A Novel Anti-Diabetic Peptide Nanomedicine Against Pancreatogenic Diabetes

BY

AMRITA BANERJEE
M.S., Birla Institute of Technology Mesra, India, 2007

THESIS

Submitted as partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biopharmaceutical Sciences in the Graduate College of the University of Illinois at Chicago, 2012

Chicago, Illinois

Defense Committee:

Hayat Onyuksel, Chair and Advisor
Francis Schlemmer
Seungpyo Hong
Hyun-Young Jeong
Pradeep Dudeja, Digestive Diseases and Nutrition
This thesis is dedicated to my family and Shubhasis Chakrabarty for their endless support and encouragement.
ACKNOWLEDGEMENTS

I would like to thank several people who have lent their immense support toward the completion of the work documented in this thesis.

At the outset, my sincerest thanks to my research advisor, Dr. Hayat Onyuksel who provided me the opportunity to work in her laboratory and guided me all throughout my dissertation research. Under her supervision, I was encouraged to think independently and conduct a proper hypothesis driven research. Most of all, I am thankful to her for allowing me choose my own research area based on my interest and for lending me intellectual as well as financial support during the period. I would also like thank Dr. Francis Schlemmer, Dr. Seungpyo Hong, Dr. Hyun-Young Jeong and Dr. Pradeep Dudeja for graciously agreeing to be a part of my thesis committee and for providing valuable inputs during committee meetings.

My thanks to Dr. Bao-Shiang Lee from Research Resources Center UIC, for synthesizing and purifying pancreatic polypeptide used in the research; and for allowing me to use the spectropolarimeter for long periods of time. I am immensely grateful to Mr. Z. Lowell Stein for his assistance during studies involving high pressure liquid chromatography and for all the interesting reading materials that he provided.

Last but not the least; I would like to thank both my present and past lab members for their invaluable help and friendship during my stay in the laboratory. I am grateful to Dr. Sok Bee Lim, Dr. Antonina Kuzmis, Fatima Khaja, Ece Gulcur, Mentor Thaqi, Stephanie Drake and Kenneth Brandenburg for teaching me to use instruments that have been used in this project.

AB
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>A. PANCREATOGENIC DIABETES</td>
<td>1</td>
</tr>
<tr>
<td>1. Causes of pancreatogenic diabetes</td>
<td>2</td>
</tr>
<tr>
<td>2. Pathogenesis of pancreatogenic diabetes</td>
<td>3</td>
</tr>
<tr>
<td>3. Current therapies for pancreatogenic diabetes</td>
<td>5</td>
</tr>
<tr>
<td>4. Limitations of current therapy for pancreatogenic diabetes</td>
<td>6</td>
</tr>
<tr>
<td>5. Pancreatogenic diabetes animal model</td>
<td>8</td>
</tr>
<tr>
<td>B. STERICALLY STABILIZED PHOSPHOLIPID MICELLES AS DRUG DELIVER SYSTEMS</td>
<td>10</td>
</tr>
<tr>
<td>1. Sterically stabilized phospholipid simple micelles</td>
<td>10</td>
</tr>
<tr>
<td>2. Sterically stabilized micelles as carriers for peptide drugs</td>
<td>15</td>
</tr>
<tr>
<td>3. Influence of electrolytes on properties of sterically stabilized micelles</td>
<td>20</td>
</tr>
<tr>
<td>C. PANCREATIC POLYPEPTIDE</td>
<td>22</td>
</tr>
<tr>
<td>1. Physiological role of pancreatic polypeptide</td>
<td>22</td>
</tr>
<tr>
<td>2. Limitations in therapeutic application of PP</td>
<td>24</td>
</tr>
<tr>
<td>3. PP analogues and current clinical trials</td>
<td>24</td>
</tr>
<tr>
<td>4. Pancreatic polypeptide in sterically stabilized micelles</td>
<td>25</td>
</tr>
<tr>
<td>D. HYPOTHESIS AND SPECIFIC AIMS</td>
<td>27</td>
</tr>
<tr>
<td>E. SIGNIFICANCE OF THE PROJECT</td>
<td>29</td>
</tr>
<tr>
<td>II. MATERIALS AND METHODS</td>
<td>31</td>
</tr>
<tr>
<td>A. MATERIALS</td>
<td>31</td>
</tr>
<tr>
<td>B. METHODS</td>
<td>32</td>
</tr>
<tr>
<td>1. In Vitro experiments</td>
<td>32</td>
</tr>
<tr>
<td>1.1. Preparation of PP-SSM</td>
<td>32</td>
</tr>
<tr>
<td>1.2. Characterization of PP-SSM</td>
<td>33</td>
</tr>
<tr>
<td>1.2.1 Particle size distribution analysis</td>
<td>33</td>
</tr>
<tr>
<td>1.2.2 Fluorescence spectroscopy</td>
<td>34</td>
</tr>
<tr>
<td>1.2.3 Circular dichroism</td>
<td>35</td>
</tr>
<tr>
<td>1.3. Influence of pH and electrolyte on peptide micelle association</td>
<td>35</td>
</tr>
<tr>
<td>1.4. Freeze-drying of PP-SSM</td>
<td>37</td>
</tr>
<tr>
<td>1.5. Assessment of stability of PP-SSM against proteolytic degradation</td>
<td>38</td>
</tr>
<tr>
<td>1.6. In vitro bioactivity assessment of PP-SSM</td>
<td>40</td>
</tr>
<tr>
<td>2. In Vivo Experiments</td>
<td>41</td>
</tr>
<tr>
<td>2.1. Glucose tolerance and insulin sensitivity tests</td>
<td>41</td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS (continued)

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2. Induction of pancreatogenic diabetes in rats</td>
<td>42</td>
</tr>
<tr>
<td>2.3. Antidiabetic efficacy study of PP-SSM</td>
<td>43</td>
</tr>
<tr>
<td>2.4. Assessment of hepatic glycogen content</td>
<td>45</td>
</tr>
<tr>
<td>C. DATA AND STATISTICAL ANALYSES</td>
<td>46</td>
</tr>
<tr>
<td>III. RESULTS</td>
<td>47</td>
</tr>
<tr>
<td>A. IN VITRO EXPERIMENTS</td>
<td>47</td>
</tr>
<tr>
<td>1. Characterization of PP-SSM</td>
<td>47</td>
</tr>
<tr>
<td>1.1. Particle size analysis</td>
<td>47</td>
</tr>
<tr>
<td>1.2. Fluorescence spectroscopy</td>
<td>49</td>
</tr>
<tr>
<td>1.3. Circular dichroism</td>
<td>50</td>
</tr>
<tr>
<td>2. Influence of pH and electrolyte on peptide loading in micelles</td>
<td>51</td>
</tr>
<tr>
<td>3. Freeze-drying of PP-SSM</td>
<td>57</td>
</tr>
<tr>
<td>4. Assessment of stability of PP-SSM against proteolytic degradation</td>
<td>63</td>
</tr>
<tr>
<td>5. <em>In vitro</em> bioactivity assessment of PP-SSM</td>
<td>66</td>
</tr>
<tr>
<td>B. IN VIVO EXPERIMENTS</td>
<td>68</td>
</tr>
<tr>
<td>1. Glucose tolerance test</td>
<td>69</td>
</tr>
<tr>
<td>2. Insulin sensitivity test</td>
<td>73</td>
</tr>
<tr>
<td>3. Assessment of hepatic glycogen content</td>
<td>76</td>
</tr>
<tr>
<td>IV. DISCUSSION</td>
<td>79</td>
</tr>
<tr>
<td>A. IN VITRO EXPERIMENTS</td>
<td>79</td>
</tr>
<tr>
<td>1. Preparation and characterization of PP-SSM</td>
<td>79</td>
</tr>
<tr>
<td>2. Effect of electrolytes on peptide loading in micelles</td>
<td>80</td>
</tr>
<tr>
<td>3. Freeze drying stability of PP-SSM</td>
<td>82</td>
</tr>
<tr>
<td>4. Assessment of stability of PP-SSM against proteolytic degradation</td>
<td>82</td>
</tr>
<tr>
<td>5. <em>In vitro</em> bioactivity assessment of PP-SSM</td>
<td>83</td>
</tr>
<tr>
<td>B. IN VIVO EXPERIMENTS</td>
<td>84</td>
</tr>
<tr>
<td>1. Glucose tolerance test</td>
<td>84</td>
</tr>
<tr>
<td>2. Insulin sensitivity test</td>
<td>85</td>
</tr>
<tr>
<td>3. Assessment of hepatic glycogen content</td>
<td>86</td>
</tr>
<tr>
<td>V. CONCLUSIONS</td>
<td>89</td>
</tr>
<tr>
<td>VI. FUTURE DIRECTIONS</td>
<td>93</td>
</tr>
<tr>
<td>CITED LITERATURE</td>
<td>96</td>
</tr>
<tr>
<td>CHAPTER</td>
<td>PAGE</td>
</tr>
<tr>
<td>----------</td>
<td>------</td>
</tr>
<tr>
<td>APPENDIX</td>
<td>96</td>
</tr>
<tr>
<td>VITA</td>
<td>108</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Peptides associated with sterically stabilized micelles</td>
<td>19</td>
</tr>
<tr>
<td>II. HPLC parameters for pancreatic polypeptide detection and quantification</td>
<td>39</td>
</tr>
<tr>
<td>III. Effect of pH and presence or absence of sodium chloride in the aqueous media on the association of PP with SSM</td>
<td>55</td>
</tr>
<tr>
<td>IV. Particle size distribution of PP-SSM before and after freeze-drying</td>
<td>59</td>
</tr>
<tr>
<td>V. Rate of degradation and half-life of PP in buffer or SSM subjected to tryptic digestion</td>
<td>63</td>
</tr>
<tr>
<td>VI. Glucose tolerance in normal, diseased and drug treated animals</td>
<td>70</td>
</tr>
<tr>
<td>VII. Insulin sensitivity in normal, diseased and drug treated animals</td>
<td>74</td>
</tr>
<tr>
<td>VIII. Hepatic glycogen content in drug treated animals</td>
<td>77</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

FIGURE PAGE

1. Pathogenesis of pancreatogenic diabetes secondary to chronic pancreatitis ........... 5
2. Chemical structure of distearoyl phosphatidylethanolamine chemically conjugated to polyethylene glycol ...................................................................................................................... 10
3. Illustration of formation of SSM. ........................................................................... 14
4. Diagrammatic representation of preparation of peptide loaded micelles .................. 16
5. Size and aggregation numbers of phospholipid monomer, and micelles prepared in presence and absence of sodium chloride ................................................................................................... 21
6. The amino acid sequence of pancreatic polypeptide.............................................. 23
7. Schema for anti-diabetic efficacy studies in rats...................................................... 46
8. Particle size distribution of PP in presence or absence of SSM............................ 48
9. Fluorescence emission of PP in presence or absence of SSM ................................. 49
10. Secondary structure of PP in presence or absence of SSM ........................................ 50
11. Saturation curves of PP-SSM in buffers of different pH in the absence of sodium chloride . 53
12. Saturation curves of PP-SSM in normal saline or buffers of different pH in the presence of sodium chloride ............................................................................................................................ 54
13. Secondary structure of PP at different pHs in presence or absence of SSM prepared in phosphate buffer .................................................................................................................................. 56
14. Representative photograph of lyophilized cake of PP-SSM .................................. 59
15. Particle size distribution of PP-SSM before and after lyophilization ....................... 60
16. Fluorescence emission of PP-SSM before and after lyophilization ......................... 61
17. Secondary structure of PP before and after lyophilization ..................................... 62
18. Tryptic digestion kinetics of PP and PP-SSM ....................................................... 64
19. Semi log plot of tryptic digestion kinetics of PP and PP-SSM ................................. 65
# LIST OF FIGURES (continued)

