

Based on this, a prediction about how each of the NS peptides will impact membrane damage can be made. Both NSx6 and NSx8 slow amylin aggregation, and therefore are expected to increase the amount of membrane damage. NSx5 and NSx9 increase the rate of aggregation and so would be predicted to show a decrease in the extent of membrane damage. NSx7 shows an ability to slow aggregation only at the lowest concentration assessed here (2 μM), which predicts that at this concentration an increase in membrane damage would be seen, while at all others the reverse would be the case.

On the other hand, if a correlation between increased membrane damage and slower aggregation rates is not observed, this could provide mechanistic details about the interactions between amylin and each of the NS compounds. Based on the model of amylin aggregation in which the membrane-bound intermediate is responsible for pore formation, and thus cell damage, a compound which preferentially binds the soluble form of amylin—i.e. before it binds to lipid membranes—could, in principle, slow aggregation without increasing membrane damage. This is not a certainty, but rather a testable hypothesis for future experiments.

Finally, binding studies should be performed to investigate the molecular interactions between NSx6, NSx8, and amylin in order to uncover the mechanism by which each is exerting its effects. In this way, this study also provides two novel lead compounds that can be tested for potential use as aggregation inhibitors. The discovery of such compounds through serendipity is one of the historic hallmarks of scientific discovery. However, it is important to note that the true aim of this experiment is to emphasize how

non-specific interactions between molecules can play a significant role in the results obtained.

Materials/ Methods

Peptide Synthesis

Samples of full-length human amylin (3905 g/mol; purchased from SynBioSci, Livermore, CA, or Anaspec, Freemont, CA) were dissolved in HFIP (hexafluoroisopropanol), aliquoted in 0.5 mg increments, frozen in dry ice/ acetone and lyophilized for storage. Regular, protected amino acids were obtained from Bachem Americas, Inc. (Torrance, CA), Anaspec (Freemont, CA), and Synthetech, Inc. (Albany, OR). Coupling agents and resins were purchased from Bachem Americas, Inc. and Midwest Biotech, Inc. (Fishers, IN). Solvents and deprotection agents were obtained from Fisher Scientific (Pittsburgh, PA) and Sigma-Aldrich Chemical Co. (St. Louis, MO). Peptides in the series investigated here were synthesized on a PS3 Automated Peptide Synthesizer from Protein Technologies (Tucson, AZ) using established protocols for Fmoc (N-α-fluorenylmethyloxycarbonyl) protected amino acids on Rink amide MHBA resin (*p*-methylbenzhydrylamine; 0.70 mmole/g) on a 0.1 mmole scale. This resin yields a C-terminal amide ('carboxamide') upon cleavage.

The deprotection solution for N-terminal amines was 20% piperidine in DMF (N,N-dimethylformamide). The coupling agent for synthesis was HBTU (O-(Benzotriazol-1yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) activated with 0.4 M DIEA (N,N-diisopropylethylamine) in DMF. Deprotection of side chains and cleavage from resin was performed simultaneously with 11 mL 90% TFA (trifluoroacetic acid)/ 10% scavenger cocktail (phenol and water) as described previously [16]. Crude peptide product was

purified by preparative reversed-phase high performance liquid chromatography (RP-HPLC) on a Waters (Milford, MA) instrument with a Phenomenex (Torrance, CA) Jupiter C18 column (2.2 x 25.0 cm, 10 mL/ min), using a linear gradient of 10% acetonitrile (0.1% TFA)/ water (0.1% TFA) to 50% acetonitrile (0.1% TFA)/ water (0.1% TFA). Fractions containing pure peptide were frozen in dry ice/ acetone bath and lyophilized for storage.

Peptide Analysis

Peptide purity was assessed by analytical RP-HPLC. Peaks were monitored at 280 nm. All synthesized peptides were ≥ 97% pure as analyzed by peak integration. Electrospray mass spectrometry, courtesy of Dr. Ruth Ann Armitage, was used to determine if peptide products matches the appropriate molecular weights.

Preparation of Large Unilamellar Vesicles (LUVs) Containing Carboxyfluorescein Dye

Lipids were purchased from Avanti Polar Lipids (Alabaster, AL), and carboxyfluorescein dye from Sigma-Aldrich (St. Louis, MO). Lipid vesicles were created in order to encapsulate the dye. Disruption of the vesicle membrane causes the dye to leak into the surrounding buffer, providing a straightforward way to measure the amount of amylin-induced membrane damage. Baseline controls were compared to runs with added peptide and added detergent (which acted as a positive control to give 100% leakage).

The protocols for LUV preparation and the dye leakage assay have been described previously [16]. Briefly, vesicles were prepared by taking 5 mg of a 7:3 ratio of the lipids

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-(phospho-L-serine) (DOPS), respectively, and dissolving it in 2 mL of chloroform. The chloroform was evaporated by a gaseous nitrogen stream, creating a thin film of lipid on the side of a test tube, which was then dried in a vacuum desiccator overnight. 500 μL of 30 mM carboxyfluorescein dye in a pH 7.5 sodium phosphate buffer solution was added to the dried lipid to make multilamellar vesicles (MLVs). This solution was vortexed thoroughly to mix the components and subsequently frozen using dry ice and acetone and thawed five times consecutively. The solution was then extruded 21 times through polycarbonate filters (pore size 200 nm) using a mini-extruder from Avanti Polar Lipids, fitted with two 1000 μL Hamilton gastight syringes, producing large unilamellar vesicles (LUVs). To remove non-encapsulated carboxyfluorescein, the LUV solution was placed on a Sephadex G50 gel exclusion column and the first colored fragment, the separated dye-containing vesicles, was collected.

