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Balakrishna Kurva

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By Balakrishna Kurva

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Dr. Deborah Heyl-Clegg, PhD, Chair

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Thesis Approval Form

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Approved:

_____	_____
Dr. Deborah Heyl-Clegg, PhD Thesis Chair	Date
_____	_____
Dr. Maria Millette, PhD Committe Member	Date
_____	_____
Dr. Tim Friebe, PhD Commitee Member	Date
_____	_____
Dr. Ross Nord, PhD Department Head	Date
_____	_____
Dr. Deborah deLaski-Smith Dean of the Graduate School	Date

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Abstract

Human islet amyloid polypetide (hIAPP) is a 37 amino acid peptide, co-secreted along with insulin in the islets of Langerhans of pancreatic beta cells. Aggregation of hIAPP fibrils is believed to be toxic to beta cells and responsible for beta cell dysfunction and death associated with type 2 diabetes. Insulin has been found to act against the actions of hIAPP. The internal sequence of insulin, HLVEALYLV, recognizes and binds the 10-19 region of hIAPP. Since insulin reportedly blocks the formation of amyloid fibers, insulin analogs may protect cell membranes from damage. In an effort to study this effect, truncated analogs of insulin, namely HLVEALYLV and LVEALYLV, were synthesized and tested in the presence of hIAPP. Carboxyfluorescein-encapsulating vesicles that mimic the β-cell were created using 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 the percent leakage of fluorescent dye from the vesicles in the presence of the peptides was calculated as compared to a 100% Triton-X detergent-treated control. The activity of the truncated analogs was compared to that of insulin under the same conditions. Whereas insulin was somewhat protective of the effects of hIAPP, the shorter analogs were found to increase the damage caused by hIAPP rather than reducing it, possibly by co-aggregating with the hIAPP.

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1. Introduction

1.1 Diabetes Mellitus

Diabetes is a chronic disease in which the body cannot use the insulin hormone properly, due to the lack of insulin or because the somatic cells are insensitive to the insulin produced in the body. The insulin hormone is useful for the transportation of glucose from the blood into the somatic cells. The insulin actually activates the receptor for glucose to come to the surface of the body cells. Then eventually glucose is converted into the ATP, which acts as the energy for the body. The hormone insulin is secreted by the beta cells of the pancreas. The decreased production of insulin results in diabetes mellitus. It is mainly classified in two forms: Type 1 diabetes and Type 2 diabetes.

Type 1 Diabetes is also known as Insulin Dependent Diabetes (IDDM) or Juvenile-onset Diabetes. In this disease, the pancreas produces little or no insulin. This usually is seen in children of ages 10-14 years. The factors affecting the disease are environmental, genetic, or auto-immune. The development of Type 1 diabetes may be due to infection or environmental agents. Infectious agents attack the beta cells; these include viruses like mumps, rubella, coxsackie B4, and toxic chemicals and cytotoxins released in the body.[1] In some cases, exposure of children to cow's milk in infancy may lead to Type 1 diabetes. The cow milk contains an albumin serum protein called Bovine Serum Albumin (BSA), which acts as the environmental trigger to produce antibodies and results in the destruction of the insulin producing cells[1]. Some people are genetically susceptible to environmental factors, which cause changes in the genetic code and decreased production of insulin, resulting in the disease. Type 1 diabetes can be seen both in children and adults but usually starts in childhood. It is usually treated by careful monitoring of blood glucose and injecting insulin and by changing dietary habits.

Type 2 Diabetes is also known as Non-Insulin Dependent Diabetes Mellitus (NIDDM) or adult-onset Diabetes. Ninety percent of the world population diagnosed with diabetes is suffering from Type 2 Diabetes. Type 2 Diabetes is mostly observed in older people and people who are suffering with hypertension, obesity, and high levels of blood cholesterol. This is the sixth most prevalent disease that is responsible for high morbidity in the United States of America. It results from a combination of decreased insulin secretion and the development of insensitivity of the body cells to the insulin in the body. The formation of amyloid fibrils in the beta cells of the pancreas is a characteristic feature of Type 2 Diabetes. These amyloid fibrils are thought to be cytotoxic to the beta cells of the pancreas, which leads to the death of the beta cells. Insulin resistance and hypertension are symptoms associated with syndrome X, also called metabolic syndrome.[2]

1.2. What is Amylin?

Amylin is also known as islet amyloid polypeptide. Islet amyloid polypeptide (IAPP) is present in the β-cells of the islets of Langerhans of the pancreas. This hormone is co-synthesized and co-secreted along with insulin (approximately 1:100 ratio) in the β-cells of the pancreas.[3] Thus it acts as the synergistic partner to insulin in inhibiting the appearance of glucose in the plasma.[4] It is a 37 amino acid long chain that selectively inhibits insulin stimulated glucose utilization and glycogen deposition in muscles, but it does not affect adipocyte glucose metabolism.[5] Amylin fibers or islet amyloid polypeptide fibers are present in the islets of pancreatic cells of patients suffering from Type 2 Diabetes.[6] Similar protein fibers are associated with Alzheimer's disease and Prion diseases.[1]

1.3. Genes for Amylin

Synonyms for the amylin gene are DAP and IAPP. The human amylin gene is located on chromosome 12 of the genome[7] (Entrez Gene ID: 3375). It consists of three exons, two introns, a TATA-box, a CCAAT-sequence and a GT-element.[8] Its mRNA consists of 1482 base pairs. The Refseq mRNA ID is NM_00415.1. The Blast n results for Human amylin against refseq_rna got 111 blast hits and 98% identities for Chimpanzee (XM_001144800.1), 77% identities for Dog (NM_001003233.1), and 80% identities for Cat (NM_001043338.1).

The amylin precursor protein consists of 89 amino acid residues (NP_000406.1). Its structure is closely related to the calcitonin family proteins (PF00214). It has a disulfide bond between positions 35 and 40 and a proteolytic cleavage site at the 73rd position.[9] According to the Homologene, the protein is closely related to amylin of Chimpanzee (GeneID: 741937). The Clustal w showed that amylin peptide is not exactly the same in all mammalian species.[10] The active part of amylin is different from species to species.[11] Comparison of the similarities between IAPP and the P fam family (Accession number: PF0024) peptide sequences shows that these peptides may bind to structurally related receptors.[12]

1.4. Amylin/ hIAPP Function

Amylin's function is glycemic control in the body. Insulin lowers the blood glucose levels, and glucagon increases the blood glucose level in the plasma.[13] Amylin supports the insulin hormone from the inhibition of blood glucose levels' appearance by slow degradation in the body.[14] Amylin mainly stops glucagon secretions during hyperglycemia and therefore reduces the total insulin demand.[15]

1.5. *Amylin role in Diabetes Mellitus*

Amylin has been isolated from pancreatic amyloid fibrils in patients suffering with Diabetes Mellitus 2 and insulinomas.[16] In the initial stage of the disease, the body becomes insensitive to insulin, and in the later stages of the disease the degradation of the beta cells increases and leads to the decreased production of insulin in the body. However, the exact mechanism involved in amylin causing Diabetes Mellitus 2 is unknown. A specific immune reactivity of antibodies to IAPP has been found in amyloid plaques and in the islets of Langerhans.[17] Amylin forms amyloid fibers in beta islet cells and induces the death of beta cells.[17] This results in the reduced production of insulin and causes Diabetes Mellitus.[18]

1.6. *Active regions in the hIAPP*

Figure1 a) shows the full length of the human IAPP sequence and 1b) shows the full length of the rat IAPP sequence with the three proline amino acid residues at the 25^{th}, 28^{th}, and 29^{th} positions. Human IAPP (hIAPP) and rat IAPP (rIAPP) are very similar except for a few amino acids. The hIAPP forms amyloid fibrils, but rIAPP cannot form amyloid fibrils due to presence of the three proline residues in its sequence. The residues from 20 to 29 are the active region for the formation of the fibril because this region initially will form the anti-parallel beta pleated sheets that result in amyloid fibril formation. The 20 – 29 region of this polypeptide is therefore critical for the formation of the fibrils. Any change in the sequence or replacement of amino acids from 20 to 29 residues might help in the reduction of fibril formation. The other potential regions that might affect the amyloigenicity, apart from 20 to 29, are the 30 to 37 and 8 to 20 regions. These regions allow the intramolecular beta sheet formation in hIAPP but not in rIAPP due to the presence of the proline amino acids in the rat form.

a) Human/hIAPP$_{1-37}$: $^{+}H_3N$-KCNTATCAT QRLANFLVHS SNNFGAILSS TNVGSNTY-$CONH_2$

b) Rat/rIAPP$_{1-37}$: $^{+}H_3N$-KCNTATCAT QRLANFLVRS SNNLG**PV**L**PP** TNVGSNTY-$CONH_2$

Figure 1: The amino acid sequence of human and rat IAPP are illustrated.