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>20. Inhibition of forskolin stimulated cyclic AMP production by PP and PP-SSM</td>
<td>67</td>
</tr>
<tr>
<td>21. Glucose tolerance in normal animals, diseased animals and animals subjected to various treatments</td>
<td>71</td>
</tr>
<tr>
<td>22. Glycemic excursions in animals subjected to different treatments after glucose injection</td>
<td>72</td>
</tr>
<tr>
<td>23. Insulin sensitivity in normal animals, diseased animals and animals subjected to various treatments</td>
<td>75</td>
</tr>
<tr>
<td>24. Glycogen content in livers of animals subjected to different treatments</td>
<td>78</td>
</tr>
</tbody>
</table>
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenoside monophosphate</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
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<tr>
<td>Cmax</td>
<td>Concentration maximum</td>
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<tr>
<td>CP</td>
<td>Chronic pancreatitis</td>
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<td>CMC</td>
<td>Critical micelle concentration</td>
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<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
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<tr>
<td>DM</td>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>DSPE-PEG\textsubscript{2000}</td>
<td>1,2-Distearoyl-\textit{sn}-glycero-3-phosphoethanolamine-\textit{N}-methoxy-[poly(ethylene glycol); molecular weight 2,000]</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle’s minimum essential media</td>
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<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>EPR</td>
<td>Enhanced permeability and retention</td>
</tr>
<tr>
<td>FD</td>
<td>Freeze drying</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GIP</td>
<td>Glucose dependent insulinotropic polypeptide</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon like peptide-1</td>
</tr>
<tr>
<td>GTT</td>
<td>Glucose tolerance test</td>
</tr>
</tbody>
</table>
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES</td>
<td>(N- [2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid])</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-Isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin like growth factor</td>
</tr>
<tr>
<td>IGT</td>
<td>Impaired glucose tolerance</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>IST</td>
<td>Insulin sensitivity test</td>
</tr>
<tr>
<td>MODY1</td>
<td>Maturity onset diabetes of young type 1</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>PACAP&lt;sub&gt;1-38&lt;/sub&gt;</td>
<td>Pituitary adenylate cyclase-activating peptide&lt;sub&gt;1-38&lt;/sub&gt;</td>
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<tr>
<td>PB</td>
<td>Phosphate buffer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PD</td>
<td>Pancreatogenic diabetes</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PP</td>
<td>Pancreatic polypeptide</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Peroxisome proliferators activated receptor gamma</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reverse-phase high performance liquid chromatography</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SSM</td>
<td>Sterically stabilized micelles</td>
</tr>
</tbody>
</table>
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSMM</td>
<td>Sterically stabilized mixed micelles</td>
</tr>
<tr>
<td>T1DM</td>
<td>Type 1 diabetes mellitus</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>T3cDM</td>
<td>Type 3c diabetes mellitus</td>
</tr>
<tr>
<td>tmax</td>
<td>Maximum time</td>
</tr>
<tr>
<td>TREM</td>
<td>Tiggering receptor expressed on myeloid cells-1</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
</tr>
</tbody>
</table>
SUMMARY

This dissertation research aimed to develop a novel and safe nanomedicine of human pancreatic polypeptide (PP) for management of pancreatogenic diabetes (PD). PD is a sub-type of diabetes mellitus which occurs secondary to pancreatic disorders such as chronic pancreatitis, pancreatic neoplasia and resection of the pancreas amongst others. As a result, endocrine dysfunction is observed which leads to development of diabetes. Patients suffering from this disease have reduced levels of insulin, glucagon, PP and glucagon like peptide-1 (GLP-1) in their body. However, PD is associated with only mild hyperglycemia as exocrine insufficiency observed due to damage to pancreas also leads to malabsorption of nutrients from the gut that counterbalances the impaired glucose clearance from the blood due to deficiency of insulin. The two pathological hallmarks of PD are impaired glucose tolerance (IGT) and hepatic insulin resistance. Impaired glucose tolerance is a clinical condition where a person has slightly higher blood glucose level than normal but not sufficiently high to be severely hyperglycemic. IGT develops as a result of decreased insulin secretion from the pancreas and insulin resistance. Insulin resistance observed in PD is a physiological condition wherein the body is unable to utilize insulin effectively due to various reasons such as presence of antibodies against insulin, lack of expression of insulin receptors, decreased interaction of insulin with insulin receptors due to presence of antibodies binding to the receptors and post receptor signaling failure amongst others. In PD, only hepatic insulin resistance is observed while peripheral insulin sensitivity (at muscle and adipose tissues) is normal. This feature differentiates PD from type 2 diabetes mellitus where both hepatic and peripheral insulin
resistance is observed. PD is distinct from type 1 diabetes mellitus from the fact that ketoacidosis, that is a common occurrence in type 1 diabetes mellitus, is not detected in PD. In addition, in type 1 diabetes mellitus, only a deficiency of insulin is observed whereas in PD, there is a lack of secretion of all endocrine hormones. This factor complicates the management of PD as insulin injections often leads to dangerously low blood glucose level in the body due to absence of counter-regulatory hormone glucagon. Therefore PD is also termed as ‘brittle’ diabetes and PD patients on insulin therapy need to be monitored carefully for development of acute hypoglycemia.

The pathophysiological basis of the disease is destruction of pancreatic architecture. The acinar cells are damaged due to underlying pancreatic disorder such as pancreatitis. Existence of chronic inflammatory conditions causes fibrosis of the pancreatic tissues, blockage of pancreatic ducts and separation of endocrine cells into small dysfunctional lobules. Owing to this, the pancreas loses its exocrine as well as endocrine function and the latter is responsible for development of PD. Occurrence of this disease is quite common in southeast Asian countries where there is a high prevalence of tropical and fibrocalcific pancreatitis. In North America, PD occurs mostly because of alcoholism which results in bouts of acute pancreatitis episodes, culminating in the development of chronic pancreatitis. Patients suffering from PD are at a high risk of contracting pancreatic cancer which amplifies the mortality index of such patients. However, it must be noted that no clear consensus is available on the number of
mortalities due to PD as the disease is complex disorder and death cannot be solely attributed to diabetes.

Management of PD requires special considerations because of its ‘brittle’ nature and the risk of development of pancreatic cancer. There are no specific guidelines for treatment of PD but drugs that are prescribed for type 2 diabetes mellitus are used for therapy. These drugs include insulin and insulin secretagogues, metformin, peroxisome proliferator activated receptor gamma (PPAR γ) agonists and alpha glucosidase inhibitors. Insulin therapy, apart from standing the risk of causing severe life threatening hypoglycemia, has also been found to increase the risk of development of cancer in patients with chronic pancreatitis. Since chronic pancreatitis is the most important precipitating factor for PD, it can be construed that insulin therapy for PD patients is fraught with many risks. Metformin on the other hand demonstrates antineoplastic activity and reduces the risk of cancer in diabetic patients, but use of metformin is contraindicated in alcholism which is the primary reason for development of CP, therefore metformin is not suitable for treatment of CP induced PD. In addition, metformin also acutely increases the secretion of GLP-1 (an insulin secretagogue) from the pancreas. PPARγ agonists on the other hand, are associated with risks such as edema due to significant fluid retention and congestive heart disease. Alpha-glucosidase inhibitor drugs decrease the absorption of glucose from the gut which in the case of PD, can only encumber upon the underlying malabsorption problem associated with this disease. Therefore, great caution must be exercised while treating PD patients with drugs that are conventionally used for management
of type 2 diabetes mellitus. Currently there is no drug available in the clinics that will effectively address the problem of impaired glucose tolerance and hepatic insulin resistance in PD without any significant risk.

To address this problem, we used PP for the management of PD. Hepatic insulin resistance in PD has been attributed to the deficiency of PP in PD patients. PP is a 36 amino acid long peptide that is secreted by the F cells of the pancreas mostly as a response to food intake. It exerts its anti-diabetic activity by increasing the expression of insulin receptors in the hepatocytes. Therefore, not surprisingly, lack of this hormone results in development of hepatic insulin resistance. Moreover, the peptide inhibits the production of resistin (a hormone that is strongly implicated in the development of insulin resistance). Exogenous administration of this peptide does not increase insulin levels in the body, therefore treatment of PD using PP can be considered safe.

However, the peptide suffers from the problem of short biological half-life that curtails its therapeutic application and necessitates administration of PP as continuous intravenous infusion which is both patient non-compliant and can lead to development of adverse effects due to higher total dose administered that may interact with off target site receptors in the body. Formulations of free PP, such as PP in saline/buffer have limited usefulness due to self-aggregation propensity of the peptide. Administration of aggregated peptides reduces the total dose available for therapy since aggregated peptides are rapidly cleared from the body and only
peptide monomers are able to interact with the receptors to bring about a pharmacological response.

To overcome these problems, our lab has developed an innovative nanomicellar system to improve stability of PP in vivo. We have earlier shown that association of amphiphilic peptides such as vasoactive intestinal peptide (VIP) with sterically stabilized micelles (SSM) diminishes its degradation by proteolytic enzymes and allows its extravasation only at the disease site that minimizes development of adverse effects. The formulation of PP in SSM (PP-SSM) can be passively targeted to the liver through its fenestrations where it can be readily taken up by the receptors of the peptide (Y4 receptors) expressed on the hepatocytes. Polyethylene glycol (PEG) layer of the micelles will provide steric hindrance to the formulation, thus help evade the reticulo-endothelial system, making the formulation long-circulating. The altered pharmacokinetics and biodistribution of PP-SSM, can lead to higher total accumulation of the peptide in the liver, that will reduce the total dose required for therapy. We have earlier observed that upon association with SSM, amphiphilic peptides such as VIP and GLP-1 adopt an alpha-helical conformation that is not only the most favorable conformation for receptor interaction for these peptides but also increases the stability of the peptide against proteolytic degradation in vivo. Increased stability of peptide molecules in micelles precludes the need of administering large peptide doses for achieving therapeutic efficacy with no toxicity. Similar to VIP and GLP-1, interaction of PP with its receptors (Y4 receptors) also requires alpha-helical conformation that further asserts the suitability of delivery of PP in SSM. Furthermore, peptides
associate with SSM as monomers that subvert the pre-disposition of these biomolecules toward self-aggregation. In addition, SSM are easy to prepare, can be sterile filtered, lyophilized without the use of any cryo/lyoprotectant and scaled-up for large scale production. SSM are also considered a safe delivery vehicle as it is composed of phospholipids which are an essential part of all living cells and the PEGylated phospholipid molecule used for preparation of SSM has been approved for human use in another parenteral formulation (Doxil®). Based on these rationales, the hypothesis of my dissertation research was that PP will self-associate with SSM to form a novel, anti-diabetic nanomedicine against pancreatogenic diabetes. The specific aims designed to test this hypothesis were as follows:

1. Prepare and characterize a formulation of PP in sterically stabilized micelles
2. Evaluate in vitro stability and bioactivity of PP in micelles
3. Evaluate anti-diabetic activity of PP-SSM in vivo

Results from the characterization studies indicated that PP self-associated with SSM. This thwarted the self-aggregation of the peptide molecules as observed in formulation of PP in buffer. Fluorescence emission from the tyrosine amino acids of the peptide was monitored to confirm association of PP with SSM. The fluorescence emission of PP in SSM was approximately three fold higher than PP in buffer. We believe that this was due to association of PP with SSM as monomers that decrease the self-quenching observed in PP aggregates. The native peptide
demonstrated some degree of alpha-helical conformation but SSM increased the alpha-helicity of the peptides, thus provided conformational stability to the molecules.