Dye Leakage/ Membrane Damage Assay (Carboxyfluorescein Assay)

Peptide stock solutions for the dye leakage assay were prepared as follows. A 0.70 mM stock solution of amylin was formed by dissolving a single 0.5 mg aliquot in 40 μL dimethyl sulfoxide (DMSO) and diluting with 144 μL sodium phosphate buffer (pH 7.5). Corresponding 0.70 mM stock solutions of each peptide in the NS series were created by dissolving 1.83 μmol of pure, lyophilized peptide in 2620 μL DMSO.

To a 3 mL test tube containing an appropriate amount of buffer, aliquots of each NS peptide were added in increasing amounts per sample while amylin was added at a fixed volume to produce a final concentration of 10 μM amylin. Once both peptides were

added to the test tube, a 20 μL aliquot of dye-containing vesicle solution was added. The total assay volume was 1.5 mL. NS compound concentrations ranged from 0 to 100 μM, allowing us to look at their impact on amylin-induced membrane damage over a large concentration range and compare that to what occurs with amylin itself. Control runs with only NS peptide and vesicles were run concurrent with test reactions to investigate whether or not our novel peptides are themselves capable of damaging membranes.

Once all assay components were added, the test tubes were mixed by inversion, and 300 μL from each tube was transferred to a well in a 96 well plate, which was inserted into a FLx 800 Fluorescence Microplate Reader (BioTek Instruments, Winooski, VT) with KC4 software (filters set to 485 nm excitation, 20 nm bandwidth, 528 nm emission, 20 nm bandwidth). A time-course fluorescence spectrum was taken over 180 minutes. The control used to determine 100% leakage was detergent, 80 μL Triton X (20% v/v in buffer), which induced the release of any remaining dye from vesicles, resulting in the highest possible fluorescence. Dye leakage was reported by the equation:

$$\text{Percentage of dye leakage} = (F - F_{baseline})/(F_{detergent} - F_{baseline})$$

where $F_{baseline}$ was the fluorescence of the LUVs in the absence of peptide (buffer only). The spectra were saved and compiled in Microsoft Excel. Since little time dependence was observed, the percentage of dye leakage was averaged over the three hour time period. All assays were run in triplicate and average values are reported.

Thioflavin T (ThT) Assay

Thioflavin T was purchased from Sigma-Aldrich (St. Louis, MO). The ThT assay has been described previously [13,16]. Briefly, vesicles of 7:3 DOPC/DOPS were prepared as

described above, except that carboxyfluorescein dye was absent. The presence of lipid vesicles in this assay serves to catalyze amylin fiber formation and more closely mimic the *in vivo* environment in which amylin is thought to aggregate.

It was necessary to adjust the total assay volume to 1.3 mL for our series in order to minimize the amount of amylin consumed. Both amylin and each NS peptide were used from 1.28 mM stock solutions. The amylin stock was prepared by dissolving a single 0.5 mg aliquot in 40 μL DMSO and 60 μL of sodium phosphate buffer (pH 7.5). NS peptide stocks were created by dissolving 1.96 μmol of each peptide in 100 μL DMSO then diluting up to 1.5 mL by slow addition of sodium phosphate buffer with mixing to avoid precipitating the peptide. Only the NSx7 peptide (sequence YFKPKFY) did not dissolve in 100 μL DMSO; instead 500 μL DMSO were needed, diluted with 1000 μL of buffer.

Each assay reaction was assembled as follows in a 3 mL test tube. Increasing amounts of NS peptide were added to buffered solutions containing thioflavin T (ThT) dye (final dye concentration: 25 μM). As with the dye leakage assay, NS peptide concentrations ranged from 0 to 100 μM. Subsequently, sufficient amylin was added to each tube to achieve a final, fixed concentration of 10 μM. Finally, 30 μL of the vesicle solution containing LUVs without dye was added to catalyze fiber formation. Each solution was mixed by inversion, then 300 μL from each tube was transferred to a well in a 96 well plate, which was inserted into a FLx 800 Fluorescence Microplate Reader (BioTek Instruments, Winooski, VT) with KC4 software (filters set to 440 nm excitation, 30 nm bandwidth, 485 nm emission, 20 nm bandwidth). A time-course fluorescence spectrum was taken over a minimum of 4 hours, 30 minutes. Each assay was run in triplicate. A sigmoidal increase in fluorescence intensity over time indicated fiber formation. The $t_{½}$ of

each run was calculated from the plot to assess the impact of each peptide on the kinetics of amylin fiber formation. Control runs with DMSO, vesicles, and each NS peptide showed that none of the assay components showed fluorescence under the reaction conditions used here.

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