1.7. *The Three Dimensional Structure of Amylin*

Previous studies suggest that hIAPP exhibits a random coil structure with small components of α- helical nature, which then transforms to β sheet fibrils upon interaction with the cell membrane. A recent study confirmed that soluble hIAPP has mainly an unordered backbone structure. Oligomers of hIAPP represent intermediates in the path of fibril formation. These also contain α- helical structures. The three-dimensional structure of the hIAPP has been studied with high resolution techniques such as electron microscopy, X-ray diffraction and electron diffraction. These studies clearly reveal that hIAPP contains a significant amount of well ordered cross β-structures. During fibril formation, hIAPP initially undergoes a change from a random coil to a mixture of β sheet and α- helical structure conformations.[18]

1.8. *Importance of Presence of Proline Residues*

The comparison of the human IAPP sequence and the rat IAPP sequence within the 20 - 29 region shows the difference to be the presence of three proline residues in the rat IAPP sequence. Some studies suggest that the presence of proline residues in the rat IAPP sequence destabilizes the formation of the beta sheets so that the rat cannot develop amyloid fibrils.

1.9. *Role of Histidine in Amyloid Formation*

The hIAPP contains a single Histidine residue at the 18th position, and its protonation state depends on pH, as it changes from the intragranular to extra granular part of the beta cells. The aggregation of amylin monomers is considerably slower at the lower pH of 4 than the higher pH of 8.8 of the beta cells.[18] The rate of aggregation of the fibrils increases drastically at the lower pH in the presence of salt. The single Histidine amino acid residue is protonated at the lower pH 4. This protonated state can affect beta sheet folding as well as reduce zinc binding. Since Zn is positively charged, protonation would decrease the Zn binding, whereas the deprotonated form at the higher pH 8.8 can interact with the metal ion. At this point, the unstructured monomers tend to form the beta sheet structure of the fibril. Above the pKa value of the histidine, the hAIPP readily aggregates to form amyloid while the aggregation is slower at lower pH.[18]

1.10. *Effect of pH and Calcium Concentrations*

pH is the important factor for maintaining the active forms of the hIAPP and insulin in the beta cells. If there is a change in the pH of the beta cells, then it will lead to fibril formation of the hIAPP. Islet amyloid may also form due to an inability to clear the IAPP from the beta cells. A change in the pH in the beta cells also results in fibrillogenesis. In a medium of acidic pH, amylin is in the active form, and if there is an increase in pH, such as to 7.4 in the extracellular region, then there is greater possibility for the formation of the fibrils in the beta cells. These hIAPP fibrils allow the entry of an excess concentration of extracellular calcium ions, which leads to oxidative stress and apoptosis of the beta cells.[19]

1.11. *Steps Involved in the Formation of the Amyloid Fibrils*

Figure 2 explains the steps in the formation of the amyloid fibrils. This figure is adapted from reference 18.

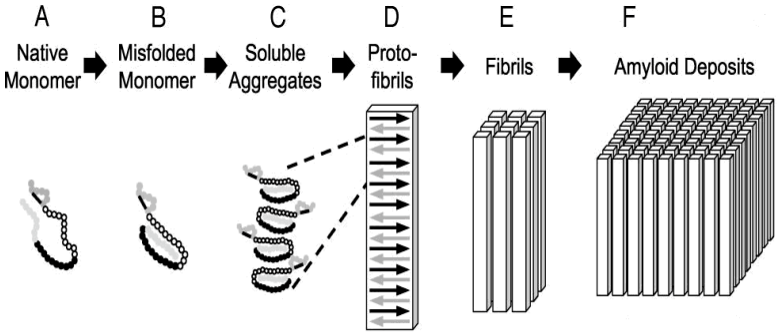

Figure 2: Steps involved in the formation of the Amyloid Fibrils are illustrated.

Initially, the single native monomers [A] of the IAPP misfold and form the misfolded monomers [B], and these misfolded monomers cling to each other and form aggregates [C]. These aggregates align parallel to one another and form a beta sheet formation. This results in the formation of the protofibrils [D] and conversion of the protofibrils into fibrils [E]. Subsequently, more fibrils accumulate to form amyloid deposits [F].

1.12. *Possible Types of Lipid Membrane Disruptions Caused by the Peptides*

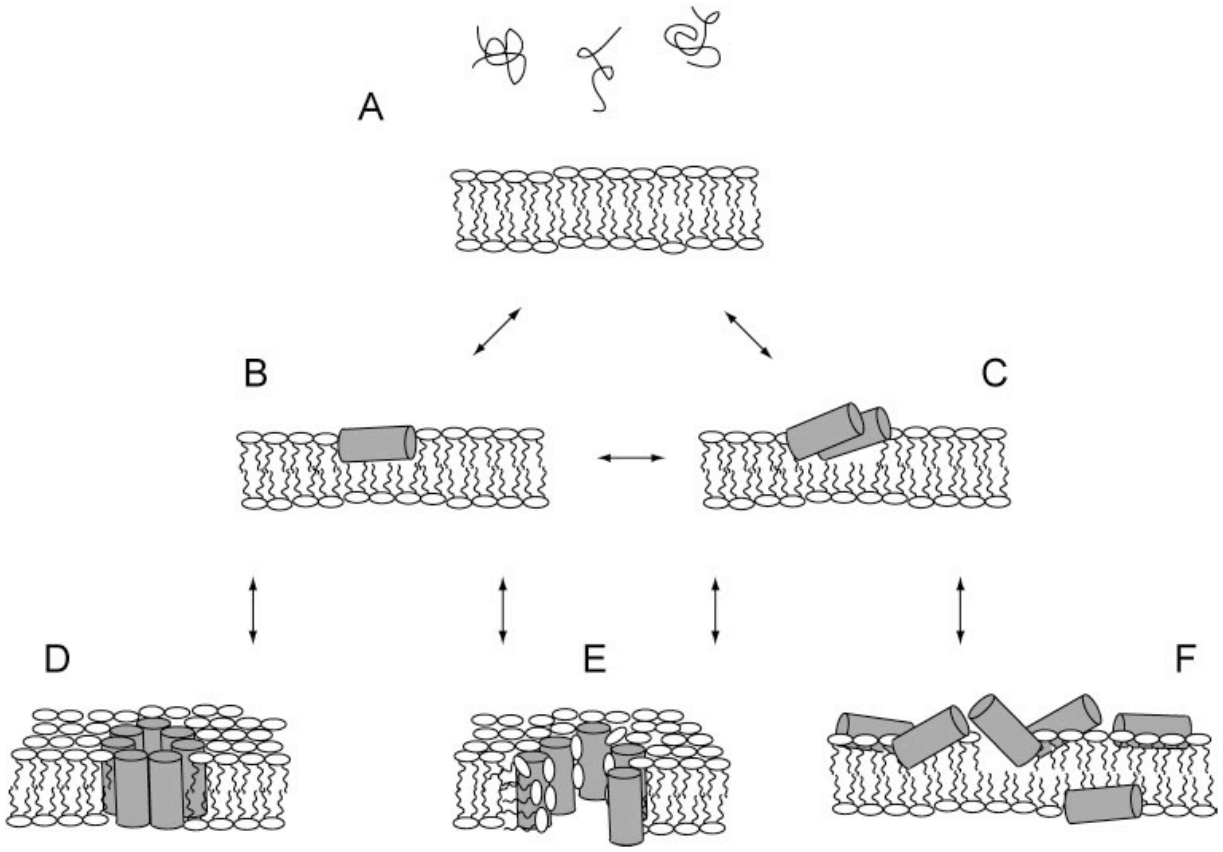

Figure 3: Types of peptide and lipid membrane interactions and possible membrane disruption mechanisms.

This figure is adapted from reference [3]. An overview of the general mechanisms of lipid membrane disruption is as follows. [A] The unstructured peptides approach the lipid membrane. [B] The single monomer folds and attaches to the lipid bi layer membrane. [C] More monomers join the peptide on the membrane surface. [D] The "Barrel-stave model" is forming a pore across the membrane. [E] The "Toroidol wormhole" is where the peptides and lipids are aligning in the pore of the lipid membrane. [F] The "Detergent-like" or "carpet model" is where the high

concentration of the peptides are on the surface of the lipid, resulting in the disruption of the lipid membrane.

In general, peptides permeabilize lipid membranes by any of three types of possible mechanisms. At higher concentrations, peptides may self-assemble, which results in the formation of the fibrils. The fibrils approach the lipid membranes and disrupt in any one of the three mechanisms. The first one is the "Barrel-stave model" in which the peptides form transmembrane pores[2] [Fig 3D]. The second one is the "toroidal wormhole" model in which the peptides bind to the parallel surfaces of the lipid membranes and create positive curvatures along the membranes. This results in the formation of pores. Then the lipid and fibrils align inside the pores of the membranes and cause the destabilization in the membranes [Fig 3E]. The third one is called the "carpet model" or "detergent-like" model where the peptides reside on the surface of the membranes and use the diffusion principle, pulling apart the phospholipids, resulting in the disruption [Fig 3F] .[18]

1.13. *The Process of Membrane Disruption by hIAPP*

The anionic lipids in the phospholipid membranes are more attractive regions for the attachment of the hAIPP amyloid fibrils. The positively charged N-terminus of hIAPP is attracted to the negative membrane. The hydrophobic nature of the hIAPP allows it to aggregate with other molecules of itself, and this results in the accumulation. Subsequently, the nucleation of the peptide results in the leakage of the contents of the beta cells, due to alteration of both membrane structure and function, and finally leads to the cell's death.

