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Experimental simulation of human islet amyloid polypeptide (hIAPP)-pancreatic beta cell membrane interactions: Inferences and implications in the etiopathogenesis of diabetes mellitus type II

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Experimental Simulation of Human Islet Amyloid polypeptide (hIAPP)-Pancreatic Beta Cell Membrane Interactions: Inferences and Implications in the Etiopathogenesis of Diabetes mellitus Type II

Sarika Pamarthy
Experimental Simulation of Human Islet Amyloid polypeptide (hIAPP)-Pancreatic Beta Cell Membrane Interactions: Inferences and Implications in the Etiopathogenesis of Diabetes mellitus Type II

by

Sarika Pamarthy

Thesis

Submitted to the Department of Chemistry
Eastern Michigan University
In partial fulfillment of the requirements
For the degree of
MASTER OF SCIENCE
in Chemistry

Deborah Heyl-Clegg, PhD, Chair
April 17, 2012
Ypsilanti, Michigan
This thesis work is dedicated to
Sai Baba, the wirepuller and caretaker of all living creatures.
Experimental Simulation of Human Islet Amyloid polypeptide (hIAPP)-Pancreatic Beta Cell Membrane Interactions: Inferences and Implications in the Etiopathogenesis of Diabetes mellitus Type II

by

Sarika Pamarthy

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Committee Member

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Department Head

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Deborah de Laski-Smith, PhD Date

Dean of the Graduate School
ACKNOWLEDGEMENT

Writing my thesis, the journey: The timing of my research completion perfectly coincided with our scientific publication, providing a strong impetus with which I could lay down the initial framework of my thesis. I must admit the journey has been arduous, fraught with several dilemmas and painful moments. Time management during multitasking, critical analysis of our own work in the light of available literature, bridging theory and application, dealing with people both inside and outside our department, learning new research techniques, operating independently and as part of a team and most importantly writing, communication and presentation skills were several components that made this process extremely challenging but well worth the effort. In retrospect, I am thankful for the invaluable lessons and practical experience that I believe will propel me forward in the scientific arena.

First and foremost, I would like to thank Sai Baba, my spiritual master, for directing the course of my life as the great wirepuller. I feel very privileged to study a microcosm of HIS creation, the universal laboratory, through protein chemistry. I am thankful for Baba’s grace, which saw me through the completion of this project in HIS perfect time.

I would like to thank Dr. Deborah Heyl-Clegg from the bottom of my heart for her unwavering support as a mentor, both within and outside the lab. Right from the early days of my association with Dr. Heyl-Clegg, I never failed to receive undivided attention and guidance despite her busy schedules and innumerable students seeking her time.
She has always been extremely approachable, and her ever-pleasant disposition makes her an ideal role model worth emulating. I feel very fortunate to have been associated with her and wish well for her and her family.

I would like to gratefully acknowledge Dr. Timothy Brewer for being a wonderful graduate coordinator. After several semesters of interaction with Dr. Brewer, I feel extremely fortunate to have experienced his simplicity, fairness, and accommodating nature. I would also like to thank Dr. Brewer for accepting responsibility as one of my thesis committee members and for his critical review of my thesis work. I could not have asked for a better guide as an international student with little knowledge of graduate school dynamics in the U.S.

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I would like to highly commend the Halle Library resources and excellent staff who are ready to help often at odd hours of the day and night (!). The times spent at the library will not be forgotten.
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Abstract

Pancreatic beta cells secrete insulin, an endocrine hormone that regulates blood glucose levels and maintains normal physiological activity in humans and animals. Diabetes mellitus type II is a consequence of the gradual destruction of these important cells, likely by human islet amyloid polypeptide (hIAPP) that is co-secreted with insulin. Increasing health care costs, coupled with the World Health Organization’s prediction of a worldwide diabetic epidemic by year 2030, make experimental diabetes research a crucial prologue to future clinical trials in prevention, diagnosis, and treatment of Diabetes mellitus type II.

Our experimental set-up simulates hIAPP peptide fragment and pancreatic beta cell membrane interactions, and it uses density functional methods and circular dichroism spectroscopic analysis of the hIAPP molecule to uncover factors that initiate and promote progression of beta cell death. Results from our study establish the potential role of hIAPP and a two-step molecular mechanism of pancreatic beta cell damage in diabetes mellitus type II.
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1. Introduction

Diabetes mellitus: an overview and current statistics

1.1 Normal anatomy and physiology of the pancreas

The pancreas (Figure 1) is an important abdominal organ located in close relationship to the duodenum and small intestine in most animals, with exocrine and endocrine functions. The exocrine part of the pancreas produces enzymes, most importantly trypsin, amylase, and lipase, that aid digestion of proteins, carbohydrates, and fat in food within the gastrointestinal tract. The endocrine portion secretes multiple hormones directly into the blood stream, most importantly, insulin and glucagon to modulate blood glucose levels and maintain normal physiological activity in humans and animals (1).

Figure 1. Gross anatomy of the pancreas.
1.2 Cytoarchitecture of the pancreas

The “islets of Langerhans” are small clusters of cells that constitute the functional units of the endocrine pancreas. While a German pathological anatomist Paul Langerhans discovered these islets during his doctoral studies at the Berlin Pathological Institute in the year 1869, a French pathologist and histologist, Edouard Laguesse of Lille, named them the “islets of Langerhans” much later in 1893. Laguesse also postulated that these cells produced an internal secretion, which was named “isletin” after the islets of Langerhans and later changed to insulin in about 1923. Laguesse’s findings were considered to be a major step in the discovery of hormones in the early days of endocrinology (2,3).

A healthy adult human pancreas contains about one million islets of Langerhans, found in higher concentrations within the pancreatic tail region. The mass of the islets is approximately 1-1.5 grams, making up 1-2 % of the weight of the pancreas, which is roughly about 100 gm in a person weighing about 70 kg (4,5).

The “islets of Langerhans” contain multiple cell types including alpha, beta, delta, epsilon, and F cells, responsible for production of several peptide hormones: glucagon, insulin and amylin, somatostatin, pancreatic polypeptide, and ghrelin, respectively. These peptide molecules, most importantly insulin and glucagon, play a vital role in the tight regulation of blood glucose metabolism required for normal homeostasis. The histology of the normal pancreas can be seen in Figure 2.
1.3 Insulin and Amylin

Small quantities of Amylin or human Islet Amyloid Polypeptide (hIAPP) are co-secreted by the pancreatic beta cells with insulin, in an approximate ratio of 1:100. The presence of excess amounts of hIAPP, a 37 amino acid peptide, is implicated as an important etiological factor in pancreatic beta cell destruction.
The clinical disease Diabetes mellitus type II occurs as a terminal consequence of the gradual and irreversible destruction of these cells. This is a progressive disease associated with insufficient insulin production and insulin resistance. In the first phase of the disease, the body becomes insensitive to insulin, which may be partly compensated for by increased insulin secretion from pancreatic beta cells. In the second phase, the production of insulin declines as these insulin producing beta cells begin to die. Amyloid deposits in pancreatic islets have been commonly found in Diabetes mellitus type II patients.

1.4 History of Amyloid discovery and association with Diabetes mellitus.

Rudolph Virchow, a German physician scientist, introduced the term “amyloid” in 1854. Based on the pale blue staining of cerebral corpora amylacea on treatment with iodine, he concluded that the given substance was likely starch or cellulose (carbohydrate). He named it “amyloid”, derived from the Latin amylum and the Greek amylon. A few years later in 1859, after several chemical investigations, Carl Friedreich and August Kekule demonstrated two important things: the presence of protein in a “mass” of amyloid and the apparent absence of carbohydrate based on the high nitrogen content. Amyloid was then considered a protein and subsequently a class of proteins, with a propensity to undergo conformational changes resulting in fibril formation.

It was not until the year 1901 that Eugene L. Opie, a medical student, first discovered the association between hyaline material deposited in the pancreatic islets and diabetes. The histopathology of this hyaline material was ascertained as amyloid in 1943 and later confirmed by alkaline Congo red staining and electron microscopy identification of fibrillar structure (6,7,8).
1.5 hIAPP gene structure

The total size of the gene for hIAPP is 6599 base pairs (Entrez gene id: 3375). The precursor protein, “preprohIAPP,” contains 89 amino acids confirming its cellular secretion/origin (Refseq: NP_000406). Its mRNA is 1462 base pairs long and contain 3 exons (NM_000415). It is located on chromosome 12 (NC_000012.10) of the human genome, gene locus is 12p12.3-p12.1. The peptide is unique but shares common amino acids with calcitonin (9).

1.6 Diabetes mellitus: basic concepts

The lack of normal concentration of insulin in the bloodstream of a human or animal leads to a disease condition known as Diabetes mellitus. It is one of the most common chronic human metabolic diseases and is of two major types.

Diabetes mellitus type I: commonly referred to as insulin-dependent or childhood-onset diabetes. This subset has a younger age of onset and results from insufficient production of insulin (quantitative defect) and accounts for 5-10% of the cases. There is gradual destruction and decrease in number of pancreatic beta cells either directly by viral infections (EMC virus, Mengo virus 2T, Coxsackie B4 viruses) or autoimmune mechanisms.

Diabetes mellitus type II: commonly referred to as non-insulin-dependent or adult-onset diabetes. This subset has an older age of onset, is associated with genetic factors, obesity, and physical inactivity, and usually makes up 90-95% of the cases. These diabetogenic factors lead to increasingly worsening pancreatic beta cell defects and insulin resistance.
The initial decreased tissue responsiveness or resistance to normal amounts of secreted insulin paralleled by gradual destruction of pancreatic beta cells leads to an irreversible phase of insulin deficit (initial qualitative defect followed eventually by a quantitative deficit).

Figure 3. Brief mechanism of beta cell damage by hIAPP.

These two processes occur simultaneously, leading to ultimate beta cell failure, explained graphically in Figure 3. Pancreatic beta cell defects cause decreased insulin release, leading to gradual increase of blood glucose concentration (hyperglycemia) above normal physiologic levels. This hyperglycemia imposes an increased insulin demand on the pancreatic beta cells with coincidental increased release of amylin (hIAPP), normally cosecreted with insulin. As both these processes occur simultaneously, it leads to a gradual spiralling of the disease process, resulting in the clinical symptoms and physical manifestations of Diabetes mellitus.
The hIAPP polypeptide is postulated to be the causative factor in pancreatic beta cell damage and is associated with lowered insulin secretion in Diabetes mellitus type II. hIAPP has been identified in postmortem pancreatic islet specimens of Diabetes mellitus type II. In humans, amyloid is found more frequently in older diabetic patients, especially in severe diabetes requiring insulin therapy (10). hIAPP forms plaques via sequential fibril formation and aggregation, processes that are intimately linked to and hypothesized to cause simultaneous disruption of beta cell membranes leading to eventual cell death. The terminal consequence of this process is gradual beta cell destruction and decreasing pancreatic beta cell mass with subsequent total failure of insulin secretion due to irreversible pancreatic beta cell destruction. Interestingly, an insulin binding area has been identified on the hIAPP molecule, and it has been observed that normal levels of secreted insulin actually limit the beta cell damage caused by this peptide (11). In summary, gradual hIAPP deposition leading to islet amyloidosis can be considered both a cause and an effect of the disease process.