PP-SSM nanomedicine was further characterized for their association in different pH phosphate buffers and in the presence or absence of strong electrolytes in the buffer. We had earlier observed that strong electrolytes such as sodium chloride influence the properties of SSM. In the absence of sodium chloride, aggregation number of the micelles was reduced; small sized micelles are formed and critical micelle concentration was increased 10 fold as compared to SSM prepared in buffers containing sodium chloride. Therefore, presence or absence of sodium chloride in the aqueous media was likely to influence interaction of PP with SSM. The influence of pH on PP-SSM interaction was studied because solubility of a peptide in aqueous media is largely dependent on the pH of the media. To study the effect of these formulation parameters on peptide-micelle association, PP-SSM was prepared in pH 6.5, 7.4 and 8.0 phosphate buffers with or without 0.9% w/v sodium chloride in aqueous media. Association was characterized through determination of saturation molar ratio of association between PP and SSM and conformation of the peptide in these formulations. The saturation molar ratio of association between PP and SSM was used to calculate the maximum number of peptide molecules that associated with each micelle. We found a strong influence of presence or absence of sodium chloride on the association molar ratio of PP-SSM. In the absence of sodium chloride, the association ratio was lower ranging between 8:1 and 11:1 lipid: peptide ratio. On the contrary, the molar ratio of association of PP-SSM prepared in buffers containing sodium
SUMMARY (continued)

chloride was much higher and ranged between 32:1 and 49:1 lipid: peptide ratio. This suggested a rapid and higher degree of association between PP and SSM in the absence of sodium chloride. However, the number of peptide molecules per micelle was found to be similar in both cases (~2-3 per micelle). This was determined based on the difference in the property of SSM such as size and aggregation number in the presence and absence of sodium chloride. In the absence of sodium chloride, there is no screening of charges on the phospholipid head group of micelles. As a result electrostatic repulsion between phospholipid molecules occur that decreases the aggregation number, hence small sized micelles (~7.7 nm) are formed. Due to electrostatic repulsion between phospholipid molecules, there is more available room for association of PP with each micelle. Charges on the peptide molecules can also potentially drive electrostatic interaction between PP and SSM. These account for the rapid and higher degree of association between PP and SSM, but small size of the micelles limits association to only 2-3 PP molecules per micelle. In the presence of sodium chloride, the charges on both phospholipid head groups and peptide molecules are masked. Due to absence of electrostatic repulsion between phospholipid molecules, the aggregation number is increased and large sized micelles are formed (~14 nm). Increased stacking of phospholipid molecules per micelle results in lesser room for association of PP molecules with SSM but the large size of micelles counterbalances this shortcoming. Therefore despite a lower degree of association between PP and SSM, the number of peptide molecules per micelle in presence of sodium chloride is similar to that observed for PP-SSM prepared in the absence of sodium.
SUMMARY (continued)

chloride. Change in pH of the aqueous media did not bring about any significant change in peptide-micelle association.

With regard to stability of PP-SSM toward lyophilization, we observed that freeze-drying stability also depended on the presence or absence of sodium chloride in aqueous media. PP-SSM prepared and lyophilized in phosphate buffered saline and reconstituted using water demonstrated significant loss of peptide-micelle association whereas PP-SSM prepared and lyophilized in phosphate buffer without sodium chloride but reconstituted in normal saline, retained peptide-micelle association. Hence it was considered to be stable to lyophilization.

Results from in vitro stability and bioactivity studies of PP-SSM indicated that SSM significantly improved the stability of PP against proteolytic degradation. PP in SSM subjected to a 2h long tryptic digestion, demonstrated a 2.5 fold increase in half-life than PP in buffer. The bioactivity of the peptide was also found to be retained in micelles. Bioactivity was evaluated through assessment of the ability of PP to bind and activate its receptor. PP inhibits forskolin stimulated cyclic adenosine monophosphate (cAMP) production in cells. This pharmacological response was utilized to evaluate the bioactivity of PP. PP in SSM inhibited forskolin stimulated cAMP production to a similar extent as PP in buffer, therefore demonstrating that association of PP with micelles does not alter the ability of the peptide to bind and activate its receptors.

These promising results, prompted us to evaluate the efficacy of the nanomedicine for the treatment of pancreatogenic diabetes in vivo. To this end, a rat model of chronic
pancreatitis induced pancreatogenic diabetes was utilized. In this model, PD develops as a result of daily intraperitoneal injections of L-arginine. Animals demonstrate impaired glucose tolerance and have deficiency of insulin by 4 weeks of L-arginine injections. In our study, 6 week old male Wistar rats developed impaired glucose tolerance and loss of insulin sensitivity within ~2-3 weeks of daily arginine injections. These animals were then administered PP/PP-SSM and controls such as buffer, empty SSM, insulin and metformin for a period of 5 days. Glucose tolerance and insulin sensitivity tests conducted at the end of treatment period showed that PP-SSM and metformin treated group significantly improved glucose tolerance and insulin sensitivity in the animals as compared to other treatment groups. All PP treated animals did not show significant improvement in glucose tolerance and insulin sensitivity. Quantification of hepatic glycogen content after subjecting the animals to glucose challenge also demonstrated a similar result. PP is known to aid the uptake of glucose from the blood that is eventually stored in the liver as glycogen. The glycogen content of livers harvested from animals treated with PP-SSM was significantly higher than that of PP treated animals. Results of this study further corroborated the notion that PP demonstrates greater biological activity when delivered in SSM. Overall, the data indicated that delivery of PP in SSM brings about significant improvement in anti-diabetic efficacy for the treatment of PD, potentially through decreased aggregation of PP in aqueous media and increased stability of this enzyme-labile peptide against degradation in vivo.
SUMMARY (continued)

To summarize, we have developed and characterized a novel formulation of pancreatic polypeptide in sterically stabilized phospholipid micelles that demonstrated significant anti-diabetic activity in a rodent model of pancreatogenic diabetes. This was the first time that a micellar formulation of pancreatic polypeptide was investigated for the management of pancreatogenic diabetes. Since these micelles are composed of phospholipids that are approved by United States Food and Drug Administration for use in humans, we believe that transition of this nanomedicine to the clinics will be smooth and easy. We foresee a significant contribution of PP-SSM nanomedicine in the effective management of pancreatogenic diabetes without the risks associated with the current therapy for this disease.
I. INTRODUCTION

A. PANCREATOGENIC DIABETES

Pancreatogenic diabetes (PD), also known as type 3c diabetes mellitus (T3cDM), encompasses all diabetes mellitus (DM) cases that occur as a result of pancreatic diseases (American Diabetes Association 2011). This disease is caused by pancreatic endocrine dysfunction that results in impaired glucose tolerance and hepatic insulin resistance, the two hallmarks of PD (Yki-Jarvinen et al., 1986). However, the disease has been often misdiagnosed as type 2 DM. In a recent study, PD was prevalent in 9.2% of patients diagnosed with type 2 diabetes mellitus (Ewald et al., 2012). Overall, PD accounts for approximately 8% of all diabetic patients worldwide (1-2% of diabetic patients in North America and 15-20% of diabetic patients in Southeast Asian countries due to high prevalence of tropical and fibrocalcific pancreatitis in those countries) (Cui and Andersen 2011).

The pathophysiology of PD differs from both type 1 and type 2 DM. Type 1 diabetes mellitus (T1DM) is considered an autoimmune disease where the presence of autoantibodies against pancreatic beta cells in the body causes beta cell atrophy (Bach 1994). T1DM is characterized by severe hyperglycemia and ketoacidosis which are rarely observed in patients with PD (Maeda and Hanazaki 2011). Apart from this, hepatic insulin sensitivity as well as levels of glucagon, pancreatic polypeptide (PP), glucose dependent insulinotropic polypeptide (GIP) and glucagon like peptide-1 (GLP-1) are reduced in PD while they are normal in T1DM (Cui and Andersen 2011). This difference in the enteric glucoregulatory hormone levels also
distinguishes pancreatogenic diabetes from type 2 diabetes mellitus (T2DM) (Cui and Andersen 2011). In addition, insulin resistance observed in T2DM occurs as a result of loss of both hepatic and peripheral insulin sensitivity whereas in PD, only the former is observed (Cui and Andersen 2011).

As aforementioned, loss of endocrine function in PD decreases levels of insulin and counter regulatory hormone glucagon in the body. Therefore, exogenous administration of insulin often leads to development of severe hypoglycemia and, therefore, PD is also termed as ‘brittle’ diabetes (Maeda and Hanazaki 2011). This may lead to hypoglycemic shock, irreversible damage to central nervous system and occasional deaths (Maeda and Hanazaki 2011; Kahl and Malfertheiner 2004; Hutchins et al., 2002). When not treated appropriately, such patients develop diabetic nephropathy, neuropathy and retinopathy (Deckert 1960). Patients with PD are also at a higher risk of suffering from pancreatic cancer that largely increases the mortality of these patients. Moreover, exocrine pancreatic dysfunction due to underlying pancreatic disorder causes nutritional deficiencies that lead to development of malnutrition and metabolic bone disease (Rehorkova 2006; Dresler et al., 1991).

1. **Causes of pancreatogenic diabetes**

   Pancreatogenic diabetes is caused by perturbation in the exocrine and endocrine environment of the pancreas. The disorder occurs secondary to diseases related to the
pancreas such as chronic pancreatitis, hemochromatosis, pancreatic neoplasia, pancreatic resection and cystic fibrosis (Cui and Andersen 2011).

Chronic pancreatitis (CP) accounts for approximately 76% of all pancreatogenic diabetes cases (Cui and Andersen 2011). CP is a clinical disorder where progressive inflammation of the pancreas leads to irreversible changes in the architecture and function of the pancreas. The most common causative factor for development of CP in North America is alcohol abuse (Braganza et al., 2011). In a study including 500 patients with CP developed due to alcoholism, nearly 83% developed PD within 25 years of onset of CP (Malka et al., 2000). Chronic pancreatitis is also a major risk factor for pancreatic cancer (Maisonneuve and Lowenfels 2002). Conversely, PD develops in nearly 30% of all patients with pancreatic cancer (Cui and Andersen 2011; Pannala et al., 2008). PD due to pancreatic resection has been observed in nearly 40-50% of patients during follow-up examinations (King et al., 2008; Slezak and Andersen 2001).