1.14. *Antagonistic Nature of Insulin in the Fiber Formation*

Insulin has an A chain and B chain in its structure. The B chain is the important region in the inhibition of the amyloid formation. Insulin is a 51 amino acid residue, which can regulate the body glucose levels and is a potent inhibitor of the IAPP fiber formation. Previous studies indicate that stoichiometric quantities of insulin can inhibit the formation of the IAPP fibers. Insulin may bind to a recognition site on hIAPP, interfering with its ability to self-interact to form fibers.[21]

1.15. *Research Goals and Objectives*

Type 2 Diabetes Mellitus affects 150 million people worldwide and has become a leading global health risk in the 21st century. Diabetes is at epidemic proportions and continues to augment globally. It needs urgent attention to suppress its dominance throughout the world. The pathology of Type 2 Diabetes Mellitus is the deposition of the amyloid deposits in the beta cells of the pancreas. These deposits are found in the beta cells of 90% of Type 2 Diabetic patients after their death. Furthermore, it has been assumed that there is a relationship between the hIAPP fibril formation and development of the Type 2 diabetes. The link between hIAPP fibril formation and beta cell death have led to increased interest in the aggregation of the amylin and in the mechanism of fibril formation. Insulin is co-synthesized and co-secreted along with hIAPP in the beta cells of the pancreas, but it antagonizes the property of fibril formation in the beta cells of the healthy individual. In fact, its presence probably helps suppress fibril formation within the secretory vesicles. Insulin has two chains, the A chain and the B chain, which attach to each other by disulfide bonds at the cysteine residues located at 2nd and 7th positions. [20] The B chain of the insulin acts as a potent inhibitor of hIAPP oligomerization and fibril formation.

According to Gilead et al., a decapeptide within insulin, HLVEALYLV, is considered to be the core region of insulin which can make contact with the 10-19 region of hIAPP and prevent fibril formation.[21]

In this research, the recognition region of the natural inhibitor insulin was used to design smaller analogs in an attempt to slow the fiber formation and membrane damage. We synthesized both insulin analogs, HLVEALYLV and the truncated LVEALYLV, in the laboratory using an automated PS3 peptide synthesizer. These peptides were cleaved from the solid support resin and purified, and the peptide masses were confirmed by using mass spectroscopy. Shorter sequences are economically and synthetically advantageous and may serve as a starting point for the design of small molecules that inhibit hIAPP toxicity.

The inhibitory studies of the insulin-based peptides on hIAPP analogs in lipid membrane models were then determined by using a dye leakage assay method. The intensity of the disruption caused by the active hIAPP peptides on the membrane model is directly proportional to the measured fluorescence intensity of the dye released from preformed liposomes.

2. Experimental Methods

The insulin analogs, HLVEALYLV and LVEALYLV, were prepared using solid phase peptide synthesis by an automated peptide PS3 synthesizer. The single coupling method was used for the formation of the peptide bonds of the desired amino acid sequence. All of the solvents used for the synthesis of the peptides were ACS grade, and Fmoc protected amino acids were purchased from Midwest Biotech, Inc., and Protein Technologies, Inc. The N, N-dimethylformamide (DMF) and 20% piperidine solution (v/v) in DMF, O- (Benzo-triazol-1-yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate (HBTU) and N, N-diisopropylethylamine in DMF were purchased from Fischer Scientific. The target peptides were purified from branched peptides and other impurities using reverse phase high performance liquid chromatography (RP-HPLC). The purity of the peptides was confirmed by using analytical HPLC, and molecular weights by electrospray mass spectrometry. The performance of the peptides on the single layered vesicles was tested using a dye-leakage assay method with the help of a FLx 800 Fluorescence Microplate Reader with KC4 software.

2.1. Solid Phase Peptide Synthesis

Solid Phase Peptide Synthesis (SPPS) was developed by R. B. Merrifield for the synthesis of peptides and small proteins using chemicals in the laboratory. [22] Two major forms of peptide synthesis use SPPS. The Fmoc (fluorenylmethyloxycarbonyl) method utilizes a base liable alpha-amino protecting group, and the t-Boc (t-butyloxycarbonyl) method uses an acid liable protecting group.[22] Of these two methods, the Fmoc method has a higher yield and produces purer peptides. The general principle involved in solid phase peptide synthesis is that one end of the peptide is

attached to the solid support resin, and the desired sequence of N- α amino acids is built backward from the C-terminus. After the synthesis of the peptide sequence, the solid resin support can be detached from the peptide using the cleavage procedure. An outline of the procedure can be seen in Figure 4.

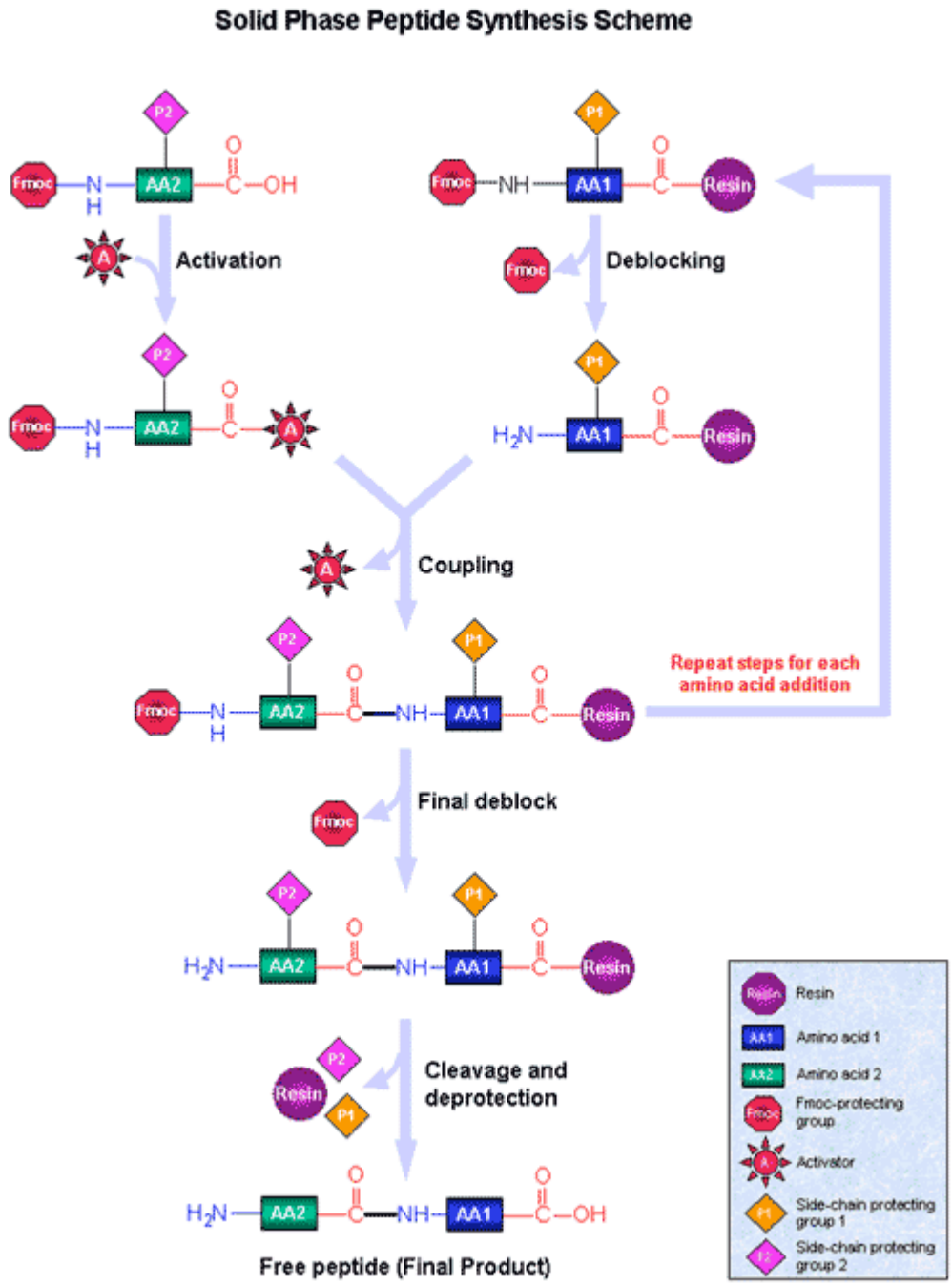

Figure 4: Solid Phase peptide synthesis scheme. This figure is adapted from reference [23].

The general principle involved in SPPS is repeated coupling and deprotection of the amino acids. The free N-terminal amine of the solid phase peptide growing chain is coupled to

the single incoming N-protected amino acid to form an amide bond. A basic reagent (piperidine) then causes it to be deprotected again so it is ready for the next coupling. A 20% piperidine solution (v/v) in DMF was used for deprotection. O-(Benzotriazol-1-yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate (HBTU) was used as a coupling agent, activated by 0.4 M N, N–diisopropylethylamine in DMF.[6] DMF is used to wash the peptide-resin between coupling and deprotection steps. A diagram of the peptide synthesizer is shown in Figure 5.