1.7 Amyloidosis and Amyloid associated diseases

In Diabetes mellitus type II and multiple other diseases, inflammatory responses responsible for the disease are postulated to occur either due to amyloid formation or misfolding of normal protein molecules (12,13). Table 1 below lists the disease and the respective protein implicated in each disease process. About 23 different subtypes of amyloid associated proteins have been described with symbols, A standing for amyloid and the following letter for the precursor protein, e.g. Aβ protein in Alzheimer's disease is Amyloid beta protein.
Table 1. Amyloid associated diseases and the implicated proteins.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes mellitus type II</td>
<td>Amylin or hIAPP</td>
</tr>
<tr>
<td>Alzheimer’s disease</td>
<td>Aβ protein</td>
</tr>
<tr>
<td>Parkinson’s disease</td>
<td>α Synuclein</td>
</tr>
<tr>
<td>Huntington’s disease</td>
<td>Huntingtin</td>
</tr>
<tr>
<td>Secondary systemic amyloidosis</td>
<td>Amyloid A</td>
</tr>
<tr>
<td>Senile systemic amyloidosis</td>
<td>Transthyretin</td>
</tr>
<tr>
<td>Creutzfeldt Jacob/ “Mad cow” disease</td>
<td>Prion protein</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>Immunoglobulin heavy chain</td>
</tr>
<tr>
<td>Hemodialysis related amyloidosis</td>
<td>Aβ2 Microglobulin</td>
</tr>
</tbody>
</table>

An underlying common mechanism of amyloid- and other protein-induced cell death in various organ systems of the body has been proposed and investigated by researchers. This would help identify a common etiological basis and unifying mechanism that would indirectly lead to the formulation of a common solution to multiple diseases.

In our study, exploration of the structural characteristics and activity of various hIAPP fragments would establish the potential role of the suspected disease causing regions within the molecule. Based on this information, crucial applications in prevention and treatment of this irreversible disease process can be formulated.
1.8 Diabetes mellitus: worldwide distribution and health care costs.

World Statistics on Diabetes mellitus:

Once thought of as a disease of affluent countries, the WHO and International Diabetes Federation now predict a “Diabetic epidemic” with globalization of the disease by the year 2030. The global and regional distribution of Diabetes is shown in Tables 2 and 3, respectively.

**Table 2. Global burden: Prevalence and Projections, 2010 and 2030:** This information was obtained from the Diabetes Atlas maintained by the International Diabetes Federation (IDF) (14).

<table>
<thead>
<tr>
<th></th>
<th>2010</th>
<th>2030</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total world population (billions)</td>
<td>7.0</td>
<td>8.4</td>
</tr>
<tr>
<td>Adult population (20-79 years, billions)</td>
<td>4.3</td>
<td>5.6</td>
</tr>
<tr>
<td>Diabetes and Impaired glucose tolerance (IGT) (20-79 years) Global prevalence (%)</td>
<td>6.6</td>
<td>7.8</td>
</tr>
<tr>
<td>Comparative prevalence (%)</td>
<td>6.4</td>
<td>7.7</td>
</tr>
<tr>
<td>Number of people with diabetes (millions)</td>
<td>285</td>
<td>438</td>
</tr>
</tbody>
</table>
Table 3. Regional distribution of Diabetes, comparing numbers in 2010 and 2030.

<table>
<thead>
<tr>
<th>Region</th>
<th>2010 Population (20-79 years)</th>
<th>2010 No of people with diabetes</th>
<th>Comparative diabetes prevalence</th>
<th>2030 Population (20-79 years)</th>
<th>2030 No of people with diabetes</th>
<th>Comparative diabetes prevalence</th>
<th>Increase in the No of people with diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAC</td>
<td>220</td>
<td>27.4</td>
<td>10.2</td>
<td>210</td>
<td>23.2</td>
<td>12.1</td>
<td>42.4%</td>
</tr>
<tr>
<td>MENA</td>
<td>344</td>
<td>20.6</td>
<td>6.3</td>
<td>333</td>
<td>61.7</td>
<td>10.6</td>
<td>94.9%</td>
</tr>
<tr>
<td>SEA</td>
<td>638</td>
<td>69.7</td>
<td>7.6</td>
<td>1,200</td>
<td>101.0</td>
<td>9.1</td>
<td>72.1%</td>
</tr>
<tr>
<td>EUR</td>
<td>696</td>
<td>16.2</td>
<td>9.9</td>
<td>909</td>
<td>36.7</td>
<td>8.1</td>
<td>49.9%</td>
</tr>
<tr>
<td>SACA</td>
<td>287</td>
<td>18.6</td>
<td>6.5</td>
<td>382</td>
<td>29.6</td>
<td>7.8</td>
<td>69.1%</td>
</tr>
<tr>
<td>WP</td>
<td>1,531</td>
<td>16.7</td>
<td>4.7</td>
<td>1,772</td>
<td>11.5</td>
<td>5.7</td>
<td>47.0%</td>
</tr>
<tr>
<td>AFR</td>
<td>379</td>
<td>17.1</td>
<td>3.3</td>
<td>653</td>
<td>21.9</td>
<td>4.7</td>
<td>99.1%</td>
</tr>
<tr>
<td>Total</td>
<td>4,568</td>
<td>244.6</td>
<td>8.1</td>
<td>5,888</td>
<td>438.4</td>
<td>7.7</td>
<td>58.0%</td>
</tr>
</tbody>
</table>

(Legend: AFR-Africa, EUR-Europe, MENA-Middle East and North Africa, NAC-North America and Caribbean, SACA-South and Central America, SEA-South east Asia, WP-Western Pacific.)

A 2008 report by the World Health Organization (WHO) estimates about 2% of all non-communicable disease (NCD) deaths worldwide to Diabetes (Figure 4), with cardiovascular diseases responsible for the largest proportion of NCD deaths under the age of 70 (39%), followed by cancers (27%), chronic respiratory diseases, digestive diseases, and other NCDs together responsible for approximately 30% of deaths (15).
Figure 4. Worldwide distribution of non-communicable disease according to a 2008 W.H.O. report.

In Diabetes mellitus, excess glucose in the bloodstream is gradually deposited and transformed to fatty plaques leading to progressive damage of vital organs like the kidney, heart, brain, and eyes. This multisystem damage mainly occurs via involvement of the blood vessels supplying these organs (16).

**National Statistics in the USA:** A short summary of the compilation of disease incidence and prevalence by the Center for Disease Control (CDC), Atlanta, using multiple data systems, is shown below.

1) Among U.S. residents aged 65 years and older, 10.9 million, or 26.9%, had diabetes in 2010.

2) About 215,000 people younger than 20 years had diabetes (type 1 or type 2) in the United States in 2010.

3) About 1.9 million people aged 20 years or older were newly diagnosed with diabetes in 2010 in the United States (16).
Diabetes is the leading cause of kidney failure, nontraumatic lower-limb amputations, and new cases of blindness among adults in the United States. Diabetes is also a major cause of heart disease and stroke and is the seventh leading cause of death in the United States. Lack of awareness of the disease process is partly due to the chronic and often gradual destruction of beta cells, causing it to go unnoticed. There is little that can be done to reverse full-blown disease. Hence, there is a great need for research in this area due to the aforementioned reasons.

Total costs, including direct medical costs and indirect costs due to disability, work loss, and premature mortality, are approximately $174 billion per year in the United States (2007). As the population gets older, the impact of these diseases is expected to grow dramatically.

1.9 Brief introduction of our laboratory research

The causative role of various regions of the 37 amino acid hIAPP molecule in pancreatic beta cell membrane disruption has yet to be fully elucidated to understand the pathogenesis of Diabetes mellitus. Our experimental set-up simulates interactions between individually synthesized, overlapping hIAPP fragments (1-19, 10-29, 10-19, 20-29) and liposomes (analogous to artificial pancreatic beta cell membranes) in two types of fluorescence-based assays that examine and uncover possible factors that initiate pancreatic cell membrane damage.
Cross comparison of individual fragment behaviour to the activity of the full-length hIAPP molecule, with positive controls (100% destruction of lipid vesicles exposed to known strong detergent agents) and negative controls (lipid vesicles that were neither exposed to detergent, hIAPP nor hIAPP fragments), helped to establish the validity of our experimental method.

Liposome disruption equivalent to pancreatic beta cell membrane damage, predominantly caused by particular fragments of the hIAPP molecule, was identified by the carboxyfluorescein dye leakage assay. Amyloid fibril forming capability, an indirect measure of the individual fragments’ tendency to build upon initial membrane damage and eventually cause progressive beta cell death, was analyzed via fragment activity in a Thioflavin based assay.

Additionally, computational work using density functional methods was used to construct individual hIAPP fragment conformations and study the electron/charge distribution along these fragment models to predict relative activity of fragments. This theoretical explanation of fragment activity performed by another independent research team, blinded to our wet lab work, strongly validated our experimental data and results.

Last, a fourth independent method, circular dichroism (CD) spectroscopy, was used to conclude our investigation of the hIAPP molecule. CD spectroscopic analysis of structural conformation of individual fragments was used to correlate structure-activity relationships over fixed time periods consistent with the time scale used for the previous assays and also with the theoretical data from the computational study.
These results were used to establish, with a very high degree of certainty, the sinister areas or culprit fragments of hIAPP involved in initiation and acceleration of the pancreatic beta cell membrane destruction that culminates in the pathologic state of Diabetes mellitus type II.

The results from all the four independent and established scientific methods are used to compare and contrast between activity levels and to clearly delineate the potential role of various fragments of hIAPP in the disease process. Finally, a coherent sequence of possible molecular mechanisms causing pancreatic damage can be formulated, reiterating contemporary work by other research groups in the synthetic peptide chemistry field. Comparison with other large studies has also been made to standardize our analysis.

In light of the World Health Organization’s prediction of a worldwide diabetic epidemic by year 2030, coupled with an ever-increasing global need for cost-effective health care, experimental diabetes research is a crucial prologue to future clinical trials in prevention, diagnosis, and treatment of Diabetes mellitus type II. The extremely small mass of the pancreatic beta cells relative to the body weight, their critical responsibility in glucose metabolism during the entire lifetime of an organism, and the clinical, financial consequences of Diabetes mellitus type II on society necessitates extensive research in this area.
2. Research Goals

The working hypothesis of our research is that the presence of plaques and deposits of hIAPP within the pancreas of patients with Diabetes mellitus type II suggests its likely role in the etiology and pathogenesis of this disease process. Interestingly, amyloid has also been implicated in Parkinsonism, Alzheimer’s disease, multiple myeloma, rheumatoid arthritis and Huntington’s disease. An underlying common mechanism of amyloid-induced cell death in various organ systems of the body has led to a new disease entity, “Amyloid associated disease,” which seeks to bring together a common etiological basis that would indirectly lead to the formulation of a common solution for all these disease processes.