2. **Pathogenesis of pancreatogenic diabetes**

PD results from progressive endocrine dysfunction that leads to a deficiency of insulin, glucagon, PP and incretin hormones such as GIP and GLP-1 (Cui and Andersen 2011). Onset of PD is dependent on destruction of pancreatic parenchyma which is largely influenced by extent of inflammation mostly caused by alcoholism (Raue and Keim 1999). The pathogenesis of PD includes progressive destruction of alpha, beta and F cells of the pancreas, glandular atrophy, blockage of pancreatic ducts as well as and loss of perisinusoidal diffusion that result in
impaired glucose homeostasis and development of PD (Figure 1) (Raue and Keim 1999). Deficiency of enteric glucoregulatory hormones causes mild hyperglycemia, impaired glucose tolerance and blunted hepatic responsiveness to insulin. One of the principle reasons for decreased hepatic sensitivity toward insulin in PD secondary to CP is lower expression of insulin receptors in the liver that leads to ineffective utilization of circulating insulin in the body (Tiwari et al., 2007). Additionally, a contributing factor toward development of insulin resistance is elevated levels of serum resistin that is observed in CP (Adrych et al., 2009). Resistin is a hormone secreted by the adipose tissues and has been recently implicated to play a major role in the development of insulin resistance most prominently in the liver (Adrych et al., 2009). PD secondary to CP is also associated with a lack of suppression of hepatic glucose production upon injection of insulin (Cui and Andersen 2011). This has again been attributed to deficiency of insulin receptors and glucose transport protein GLUT 2 in chronic pancreatitis (Nathan et al., 2001; Andersen et al., 1994).
3. **Current therapies for pancreatogenic diabetes**

Currently, there are no set guidelines for management of pancreatogenic diabetes and drugs that are used for treatment of T2DM are generally recommended for treatment of PD (Cui and Andersen 2011). This includes metformin, insulin and insulin secretagogues such as sulphonylureas and drugs that primarily influence the absorption of glucose such as peroxisome proliferator-activated receptor (PPARγ) agonists (Examples: rosiglitazone and pioglitazone) and alpha glucosidase inhibitors (Example: acarbose)(Cui and Andersen 2011; Berstein 2005).
Metformin serves to decrease hepatic glucose production by reducing gluconeogenesis (Hundal et al., 2000). Insulin secretagogues stimulate the secretion of insulin while PPARγ agonists enhance insulin sensitivity of skeletal muscles and adipose tissues. Alpha glucosidase inhibitors delay carbohydrate digestion and absorption from gastrointestinal tract thereby decreasing the glucose level in the blood (Murphy and Holder 2000; Bischoff 1995). However, it must be noted that despite the multitude of anti-diabetic drugs available for treatment, only a few studies have been conducted to examine the efficacy of these anti-diabetic drugs in T3cDM as compared to T2DM (Cui and Andersen 2011). For management of exocrine insufficiency, pancreatic enzyme supplementation is provided to prevent metabolic bone disorder and nutritional complications (Cui and Andersen 2011).

4. **Limitations of current therapy for pancreatogenic diabetes**

Treatment of PD requires special consideration due to a high risk of development of pancreatic cancer in patients suffering from chronic pancreatitis induced PD. It is estimated that chronic pancreatitis patients are at a 10-20 fold higher risk of developing pancreatic cancer as compared to the general population (Lowenfels et al., 1993). In addition, recent studies bring about a clear correlation between insulin and insulin like growth factor (IGF) in the regulation of cancer progression (Pollak 2008; Rozengurt et al., 2010). Hyperinsulinemia as observed in insulin resistance has been recently found to increase the risk of many malignancies and not surprisingly insulin receptors (IR) and IGF receptors are overexpressed in many tumors (Pollak 2008; Rozengurt et al., 2010). Under such conditions, insulin can bind to IGF receptors and
initiate the IGF signaling cascade (Belfiore 2007). IGF is a growth factor implicated in transformation of cells to malignant state, tumor progression, metastasis, suppression of apoptosis (Frasca et al., 2008). A clinical trial conducted by Bonelli et. al. found that treatment of diabetes using insulin or insulin secretagogues increased the risk of development of pancreatic cancer in patients by greater than threefold as compared to patients treated without insulin (Bonelli et al., 2003). These studies suggest that therapy of PD based on insulin or insulin secretagogue can escalate the already enhanced risk of development of pancreatic cancer in such patients. Therefore the use of insulin and insulin secretagogues should be avoided where possible due to mounting evidence suggesting the role of insulin in increasing the risk of pancreatic cancer in diabetes (Bonelli et al., 2003). On the other hand, inability of pancreas to secrete counter-regulatory hormone glucagon, increases the risk for development of severe hypoglycemia as a result of insulin therapy in patients suffering from PD (Cui and Andersen 2011).

Metformin, the other commonly used drug for treatment of insulin resistance decreases the risk of cancer in diabetic patients and therefore is favored for management of T2DM (Landman et al., 2010; Evans et al., 2005). However, a well known side effect of metformin therapy is lactic acidosis which is a physiological condition that occurs due to anaerobic breakdown of glucose leading to accumulation of lactic acid in the blood and decrease in blood pH and maybe fatal (Chang et al., 2002; Misbin et al., 1998). This is a more serious problem in PD secondary to CP there is an underlying problem of acid-base imbalance hence metabolic acidosis (Eovaldi and Zanetti 2011). In addition metformin therapy is contraindicated in
alcoholism which is the primary reason for development of CP in North America, therefore metformin therapy is not suitable for treatment of patients with PD secondary to CP (Pillans 1998). Apart from this, metformin therapy has been shown to acutely increase levels of GLP-1 (an insulin secretagogue) in the body (Maida et al., 2011).

Other anti-diabetic drugs such as PPARγ can cause significant fluid retention resulting in edema and also congestive heart disease, therefore this treatment option is fraught with serious adverse effects (Nesto et al., 2003). Alpha glucosidase inhibitors reduce glucose digestion and absorption from the gut and therefore are not very helpful for treatment of PD where there is a pre-existing problem of malabsorption of nutrients from the gut. On these bases, it is evident that there is a need for better treatment option for effective management of PD without the associated risks of current therapies.

5. **Pancreatogenic diabetes animal model**

The most common etiology of pancreatogenic diabetes is chronic pancreatitis. Therefore most animal models of pancreatogenic diabetes are based on animals that have chronic pancreatitis. CP in animals can be induced either surgically or non-surgically. Invasive or surgical models of CP include infusion of sodium taurocholate or oleic acid into the pancreatic ducts of rats (Otsuki et al., 2010). Ligation of pancreatic duct has also been reported to cause pancreatic atrophy. Non-invasive CP animal models include repeated subcutaneous or intraperitoneal injection of caerulein or repeated intraperitoneal injection L-arginine that cause pancreatitis
(Otsuki et al., 2010). Repeated injection of caerulein leads to mild damage to pancreatic parenchyma and infiltration of inflammatory mediators within a month of the first caerulein injection, however pancreatitis induced by this chemical is transient. Within a week of stoppage of injection, the histology of pancreas appears normal (Yamaguchi et al., 2005). CP induced by sodium taurocholate infusion has also been found to be transient where animals recover within 14 days of stoppage of infusion. However, CP induced due to L-arginine or intraductal oleic acid infusion has been reported to be persistent (Weaver et al., 1994). Besides being non-invasive, L-arginine induced CP model is also a reproducible model for PD secondary to CP. Repeated intraperitoneal injection of high concentrations of L-arginine causes regular episodes of acute pancreatitis that leads to development of chronic pancreatitis similar to that observed in humans (Aghdassi et al., 2011; Otsuki et al., 2010). The exact mechanism by which L-arginine causes pancreatitis is not well-known, however studies suggest the role of inflammatory modulators, oxygen free radicals and nitric oxide generated by L-arginine in the induction of pancreatitis. Studies have shown that repeated injections of L-arginine cause acinar cell necrosis within 48h of first injection (Tani et al., 1990) while serial injection of the chemical for 5 consecutive days causes 90% destruction of acinar cells and replacement with adipose tissue (Delaney et al., 1993). Weaver et. al. have demonstrated that repeated injection of 350 mg/100g body weight L-arginine to rats resulted in significantly lower insulin levels in the animals and impaired glucose tolerance within first week of arginine injection, leading to development of pancreatogenic diabetes (Weaver et al., 1994). All these studies indicate that,
L-arginine induced pancreatogenic diabetes is an appropriate model for conducting anti-diabetic efficacy studies in animals.

B. STERICALLY STABILIZED PHOSPHOLIPID MICELLES AS DRUG DELIVER SYSTEMS

1. Sterically stabilized phospholipid simple micelles

Sterically stabilized micelles (SSM) are phospholipid micelles prepared using polyethylene glycolated (PEGylated) lipids such as DSPE-PEG$_{2000}$ (distearoyl-phosphatidylethanolamine chemically conjugated to PEG$_{2000}$) (Figure 2).

![Chemical structure of distearoyl phosphatidylethanolamine chemically conjugated to polyethylene glycol.](image)

**Figure 2.** Chemical structure of distearoyl phosphatidylethanolamine chemically conjugated to polyethylene glycol.
By definition, a micelle is a self-aggregated complex of amphiphilic molecules, which is a molecule possessing both nonpolar and polar portions. In aqueous media, above certain concentration known as the critical micellar concentration (CMC), the molecules aggregate such that the polar parts of the molecule are directed toward water and remain hydrated while the non-polar parts cluster together and are oriented away from water for an energetically favorable arrangement. Micelles are not static entities but are formed spontaneously in a reproducible manner with same size and aggregation number, for a given aqueous media condition. They exist in a dynamic equilibrium where there is a rapid exchange between monomers from the solution and micelle. CMC of micelles is constant at a given temperature and pressure and is represented in concentration terms such as molarity. The lower the CMC of a micellar species, the more readily the micelles are formed and more stable are the micelles to dilution.

A fundamental property of a micelle is its ability to solubilize a wide variety of organic solutes with distinct hydrophobicities and associate with amphiphilic peptide molecules. The primary assembly of a micelle provides an inner hydrophobic core in which drugs are solubilized through hydrophobic interactions. The hydrophilic outer layer prevents interaction of the solubilized drugs with aqueous bulk phase providing stability to drugs encapsulated in the core. Also based on the physico-chemical properties of the drug and micelle core properties, different amounts of drug can be solubilized in a micelle. PEG forms the hydrophilic corona of the micelles and increases the aqueous solubility as well as imparts stealth characteristics to them by preventing recognition by opsonins in vivo. This ‘steric barrier’ makes the micelles long
circulating. These PEGylated phospholipid micelles are quite stable to in vivo dilution due to very low CMC (0.5-1 µM) compared to more conventional detergents such as sodium dodecyl sulfate and cetyl trimethyl ammonium bromide (Ashok et al., 2004; Erogbogbo et al., 2008). When these phospholipids are dispersed in water above their CMC, they spontaneously self associate to form SSM with an average diameter of ~ 15 nm (Figure 3) (Ashok et al., 2004). Incorporation of 10% phosphatidylcholine (PC) results in the formation of sterically stabilized mixed micelles (SSMM). SSM have been used to solubilize water-insoluble drugs without the use of any toxic solvents or detergents (Onyuksel and Banerjee 2011). The solubilized drugs are chemically unmodified and hence, bioactivity is not compromised. DSPE-PEG micelles have recently been used as delivery systems for many hydrophobic drugs such as paclitaxel (Krishnadas et al., 2003b), diazepam (Ashok et al., 2004), camptothecin (Koo et al., 2011), 17-AAG (Önyüksel et al., 2009) and indisulam (Cesur et al., 2009).