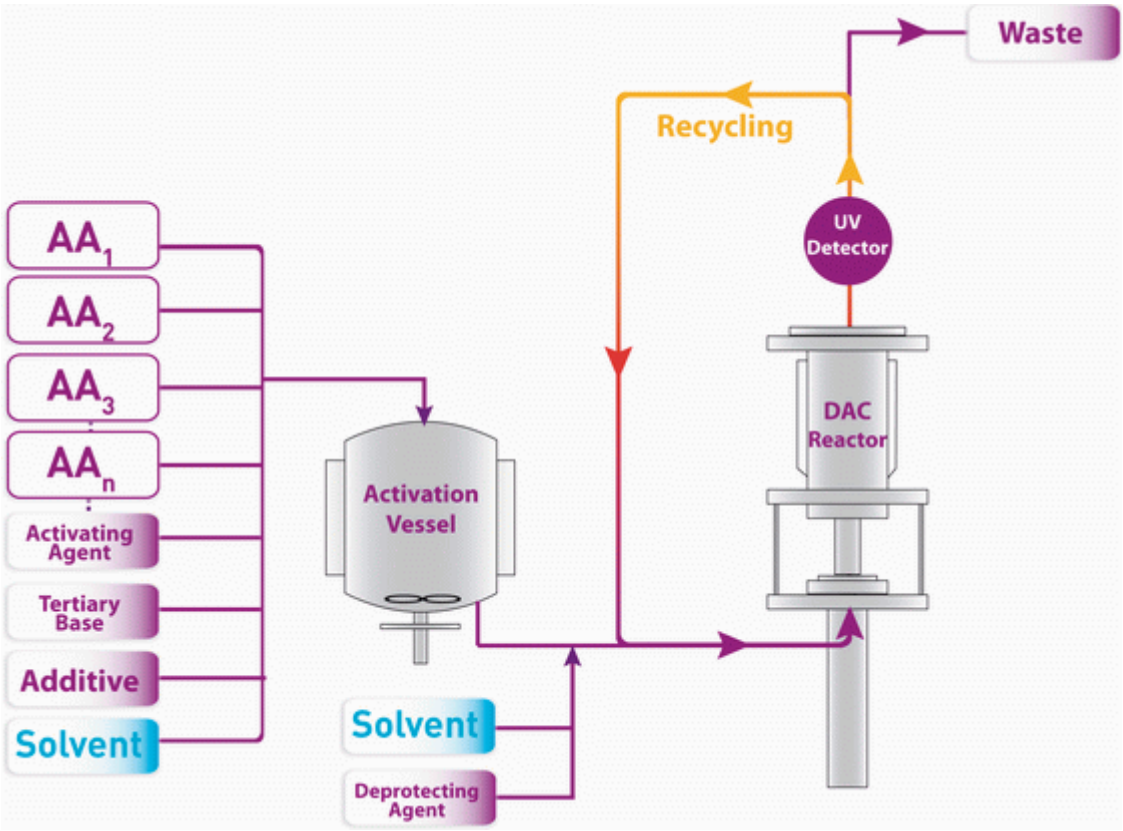

Figure 5: Diagram of the Automated Solid Phase Peptide Synthesizer.

This figure is adapted from reference [24].

2.2. Synthesis of Insulin Analogs (HLVEALYLV and LVEALYLV)

The amino acids in the sequence (0.4 mmoles each) were accurately weighed and transferred to the respective vials. Then HBTU 0.152g (0.4mmoles) was added to each vial. The respective vials were placed in the carousel beginning with the C- terminal end amino acid, proceeding to the N- terminal position. Then 0.16g (0.1mmole) of MBHA (methyl benzhydryl amine) resin was weighed and transferred to the reaction vessel. The PS3 was programmed to complete the sequence, and synthesis of the peptide was continued to produce the desired peptide sequence in the reaction vessel. Once the synthesis was completed, the peptide resin was taken out of the reaction vessel and subjected to cleavage.

2.3. *Peptide Cleavage*

The peptide-resin was washed off using DMF and methylene chloride, and dried under the vacuum line for approximately one hour. The cleavage cocktail was prepared in a 50 ml beaker in an ice bath by adding 0.5 ml of water, 0.5 ml of anisole, a small amount of phenol crystals, and 10 ml of trifluoroacetic acid. The resin-peptide was transferred to the cocktail contained in the beaker, and it was subjected to stirring for approximately 2 hours at room temperature. The cleavage mixture was transferred to a coarse fritted glass funnel, removing the now uncharged resin, and the peptide solution was collected in a side-armed flask. The collected peptide solution was mixed with 50 ml cold diethyl ether, causing the peptide to precipitate from the solution. The precipitated peptide solution was slowly filtered through the fine fritted glass funnel using the vacuum line. The filtered peptide was dried for a few minutes and was easily scraped out from the funnel using a spatula. The collected peptide was then transferred to a lyophilization flask containing a 70% acetonitrile and 30% water mixture and dissolved. Next an

equal volume of distilled water was added to dilute the solution. Then the flask was frozen by being kept in the -80^{o} C freezer for approximately an hour and then subjected to lyophilization overnight.

2.4. *Peptide Purification and Analysis*

For the purification of the peptide, reversed phase HPLC was used. Reversed phase HPLC works based on the hydrophobic nature of the peptides. The solvents used were 0.1% TFA-water and 0.1% TFA-acetonitrile. The solutions were mixed and eluted in a linear gradient through a C18 column. The crude peptides were purified by preparative reversed-phase high performance liquid chromatography (RP-HPLC) on a Waters (Milford, MA) instrument. A Phenomenex Jupiter column (C18, 10µm, 250 x 21.20 mm, 300Å) was used to obtain purified peptide from the impurities. The mobile phase solvents were made to run with a linear gradient of 10-50% organic component over two hours, having a 10ml/min flow rate. The crude peptide was weighed to check the mass and then dissolved in trifluoroacetic acid. Then the peptide was injected into the HPLC. The pure peptide was collected in test tubes based on the detection of the absorbance at 254nm, and tubes containing pure peptide (as monitored by UV absorbance) were combined, frozen, and lyophilized.

2.5. *Analytical HPLC*

The purity of the peptides was determined using analytical RP-HPLC with a mobile phase made up of 0.1% TFA-water and 0.1% TFA-acetonitrile solutions on a Phenomenex Jupiter column (C18, 5µm, 250 x 4.6 mm) with a flow rate of 1ml/min. The purity was measured

for both peptides, HLVEALYLV and LVEALYLV, and found to be ≥ 95% by peak integration. The molecular weight of the peptides was found using electrospray mass spectrometry. This confirmed correct molecular weights for HLVEALYLV and LVEALYLV as 1055.3Da and 918.1 Da, respectively.

2.6. Preparation of Lipid Unilamellar Vesicles and Dye Leakage Assay

The materials used were carboxyfluorescein dye (purchased from Sigma-Aldrich), DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) and DOPS (1,2-dioleoyl-sn-glycero-3-(phospho-L-serine)). These lipids were purchased from Avanti Polar Lipids, and structures are shown in figures 6-7. Triton-X was used as a detergent (purchased from Sigma-Aldrich).

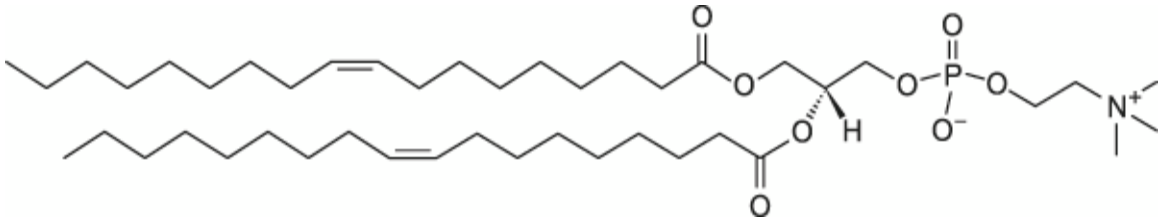

Figure 6: Structure of DOPC[26] (a neutral lipid)

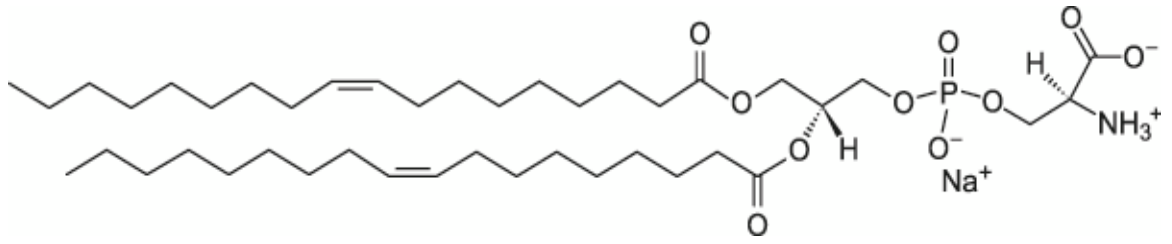

Figure 7: Structure of DOPS [27](a negatively charged lipid)

The dye-lipid vesicles were prepared by entrapping the carboxyfluorescein dye in the single layered lipid vesicles. For this preparation, 5mg of a 7:3 mixture of DOPC: DOPS were taken in test tubes and mixed with 2ml of chloroform. After 10 minutes, the chloroform was evaporated from the test tubes using nitrogen gas, and the test tubes were placed in a vacuum dessicator overnight.