We had two main goals for our project. Our experimental set-up simulates hIAPP and pancreatic beta cell membrane interactions to uncover factors that initiate and promote progression of beta cell death.

We also explored two important mechanisms of pancreatic cell death: cell destruction via membrane pore formation by various hIAPP fragments and amyloidogenic fibril formation by fragments of hIAPP leading to fibril-dependent cell membrane destruction. The observations and results of our study establish the precise portions of hIAPP that can cause pancreatic damage and help elucidate the molecular mechanisms of activity by the implicated toxic species. It also provides an opportunity to explore the cellular environment affecting the formation of hIAPP aggregates and devise methods to intervene and prevent hIAPP aggregation and subsequent pancreatic cell death that leads to Diabetes mellitus type II.
3. Materials and Methods

3.1 Peptide synthesis

3.1.1 Discovery of the amide or peptide bond.

The peptide bond is integral to the biochemistry of all living organisms as it forms a link between two amino acids, the building blocks of proteins. In 1902, Emil Fischer, a German chemist, became a Nobel laureate for his work in the field of carbohydrate chemistry; at this time he also made great contributions to the knowledge of proteins and amino acids. He studied the synthesis of proteins by obtaining the various amino acids in an optically active form. His initial synthesis of glycy1-glycine in 1901 represents the first synthesis of a free peptide. Fischer later reported the synthesis of an octadecapeptide (Leu-Gly3-Leu-Gly3-Leu-Gly9) in 1907. Additionally, Fischer also initiated peptide nomenclature by naming his first byproduct a “dipeptide,” thereby defining the terminology of peptide chemistry that is used to date (17). Fischer’s discovery was futuristic and laid the cornerstone for peptide research and peptide therapeutics leading to development of innumerable drugs and diagnostic methods.

3.1.2 History of solid phase peptide synthesis techniques: R.B. Merrifield and his contributions.

Dr. Robert Bruce Merrifield, an American biochemist, is the pioneer of solid phase peptide synthesis and received the Nobel Prize for this invention in 1984. His initial discovery of a dinucleotide peptide growth factor during his graduate studies later led to the development of the solid peptide synthesis technique in 1959.
The methods available until then were fraught with technical difficulties related to solubility and purification and were not ideally suited for long chain polypeptide synthesis. Dr. Merrifield investigated a new approach to peptide synthesis in an effort to overcome some of these difficulties. He was the sole author of a paper describing this technique to synthesize a model tetrapeptide, \textbf{L-leucyl-L-alanyl-L-Valine}, in the \textit{Journal of the American Chemical Society}, the fifth most cited paper in the journal's history (18).

Subsequently, Dr. Merrifield's laboratory first synthesized bradykinin, angiotensin, desamino-oxytocin, and insulin. In 1969, Dr. Merrifield and Bernd Gutte first synthesized the enzyme ribonuclease A, proving the chemical nature of enzymes. This was a significant step that demonstrated the functional influence of the three-dimensional, tertiary structure of a peptide or protein containing a linear sequence of amino acids joined by peptide bonds.

Dr. Merrifield's method allowed systematic exploration of the structural basis of peptide chemistry. The study of enzymes, hormones, and antibodies greatly stimulated progress in biochemistry, pharmacology, and medicine. The development and applications of the technique have been expanded to include solid phase synthesis of nucleotides and saccharides. In 1993, he published his autobiography, \textit{Life During a Golden Age of Peptide Chemistry}. He also received the Association of Biomolecular Resource Facilities (ABRF) Award for outstanding contributions to Biomolecular Technologies in 1998. ABRF members represent 41 different countries across the world.
The abstract of Dr. Merrifield’s paper reads, A new approach to the chemical synthesis of polypeptides was investigated. It involved the stepwise addition of protected amino acids to a growing peptide chain, which was bound by a covalent bond to a solid resin particle. This provided a procedure whereby reagents and by-products were removed by filtration, and the recrystallization of intermediates was eliminated. The advantages of the new method were speed and simplicity of operation. The feasibility of the idea was demonstrated by the synthesis of the model tetrapeptide \textit{L-leucyl-L-alanyl-glycyl-L-valine}. The peptide was identical with a sample prepared by the standard p-nitro-phenyl ester procedure.

To explain further, Dr. Merrifield proposed the use of a solid phase, an insoluble polymer with stable physical form and a functional group, for temporary covalent bond formation with the first amino acid in the peptide chain to be synthesized. This polymer surface and/or the first covalently bound amino acid provide a starting point for peptide synthesis and allow addition of a desired sequence of amino acids to the free end of the first amino acid. Finally, the peptide is separated from the solid polymer supporting it, and pure peptide is harvested and analyzed via chromatography. (Figure 5 shows the stepwise flowchart of peptide chemical synthesis, and Figure 6 shows a ball and stick model of synthesized peptide.)

The polymers available at that time were cellulose, polyvinyl alcohol, polymethacrylate, and sulfonated polystyrene. The one that worked best was a chloromethylated copolymer of styrene and divinylbenzene. The solid phase or resin, in the form of 200-400 mesh beads, possessed a porous gel structure, allowing ready penetration of reagents during chemical reaction.
In addition, it also allowed purification of intermediate peptides by washing and dissolving away the impurities instead of the usual lengthy recrystallization procedures. This greatly improved productivity and working time by simplifying multiple intermediate chemical manipulations related to the peptide synthesis flowchart. Dr. Merrifield’s methodology, which has now lent itself to automation, also provides a tireless route to the synthesis of higher molecular weight polypeptides, inaccessible by conventional procedures.

The important limiting factors of this method, diffusion and steric hindrance, were not serious enough to prevent completion of the required chemical reactions. Though individual peptide bond reaction rates were slower than corresponding ones in solution, the conditions in this method permitted all of the reactions to occur at useful rates. Since the growing peptide chain was in the completely insoluble solid phase at all times, the term solid phase peptide synthesis was coined to describe this method. The success of these initial experiments ultimately demonstrated the feasibility of solid phase peptide synthesis. The real value of this method lies in its later application to production of much longer peptide sequences, especially those with biological activity.

3.1.3 Experimental hIAPP-pancreatic beta cell membrane interactions.

Dye leakage assays: Synthesis of hIAPP fragments and dye containing liposomes.

Several standard and proven methods used within our experimental set-up allowed us to achieve our project goals. While earlier studies suggested that the 20-29 residues of the 37 amino acid long hIAPP mainly influence its aggregation during amyloidogenesis, the novel part of our study included synthesis and testing activity of multiple shorter fragments of the hIAPP molecule with varying peptide lengths (1-19, 10-19, 20-29, and 10-29 fragments).
These fragments allowed us to examine the experimental behavior of various portions of the hIAPP molecule during their interactions with the dye containing lipid vesicles or liposomes that are analogous to pancreatic beta cell membranes in our study.

There were two types of dye assays used in our study: the carboxyfluorescein and Thioflavin T assays, each with a different purpose. In the carboxyfluorescein dye assay, the goal of the assay was to quantify the extent of liposome damage by individual hIAPP fragments based on the percentage of carboxyfluorescein dye leakage. A spectrofluorometer, a fluorescence sensor instrument, measured the amount of dye leakage. The Thioflavin T assay was used to specifically identify amyloidogenic potential of each fragment after liposome disruption. Thioflavin is a chemical dye that is widely used to visualize and quantify the presence of misfolded protein aggregates such as amyloid. The chemical Thioflavin, upon binding with or having specific chemical interaction with beta sheet rich structures like those in amyloid aggregates, shows enhanced fluorescence and a characteristic red shift of its emission spectrum identified on the spectrofluorometer.

**Translation of research goals and the ideology behind our experimental set-up:**

Our goal was to confirm previous reports (19, 20, 21) related to activity of various regions within the hIAPP molecule. The N-terminus of the hIAPP peptide was implicated in the initiation of vesicle damage by insertion into the lipid vesicles, to be identified by liposome leakage of carboxyfluorescein or Thioflavin T dye in the two different assays.
Accordingly, the full-length peptide and the 1-19 fragment must be able to cause significant vesicle damage, while the 10-19 fragment (containing the insulin binding region) and the 20-29 fragment (containing the amyloid-causing portion) should have lower levels of liposome disruption. A second observation was the requirement of the amyloidogenic region (20-29 fragment) in causing amyloidogenesis, which should be seen maximally with the full-length amylin/hIAPP, followed by the 10-29 and 20-29 fragments. The 1-19 and 10-19 fragments, which lack this region, should intuitively show weaker amyloidogenesis.

The main focus of my research project revolved around the activity of the 20 amino acid long 10-29 fragment that has overlapping activity with the three other fragments: 1-19, 10-19, and 20-29. This fragment showed overlapping behavior traits with other fragments due to the partial presence of the N terminal amino acid residues and complete presence of the insulin binding and amyloidogenic 20-29 regions. We were able to successfully study individual interactions of the fragments with the liposomes during the experimental simulation. We were also able to identify whether or not the overlapping portions of the fragments had any significant influence on the degree of membrane disruption and the potential for amyloidogenesis.

3.1.4 Brief summary of the experimental procedure

The experimental procedure began with customized peptide fragment synthesis from individual amino acids utilizing the PS3 Solid Phase Peptide Synthesizer. Peptide cleavage from the resin was followed by filtration and lyophilization (in simple terms, freeze drying the solution to obtain the peptide in solid form). High levels of peptide purification were achieved (>95%) using RP-HPLC (Reverse Phase-High Performance Liquid Chromatography) techniques, and peptide identity was confirmed via Electrospray Mass Spectrometric evaluation of molecular weights of the individual hIAPP peptide fragments.
The next step involved modeling cell membranes with uniformly sized unilamellar lipid vesicles, with a similar composition as that of the pancreatic beta cell membrane, using a 7:3 ratio of DOPC: DOPS (1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-(phospho-L-serine) (DOPS)). Carboxyfluorescein dye was encapsulated within these overall negatively charged phospholipid vesicles.

Varying concentrations (0.5-50 μM) of the synthesized hIAPP fragments were exposed to fixed concentrations of the dye encapsulated lipid vesicles in an experimental simulation of hIAPP-pancreatic beta cell membrane interactions that occur in vivo. Peptide and vesicle ratios were within biological range, and controls were used for comparison.

The Spectrofluorometer quantified dye leakage from disrupted lipid vesicles, recorded every minute over 4-6 hour intervals. The extent of fluorescence detected is suggestive of the level of interaction between the fragments and the liposomes, with higher values suggesting greater membrane destruction by specific fragments.
Figure 5. Flowchart of solid phase peptide synthesis.