These nanosized micelles can be targeted to desired site both passively utilizing the enhanced permeation and retention (EPR) effect and actively through receptor mediated internalization. Tumors and inflammation sites are characterized by the presence of leaky vasculature which occurs as a result of formation of blood vessels with aberrant basement membranes (Kim and Bae 2009). Drug loaded micelles cannot cross normal vasculature but can extravasate out of circulation only at the leaky vasculature at tumor and inflammation sites. Drug delivery through these nanocarriers therefore decreases both the volume of distribution as well clearance of the drug. Attaching a targeting ligand to micelles enables efficient intracellular delivery of the carrier. Several targeting ligands have been identified such as
vasoactive intestinal peptide (VIP), the receptors for which are overexpressed in most cancers and in rheumatoid arthritis. VIP can be chemically conjugated to DSPE-PEG\textsubscript{2000} and the resulting micelle can be used for delivery of treatment drugs to tumor and inflammation sites. Moreover, it is observed that VIP adopts alpha-helical conformation when associated with SSM, which is the preferred conformation for receptor interaction and also protects the peptide from enzymatic degradation \textit{in vivo}. In our laboratory we have found that delivery of drugs utilizing these internalizing VIP receptors enhances the cytotoxic effect of paclitaxel while decreasing distribution to other organs as compared to Taxol (Krishnadas et al., 2004). The unique advantage of using VIP as a targeting moiety over other targeting ligands is that VIP is not expressed in the vascular endothelium (Reubi 2003). Therefore, VIP labeled micelles can only interact with its receptor once it extravasates out from circulation, thereby significantly reducing adverse effects due to interaction with normal tissues. Targeting of the drug loaded micelles increases the therapeutic efficacy of the drug while decreasing the dose required for obtaining a therapeutic effect and minimizing toxicity observed due to non-specific biodistribution to other parts of the body.

SSM are considered safe delivery vehicles as phospholipids are natural components of the body that minimizes toxicity concerns such as immunogenic reactions due to the presence of foreign molecules in the body. SSM are easy to prepare and can be scaled-up for large scale production in a reproducible manner. They have also been found robust to lyophilization without the use of any lyoprotectant or cryoprotectant (Lim et al., 2008). This is a significant step toward easy transitioning of SSM based formulations to the clinics. In addition, DSPE-
PEG\textsubscript{2000} is approved by United States Food and Drug Administration (USFDA) in a parenteral product (Doxil\textsuperscript{®}) for human use, that further substantiates the safety of these micelles (Lim et al., 2012).

**Figure 3.** Illustration of formation of SSM. Adopted from (Thaqi 2011).
2. Sterically stabilized micelles as carriers for peptide drugs

Peptides are an important class of therapeutic agents but their development into commercial products is often hampered due to their inherent physico-chemical and biological instabilities. Issues such as aggregation, oxidative and hydrolytic degradation in vitro, short biological half-lives and non-specific biodistribution of the molecules in vivo that generate adverse effects often impede the development of these biomolecules into therapeutic agents. In order to circumvent these issues, our laboratory has employed SSM as a delivery platform for peptide drugs to counteract the physico-chemical/biological instability of peptides as well as target them to sites of action without any modification in their chemical structure that can compromise bioactivity of these molecules (Lim et al., 2011; Kuzmis et al., 2011). Our work has however been limited only to unstructured amphiphilic peptides with amino acid numbers ranging between 17 and 36 (Table I). These peptide molecules being amphiphilic in nature are postulated to associate with the PEG palisade of micelles. These peptide nanomedicines are prepared by adding an aqueous solution of amphiphilic peptides to preformed micelles followed by a period of equilibration to obtain the final product (Figure 4).
We have earlier observed that SSM prevents aggregation of peptide drugs by accommodating individual peptide monomers and stabilizing them in the PEG palisade through electrostatic and hydrophobic interactions. We have observed that peptides such as GLP-1, PP and neuropeptide Y (NPY) form large heterogeneous aggregates in aqueous media which is prevented when they are incubated with SSM (Lim et al., 2011; Kuzmis et al., 2011; Banerjee and Onyuksel 2012a). In addition, association of peptides with phospholipid micelles brings forth a change in the peptide secondary structure that plays a pivotal role in improving stability.
of peptides against enzymatic degradation. Peptide drugs such as VIP, pituitary adenylate cyclase-activating peptide\textsubscript{1-38} (PACAP\textsubscript{1-38}), GLP-1 and secretin that have random coil conformation in aqueous media were found to adopt alpha-helical secondary structure when associated with micelles (Lim et al., 2011; Onyuksel et al., 2006; Krishnadas et al., 2003a). This is greatly advantageous since the peptides are stabilized in micelles in their active conformation required for receptor interaction and the amino acid residues that are susceptible to proteolytic cleavage are also effectively buried in the PEG palisade and retained in the circulation. Peptides associated with phospholipid micelles also exhibit enhanced therapeutic efficacy for the same dose as compared to peptide in buffer, due to more availability of peptides in stable and active conformation at the target site. This was demonstrated using GLP-1-SSM and VIP-SSM formulations. GLP-1-SSM administered to an acute lung injury mice model for evaluation of its anti-inflammatory potential demonstrated significantly decreased accumulation of neutrophils and production of pro-inflammatory cytokines such as interleukin-6 and tumor necrosis factor compared to GLP-1 in saline (Lim et al., 2011). Potentiation of therapeutic effect was also demonstrated in VIP-SSM through evaluation of vasodilatory effect of VIP in hamster cheek pouch arteries (Onyuksel et al., 1999). An approximate threefold increase in potency and duration of vasodilation was observed with VIP-SSM compared to VIP alone. In addition to targeting of peptide molecules to the diseased sites, phospholipid micelles reduce generation of adverse effects of the peptides. When VIP, a potent vasodilator, was administered to healthy animals, no decrease in blood pressure was observed whereas a significant and rapid hypotensive effect was noted when VIP was administered alone (Koo et al., 2005).
Besides SSM, we have also studied the interaction of peptide molecules with SSMM to determine if peptides interact in a similar or different manner with SSMM. Using VIP and GLP-1 as our model peptides, we observed no significant difference between SSM and SSMM in terms of peptide loading capacity and conformational changes and, hence, concluded that SSMM was comparable to SSM as a peptide nanocarrier (Lim et al., 2007). In view of the additional steps required to prepare SSMM that can hamper large-scale production and no advantage in peptide loading, SSM was picked as the better carrier for peptide delivery.

These studies clearly indicate that peptide molecules that suffer from physico-chemical and biological instability issues can be effectively delivered in SSM to assist their future development into commercial products.
<table>
<thead>
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<th>Name</th>
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<th># Amino acids</th>
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<td>Blood glucose lowering, anti-inflammatory</td>
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<td>Glucose-dependent insulinoctropic peptide (GIP)</td>
<td>Glucose-dependent insulin secretion modulation</td>
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<td>Neuropeptide Y (NPY)</td>
<td>Anti-anxiety, anti-cachexia activities</td>
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<tr>
<td>Pancreatic Polypeptide (PP)</td>
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<td>36</td>
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<td>Pituitary adenylate cyclase activating peptide (PACAP1-38)</td>
<td>Neurotransmitter, neurohormone, possesses immunomodulatory activity</td>
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<td>Secretin</td>
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<td>A synthetic peptide under investigation for anti-inflammatory activity</td>
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<td>Vasoactive Intestinal Peptide (VIP)</td>
<td>Anti-inflammatory, vasodilation</td>
<td>28</td>
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3. **Influence of electrolytes on properties of sterically stabilized micelles**

The nature of electrolytes in the aqueous media is known to greatly influence CMC, aggregation number as well as size of micelles (Abe et al., 1989). In order to determine the effect of electrolytes on properties of SSM, we have recently characterized SSM prepared in pure water and in 166mM in HEPES (N-([2-Hydroxyethyl] piperazine-N’-[2-ethanesulfonic acid]) HEPES buffered saline in terms of their hydrodynamic diameter, aggregation number, CMC and viscosity through experimental and molecular dynamic simulation studies (Vukovic et al., 2011). In the absence of strong electrolyte such as sodium chloride, micelles of size approximately 4-5 nm were formed whereas in the presence of sodium chloride such as in HEPES buffered saline, larger micelles with size around 14 nm were observed. It is believed that in the absence of strong electrolyte such as sodium chloride, electrostatic repulsion between the phosphate head groups of the phospholipid molecules decrease the aggregation number of the micelles; thus leading to the formation of small sized micelles. Conversely, in aqueous media containing electrolytes, the negative charge on the phospholipid head group is screened that result in increased stacking of phospholipid molecules, higher aggregation number and consequently larger sized micelles (Borisov and Zhulina 2002). *In silico* studies using molecular dynamics simulation confirmed this viewpoint and demonstrated that aggregation number of SSM is much lower in pure water as compared to SSM prepared in presence of sodium chloride (Figure 5). An approximate 10 fold increase in CMC of SSM was observed for micelles prepared in pure water (10µM in pure water vs 1µM in HEPES buffered saline). Masking of negative phosphate group charge by electrolytes, stabilizes the micelle and decreases CMC. Apart from
this, strong electrolytes decrease the number of water molecules available for interaction with phospholipids, thus decreases the solubility of the molecules and consequently reduces the CMC of micelles (Moreira and Firoozabadi 2010).

**Figure 5.** Size and aggregation numbers of phospholipid monomer, and micelles prepared in presence and absence of sodium chloride (Vukovic et al., 2011)

Molecular dynamics simulations showing snapshots of PEGylated phospholipid monomer and equilibrated micelles in pure water or in HEPES buffered saline (0.166 M NaCl) with their corresponding aggregation numbers.
C. **PANCREATIC POLYPEPTIDE**

1. **Physiological role of pancreatic polypeptide**

Pancreatic polypeptide (PP) is an endogenous peptide hormone secreted mostly postprandially by F cells, also called PP cells of the islets of Langerhans of pancreas (Lonovics et al., 1981). The primary role of this 36 amino acid long peptide (Figure 6) is to modulate digestion of food by inhibition of gastric emptying as well as biliary secretion (Schmidt et al., 2005; Langlois et al., 1990). PP binds with picomolar affinity to its constitutive Y4 receptor (Lerch et al., 2005) that is expressed in the liver, intestine, adrenal gland and parts of the central nervous system (Bard et al., 1995). The peptide is reported deficient in chronic pancreatitis; while its receptors (Y4 receptors) are overexpressed in the same disease (Seymour et al., 1998; Hennig et al., 2002; Adrian et al., 1979).

PP possesses significant glucose regulatory function. The peptide sensitizes the liver to the action of insulin by increasing the number of insulin receptors in the hepatocytes (Seymour and Andersen 1999; Seymour et al., 1996; Seymour et al., 1995). PP has also been found to decrease the expression of resistin hormone that plays a major role in the development of hepatic insulin resistance and elevated levels of resistin is reported in CP (Adrych et al., 2009; Asakawa et al., 2003; Steppan and Lazar 2002). Deficiency of the peptide results in significant hepatic insulin resistance (Seymour and Andersen 1999; Andersen 2007) as well as glucose intolerance which can be attributed to the impairment of hepatic sensitivity to insulin and up regulation of resistin hormone. In patients with diabetes secondary to CP, administration of PP reverses insulin resistance and improves glucose tolerance (Sun et al., 1986; Seymour et al., 1995).
A major advantage of using PP for management of pancreatogenic diabetes is that administration of PP does not increase serum insulin levels (Brunicardi et al., 1996) which is otherwise a shortcoming in the current therapy for pancreatogenic diabetes. Coelle et. al. investigated the role of PP in management of pancreatitis and found a beneficial effect of pretreatment with PP on mortality and pancreatic histology in mice (Coelle et al., 1983). Administration of PP before induction of pancreatitis resulted in significant decrease in mortality as well as maintenance of normal pancreatic histology compared to saline treated animals. This study demonstrated the protective role of PP and suggested that exogenous administration of PP may prevent further damage of pancreatic architecture in CP. Overall, experimental data from the literature suggest that pancreatic polypeptide has immense potential for treatment of diabetes secondary to chronic pancreatitis.