These dried lipids could be stored in the freezer for long periods of time. When needed for the assay, the lipids were removed from the freezer and thawed at room temperature for about 30 minutes prior to the assay. Then 5mg of the carboxyfluorescein dye, which was dissolved in 500 µL of sodium phosphate buffer at pH 7.5, was mixed with the dried lipid. The whole solution was vortexed for approximately one minute to mix all the components of the solution. Then the solution was subjected to freeze and thaw cycles for 5 times successively using dry ice/acetone. The thawed solution was extruded 21 times to cause vesicles to become a uniform size using a Mini-extruder (Figure 8) (purchased from Avanti Polar Lipids). The extrusion process was difficult due to the small pore size (0.1 micron) of the polycarbonate filters. Once the extrusion process was completed, the solution was transferred into a Sephadex G50 gel exclusion column.[9] Gel filtration chromatography separates the lipid vesicles in solution based on

size in order for the dye-containing lipid vesicles to separate and elute from the remaining free dye. The first small band of yellow color was collected in a separate container and saved for the dye leakage assay.

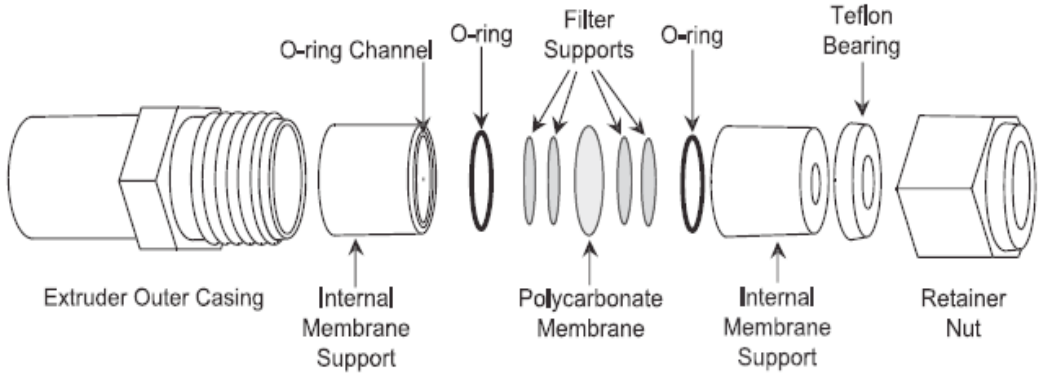

Figure 8: Mini extruder used for the size uniformity of the vesicles.

The insulin analogs (HLVEALYLV or LVEALYLV) were weighed to 1.4 mg and dissolved in 350 µL of dimethyl sulfoxide (DMSO) solvent, then sonicated for approximately one minute. In the same way, the hIAPP peptide analog (1mg) was weighed and dissolved in 350 µL of dimethyl sulfoxide (DMSO) solvent to make a stock solution. The other solutions used for the assay were pH 7.5 sodium phosphate buffer, and Triton X-100, a detergent solution, which was used as the positive control to obtain 100% dye leakage from the lipid vesicles.

To study the effect of hIAPP itself, the sample test tubes concentrations are shown in Table 1.

Table 1: Volume of Components in Sample Tubes of hIAPP Alone

Trial	**Sodium phosphate buffer (µL)**	**hIAPP (µL)**	**Lipid vesicles (µL)**	**Concentration of hIAPP (µM)**
1	1441	1	20	0.5
2	1464	2.5	20	1.2
3	1489	5	20	2.4
4	1465	10	20	4.8
5	1449	12.5	20	6
6	1451.5	25	20	12
7	1479	37.5	20	18
8	1483.5	50	20	24

To study the effects of variable amounts of insulin/analog with constant hIAPP, concentrations are shown in Table 2.

Table 2: Insulin/Analog Combined Activity Assay; Amylin conc. 10 μM.

Trial	**Buffer (μL)**	**hIAPP (μL)**	**Insulin/ Analog (μL)**	**Lipid vesicles (μL)**	**Conc. Insulin (μM)**	**Ratio of Insulin: hIAPP**
0	1475	5	0	20	0	N/A
1	1474	5	1	20	0.5	1:5
2	1472.5	5	2.5	20	1.2	1:2
3	1470	5	5	20	2.4	1:1
4	1465	5	10	20	4.8	2:1
5	1462.5	5	12.5	20	6	2.5:1
6	1450	5	25	20	12	5:1
7	1437.5	5	37.5	20	18	7.5:1
8	1425	5	50	20	24	10:1

The volume of components in detergent and control tubes are shown in Table 3.

Table 3: Control and Detergent Tube Composition

Trial	hIAPP	Insulin	Vesicles	Buffer	DMSO	Detergent
Detergent	0	0	20	1390	50	40
Control	0	0	20	1430	50	0

2.7. Fluorescent Dye Leakage Assay

The general principle involved in the dye leakage assay was that membrane active peptides can cause the disruption of the vesicles, resulting in the leakage of the fluorescent dye from the vesicles. In turn, this increases the monitored fluorescence over time. A FLx 800 fluorescence micro-plate reader (BioTek Instruments) was used to analyze the studies of the leakage caused by the peptides in the lipid vesicles at an excitation of 485 nm and an emission of 528 nm over a period of 3 hours. The 7: 3 mixture of DOPC: DOPS lipid mimics the actual beta cell membrane composition and can be used as a model for pancreatic beta cells. The composition of assay tubes is listed in Tables 1-3. Samples were transferred to a 96-well plate for reading. Dye leakage is calculated by the equation:

Percent Fraction Leaked = [(Value-Control)/ (Detergent-Control)]*100

The percent fraction leaked (% fluorescence) was plotted against time for each concentration of each peptide to determine the percent membrane disruption. The hIAPP was held constant while the concentration of synthesized inhibitor was varied, with ratios of inhibitor to hIAPP ranging from 0.2:1 to 10:1.

3. Results and Discussion

The inhibitory ability of the insulin analogs was studied on the hIAPP-induced leakage on single layered vesicles. The 7:3 DOPC/DOPS lipid vesicles were used for the inhibitory studies. These vesicles imitate the somatic body cells and are commonly used in liposome models of membrane systems. DOPC is zwitterionic, while DOPS is negatively charged. The 7:3 mixture of DOPC: DOPS is considered a good model for pancreatic beta cell studies, with 30% negatively charged lipids.

The data in Figure 9 were gathered through a dye leakage assay showing the interaction between the β-cell mimics and twelve concentrations of hIAPP. Fluorescence values were taken every minute for three hours, and the graph was produced by using the previously described formula for percent leakage, plotted versus time for each concentration. It should be noted that membrane leakage increased over the time period observed, indicating the possibility of aggregate formation. It is clear that significant damage to the vesicles is caused by high concentrations of hIAPP as the percent leakage approaches 100% at 50 mM. Figure 10 was then produced by averaging the percent membrane disruption at each time point and plotting these values against concentration. This figure shows concentration dependent damage to the β-cell membrane mimics by hIAPP. A logarithmic regression is shown with a correlation coefficient of 0.87.