Figure 6. Ball and stick visualisation of solid phase peptide synthesis; peptide bonds linking ball and stick forms of amino acids in various colors.

http://en.wikipedia.org/wiki/Peptide_synthesis
3.1.5 Raw material required for solid phase peptide synthesis:

1) Amino acids: The initial part of our laboratory investigation was based upon interactions between individually synthesized, overlapping hIAPP fragments (1-19, 10-29, 10-19, 20-29) and dye containing lipid vesicles or liposomes (analogous to artificial pancreatic beta cell membranes) in two types of fluorescence-based assays using carboxyfluorescein and Thioflavin T. These assays were helpful in evaluation of varying levels of activity of the different fragments of the hIAPP molecule in the initiation and progression of pancreatic cell membrane damage in Diabetes mellitus type II.

Table 4. hIAPP full-length molecule and fragments overview: Amino acid sequence, length and significance.

<table>
<thead>
<tr>
<th>hIAPP Fragment</th>
<th>Amino acid length</th>
<th>Amino acid sequence</th>
<th>Significance of fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-19</td>
<td>19 amino acids</td>
<td>KCNTATCATQRLANFLVHS</td>
<td>N-terminus and insulin binding region</td>
</tr>
<tr>
<td>10-29</td>
<td>20 amino acids</td>
<td>QRLANFLVHSSNNFGAILSS</td>
<td>Insulin binding and fibril forming amyloidogenic region</td>
</tr>
<tr>
<td>20-29</td>
<td>10 amino acids</td>
<td>SNNFGAILSS</td>
<td>Fibril forming amyloidogenic region</td>
</tr>
<tr>
<td>10-19</td>
<td>10 amino acids</td>
<td>QRLANFLVHS</td>
<td>Insulin binding region</td>
</tr>
<tr>
<td>Full length Amylin or hIAPP</td>
<td>37 amino acids</td>
<td>KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY</td>
<td>1-37 amino acid sequence from N to C terminal</td>
</tr>
</tbody>
</table>

25
The smaller customized fragments of the hIAPP molecule were synthesized using individual samples of the required regular N-α-fluorenlymethyloxycarbonyl (Fmoc) protected amino acids, which were purchased from various chemical manufacturers including Bachem Americas, Inc. (Torrance, CA), Anaspec (Freemont, CA), and Synthetech, Inc. (Albany, OR). About fifteen of the twenty known amino acids were used in chemosynthesis of the four hIAPP fragments (1-19, 10-29, 19-19, 20-29) with an amidated C-terminus and an acetylated N-terminus, as described earlier.

Synthesis of the longer full-length human amylin (37 amino acids) requires a tightly controlled environment and advanced instrumentation to ensure proper coupling and purification of the long, aggregating sequence. Since this was not feasible in our academic lab environment, a readymade commercially synthesized product was purchased from SynBioSci (Livermore, CA). The hIAPP samples dissolved in hexafluoroisopropanol (HFIP) were aliquoted, frozen, and lyophilized for use along with the shorter fragments of hIAPP synthesized in our lab. Additionally, another mammalian species, the rat IAPP, was studied and compared mainly with the hIAPP during molecular modeling experiments in our project. The rats are supposed to be less prone to Diabetes mellitus type II than humans mainly due to the differences in amino acid composition of IAPP and secondary structure, which indirectly influence physiologic function.
2) Resins and Coupling agents: These were purchased from Bachem Americas, Inc., and Midwest Biotech, Inc. (Fishers, IN). The role of the resin or solid phase material is similar to a virtual platform where individual amino acids are lined up for peptide bond formation. Additionally, during cleavage from the peptide, the resin also contributes the carboxamide C terminus of a peptide fragment. The resin that was used is p-methylbenzhydrylamine (MBHA).

Coupling agents are chemicals that interact with functional groups on neighbouring amino acids (amino, -NH$_2$ and carboxylic acid, -COOH groups) to result in the formation of a peptide bond. At the completion of peptide bond formation between all of the amino acids arranged in a predetermined sequence, the resin and coupling agents need to be removed or washed out.

In simple words, the creation of a peptide fragment with solid phase techniques can be likened to threading beads on a string (Figure 7). The "beads" or individual amino acids, are held together in a predetermined sequence by newly formed peptide bonds, which constitute the invisible "string" in this analogy.

Figure 7. Sequence of the Human IAPP.
3) Solvents and deprotecting agents were obtained from Fisher Scientific (Pittsburgh, PA) and Sigma-Aldrich Chemical Co. (St. Louis, MO). Solvents are agents that create a liquid environment for chemical interaction between the individual amino acids while removing excess reagent surrounding the growing peptide in intermittent wash cycles, while allowing the peptide itself to remain covalently attached to the insoluble resin. Deprotecting agents used were helpful in uncovering a new N-terminal amino group to which the next amino acid may be attached. Thus, repeated cycles of amino acid coupling followed by solvent wash cycle, deprotection, and wash occur in quick succession during the synthesis of a peptide chain in the solid phase peptide synthesis technique.

3.1.6 Instrumentation and Procedural Detail of Solid Phase Peptide Synthesis:

Solid phase peptide synthesizer – chemosynthesis of hIAPP peptide fragments.

The peptides were chemically synthesized on a PS3 Automated Peptide Synthesizer (Figures 8, 9, 10.) from Protein Technologies (Tucson, AZ) using standard solid phase techniques (18) for N-α-fluorenlymethyloxycarbonyl (Fmoc) protected amino acids on Rink amide p-methylbenzhydylamine (MBHA) resin (0.64 mmole/g) on a 0.1 mmole scale. A novel double couple protocol, set at a 0.4-mmol scale that requires four times the usual amount of individual amino acids (raw material), was used for synthesis of the longer fragments, 10-29 and 1-19. This method ensures more complete coupling than the single couple method and improves the yield for longer sequences.
Thus each amino acid was added in major excess (4x times), and peptide bond formation between amino acids was highly optimized by a series of well-characterized chemical agents. The purpose of utilizing large amounts of individual amino acids for peptide fragment synthesis was to generate extremely high yield in each step. For example, if each coupling step were to have 99% yield, a 26-amino acid peptide would be synthesized in 77% final yield (assuming 100% yield in each deprotection); if each step were 95%, it would be synthesized in 25% yield.

The MBHA resin contributes the amine part of the C-terminal carboxamide group of the peptide fragment upon cleavage. The side chains of Ser and Thr were protected as the t-butyl derivatives, Asn, Gln, His, and Cys as trityl, Lys as t-butyloxy carbonyl (Boc), and Arg as the 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc) form. For the Ala-Thr sequence in the hIAPP 1-19 analog, a pseudoproline/oxazolidine derivative [Fmoc-Ala-Thr (ψMe,Me pro)-OH] purchased from EMD Biosciences, Inc. (LaJolla, CA) was utilized (22). The deprotection solution for the N-terminal amine was 20% piperidine in N,N-dimethylformamide (DMF). O-(Benzotriazol-1yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was used as a coupling agent, activated by 0.4 M N,N-diisopropylethylamine (DIEA) in DMF.

The N-terminal Fmoc group was removed, and simultaneous deprotection and cleavage from the resin were accomplished by treatment with 11 ml 90% trifluoroacetic acid (TFA)/10% scavenger cocktail (anisole, thioanisole, phenol, water). The reaction was begun at 0°C, allowed to warm to room temperature and stirred for 2 hours. The uncharged resin was separated from the solution by filtration. The peptide was precipitated with cold diethyl ether, filtered, redissolved in 30% acetonitrile/70% water and lyophilized.
Figure 8. PS3 peptide synthesizer with components.

Figure 9. Reaction vessels with raw material.

Figure 10. Peptide filled vials arranged in required sequence.

Figure 11. Peptide-Resin cleavage.

Figures 12, 13. Lyophilization-freeze dried peptide.

Figure 14. Final peptide product.
3.1.7 Instrumentation and Purification of crude peptides obtained from Solid Phase:

1) Preparative Reverse-Phase High Performance Liquid Chromatography (RP-HPLC):

   The crude peptides were purified to homogeneity 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 9 25.0 cm, 10 ml/minute). A linear gradient of 10% acetonitrile (0.1% TFA)/water (0.1% TFA) to 50% acetonitrile (0.1% TFA)/water (0.1% TFA) was employed, followed by lyophilization. The 1–19 fragment was oxidized in the presence of 20% dimethyl sulfoxide (DMSO) to form the disulfide (23).

2) Analytical Reverse Phase – High Performance Liquid Chromatography (RP-HPLC) for analysis of peptide purity:

   The peptide purity was assessed by analytical RP-HPLC. Peaks were monitored at 214, 230, 254 and 280 nm. The peptides were 97% pure as analyzed by peak integration (Figure 15 below). Electrospray mass spectrometry confirmed the appropriate molecular weights (Figure 16 below).
Figure 15. Reverse phase – High Performance Liquid Chromatography (RP-HPLC)- analysis of peptide purity.

Figure 16. Electrospray mass spectrometry confirmation of the purity of the 10-29 peptide fragment, peak readings of molecular weight of peptide tested measured 1087.7 (1087.7 x 2 = 2174.4) corresponding to the weight of the 10-29 fragment.

3.1.8 Preparation of Large Unilamellar Vesicles (LUVs) and Dye Leakage Assay

Lipids were purchased from Avanti Polar Lipids (Alabaster, AL), and carboxyfluorescein was purchased from Sigma-Aldrich (St. Louis, MO). Lipid vesicles were created to encapsulate carboxyfluorescein, which upon disruption of the membrane leaks into the surrounding buffer.

Carboxyfluorescein is a highly fluorescent molecule, which allows its concentration to be measured by a constant scanning spectrofluorometer. This concentration then gives an indication as to the degree to which the membrane is permeabilized by the peptide. Baseline controls were compared to runs with added peptide and added detergent (which acted as a positive control to give 100% leakage). Percent leakage could then be plotted versus time to monitor membrane disruption.
The 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 (a zwitterionic and a negatively charged phospholipid mixture that mimics the β-cell membrane), and dissolving in 2 ml of chloroform. The chloroform was evaporated by a gaseous nitrogen stream to create a thin film of lipid on the side of a test tube, which was then dried in a vacuum dessicator overnight. 500 μl of 30 mM carboxyfluorescein dye in pH 7.5 sodium phosphate buffer solution was added to the previously prepared dried lipid to make multilamellar vesicles (MLVs). This solution was vortexed thoroughly to mix the components and then subsequently frozen using liquid nitrogen and thawed five times consecutively.

This solution was then extruded 21 times through a polycarbonate filter (pore size 100 nm) using a mini-extruder from Avanti Polar Lipids, fitted with two 0.5 ml Hamilton gastight syringes, producing large unilamellar vesicles (LUVs). To remove nonencapsulated 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. A vesicle solution without dye was made the same way except that it lacked carboxyfluorescein and was not run through the gel exclusion column.