Figure 6. The amino acid sequence of pancreatic polypeptide.
2. **Limitations in therapeutic application of PP**

Despite all promising therapeutic functions of PP and initial enthusiasm of utilizing the peptide for management of pancreatogenic diabetes, there is, unfortunately, no marketed formulation of the peptide in the clinics. This is most likely because the peptide is subjected to enzymatic degradation and rapid clearance *in vivo* with a half-life of a few minutes (Adrian et al., 1978; Hagopian et al., 1983). To achieve therapeutic efficacy, the peptide needs to be administered continuously which is patient non-compliant and can lead to adverse effects due to higher total dose administered and interaction of the peptide with receptors present in off target sites of the body. Self-aggregation of PP molecules in aqueous solution is another serious delivery issue associated with this peptide. Therefore, *in vivo* delivery and therapeutic application of PP pose a major challenge.

3. **PP analogues and current clinical trials**

Recent attempts have been made to minimize the bottleneck in the development of PP as a therapeutic moiety by synthesizing its analogues that mimic the activity of the native peptide while resisting its degradation by proteolytic enzymes; however the safety of such analogues is questionable and needs to be thoroughly investigated (Lim et al., 2012; Bush 2011). In a clinical trial for the treatment of obesity, an analogue of PP named as PP 1420 has been tested for phase 1 studies (Tan et al., 2012). This PP analogue is made through modification in the primary amino acid structure of PP by addition of a glycine residue at position 0 and substitutions for 5 other amino acid residues in PP. Outcome of the phase 1
clinical trial indicated that the molecule was well tolerated in humans at doses 2, 4 and 8 mg and the analogue demonstrated improved pharmacokinetic profile than unmodified peptide. A single subcutaneous injection of PP 1420 increased the half-life of PP from a few minutes to approximately 2.5 h. A lipidated analogue of PP has also been synthesized to improve the biological half-life of the peptide for the treatment of obesity. The analogue increased the half-life of PP by 6 fold in vivo compared to the native peptide and high uptake of the molecule in the liver was observed (Bellmann-Sickert et al., 2011). A phase 2 clinical trial of formulation of PP in saline is currently being conducted for treatment of type 1 diabetes mellitus and PD secondary to pancreatectomy (Elahi, 2010). In this trial, 2 pmol/kg/min of PP was continuously administered through intravenous infusion for a period of 72 h followed by evaluation of glycemic excursions in the patients. However, it must be noted that these molecules being small in size, can easily extravasate out of circulation and interact with receptors expressed in other sites; raising toxicity concerns. Chemical modification of a peptide can alter peptide bioactivity, raise immunogenicity concerns and in most cases do not resolve the non-specific biodistribution of peptides. In addition, chemical modification makes scaling-up difficult and encumbers the already inflated manufacturing cost of peptide therapeutics.

4. **Pancreatic polypeptide in sterically stabilized micelles**

To overcome the problems associated with delivery of PP, sterically stabilized micelles can be utilized to improve the physico-chemical and biological instability problem of PP. As discussed earlier, we have shown that association of amphiphilic peptides such as VIP with SSM
diminishes its degradation by proteolytic enzymes and enables its extravasation only at the disease site that minimizes development of adverse effects. Likewise, formulation of PP in SSM (PP-SSM) can be passively targeted to the liver through its fenestrations for uptake by the hepatocytes where receptors of the peptide (Y4 receptors) are overexpressed (Seymour et al., 1998). Targeting of the nanomedicine to the liver will also prevent interaction of PP with its receptors expressed in other parts of the body, thereby eliminating undesirable physiological effects of PP. Steric hindrance imparted by the PEG layer of the micelles will help evade the reticulo-endothelial system, making the formulation long-circulating. Targeted delivery and increased in vivo stability of the peptide will also help decrease the required dose for achieving effective anti-diabetic therapy. These multiple reasons make SSM an ideal delivery platform for PP that suffers from issues such as short half-life and self-aggregation. On the basis of these rationales, we attempted to develop a formulation of PP in SSM for its future application as a novel nanomedicine for the treatment of diseases associated with PP deficiencies such as pancreatogenic diabetes.
D.  **HYPOTHESIS AND SPECIFIC AIMS**

Our hypothesis is that PP can be formulated as a nanomedicine using SSM to improve glucose tolerance and overcome insulin resistance, thereby provide an effective therapeutic intervention for pancreatogenic diabetes. In this research project, we prepared and characterized PP-SSM and tested its efficacy as an anti-diabetic agent *in vivo* for the treatment of pancreatogenic diabetes. To this end, the specific aims for this project were:

1. Prepare a formulation of PP in sterically stabilized micelles

   1.1. Prepare and characterize PP-SSM by i) dynamic light scattering, ii) fluorescence spectroscopy, and iii) circular dichroism

   1.2. Study the effect of pH and sodium chloride on association of peptide with micelles

   1.3. Determine stability of PP-SSM during lyophilization

2. Evaluate *in vitro* stability and bioactivity of PP in micelles

   2.1. Determine stability of PP in micelles against proteolytic degradation *in vitro*

   2.2. Determine receptor binding and activation of PP specific Y4 receptors by PP-SSM in cell culture system

3.1. Determine the efficacy of PP-SSM in improving glucose tolerance, overcoming hepatic insulin resistance and increasing hepatic glycogen content
E. **SIGNIFICANCE OF THE PROJECT**

There is no definitive therapy for management of pancreatogenic diabetes. The common practice is to use insulin and metformin as the first line of therapy for the disease (Cui and Andersen 2011; Bonelli et al., 2003). However, insulin therapy either through exogenous insulin administration or by using insulin secretagogues is not appropriate for management of pancreatogenic diabetes. Evidence from clinical studies suggest that insulin therapy increases the risk for development of pancreatic carcinoma by greater than threefold as compared to non-insulin based therapy (Bonelli et al., 2003). This is because CP often sets the stage for pancreatic cancer where overexpression of insulin like growth factor-1 (IGF-1) receptors is observed. These receptors are implicated in several cancers (Pollak 2008; Ryan et al., 2007; Brady et al., 2007; Pollak 2012). Chronic hyperinsulinemia due to insulin resistance leads to activation of signaling pathways of IGF-1 receptors that enhance the development and growth of cancer. Therefore, therapies that enhance insulin levels increase the risk of pancreatic cancer and should be avoided in the management of diabetes secondary to CP (Cui and Andersen 2011). Where insulin therapy becomes mandatory due to poor glycemic control, it is recommended to be used with extreme caution and along with metformin which apart from being anti-diabetic agent is also a known anti-neoplastic agent. Moreover, insulin therapy should be carefully monitored because deficiency in glucagon impairs the body’s intrinsic counter-regulatory function that may cause severe hypoglycemia. Metformin therapy on the other hand, can cause lactic acidosis that negates its beneficial effect in the management of insulin resistance (Maida et al., 2011). Besides metformin acutely increases the plasma levels of
GLP-1 in the body. A better treatment approach with lesser associated risks that will effectively manage the disease is therefore essential. To this end, recently pancreatic polypeptide is being tested to treat pancreatogenic diabetes. The peptide can be used to restore impaired glucose tolerance and overcome insulin resistance and without the associated risks of currently available therapies. However, even though PP can be an effective therapy for PD, its delivery may be problematic that may prevent its commercialization.

Currently there is no marketed formulation of pancreatic polypeptide but it is being investigated in clinical trials for treatment of patients with pancreatogenic diabetes. Our proposed nanomedicine PP-SSM will improve the stability and efficacy of PP to achieve enhanced glucose tolerance and hepatic insulin sensitivity, thus allowing better utilization of circulating insulin. This will prevent chronic hyperinsulinemia resulting from insulin resistance, therefore decreasing the risk of pancreatic cancer in patients with PD. Additionally, PP does not increase insulin levels in the body, therefore management of pancreatogenic diabetes with PP will be safe and improve the quality of life in patients suffering from this disease. If successful, it would be a great step toward therapeutic intervention of this chronic disorder. Also, PP-SSM comprises biocompatible and biodegradable materials that are FDA-approved, albeit in other products. Thus, if we are able to demonstrate pre-clinical efficacy of our novel nanomedicines, it would provide an opportunity for further research and development of PP-SSM, finally culminating in its transition to a clinical setting.
II. MATERIALS AND METHODS

A. MATERIALS

1,2-Distearoyl-sn-glycero-3-phosphatidylethanolamine-N-[methoxy(polyethyleneglycol)-2000] sodium salt (DSPE-PEG$_{2000}$) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Human pancreatic polypeptide (PP) was prepared at protein research laboratory, research resources center of University of Illinois at Chicago (> 95% purity as determined by reverse phase high performance liquid chromatography). Phosphate buffered saline (PBS), pH 7.4 was purchased from Mediatech Inc, Manassas, VA. Normal saline (0.9% w/v sodium chloride injection USP) was purchased from Baxter Healthcare Corporation (Deerfield, IL). All other buffers were prepared in the laboratory using chemicals purchased from Fisher Scientific (Itasca, IL) or Sigma-Aldrich (St. Louis, MO). Cyclic AMP EIA kit was purchased from Cayman Chemical Company (Ann Arbor, MI). Eagle’s minimum essential media (EMEM) was purchased from American Type Culture Collection (Manassas, VA). The HPLC column used (Varian, Serial No. 379013, Pursuit XRs) had the following specifications - C18, 4.6 x 250 nm, 5 μm. Trypsin-EDTA (0.25% with 0.53 mM EDTA) was purchased from Mediatech, Inc. (Manassas, VA). Acetonitrile HPLC grade, trifluoroacetic acid HPLC grade and phosphoric acid HPLC grade were purchased from Fisher Scientific (Itasca, IL). Freestyle lite glucose meter and strips, Humalog® (Insulin lispro injection), Humulin® (Regular insulin human injection) and anesthetic agents Ketamine hydrochloride® Injection USP and Anased® Injection (Xylazine hydrochloride) were purchased from pharmacy store at the University of Illinois Chicago. L-arginine, D-glucose,
Amyloglucosidase and Metformin hydrochloride® were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Line: SK-N-MC cells (HTB-10) was purchased from American Type Culture Collection (Manassas, VA)

Animals: 6 weeks old male Wistar rats were purchased from Charles River Laboratories (Strain code: 003)

B. METHODS

The animal experiments were conducted in accordance to the institutional animal care committee guidelines (ACC protocol # 11-210) and to the Guide for the Care and Use of Laboratory Animals, prepared by the Committee of Care and Use of Animals of the Institute of Laboratory Animal Resources, National Research Council.

1. In Vitro experiments

1.1. Preparation of PP-SSM

The formulation of PP associated with SSM (PP-SSM) was prepared based on our previous work with other peptides and SSM (Lim et al., 2011; Kuzmis et al., 2011; Onyuksel et al., 2006). Briefly, weighed quantity of DSPE-PEG$_{2000}$ was added to phosphate buffer or saline, vortexed for 2 minutes, (Thermolyne Maxi Mix II) sonicated for 5 minutes (Branson Ultrasonic
cleaner) and allowed to equilibrate in the dark for 1h at 25°C to form micelles at a concentration of lipid above its CMC. Weighed quantity of PP in respective phosphate buffers or normal saline was added to the SSM dispersion and allowed to equilibrate for 2h in the dark at 25°C.