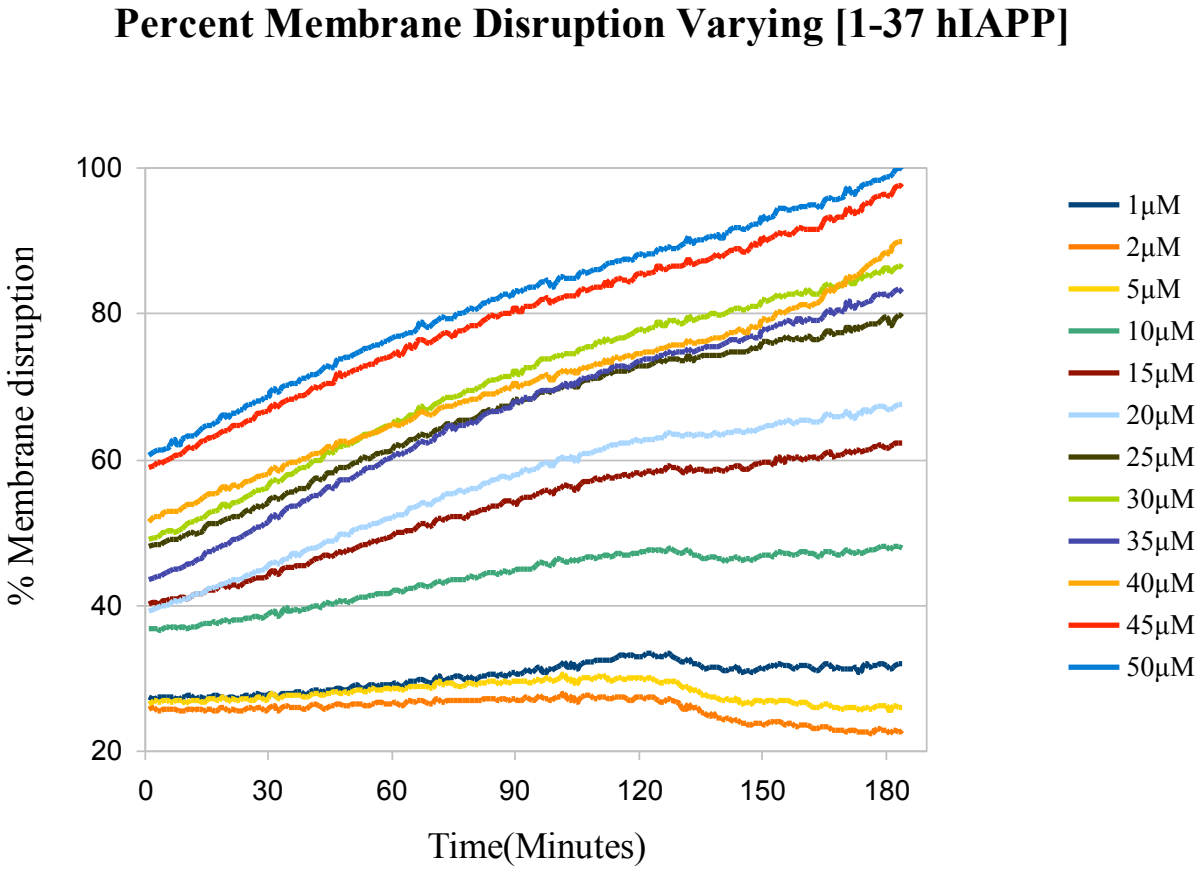

Figure 9: Dye leakage from 7:3 DOPC/DOPS vesicles by varying concentrations of hIAPP over time.

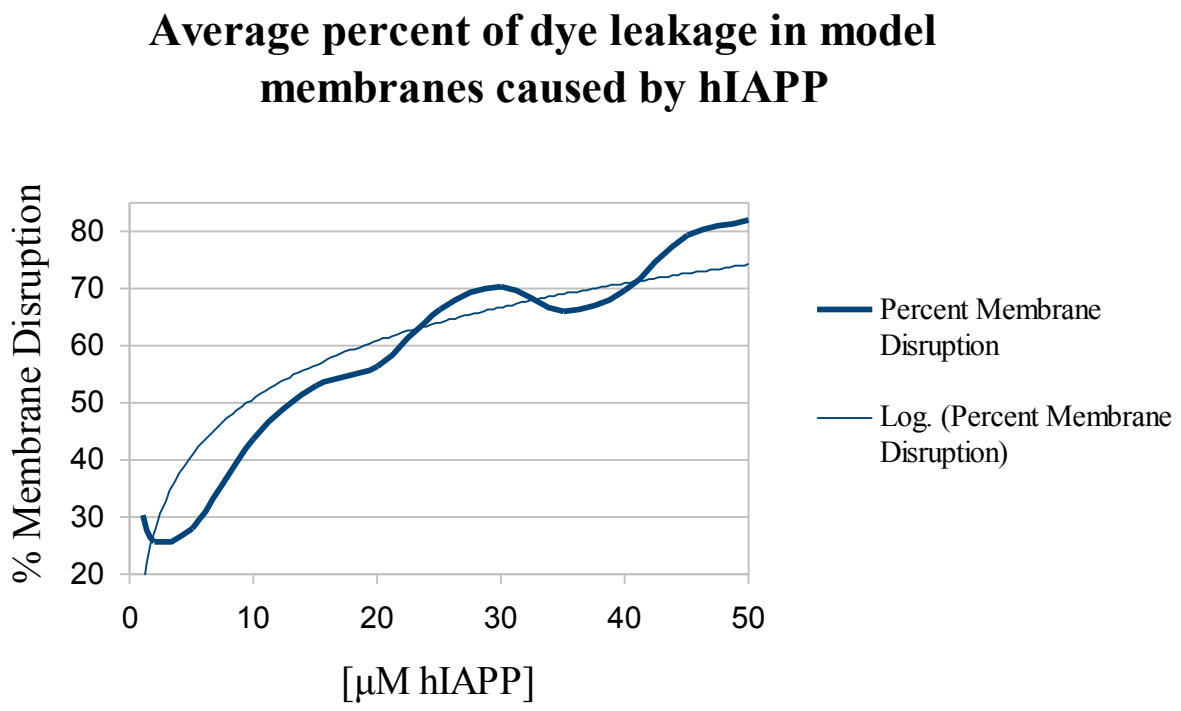

Figure 10: Average percent of dye leakage in model membranes caused by hIAPP.

The data displayed in Figure 11 and Figure 12 were gathered through a dye leakage assay where twelve concentrations of insulin ranging from 0 μM to 100 μM were run against a constant 10μM hIAPP concentration, and fluorescence readings were taken every minute for three hours. This concentration of hIAPP was chosen since it produced intermediate damage and was a reasonable starting point, where both increases and decreases in dye leakage could be noted. Figure 11 shows a variable effect by insulin, with no clear concentration dependence. It is notable that the 10 μM hIAPP itself (no insulin) showed a much greater time dependence for damage than the samples treated with insulin; the insulin seemed to have a greater effect as time elapsed, which could indicate its role in slowing time dependent fiber formation in the later phases of membrane damage. The plot in Figure 12 was produced by averaging the percent

membrane disruption over time at each insulin concentration (displayed as the ratio of the concentration of insulin to the concentration of hIAPP). It can be observed that the trial without any insulin produces the greatest membrane disruption. This confirms that insulin does inhibit membrane damage in this assay. However, the relationship between membrane disruption and the ratio of insulin to hIAPP is variable. A sharp spike can be noted at the ratio of 0.2[Insulin]:1[hIAPP]. This ratio maximizes the extent of inhibition induced by insulin. At this ratio, insulin inhibits membrane disruption by 13%. As the insulin: hIAPP ratio is increased beyond this point, membrane disruption quickly increases to varying degrees but never significantly surpasses the level of damage caused by hIAPP by itself.

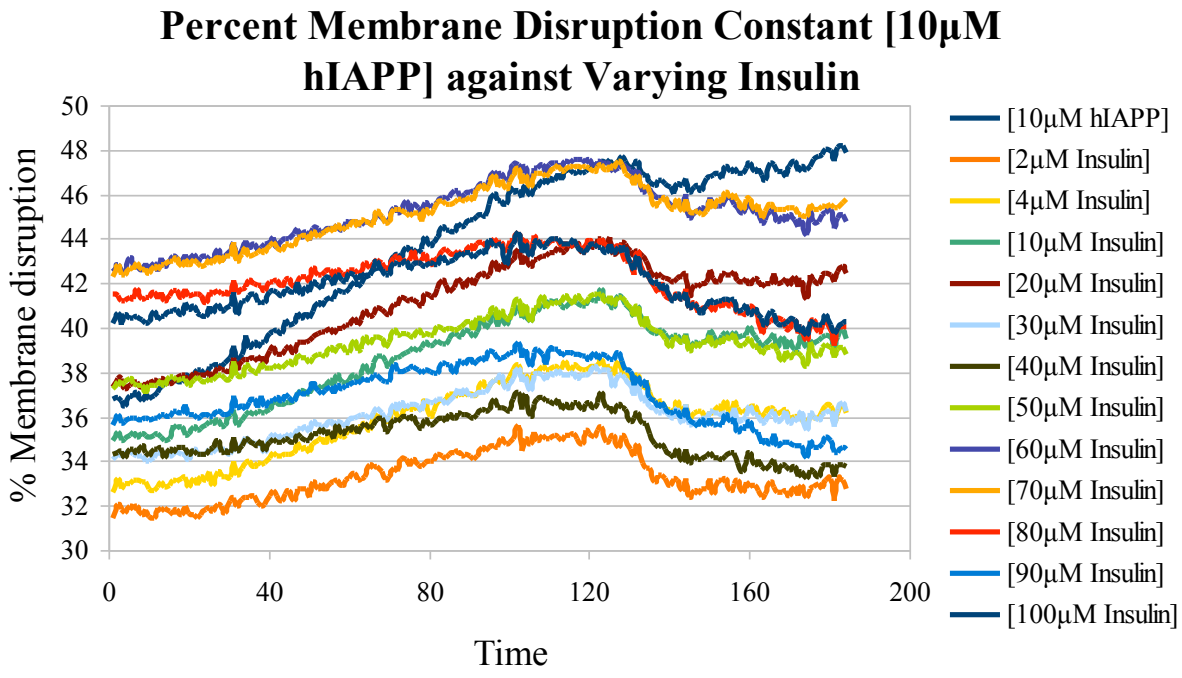

Figure 11: Percent of dye leakage from model 7:3 DOPC/DOPS liposomes in the presence of varying concentrations of insulin and 10 µM hIAPP over time.