For the assay, a small amount of the dyed vesicle solution was diluted with the vesicle solution without dye so that the stock vesicle concentration was 15.5 ± 1.5 mM, and the concentration of lipid in the well was roughly 207 μM. In the wells, the ratio of lipid to peptide therefore ranged from approximately 200:1 to 2:1.
The isolated LUVs containing the carboxyfluorescein were analyzed using scanning FT–Vis spectroscopy to determine that the wavelength of maximum absorption was 490 nm. Using a scanning spectrofluorometer, the wavelength of maximum absorbance was set as the excitation wavelength and the emission wavelength was determined to be 520 nm.

To make the peptide solution, a weighed sample of the purified peptide was dissolved in a measured volume of dimethyl sulfoxide (DMSO) and sonicated for 1 minute. Previous control runs in the absence of peptide confirmed that DMSO itself did not induce dye leakage. An aliquot of 40.0 μl of the vesicle solution was added to sodium phosphate buffer to a final volume of 3.00 ml in a 5 ml test tube. The peptide solution was added in aliquots, in increasing amounts per sample; final concentrations varied from 0.5 to 50 μM for most peptides (90 μM for the smaller fragments). Upon adding the peptide solution, the test tubes were mixed by inversion, and 300 μl from each tube were transferred to a well in a 96 well plate, which was inserted into the FLx 800 Fluorescence Microplate Reader (BioTek Instruments, Winooski, VT) with KC4 software (filter set to 485 nm excitation, 528 nm emission). A time-course fluorescence spectrum was taken over 180 minutes (3 hours). The control used to determine 100% leakage was detergent, 40.0 μl Triton X (10% v/v in buffer), which induced the release of any remaining dye from vesicles, resulting in the highest possible fluorescence. The spectra were saved and numeric values were compiled in Microsoft Excel. All assays were run three times in triplicate and average values were reported.

Dye leakage was calculated using the following equation:

\[
\text{Percentage of dye leakage} = \frac{F - F_{\text{baseline}}}{F_{\text{detergent}} - F_{\text{baseline}}},
\]

where \(F_{\text{baseline}}\) is the fluorescence of the LUVs in the absence of peptide (solvent only).
3.2 Thioflavin T (ThT) Assay

The Thioflavin T assay is similar in procedural detail to the carboxyfluorescein assay described in earlier sections except that Thioflavin (ThT) is used to identify the degree of amyloidogenesis following liposome damage. In short, liposomes or lipid vesicles of 7:3 DOPC/DOPS were prepared exactly as described in the carboxyfluorescein assay with the Thioflavin T dye replacing carboxyfluorescein in the center of each vesicle.

Thioflavin T, a cationic benzothiazole dye was first introduced by Vassar and Culling in 1959 to demonstrate the presence of amyloid in tissue sections. The enhanced degree of fluorescence of ThT upon its binding with amyloid was used as a positive indication of the presence of amyloid. The detection of amyloid by ThT in kidney sections after differentiation in acidic solutions was also proven to be highly specific by these two scientists. Later, in 1967, two pathologists Saeed and Fine confirmed these findings and established the superiority of ThT in amyloid detection by careful comparison of Congo red, crystal violet, van Gieson, and ThT dyes and their binding to several positive and negative control tissues. ThT was found to have the highest quantitative sensitivity for detection of amyloid in tissue samples (from clinical cases of amyloidosis) although green birefringence with Congo or Sirius red was more specific (24).

The potential application of ThT for amyloid identification has been exploited ever since. It is interesting to note that more recently in 2004, uncharged derivatives of ThT that readily enter the brain have been used in vivo to establish the presence of Beta amyloid, a peptide implicated in the causation of Alzheimer’s disease (25).
The chemical structure of Thioflavin T, (Figure 17) has a centrally located phenyl group with a hydrophobic dimethylamino group attached on one side while the other end contains a more polar benzothiazole group (N and S). This combination of polar and hydrophobic regions supposedly allows Thioflavin T molecules to form micelles in aqueous solution. These micelles likely contain hydrophobic interiors while the externally oriented polar N and S groups interact with the solvent. Research groups in this area suggest that the thiazole nitrogen of the dye and hydroxyl groups of tissue structures most likely form hydrogen bonds (26,27) during specific binding of the dye molecules to amyloid (Figure 18).

![Thioflavin molecular structure.](image)

![Thioflavin T-Amyloid binding.](image)
The molecular mechanism of Thioflavin-T binding to amyloid fibrils is still under current investigation (28). It has been proposed by Khurana et al. (26) that Thioflavin T molecules were in micelle form at concentrations above 4 μM in aqueous solvents as observed by the increase in the specific conductance. Formation of micelles was also associated with increased fluorescence excitation and emission of ThT. Additionally, ThT micelles were observed to bind to the surface of amyloid fibrils confirmed by atomic force microscopy. Binding of ThT micelles to amyloid fibrils causes changes in the excitation spectra and enhanced emission fluorescence. Many interactions with ThT are supposedly mediated predominantly by aromatic side-chains, particularly Tyr and Phe of the amyloid fibril. The large hydrophobic surfaces provided by these side chains allow ThT binding and additional fibril π-stacking with the dye may be another key component of these amyloid-dye binding interactions.

To summarize, the chemical attribute of Thioflavin-T causing an enhanced degree of fluorescence on binding during amyloidogenesis has been exploited in this assay and aids the evaluation of the amyloidogenic potential of the hIAPP fragments. For over 50 years, the success of ThT as an amyloid dye has resulted from its broad staining capacity, extraordinary sensitivity and ease of use. These considerations have made it among the most widely used dyes for monitoring amyloid formation. ThT's robust staining properties continue to form the basis of innovative methods for the detection and analysis of fibrils both in vitro and in vivo.
Further investigations into the molecular mechanism of ThT interactions are needed to advance our understanding of amyloid formation, kinetics, structure, and pathogenesis. Insights from such investigations will assist those who design amyloid probes and those who apply them in research and diagnostic settings.

The recent intense study of the molecular mechanism of ThT binding suggests it may be possible to apply structure-guided design to modulate the specificity of ThT to particular amyloid fibrils. Moreover, as a representative amyloid dye, understanding ThT interactions in atomic detail will ultimately guide therapies to treat amyloid diseases.

Thioflavin T (ThT) was purchased from Sigma-Aldrich (St. Louis, MO). The conditions for the ThT experiments were the same as for the leakage experiments, with tubes containing 25 μM ThT, in place of carboxyfluorescein; peptide concentration was 25 μM and lipid concentration was 430 μM for this assay. The 96-well plate was placed in the FLx 800 Fluorescence Microplate Reader with KC4 software (filter set to 450 nm excitation, 485 nm emission). A time-course fluorescence spectrum was taken over 300 minutes. Each assay was run three times in triplicate. A sigmoidal increase in fluorescence intensity over time indicates amyloid fiber formation by the fragment being tested.
3.3 Circular Dichroism Spectroscopy and its application for analysis of biologically useful molecules: brief overview

The phenomenon of circular dichroism was pioneered by three scientists, Jean Baptiste Biot, Augustin Fresnel and Aime Cotton in 1895 and has been exploited in a wide range of applications in diverse fields. Circular dichroism spectroscopy (CDS) is the method of chemical analysis of molecules by making use of the differential absorption of left and right circularly polarized light by different portions of optically active chemical molecules (29,30). The phenomenon of circular dichroism is exhibited in the absorption band of many optically active molecules and ultraviolet CD spectroscopy is most commonly used to investigate secondary structures of proteins (31). Another variety, vibrational CDS uses infrared light for structural examination of small organic molecules and, most recently, proteins and DNA.

While the dextrorotary and levorotary components of molecules provide important information, the secondary structure of a protein imparts a distinct CD spectrum that is unique to the particular molecule. Hence, the alpha helix and beta sheet of proteins and double helix of DNA have CD spectral signatures that represent or provide clues to their real structures. The ability of CDS in providing a representative structural signature makes it a powerful tool in modern biochemistry with wide ranging applications.

The far-ultraviolet (UV) spectrum (200 – 122 nm) of proteins usually reveals important characteristics of their secondary structure while the near-UV CD spectrum (400-300 nm) provides information on the tertiary structure of the protein. A high pressure, short-arc xenon lamp is the usual light source in these instruments.
The CD spectra can be readily used to estimate the fraction of a molecule that is in the alpha helix, beta sheet, beta turn or random coil conformations. These fractional assessments limit the possible number of secondary conformations that the protein can assume and act as a rough guide in predicting their structure. CDS, however, cannot precisely estimate the location and number of alpha helices within a given molecule. Inspite of this, CD remains a valuable tool especially for showing changes in conformation with corresponding changes in temperature or concentration of denaturing agents interacting with molecule being examined. CDS is also a valuable tool for verifying that a protein is in its native conformation before undertaking extensive and/or expensive experiments.

Since CDS is usually used to study proteins in solution, it complements methods that study the solid state. Also, while techniques such as X Ray Crystallography and Protein NMR Spectroscopy provide specific structural detail of proteins with atomic resolution data, CD spectroscopy is a quick method that does not require large amounts of proteins or extensive data processing. Thus, CDS can be used to survey a large number of proteins with differing conditions related to temperature, pH and salinity in a relatively short time, thus increasing throughput.

**Methodology of Circular Dichroism Spectroscopic Analysis**

The lyophilized peptide samples were dissolved at a concentration of 25 μM in 10 mM sodium phosphate buffer at pH 7.5, vortexed and sonicated for 15 s and transferred to a 0.1 cm quartz cuvette. After the initial baseline spectrum of the peptide alone in solution was taken, liposome vesicles of 7:3 DOPC/DOPS (prepared as described in earlier sections) were added to the cuvette from a 40 mg/ml stock solution to achieve a final concentration of 400 μM, and another spectrum was taken after 1 hour.
Spectra were measured at room temperature (23°C) on an AVIV circular dichroism spectrometer (Lakewood, NJ) at 1 nm intervals from 190 to 260 nm at a scanning speed of 50 nm/min and a bandwidth of 5 nm. Each spectrum reported is the average of four scans (peptides alone) or eight scans (peptides plus vesicles) after subtraction of the baseline spectrum (without peptide). The results obtained from CD Spectroscopy are considered qualitative as the use of optical methods like CD are parameterized for large proteins and thus involve some uncertainty in its application to secondary structure determination of short peptides such as the peptides in our experiment (21).

3.4 Molecular Modeling Experiments:

Molecular modeling techniques encompass all the theoretical and computational methods that are used to model or mimic the behaviour of molecules. The techniques are used in diverse fields including computational chemistry, biology and material science for studying molecular systems ranging from small chemical systems to large biological molecules and material assemblies. While manual calculations can be made for the smaller and often simple systems, computer programs and methods must be utilized to achieve molecular modeling of larger systems (32,33).