1.2. Characterization of PP-SSM

1.2.1 Particle size distribution analysis

The experiment was performed to study the aggregation behavior of PP in the presence or absence of micelles with the goal of determining whether SSM mitigated the self-aggregation propensity of the peptide. Particle size distribution of empty SSM (120 μM DSPE-PEG$_{2000}$), PP (4 μM) and PP-SSM were determined by dynamic light scattering technique using Agilent 7030 NICOMP DLS/ZLS (Agilent Technologies, Santa Clara, CA). All samples were prepared in pH 7.4 phosphate buffered saline. The hydrodynamic diameters $d_H$ of the particles were obtained utilizing the Stokes-Einstein equation (Eq. 1) that calculates hydrodynamic particle size using from the translational diffusion coefficient of the particles ($\eta$ is the shear viscosity of solvent = 0.933 cP, refractive index = 1.33, and scattering angle of 90°).

(Eq. 1) \[ d(H) = \frac{kT}{3\pi\eta D} \]

$k$ represents Boltzmann’s constant (1.38 X 10$^{-16}$ erg K$^{-1}$), $T$ represents absolute temperature while $D$ is the diffusion coefficient of the particles.
Mean hydrodynamic diameter was calculated using the average of five $d_n$ values using autocorrelation function collected over at least 15 minutes. Both intensity and volume weighted particle size distribution were determined.

1.2.2 Fluorescence spectroscopy

Fluorescence emission studies were conducted to determine whether PP associates with SSM and to evaluate the extent of association of the peptide with micelles. PP contains four tyrosine amino acids that act as intrinsic fluorophores for the peptide. Fluorescence emission of tyrosine residues in PP was monitored at excitation wavelength of 277 nm and emission wavelength of 304nm (Kanazawa and Hamaguchi 1986) using SLM Aminco 8000 spectrofluorimeter (SLM Instruments, Rochester, NY). The aim of this study was to investigate whether PP self-associated with micelles and if so to determine the saturation molar ratio of association of the peptide with micelles. Studies to determine association between PP and SSM was carried out using 4 μM peptide concentration and 120 μM lipid concentration. For determination of the saturation molar ratio of association between PP and SSM, the study was conducted using peptide concentration of 4 μM and lipid concentration ranging between 8 μM and 400 μM. The maximum emission ($I$) of each sample was normalized against the fluorescence emission of PP in buffer ($I_0$) and this function ($I/I_0$) was plotted against their respective lipid: peptide molar ratio. SigmaPlot® (Systat Software Inc., San Jose, CA) was used for curve fitting of the plotted data. The saturation molar ratio of association between lipid and peptide was determined based on the lipid concentration where 90% of the plateau was
reached. The number of peptide molecules associated with one micelle at saturation was calculated using the equation given below (Eq. 2).

(Eq. 2) Number of PP molecules associated with one SSM = Aggregation number of micelles/x; where x stands for the number of lipid molecules at saturation.

### 1.2.3 Circular dichroism

Pancreatic polypeptide is known to possess some alpha helical secondary structure in aqueous media (Glover et al., 1984). The aim of this experiment was to determine if this conformation of PP is enhanced or retained in micelles (since alpha helix is the preferred structure for peptide-receptor interaction). The conformation of PP in phosphate buffered saline or when associated with SSM was determined using circular dichroism (CD) function of Jasco 710 spectropolarimeter (Jasco Inc., Easton, MD). Lipid and peptide concentration were kept constant at 1 mM and 4 μM respectively and the spectra were recorded between 190-260 nm using 1 cm path length fused quartz cuvettes and a band width of 1 nm. The number of accumulations per sample was three and the spectra were corrected for buffer and SSM scans and smoothed using Savitzky Golay algorithm.

### 1.3. Influence of pH and electrolyte on peptide micelle association

This study was conducted to determine whether changes in aqueous media conditions bring about any change in the association between PP and SSM. It is a well known fact that pH of the aqueous media influences the solubility of a peptide (Sigma-Aldrich). A peptide is least soluble around its isoelectric point (pI) which is the pH at which both positive and negative
charges on amino acids of the peptide are neutralized such that the peptide carries no net charge and therefore has least solubility at that pH (Sigma-Aldrich guidelines.). At a pH below the isoelectric point, a peptide is positively charged while at a pH above its isoelectric point, the peptide is negatively charged. Besides, change in pH can vary the secondary structure of peptides. Since altering the pH of the aqueous media changes the solubility profile of a peptide, we postulated that pH of the aqueous media can influence the interaction between PP and SSM. In this study we attempted to determine the saturation molar ratio of association between PP and SSM at different pHs. The isoelectric point of PP is in the pH range of 6 – 7 (Kimmel et al., 1975). For the study, phosphate buffers of pH 6.5, 7.4 and 8.0 (physiological pH range and within the buffer capacity of phosphate buffer) were prepared and PP-SSM interaction was evaluated through fluorescence emission and circular dichroism studies. For both studies, peptide concentration used was 4 µM while lipid concentration ranged between 8-400 µM for fluorescence studies and for circular dichroism studies 1 mM lipid was used. Saturation molar ratio of association between peptide and lipid as well secondary structure of peptide was determined as described earlier.

Recent studies in our lab have also shown that physico-chemical properties of SSM such as hydrodynamic diameter, aggregation number, critical micellar concentration and viscosity vary with the presence or absence of strong electrolytes such as sodium chloride in the aqueous media of the formulation (Vukovic et al., 2011). Likewise, these strong ions in the aqueous media can affect the solubility as well as increase/stabilize the alpha-helical secondary structure of peptides (Lazo and Downing 1998) which can ultimately influence peptide-micelle
association. This study was conducted to understand the influence of this formulation variable on peptide-micelle association. For this purpose, the saturation molar ratio of association between PP and SSM was determined in the presence or absence of 0.9% w/v sodium chloride. Peptide concentration was kept constant at 4 µM while lipid concentration varied between 8-400 µM. Saturation molar ratio of association between peptide and lipid was calculated as described earlier.

1.4. **Freeze-drying of PP-SSM**

This study was conducted to determine whether it was feasible to lyophilize PP-SSM aqueous solution and to evaluate the formulations that were prepared and reconstituted using different aqueous media (with or without sodium chloride) for their efficacy in retaining peptide load after lyophilization. SSM can be freeze-dried without the use of any cryoprotectant or lyoprotectant at 5 – 15 mM lipid concentration (Lim et al., 2008). For this study, the lipid and peptide concentration were kept constant at 5 mM and 143 µM respectively to obtain a lipid: peptide molar ratio of 35: 1. This ratio chosen was slightly above the saturation molar ratio of PP-SSM in phosphate buffered saline as determined by fluorescence experiments described earlier. Samples were prepared in pH 7.4 phosphate buffer or phosphate buffered saline and stored overnight at -80°C followed by freezing in liquid nitrogen for at least 3 minutes. The frozen samples were then lyophilized using the Labconco FreeZone® 6 Litre FreezeDry System (Labconco, Kansas, MO). After overnight lyophilization, the samples were reconstituted by adding normal saline to samples prepared in phosphate buffer.
and sterile water to those prepared in phosphate buffered saline and swirled mechanically till complete dissolution. This was followed by incubation for 2h at 25°C in the dark for equilibration. Particle size and distribution, fluorescence emission as well as secondary conformation of the samples were measured before and after lyophilization as described above. In this study, it was ensured that the final preparations of PP-SSM formulated either way, contained 0.9% w/v sodium chloride to maintain isotonicity of the injectable product with body fluids.

1.5. **Assessment of stability of PP-SSM against proteolytic degradation**

This study was performed to assess the stability of PP against enzymatic degradation when it is self-associated with SSM as compared to peptide in buffer. Trypsin serves as a model proteolytic enzyme as it is known to cleave PP to produce four different tryptic digest products (Paquette et al., 1981). In the study, 30 μM of the peptide in buffer or in SSM were incubated with 30 nM of trypsin at 37°C to obtain a 1: 1000 molar ratio of enzyme to peptide. Lipid concentration used for the study was 900 μM in order to obtain lipid: peptide ratio of 30:1 (close to the saturation molar ratio of association between SSM and PP). Samples were incubated with trypsin for time ranging between 0 - 120 minutes and tryptic digestion of PP was stopped by adding 10 μl of 40% trifluoroacetic acid to the reaction mixture. The degradation of PP was then determined using reversed phase high pressure liquid chromatography (RP-HPLC) using LC20AB Prominence Liquid Chromatograph (Shimadzu Corporation, Kyoto, Japan) followed by UV detection using SPD-M20A Prominence Diode Array Detector (Shimadzu
Corporation, Kyoto, Japan) at 276 nm. The procedure of detection and quantification was adopted and modified from previously published literature (Taylor et al., 1988) (Table II).

Mobile phase used for detection were water with 0.2 mM phosphoric acid (A) and acetonitrile with 0.2 mM phosphoric acid (B). Samples were detected through a gradient elution of 5-30% B in the first 10 minutes followed by 30 – 40% B over the next 20 minutes. To determine the degradation kinetics, % PP remaining was plotted over time and the linear portion of semi log plot of the graph was used to determine degradation rate and half-life of the peptide.

**TABLE II.**

HPLC parameters for pancreatic polypeptide detection and quantification

<table>
<thead>
<tr>
<th>Mobile phase</th>
<th>Acetonitrile : water (with 0.2mM phosphoric acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate of flow</td>
<td>1 mL/min</td>
</tr>
<tr>
<td>Sample volume injection</td>
<td>100 µL</td>
</tr>
<tr>
<td>Detection wavelength</td>
<td>276 nm</td>
</tr>
<tr>
<td>Temperature</td>
<td>25°C</td>
</tr>
<tr>
<td>Column type</td>
<td>Zorbax 300 SB-C18, 5µm pore size (250 x 4.6 mm, Agilent Technologies, Santa Clara, CA)</td>
</tr>
<tr>
<td>Sample run time</td>
<td>35 minutes</td>
</tr>
<tr>
<td>Retention time</td>
<td>11.8 minutes</td>
</tr>
</tbody>
</table>
1.6. **In vitro bioactivity assessment of PP-SSM**

The purpose of this study was to investigate whether bioactivity of PP was retained when it associated with SSM through assessment of inhibition of forskolin stimulated cAMP production in SK-N-MC (human neuroblastoma) cells which endogenously express PP specific Y4 receptors (Li and Ritter 2005). The study was performed based on previous cAMP bioactivity studies using peptide-micelle constructs conducted in our lab (Kuzmis et al., 2011). For the study, cells were seeded at a density of $2 \times 10^5$ cells/well in a 24 well plate using eagle’s minimum essential media (EMEM) containing 10% fetal bovine serum and 1% penicillin (100 U/ml) / streptomycin (100 μg/ml). At the start of the study, the growth media was replaced with serum free media containing 0.1mM phosphodiesterase inhibitor 3-Isobutyl-1-methylxanthine (IBMX) and incubated for 1h at 37°C humidified, 5% CO$_2$ atmosphere. At the end of the incubation period, PP in PBS or PP-SSM was added to the wells at different concentrations ranging from 1 to 100nM. Approximately, 3 minutes later, forskolin was added to the wells to obtain a final concentration of 10 μM in each well and the cells were incubated for additional 45 minutes at 37°C in humidified, 5% CO$_2$ atmosphere. Thereafter, the culture media was aspirated and cells were lyzed with 100 μl of 0.1 M hydrochloric acid followed by incubation for 20 minutes at room temperature. Cell lysates were collected, centrifuged to remove cell debris and the supernatant was used for quantifying cAMP using enzyme linked immunosorbent assay (ELISA) according to the manufacturer’s protocol (cAMP EIA kit). The results obtained were normalized with total protein content measured using coomassie plus bradford assay (Thermo scientific, Rockford, IL). In the study, the negative control groups (cells
that were not stimulated by forskolin) included buffer, empty micelles and PP-SSM containing highest respective concentration of lipid and peptide used in the experiment; while positive control used were cells treated with buffer alone and stimulated with forskolin.