One possible explanation for this observation is that hIAPP forms a five-unit complex to which one molecule of insulin binds. This six-unit complex is less toxic than free hIAPP, perhaps reducing its ability to interact with the membrane in a fully aggregated form. This result is conserved over many trials and is particularly interesting given that insulin is stored in secretory cells as a hexamer, so the six-unit complex may have some significance. However, even at the optimal ratio, membrane damage as measured by this assay is only reduced by ~15%. This suggests that membrane disruption may be mediated by more than simply hIAPP aggregation and fiber formation; perhaps it is caused by transient deformations in the acyl chain arrangements induced by interaction of the positively charged peptide with the overall negatively charged membrane surface.

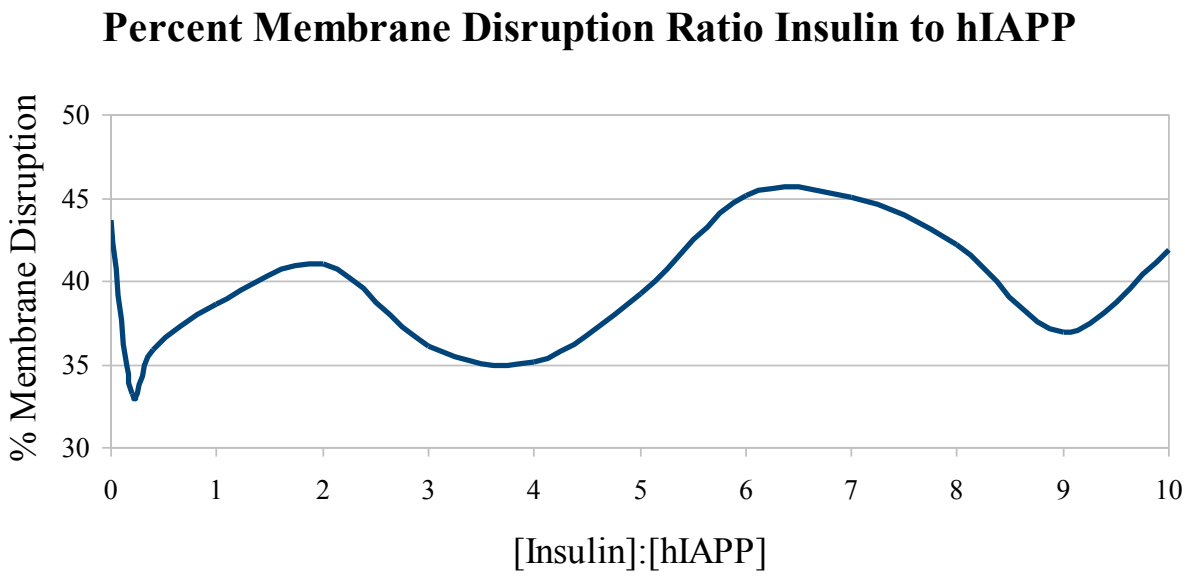

Figure 12: Average percent of dye leakage from model membranes in the presence of varying ratios of insulin to hIAPP.

Figure 13 shows the percent dye leakage from 7:3 DOPC/DOPS vesicles in the presence of 10 μM hIAPP and varying concentrations of the first inhibitor, HLVEALYLV, a sequence based on the contact area between insulin and hIAPP.

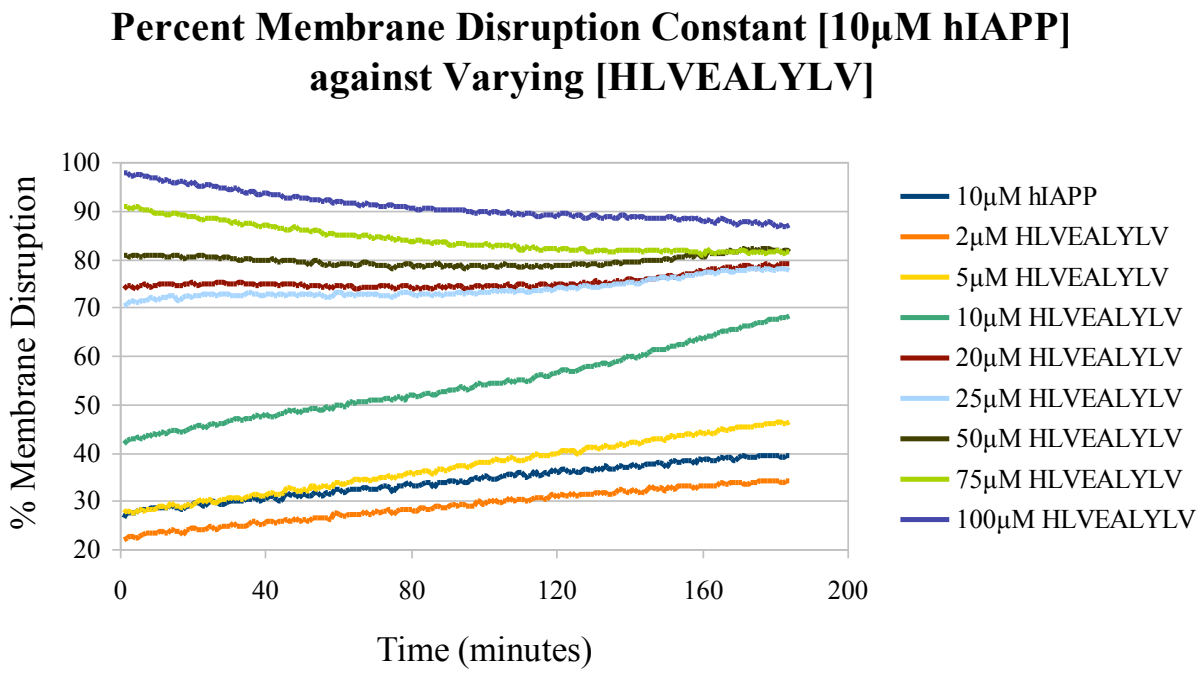

Figure 13: Percent of dye leakage from model 7:3 DOPC/DOPS liposomes in the presence of varying concentrations of HLVEALYLV and 10 μM hIAPP over time.

Concentrations of inhibitor peptide were varied from 0 to 100 μM. While general concentration dependence is visible, it can be observed that membrane damage actually increases, rather than decreases, in the presence of the inhibitor. The greater the concentration of HLVEALYV, the greater the dye leakage, ranging from about 30% with hIAPP alone to over 90% with hIAPP plus 100 μM peptide inhibitor. The decreasing damage over time for the two highest concentrations is unusual and probably due to experimental error. Overall, there is a

significant increase in dye leakage that indicates damage being caused by the synthesized peptide. Figure 14 shows a plot of the time-averaged dye leakage at each concentration versus the ratio of inhibitor peptide to hIAPP. From this graph, it is also clear that only the 0.2:1 ratio is effective in lowering membrane damage and that increasing peptide concentration actually enhances the dye leakage.

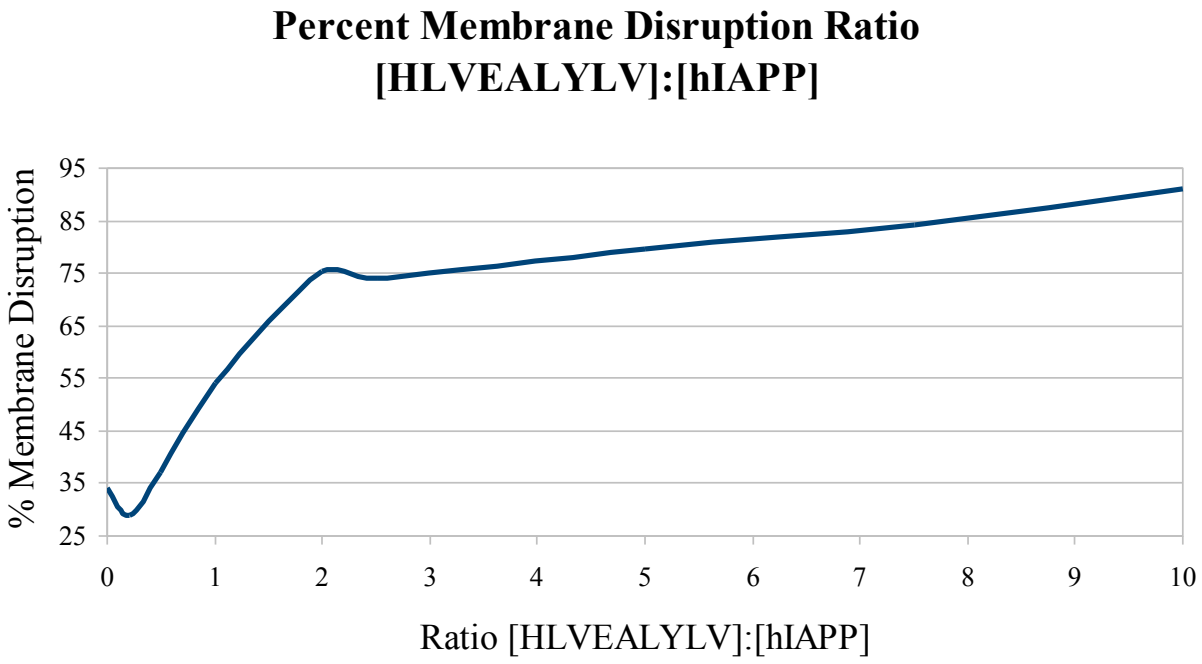

Figure 14: Average percent of dye leakage from model membranes in the presence of varying ratios of HVEALYLV to hIAPP.