The molecular modeling techniques describe molecular systems at the atomistic level where the lowest level of information is related to individual atoms or a small group of atoms. A more explicit evaluation of a system or molecule is made in quantum chemistry which makes use of electronic structure calculations and the functional influence of electron distribution within a system.
While the benefit of molecular modeling allows many more particles or atoms to be considered during simulations and reduces the complexity of a given system; quantum analysis facilitates detailed evaluation of the subatomic components by analysing the electronic distribution within a system. Molecular modeling simulations can be achieved either in vacuum, known as gas phase simulations, or in the presence of a solvent such as water, referred to as explicit solvent simulations. Simulations that do not make direct use of a solvent but theoretically estimate the effect of solvent by using empiric mathematical expression are known as implicit solvation simulations.

Molecular modeling methods are currently utilized on a routine basis for investigating the structure, dynamics, surface properties and thermodynamics of inorganic, biological and polymeric systems. Several types of biological activity have been investigated using molecular modeling including protein folding and stability, catalytic activity of enzymes, conformational changes leading to differences in biomolecular function and molecular recognition of proteins and DNA.

**Density functional theory (DFT)** is a quantum mechanical modeling method used in physics and chemistry to investigate the electronic structure of systems such as atoms, molecules and the condensed phases. Using this method, the properties of a multi-electron system can be determined by using functionals, i.e., functions of another function, which in this case is the spatially dependent electron density. Hence, the name density functional theory comes from the use of functionals of the electron density. DFT is among the most popular and versatile methods available in condensed-matter physics, computational physics and chemistry.
Modeling Calculations

All modeling calculations on our peptide fragments and the full length hIAPP were carried out using the GaussianTM suite of programs (Gaussian 03, 2004) on EMU’s Dell PWS quad-core processor, MacPro eight-core processor, or on the National Center for Supercomputing Applications’ SGI Altix cluster (34). An independent research group led by Dr. Maria Milletti and working on computational chemistry applications was involved in all the modeling calculation work related to these peptide fragments. Molecular structures of the monomers in the gas phase were constructed using the GaussView (GaussView4, 2004) visualization program and then optimized employing Density Functional methods (33,35,36). Specifically, the hybrid method B3LYP was used, which includes Becke’s three-parameter exchange-correlation hybrid functional (37) and the correlation functional of Lee, Yang, and Parr (38). The 3-21G* basis set was used for all calculations (39,40, 41,42,43,44). The Berny algorithm was employed to optimize all structures to a minimum energy (45, 46, 47, 48).
4. Results and Discussion

4.1 Liposome dye leakage assays: Carboxyfluorescein and Thioflavin T Experiments

The sequences of the synthesized fragments and purchased full length hIAPP, as well as rat IAPP (rIAPP), are shown in Figure 19.

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-29</td>
<td>SNNFGAILSS</td>
</tr>
<tr>
<td>10-19</td>
<td>QRLANFLVHS</td>
</tr>
<tr>
<td>10-29</td>
<td>QRLANFLVHSSNNFGAILSS</td>
</tr>
<tr>
<td>1-19</td>
<td>KCNTATCATQRLANFLVHS</td>
</tr>
<tr>
<td>1-37</td>
<td>KCNTATCATQRLANFLVHSSNNFGAILSSSTNVGSNTY</td>
</tr>
<tr>
<td>rIAPP</td>
<td>KCNTATCATQRLANFLVRSSNNLGPVLPPTNVGSNTY</td>
</tr>
</tbody>
</table>

Figure 19. Sequences of hIAPP fragments and rIAPP (human and rat).

In these two dye assays, membrane vesicles either filled with carboxyfluorescein or treated with Thioflavin T were allowed to react with varying concentrations of the synthesized peptide fragments. The data shown in Figures 20 and 21 were gathered from the carboxyfluorescein dye leakage assay showing the interaction between the pancreatic beta cell analogs (liposome vesicles comprised of a 7:3 ratio of the lipids DOPC and DOPS) and varying concentrations of the hIAPP fragments ranging from 0.5 to 50 µM (or higher in the cases of the shorter, lower molecular weight analogs). The fluorescence measurements indicative of dye leakage from the vesicles or dye reacting with hIAPP fibrils were recorded every minute for 3 hours, and average percent dye leakage values relative to test controls were obtained. In case of ThT assay the time of the characteristic increase of fluorescence, indicating fibril formation (if any) was noted.
Two specific time points were chosen for studying the temporal effects of the interactions between the peptide fragments and the liposomes in the carboxyfluorescein assay, the initial time point at the beginning of the experiment revealing the peptide fragments with the highest and most rapid destructive potential and the final end point 3 hours later showing the increasing destructive potential of peptide fragments with longer periods of interaction with the liposomes. Figure 20 represents the initial percent leakages, and Figure 21 shows the effect of the peptides on the integrity of the vesicles after 3 hours. Disruption of the vesicles by the peptide allows dye leakage from the vesicles into solution, leading to elimination of the self-quenching effect and increasing carboxyfluorescein fluorescence.

In our experiment we used both positive and negative controls for validating our results. The positive controls with 100% dye leakage due to total liposome disruption were set up by adding chemical detergent to solutions containing the dye encapsulated liposomes, without any peptide fragments. In contrast, the negative controls, with no expected dye leakage, were made up of liposomes alone in solution without addition of peptide fragments or detergent that would otherwise cause membrane disruption. The results obtained provide an assessment of the degree of vesicle disruption caused by each of the synthesized peptides at varying concentration levels. Baseline controls were compared to runs with added peptide (causing less than 100% leakage) and added detergent (which acted as a positive control to give 100% leakage). Percent dye leakage was then plotted against time to identify the temporal changes in peptide liposome interactions leading to worsening membrane disruption with almost all peptides and corresponding increase in fluorescence.
Results of the Carboxyfluorescein assay

Figure 20. Initial dye leakage from DOPC/DOPS (7:3) vesicles (0.21 mM) caused by varied concentrations of hIAPP peptide fragments; values represent the average of three trials.
Figure 21. Final (3 hour) dye leakage from DOPC/DOPS (7:3) vesicles (0.21 mM) caused by varied concentrations of hIAPP peptide fragments; values represent the average of three trials.

4.2 Thioflavin T assay

As discussed in the methodology section, the chemical Thioflavin T (ThT), is a dye that is commonly used for the detection and quantification of amyloid fibrils. The amyloidogenic potential or the ability of individual peptide fragments to form amyloid fibrils after liposome disruption was measured by an increase in ThT fluorescence intensity caused by Thioflavin T binding or specific chemical interaction with beta sheet rich structures found in amyloid aggregates.
The enhanced fluorescence of ThT is revealed by a characteristic red shift of the ThT emission spectrum detected by the spectrofluorometer. The time duration of this assay was also scaled to a period of 5 hours so as to measure the amount of time required for the peptide to fibrillize. It is also worthwhile to note that amyloid fibril formation for the full-length peptide showed a sharp increase at 2.5 hours and reached a maximum intensity. A relatively constant fluorescence intensity over the time period examined reflects the absence of fiber formation for the fragment peptides. Results of this assay are presented in Figure. 22.

![](image)

Figure. 22. Thioflavin T Fluorescence Intensity in the Presence of 25 μM hIAPP or Peptide Fragments Over Time.

The most obvious observation was the concentration dependence of peptide fragments on liposome vesicle damage. As anticipated, the full length hIAPP peptide was the most destructive and caused greater than 60% initial and 100% final (total vesicle destruction) dye leakage at 50 μM which is the highest concentration that was tested. A logarithmic regression for peptide concentration versus liposome damage with correlation coefficients of 0.81 and 0.87 is shown respectively, for initial (Figure. 20) and final (Figure.21) fluorescence readings.
It was also observed that membrane leakage increased significantly over the three hour time period and possibly may be attributed to amyloid aggregate formation, which worsens liposome destruction and related dye leakage. The concentration of peptide and presence of lipids influence the half life of amyloid fiber formation which has been reported as 60 minutes by earlier studies (20,49). In the ThT assay (Figure 22) a sharp spike in fluorescence was noted at 2.5 hours for the full length peptide only, suggesting amyloid fiber formation, that was maximized at three hours. In addition, Figures 20 and 21 reveal an obvious difference in the liposome leakage data between the lower peptide concentrations and 10 μM, indicating a 10 μM threshold required for inducing significant membrane damage in this assay.

As expected, the second most destructive fragment in this assay was the 1–19 segment. This fragment contains the N-terminal region, which is believed to induce transient membrane disruption by directing the binding or insertion of the peptide into the liposome membrane, inducing a first phase of damage. In the case of the full length hIAPP, this step is then followed by amyloid aggregate formation (50,51). This initial peptide-membrane interaction may be attributable to the positive charges in this region at amino acid positions 1, 11, and 18 (K, R, H) that attract it to the negatively charged phospholipid groups. However, this 1–19 segment alone does not have the capacity to cause amyloid aggregation (21), as it lacks the amyloidogenic 20–29 region. Consequently, the ThT assay (Figure 22) confirmed the absence of fiber formation for this fragment. This fragment is increasingly destructive in this assay over time and causes up to 60% dye leakage after 3 hours (only 17% initially) at 50 μM.
However, it should be noted that a clear minimal concentration is required, as effectively no dye leakage was incurred by concentrations tested that were lower than 10 µM. This pattern is similar to that observed for full length hIAPP except that the extent of damage is lower.

A logarithmic regression of the relationship between peptide concentration and percent dye leakage is shown in Figures 20 and 21 with correlation coefficients of 0.85 and 0.86, respectively. In general, the final dye leakage caused by this peptide was approximately 30% less than that caused by hIAPP itself at analogous concentrations (Figure 21), and initial dye leakage was considerably lower (Figure 20). Our results are roughly comparable to those of Brender et al. (21), who reported that the hIAPP 1–19 peptide is approximately as active as the full length peptide in POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol) liposome disruption at low concentration; however the study found that at higher concentrations, hIAPP 1–19 actually was more active than the full length peptide. The variability between results from our study and this previously published data may be attributed to the different lipid composition of the vesicles; POPG is more negatively charged than the 7:3 DOPC/DOPS mixture which is generally considered a good mimic of the islet cell. The 1–19 fragment is capable of both inducing negative curvature in lipid bilayers (52) and causing intracellular calcium release (53). Overall, it is clear that amyloid fiber formation is not a requirement for membrane damage.

The 20–29 region of hIAPP has long been speculated to mediate the aggregation of the amyloid fibrils (19, 20). This amyloidogenic region however has limited cytotoxicity on its own relative to the full length peptide, despite its ability to form fibrils (20, 54).
Data from this study support that finding, as this fragment was observed to be the least destructive of the fragments tested (Figures 20 and 21). While lower concentrations of the 20-29 peptide (below 20 μM) failed to cause any dye leakage from the vesicles, higher concentrations (up to 90 μM) induced weak dye leakage, at the maximum, up to 10%. Additionally, there was no significant increase in liposome damage over the extended 3 hour time period of the carboxyfluorescein assay and no fiber formation was observed on the ThT assay (Figure 22). This lack of activity by the 20-29 peptide fragment is in stark contrast to the extensive magnitude of liposome damage by the full length hIAPP. Our data support the theory that the 20–29 fragment may require the presence of some other region of hIAPP peptide in order to cause liposome damage. Most likely, this is the more positively charged N-terminal region of the native peptide, which is believed to direct insert into the membrane (50,51). Since this fragment lacks the positive charges that are present in the N-terminus, it does not experience a large degree of electrostatic attraction to the membrane by itself.

The 10–19 fragment contains a weakly amyloidogenic region (56); however, whether it could induce membrane disruption by itself was unknown. This fragment binds to a region of insulin, possibly via π-stacking interactions between aromatic residues, in a process that has been reported to inhibit fiber formation and limit cell membrane damage (11). Similar to the results of the 20–29 fragment from the dye leakage assay, data for this fragment demonstrate its inability to induce a great extent of dye leakage from the vesicles (Figs. 20 and 21) or to form amyloid fibers within the examined time period (Fig. 22). In fact, while the vesicle damage was slightly higher than that incurred by the 20–29 fragment (10–20% leakage), there was neither any observable concentration dependence nor significant increase of activity with time.
Despite the presence of two of the three positively charged amino acid side chains (positions 11 and 18) in the 10-19 fragment, which would theoretically allow higher electrostatic attraction to the liposomes relative to the 20–29 fragment, this fragment also fails to cause significant membrane damage. Although there is no direct evidence from this study that greater positive charge influences membrane damage, there is undoubtedly a correlation within this group of peptide fragments, where fragments with greater number of positively charged amino acids have shown higher levels of membrane destructive potential. This relationship and the theory that electrostatic interactions with membrane phospholipids accelerate hIAPP misfolding and subsequent activity have been supported by earlier studies (56). Additional support to these views is provided by results showing higher activity for all fragments with negatively charged POPG vesicles and lesser degree of activity in zwitterionic POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) vesicles compared to our vesicles composed of 7:3 DOPC/DOPS (from unpublished data obtained by our group).

Interestingly, the 10–29 fragment which is a combination of two relatively inactive and shorter fragments (the 10–19 and 20–29 peptides), showed perceptible activity in the dye leakage assay, which was noticeably higher than that seen with either of the two shorter fragments. The concentration dependence fit a logarithmic regression, with a correlation coefficient of 0.86 for the initial reading (Fig. 20) and 0.88 for the 3 hour reading (Fig. 21). The possible explanation for these findings perhaps relates to a membrane disturbing conformation adopted by the longer sequence that understandably cannot be achieved by either of the two shorter fragments. This idea was also proposed by Kajava et al. (57) who hypothesized a planar S-shaped folded structure for the 9th through the 37th amino acid residues of hIAPP, with three beta strands comprised of the segments 9–19, 20–29, and 30–37, respectively.
These strands are proposed to stack together to generate three parallel beta sheets in a cross-beta conformation. The 10–29 fragment, which contains the two smaller fragments that are required to form beta sheets according to this theory, may possibly assume a turn structure that can form similar stacked beta sheets. However, the ThT assay results for the 10-29 fragment failed to demonstrate perceptible amyloid fiber formation despite a moderate degree of liposome damage (Figs. 20, 21, 22), implying that its ability to disrupt liposomes is independent of amyloidogenesis. Alternatively, the 10–29 fragment may be long enough to insert into the membrane and still allow self-interaction at its C-terminus (the 20–29 region). At similar concentrations, this peptide was about half as active as the N-terminal 1–19 fragment and approximately one-fourth as active compared to the full length hIAPP. At the highest concentrations tested for this fragment (70 and 90 μM), which were higher than the concentrations tested for the full length peptide and the 1-19 sequence, dye leakage approached 50% after 3 hours (Fig. 21). Additionally, at all concentrations tested, there was a clear demonstration of increasing liposome damage with time. The worst degree of damage by this fragment, however, does not match up to the maximum damage impacted by the full length hIAPP. An important point to note with this 20 amino acid peptide is the presence of the His residue at the 18th position, which has been implicated in modulating the peptide-liposome membrane interaction in the human versus rat form of IAPP 1–19, thereby likely playing a key role in the cytotoxicity of non-fiber forming forms of the peptide (53). The rat form of the 1-19 peptide has the R instead of H at 18th position and renders the fragment inactive with little or no liposome destructive potential. Though we did not utilize the rat form of IAPP in our dye leakage assays, we compared results related to this peptide from earlier studies using CD spectroscopy and molecular modeling to extrapolate some of our results, in order to achieve some of our final conclusions.
In summary, our data support the two-step model where hIAPP first induces transient permeabilization and possible pore formation resulting in eventual liposome destruction. Smaller peptide fragments may not be able to adopt the same conformation as the full length analog during this process. The initial effect of peptides on the liposome membrane is observed to be destructive by itself (the first step), and is independent of amyloid fibril growth. In fact, this process may be a prerequisite to amyloid fibrillogenesis and growth, the second step of our proposed two step model. The initial step appears to be most likely mediated by the overall positively charged N-terminal 1–19 region of the peptide, as according to our data, this peptide fragment induced considerable membrane damage despite its inability to form amyloid fibers. Indeed, each short amyloidogenic region by itself did not induce extensive damage in this membrane model assay within the 3 hour time frame observed. We speculate that this may be due to a relative lack of overall positive charge required for electrostatic attraction to the membrane surface (20–29), or could be due to relatively shorter length or inability to insert into the membrane (both 10–19 and 20–29). The longer 10–29 fragment, which carries two of the three side chain positive charges of the 1–19 region, may be able to insert its N-terminus into the membrane with subsequent self-interaction of the amyloidogenic region on the membrane surface, giving it overall intermediate level activity. The other feature of this fragment could possibly relate to its ability to form a turn structure, similar to part of the proposed S-shaped fold, which can stack and generate the beta sheet structures found in amyloid fibers (57). Future experiments to determine whether surface binding is occurring will shed more light on the stepwise process of membrane interaction for these fragments.
While the positively charged N-terminal part of hIAPP appears to be necessary for the initial membrane interaction, it is the C-terminal region that most likely drives the oligomerization and subsequent amyloid fiber formation required to complete both the proposed steps in this molecular process. In the beta-structure model described above, the N-terminal 1–8 region is excluded from the serpentine turn and is free to interact with the membrane (57). It has been reported that hIAPP molecules tend to cluster in a trans-bilayer fashion in the membrane before fibrillization, with their N-termini directed toward the bilayer (56). Indeed, the reason that pre-formed fibers are not cytotoxic is likely due to their inability to insert into the membrane (51). Thus, we can conclude from the results of the dye leakage assays that a two step mechanism requires the entire hIAPP sequence. This can reasonably justify the extensive membrane damage occurring in the presence of the entire hIAPP molecule.

The association of peptide monomers into oligomers may eventually form channels through the cell membranes, leading to the leakage of cell contents and subsequent cell destruction (58). Other similar studies support a mechanism where hIAPP induces formation of small surface defects (59) or causes negative membrane curvature (52) which progresses to more extensive membrane damage. Any of these mechanisms is possible for the damage induced by the N-terminal region. It is also possible that, after hIAPP inserts into the membrane, there is a nucleation step leading to membrane fragmentation by the incorporation of lipid molecules into the amyloid fiber as it grows, and that fiber-making process accentuates the cytotoxicity (60).
4.3 Molecular Modeling and Circular Dichroism Experiments

The full length human IAPP, rat IAPP and all the previously examined fragments of the hIAPP molecule (Fig. 16) were analyzed and compared using molecular modeling. In addition, hIAPP fragments 10–19, 10–29 and 20-29 were analyzed by circular dichroism, followed by comparison to published CD data for the full length hIAPP and 1–19 analogs, to examine possible secondary structure.

The modeling calculations were mainly used to investigate two functionally relevant aspects of the fragments: charge distribution, as shown by the electrostatic potential mapped onto the total density surface, and propensity for aggregation dependent upon the overall shape/conformation attained by the fragment. Although the calculations were carried out in the gas phase, in the absence of phospholipids, the high level of theory employed here provides a very accurate depiction of the structural and electronic characteristics that ultimately influence the behavior and activity of the peptides.

In addition, the observed differences between the human and rat forms are very unambiguous, given their obvious differences and thus providing added confidence to our conclusions. According to modeling calculations the presence of predominantly positive charges at either end of the 1-19 fragment (1, 11, 18th amino acid positions) must allow this fragment greater ability to interact with the negatively charged membrane phospholipids. The dye leakage assay results reveal a high degree of liposome membrane destruction by this fragment, thus providing positive correlation with the conclusions derived from the molecular modeling calculations. The lack of the suspected amyloidogenic region, 20-29 within this fragment, correlates well with the absence of significant fibril formation in the ThT assay.
Another assumption that we can derive is that monomers exhibiting an open, mostly linear structure are able to form several intermolecular bonds (such as hydrogen bonds and electrostatic interactions), thus leading to dimer formation and eventually aggregation. This hypothesis is confirmed by comparison of the information obtained by computational analysis of the human and rat forms of IAPP, and both their respective amyloidogenic 20–29 fragments.

It is well known that rIAPP and the 20–29 fragment of rIAPP do not form amyloid deposits, unlike their human counterparts (19). In accordance with this hypothesis, we find that the optimized structure of hIAPP is linear and quite open, showing a $130^\circ$ angle at residue Leu (27th position); in contrast, the structure of rIAPP is folded onto itself, with a loop comprising residues 20–37 (Fig. 23). The same is true of the 20–29 fragments: the human peptide optimizes to a mostly linear shape, while the rat peptide folds onto itself, the folding likely contributing to its inability for dimerization/oligomerization. The slightly bent but relatively linear shape obtained for the 20-29 fragment of the hIAPP molecule agrees with several published studies. Additionally, results from our dye leakage assays also ascertain this fragment’s higher amyloidogenic potential, likely related to higher probability towards oligomerization.
Figure 23. Optimized structures of (a) hIAPP and (b) rIAPP. Hydrogen atoms are omitted for clarity.
Atomic structure data from modeling studies have revealed that residues 21–27 of hIAPP contain a small bend at the central Gly residue, facilitating the alignment of the segment in beta sheet layers upon aggregation (61). NMR and diffraction data confirm that the 20–29 segment has a mostly linear structure with a bend at Gly24 (62). In addition, when hIAPP interacts with negatively charged micelles, three amino acid residues, Phe, Ala, and Ile (23, 25, 26) form a small hydrophobic cluster that faces the hydrocarbon region of the liposomes/micelles, due to a distorted beta turn at residues Phe (F) and Gly (G) at the 23 and 24 positions of hIAPP, detected by NMR (61). This is in line with our optimized structure, where the ordered side chains of these residues are on the same side of the peptide backbone. It has also been proposed that charge–charge interactions and hydrophobic cluster formation drive the aggregation process (63). Accordingly, our optimized structure shows both charged and neutral side chain moieties that are easily accessible and prone to aggregation.