This result is interesting because the same ratio is observed for maximum activity of HLVEALYV and insulin, possibly supporting the model of the six-unit complex. However, the reduction in dye leakage is only about 5%, which may be attributable to inherent error in the assay and fluorescence readings. In addition, the very definite increase in dye leakage associated with this peptide was not observed in the insulin trial.

Figure 15 shows the percent dye leakage from 7:3 DOPC/DOPS vesicles in the presence of 10 μM hIAPP and varying concentrations of the second inhibitor, LVEALYLV, which is truncated at the N-terminus relative to the first inhibitor.

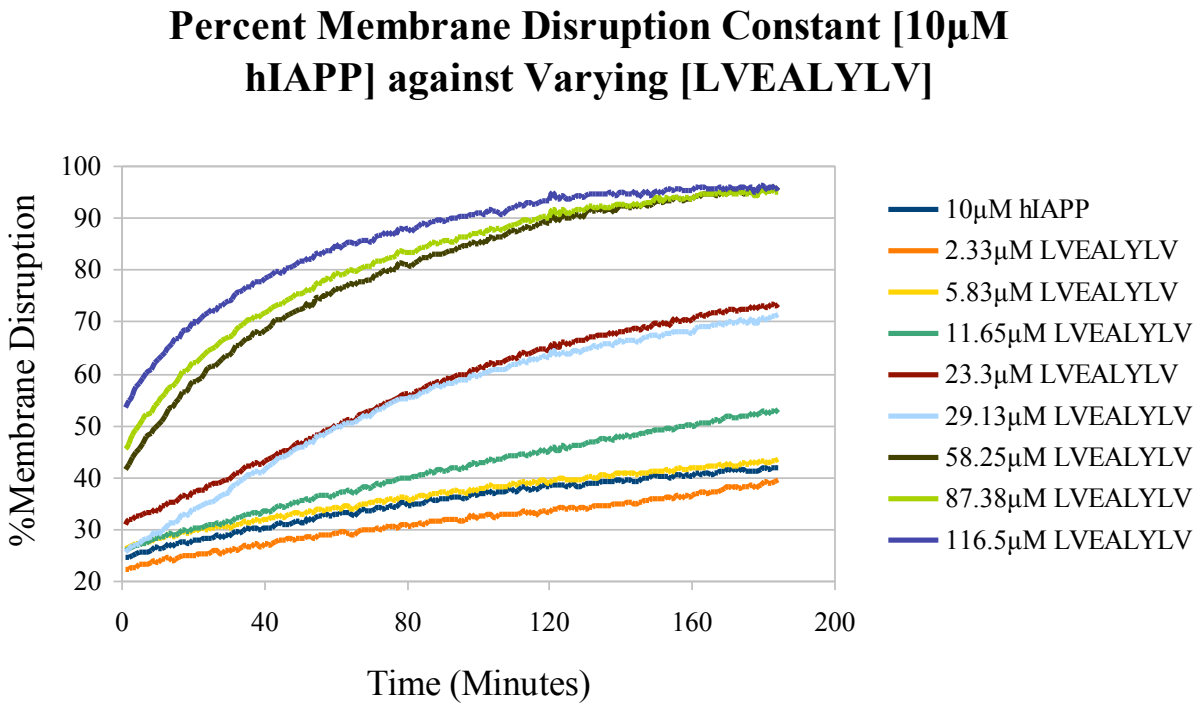

Figure 15: Percent of dye leakage from model 7:3 DOPC/DOPS liposomes in the presence of varying concentrations of LVEALYLV and 10 μM hIAPP over time.

This graph displays the same general trend as the longer inhibitor. As the concentration of LVEALYV increases, the damage to the vesicles increases. Except for the lowest concentration tested (2 μM), this peptide actually enhances the effect of hIAPP and is damaging to the vesicles. Once again, the average dye leakage over time was calculated and plotted against the ratio of inhibitor peptide to hIAPP, Figure 16. Again, only the 0.2:1 ratio is effective in protecting against damage, and only minimally so. All greater ratios cause a drastic increase

in liposome damage in a concentration dependent manner, from 35% at 0 μM LVEALYLV to almost 90% at >50 μM LVEALYV.

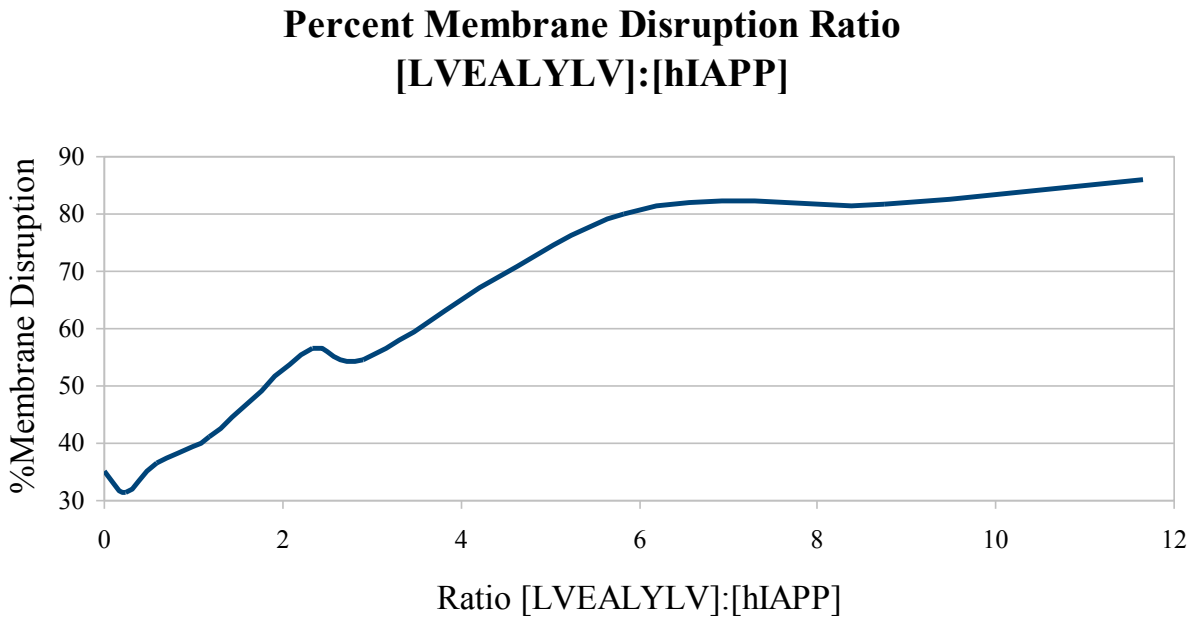

Figure 16: Average percent of dye leakage from model membranes in the presence of varying ratios of LVEALYLV to hIAPP.

Overall, the two synthesized peptides demonstrate very similar behavior, as expected, given their similar sequences. The smaller peptides, however, behave differently from insulin at ratios above 0.2:1 (inhibitor:hIAPP), increasing the damage to the liposomes. It is possible that, instead of inhibiting the aggregation of hIAPP, they actually enhance or seed the aggregation. In fact, they may co-aggregate with the hIAPP, as some researchers have reported for insulin[32]. The internal sequence of insulin at the binding interface (ALYLV) is very similar to that of hIAPP (ANFLV), so the small inhibitors may be acting unexpectedly as hIAPP analogs rather than insulin analogs.

4. Conclusion

The peptide insulin-based inhibitors HLVEALYLV and LVEALYLV were ineffective in stopping the membrane damage induced by hIAPP. Only the lowest concentrations tested, those at a 0.2:1 ratio of peptide inhibitor to hIAPP, showed inhibitory activity, and this was minimal. While insulin itself was capable of protecting the model vesicles to a small extent, the synthesized peptides actually enhanced the damage to the vesicles, with more dye leakage occurring at higher concentrations. Interestingly, both small analogs had optimal activity at the same ratio of inhibitor to hIAPP (0.2:1) as insulin, lending some support to a model of a hexamer between five units of hIAPP and one of insulin. However, it is likely that the synthesized peptides, which share some sequence homology with hIAPP at the binding interface, act more as analogs of hIAPP than of insulin, promoting hIAPP's aggregation rather than inhibiting it.

5. Future Studies

The next phase of this work will include a different assay that measures extent of aggregation rather than liposome damage to see whether they are correlated. Thioflavin T, a dye that fluoreses in the presence of aggregated peptides and fibers, can be used to study whether these synthesized peptides promote, inhibit, or have no effect on the fibrilization of hIAPP. This will shed more light on the mechanism behind the results obtained here. If aggregation is faster in the presence of synthesized peptide, that would indicate co-aggreagtion. If aggregation is actually slowed despite the increase in membrane damage, that would suggest that something more than just aggregation is occurring as the mechanism of membrane damage, possibly the formation of pores, disordering or puncturing of the membrane, or induction of curvature. Slowing hIAPP aggregation actually may prolong the presence of a cytotoxic species.

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