In terms of charge distribution, we observe that the 1–19 fragment monomer containing all the 3 positively charged amino acids has by far the most positive charge distribution at its N-terminus compared to all the fragments studied. However, the 1–19 fragment has a 110° bend at the Tyr9 residue while the 10–29 monomer fragment adopts an almost circular configuration likely due to different conformational influences. Optimized fragment structures are shown in Figure 24 on the next page. Electron density maps of the fragments and hIAPP are shown in Figure 25. In addition, we found that the shorter 10–19 and 20–29 fragments have mostly linear structures, which may be compatible with possible clustering and amyloid formation, although the ThT assay did not support fibrillization of these analogs.
Figure 24. Optimized structures of hIAPP fragments (a) 1-19, (b) 10-19, (c) 20-29 (d) 10-29. Hydrogen atoms are omitted for clarity.
Figure 25. Electron Density Maps of (a) 1-19, (b) 10-19, (c) 20-29 and (d) 10-29 Fragments and (e) hIAPP. Surfaces are constructed by mapping electrostatic potential onto the total electron density. Blue shaded regions indicate localized positive charge.
These computational results are in agreement with the experimental data which indicate that the 1–19 fragment causes the greatest liposome damage, supposedly by inserting into the membrane with its positively charged N-terminus, but does not form fibers, given its L-shaped structure. In fact, circular dichroism (CD) spectra have indicated that this fragment, similar to hIAPP 1–37, initially adopts a random coil conformation in buffer, and immediately converts to an alpha helix in the presence of negatively charged lipids. However, unlike full length hIAPP, there is no subsequent conversion to the beta sheet structure that is usually associated with amyloid fiber formation (21).

The computational data for fragment 20–29 is also in line with the accepted view, that despite low levels of liposome membrane damage due to lack of concentration of positive charge at its N-terminus (confirmed by the electron density distribution map) this fragment has better ability to form amyloid fibrils, given its mostly linear shape. Based upon CD studies, an extended beta sheet structure has been reported for this segment following four days of peptide aging in solution (64), which agrees with our linear model. While CD spectra that show a pronounced double minimum at 210 and 225 nm usually indicate an $\alpha$ helical configuration of the peptide fragment being tested, a single minimum at $\sim$220 and $\sim$195 nm reflect a $\beta$ sheet and random coil secondary structure, respectively.

Our CD analysis of the 20–29 segment, on the same 3 hour time scale as published CD studies of hIAPP 1–19 and 1–37 (21) and that of the dye leakage assay, indicates that it initially adopts a random coil structure in buffer like the other fragments (Figure 26). However, its persistent unstructured conformation an hour after the addition of lipid vesicles (Figure 27) suggests that the lipids do not influence its conformation as they do for the N-terminus containing peptides. This feature also reiterates the finding from the ThT assay that fragment 20–29 is not aggregating within this time frame.
Figure 26. CD Spectra of fragments 10-19 (green dotted line), 20-29 (blue dashed line) and 10-29 (red solid line) in pH 7.5 sodium phosphate buffer at 25 μM.

Figure 27. CD Spectra of fragments 10-19 (green dotted line), 20-29 (blue dashed line) and 10-29 (red solid line) in pH 7.5 sodium phosphate buffer at 25 μM one hour after addition of 400 μM 7:3 DOPC: DOPS vesicles.
Segment 10–19 also has a linear optimized structure and, although its charge distribution is fairly neutral and we would not expect it to be extensively attracted to the membrane, we would expect it to dimerize quite readily, as it reportedly does within the full length peptide (51). Our CD analysis indicates a random coil structure for this peptide, both in buffer alone and after the addition of liposomes (Figures 26 and 27). This is in agreement with published CD spectra for the similar analog hIAPP 8–20, which show this fragment adopting a random coil conformation in solution, with conversion to the beta sheet configuration after a four week incubation (51). The electron density map of hIAPP 10–19 shows slightly more localized positive charge near its N-terminus than the 20–29 fragment, but not as much as the 1–19 and full length peptides, which compares well with their relative activities.

The most interesting results were obtained for the 10–29 segment: this peptide had noteworthy membrane destructive activity but there was no evidence of fibril formation. The optimized conformation of this fragment in the form of an oval loop likely explains the lack of amyloid fibril formation. This conformation maximizes intramolecular interactions (between the two ends of the peptide fragment) at the expense of intermolecular interactions that are required to form dimers and eventually fibrils. However, the loop conformation of this fragment compares well with the turn of a truncated serpentine “S” shape proposed by Kajava et al. (57), so stacking of these structures is not precluded. The CD spectra of this fragment show a negative minimum intensity between 195 and 200 nm, which is indicative of a random coil arrangement in both the absence (Figure 26) and presence (Figure 27) of negatively charged lipids.
There was no evidence of conversion to either alpha helical or beta sheet structures within the 3 hour time frame monitored, which is in accordance with results from the ThT assay where there was little amyloid fibril formation even at the end of 3 hours. Though identical in length and 50% identical in sequence to the 1–19 fragment, hIAPP 10–29 does not appear to undergo the same degree of conformational change in the presence of negatively charged phospholipids, which may contribute to its relatively lower fibril forming activity. The electron density map of this fragment shows some positive charge distribution near its N-terminus, which may help attract it to the membrane and cause a moderate degree of liposome membrane damage.

In summary, when compared with previously published data in this area, our results support a two step model of pancreatic beta cell damage. The hIAPP molecule most likely exerts its damaging effects by first binding at the surface of the membrane via its positively charged N-terminal region, transiently disturbing the lipid bilayer core of the cell membranes, followed by formation of cytotoxic oligomers and eventual amyloid fibers. The first binding step induces some initial membrane damage which is later followed by the fibrillization process, gradually increasing the extent of membrane destruction. In fact, the initial membrane damage may help promote aggregation by increasing the local concentration of membrane bound hIAPP, or by influencing a conformational change in the membrane.

To further explore the correlation between positive charge distribution and liposome damage, impending studies will/can be aimed at assessing the activity of analogs where the positive charge has been masked or replaced. These studies would successfully help confirm the role of these culprit amino acid residues.
Concluding Remarks

In our study, peptide fragments of hIAPP were tested for their ability to disrupt liposomes (used as model pancreatic cell membranes) and cause amyloid fibrillization. These findings were compared to the activity of the full-length native peptide. While a maximal effect was seen for full-length hIAPP (the only peptide that formed fibers in our assay), hIAPP 1–19 was also very damaging to the liposomes. Both of these peptides showed concentration dependent damage that approached a maximum of 100% and 63% dye leakage, respectively, after 3 hours at 50 µM. The smaller, more amyloidogenic fragments (10–19 and 20–29) were much less damaging to the vesicles by themselves and displayed little concentration dependence (20% and 10% leakage maximum, respectively, after 3 hours at 50–60 µM). However, a combination of the two smaller fragments, the 10–29 sequence showed moderate liposome destruction with concentration dependent activity (30% leakage after 3 hours at 50 µM). The results of molecular modeling calculations suggest that peptides with a significant amount of positive charge at the N-terminus (containing amino acid residues 1,11,18) are more likely to cause membrane damage via increased electrostatic affinity for the negatively charged liposomes. Conversely, the overall shape of the peptide fragment influences its ability to form amyloid fibrils: while the linear segments may be more likely to aggregate, peptides that have sharp bends in their structure may have fewer sites available for binding to other peptides and thus limited amyloidogenic potential.
Future work

Translation of our research project to *in vivo* animal studies would help confirm our experimental results and be used in preclinical research development. It would lead to highly regulated temperature, pH, osmotic and chemical environment that is currently not feasible within our experimental set up.

Increasing health care costs coupled to the World Health Organization’s prediction of a worldwide Diabetic epidemic by the year 2030, make experimental diabetes research a crucial prologue to future clinical trials in prevention, diagnosis and treatment of Diabetes mellitus type II. Identifying causative factors for Diabetes mellitus Type II and extrapolation of the inferences obtained, to other amyloid associated diseases will lead to substantial reduction in morbidity and mortality and to the improvement of quality of life for people suffering from these diseases.

Research in peptide science and technology has the potential to create hitherto unknown inroads in the emerging fields of molecular imaging and molecular medicine, which will revolutionize the bench to bedside concept of translational research and clinical practice in the years to come.
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1) Figure 1 [http://en.wikipedia.org/wiki/File:Duodenumandpancreas.jpg#file](http://en.wikipedia.org/wiki/File:Duodenumandpancreas.jpg#file)

2) Figure 2

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3) Figure 3 [CREATIVE SCIENCE QUARTERLY](http://www.scq.ubc.ca/islet-amyloid-a-culprit-in-type-2-diabetes/)

4) Figure 4 Worldwide distribution of Non communicable disease (2008 W.H.O. report).
5) Figure 5. Flowchart of solid phase peptide synthesis.


6) Figure 6. Ball and stick visualisation of solid phase peptide synthesis.

http://en.wikipedia.org/wiki/Peptide_synthesis

7) Figure 7. Sequence of the Human IAPP

http://www.phoenixbiotech.net/Catalog%20Files/Amylin%20Section/02_fig1.jpg

8) PS3 peptide synthesizer with components (Laboratory photograph).

9) Reaction vessels with raw material (Laboratory photograph).

10) Peptide filled vials arranged in required sequence (Laboratory photograph).

11) Peptide-Resin cleavage (Laboratory photograph).

12) Lyophilization-freeze dried peptide (Laboratory photograph).

13) Lyophilization-freeze dried peptide (Laboratory photograph).

14) Final peptide product (Laboratory photograph).

15) Figure 15. Reverse phase – High Performance Liquid Chromatography (RP-HPLC)-analysis of peptide purity.

16) Figure 16. Electrospray mass spectrometry confirmation of peptide purity.
17) Figure 17. Thioflavin molecular structure. [http://en.wikipedia.org/wiki/Thioflavin](http://en.wikipedia.org/wiki/Thioflavin)


19) Figure 19. Sequences of hIAPP fragments and rIAPP (human and rat)

20) Figure 20. Initial dye leakage (Laboratory data)

21) Figure 21. Final dye leakage (Laboratory data)

22) Figure 22. Thioflavin T assay (Laboratory data)

23) Figure 23. Optimized structures of hIAPP and rIAPP (Laboratory data)

24) Figure 24. Optimized structures of hIAPP fragments (Laboratory data)

25) Figure 25. Electron Density Maps of hIAPP fragments (Laboratory data)

26) Figure 26. Initial CD Spectra of hIAPP fragments (Laboratory data)

27) Figure 27. CD Spectra of hIAPP fragments 1 hour after addition of liposomes (Laboratory data)