2. **In Vivo Experiments**

The purpose of this study was to determine whether PP in the presence or absence of SSM exerts any significant anti-diabetic activity for the management of pancreatogenic diabetes in a rat model of the disease. The efficacy study was performed by first determining the glucose tolerance and insulin sensitivity in normal rats, followed by disease induction and re-evaluation of glucose tolerance and insulin sensitivity. When the animals were deemed to have PD, treatment with PP/PP-SSM and their controls was initiated. At the end of treatment period glucose tolerance and insulin sensitivity was assessed again followed by quantification of hepatic glycogen content after glucose challenge.

2.1. **Glucose tolerance and insulin sensitivity tests**

6 weeks old male wistar rats were purchased (Charles River Laboratories) and allowed to acclimatize with the new surrounding for a few days upon arrival. The animals were housed in micro isolater cages in rooms where temperature and humidity were maintained within limits as recommended by the Guide for the Care and Use of Laboratory Animals and with a 14h light and 10h darkness cycle.
Throughout the study, all animals were weighed two times a week. At the start of the study, glucose tolerance and insulin sensitivity tests were performed to determine glucose homeostasis in normal animals and to quarantine any diabetic animal at the outset. For glucose tolerance test (GTT), animals were fasted for a period of 8h but given free access to water. A 30% w/v D-glucose solution was prepared and sterile filtered using 0.22 µ filter and injected intraperitoneally into the animals at a dose of 1 ml/100g body weight. Blood glucose was measured from the tail vein before glucose injection and at 30 and 60 minutes after injection. At the end of the study, all animals were provided food ad libitum. For insulin sensitivity test (IST), the animals were fasted for a period of 6h but given free access to water. Insulin solution was prepared from Humalog® at a concentration of 1 U/ml and injected intraperitoneally at a dose of 1 U/kg body weight. Blood glucose was measured from the tail vein before the insulin injection and at 30 minutes after injection and the drop in blood glucose was measured. At the end of the study, all animals were provided food ad libitum. Percent drop in blood glucose level upon insulin injection was calculated based on blood glucose level before insulin injection.

2.2. **Induction of pancreatogenic diabetes in rats**

After determining the basal glucose tolerance and insulin sensitivity of animals, pancreatogenic diabetes was induced according to the following protocol: A solution of L-arginine was prepared and injected intraperitoneally into the animals at a dose of 350 mg/kg body weight. L-arginine was injected daily and glucose tolerance and insulin sensitivity of the
animals were determined each week. The animals were considered to have impaired glucose tolerance when their blood glucose level (after 1h), was significantly different than that observed for normal animals (before disease induction). The insulin sensitivity of the animals was also considered impaired when no significant difference in the blood glucose level was observed between 0 and 30 minutes after insulin injection.

The L-arginine based animal model is a well established and accepted model of chronic pancreatitis where pancreatogenic diabetes is induced due to progressive degeneration of the pancreas. The acinar architecture of pancreas of rats treated with L-arginine gets destroyed and is replaced by adipose tissue. Thereafter, the animals develop impaired glucose tolerance and have reduced levels of insulin in the body within 4 weeks of first arginine injection. Other researchers have used this animal model to study progressive changes in the pancreas (Weaver et al., 1994; Otsuki et al., 2010).

2.3. Antidiabetic efficacy study of PP-SSM

After pancreatogenic diabetes was induced in the animals, they were divided into six groups of five animals per group such that the mean drop in percent blood glucose level (as determined from the insulin sensitivity test) was similar across the groups. The groups of animals evaluated as per treatment were:

PBS (control group)

SSM (control group)
PP in buffer (treatment group)

PP-SSM (treatment group)

Metformin® (positive treatment control)

Insulin [Humulin®] (positive treatment control)

Test samples of PP and PP-SSM were prepared as described in section in the preparation of PP-SSM section of materials and methods. PP dose of 200 µg/kg body weight was used in the study and PP-SSM samples were prepared with a lipid:peptide ratio of 30:1 (the saturation molar ratio of association between PP and SSM) so as to provide maximum peptide dose per micelle. The dose of PP used in the study was based on studies by Seymour et al. who found that a 5 day administration of PP improved glucose tolerance in rats (Seymour et al., 1995). It was estimated that upon *in vivo* administration of SSM/PP-SSM, approximately 20 fold dilution will occur after injection that would cause breakdown of some micelles and release of some peptides. Therefore in order to minimize this loss, the formulations were injected with extra lipids (to attain ~20 µM final concentration in the blood) to maintain the CMC of lipids above 1µM. Metformin dose used in the study was 30 mg/kg body weight (recommended dose for use in a clinical setting is 20-40 mg/kg body weight) while insulin (prepared from Humulin®) was used at a dose of 0.5 U/kg body weight dose. Prior to the intravenous administration of control and treatment drugs, the animals were anesthetized by intraperitoneal injection of ketamine and xylazine (50 mg/kg and 5 mg/kg respectively) and degree of anesthesia determined by toe pinch reflex. Test drugs and controls were then injected intravenously via the tail vein only after
the animals had lost their toe pinch reflex. This procedure was repeated for 5 consecutive days followed by assessment of glucose tolerance and insulin sensitivity at the end of treatment period.

2.4. **Assessment of hepatic glycogen content**

At the end of the treatment period and after evaluation of glucose tolerance and insulin sensitivity, the hepatic glycogen content in the rats was determined. For this, rats were injected with 30% D-glucose solution intraperitoneally at a dose of 1 ml/100g body weight. At 2h following glucose injections, the rats were euthanized via CO$_2$ asphyxiation followed by cervical dislocation and their livers were immediately removed and stored at -80°C. The hepatic glycogen content in the livers was measured as per the procedure described by Carr and Neff (Carr and Neff 1984). Briefly, approximately 1g of liver tissue was homogenized with pH 5.0 citrate buffer (10% w/v) and placed in boiling water and re-homogenized. After this, the homogenized mixture was divided into 2 sets and to one set 25 µl/ml of 0.5% amyglucosidase solution in citrate buffer was added. Both sets were incubated overnight at room temperature and at the end of the incubation period, the solutions were centrifuged at 28,000 g for 30 minutes and glucose content in the supernatant was determined using glucose meter. Hepatic glycogen content was evaluated as glucose concentration difference between amyloglucosidase treated samples and untreated samples.

The entire schema for *in vivo* efficacy studies is presented in Figure 7.
C. DATA AND STATISTICAL ANALYSES

All data are expressed as mean ± standard deviation (S.D.). For the statistical analysis, student’s T-test or one way analysis of variance (ANOVA) followed by Tukey’s and Fisher least significant difference post-hoc test were used and a value of p<0.05 was considered as statistical significance.
III. RESULTS

A. *IN VITRO EXPERIMENTS*

1. **Characterization of PP-SSM**

   1.1. **Particle size analysis**

   The particle size distribution of PP in the presence or absence of SSM was evaluated using dynamic light scattering by both intensity and volume weighted Nicomp analysis. In the absence of SSM, bimodal large heterogeneous aggregates of PP were observed with mean hydrodynamic diameters of 98.3±17.9 nm and 773.3±163.45 nm (Figure 8A). However, in the presence of SSM, a monomodal distribution of particles was observed with a mean hydrodynamic diameter of 13.5±0.7 nm (Figure 8B) which was not significantly different from the size of empty micelles (Figure 8C).
Figure 8. Particle size distribution of PP in presence or absence of SSM

Representative Nicomp figures showing volume weighted particle size distribution of (A) 4 µM PP in PBS; (B) PP-SSM; and (C) Empty SSM (120 µM DSPE-PEG$_{2000}$).
1.2. **Fluorescence spectroscopy**

Further evaluation of association between PP and SSM in phosphate buffered saline (pH 7.4) was performed by monitoring the fluorescence emission of tyrosine residues of the peptide that served as intrinsic fluorophores of the biomolecule. In presence of micelles, a threefold increase in fluorescence emission was observed compared to the aqueous solution of the peptide alone (Figure 9).

![Figure 9](image.png)

**Figure 9.** Fluorescence emission of PP in presence or absence of SSM

Fluorescence emission spectra of PP in phosphate buffered saline and when incubated with SSM.
1.3. Circular dichroism

The secondary structure of PP in the presence or absence of SSM was determined using near-UV circular dichroism spectroscopy. PP exhibited its characteristic alpha-helical conformation both in the presence or absence of SSM, indicating that the physiologically active secondary conformation of the peptide is retained in the micelles. Furthermore, association of PP with SSM resulted in an increase in alpha helicity as observed by increased negative CD signal for alpha helix bands at 208 and 222 nm (Figure 10).

**Figure 10.** Secondary structure of PP in presence or absence of SSM

Representative circular dichroism spectra of PP (4 µM) in phosphate buffered saline (pH 7.4) and in SSM (1 mM DSPE-PEG$_{2000}$).
2. **Influence of pH and electrolyte on peptide loading in micelles**

The effect of pH and sodium chloride on the saturation molar ratios of association between PP and SSM and subsequently the number of peptide molecules associated with each micelle was determined in normal saline (pH 4.5 – 7.0) and in phosphate buffers of pH 6.5, 7.4 and 8.0 in the presence or absence of 0.9% w/v sodium chloride. Addition of increasing amount of lipid (8 μM – 400 μM) to a constant peptide concentration (4 μM) resulted in an initial rise in the fluorescence emission, which later plateaued in the presence of excessive lipid. As evident from the saturation curves, there was a steeper increase as well as higher normalized fluorescence emission intensity in the absence of sodium chloride (Figure 11) compared to samples in aqueous media containing sodium chloride (Figure 12). Consequently, samples prepared without sodium chloride had lower saturation molar ratio of association between PP and SSM implying presence of more peptide molecules associated with micelles in such formulations. The particle size of PP-SSM prepared in the absence of sodium chloride was smaller (~7.7 nm) (Figure 15B) compared to those prepared in the presence of sodium chloride (~14 nm) (Figure 8C). Our recent molecular dynamics simulation studies using NAMD package and CHARMM27 force field indicate that DSPE-PEG\textsubscript{2000} micelles with hydrodynamic diameter of 7.7 nm have an aggregation number of 20 in pure water (Vukovic et al., 2011). Therefore in this study, the number of peptide molecules associated with each micelle in the absence of sodium chloride was determined using an aggregation number of 20 while for formulations prepared in the presence of sodium chloride an aggregation number of 90 was used (Ashok et al., 2004). Under both conditions the number of peptide molecules associated per micelle was similar *i.e.*
~2-3 peptide molecules associated with each micelle at saturation (Table III). Change in pH did
not bring about any change in the peptide-micelle interaction both in the presence or absence
of sodium chloride in the aqueous media. The secondary structure of the peptide was enhanced
in SSM compared to free peptide at all pHs studied (Figure 13).
Figure 11. Saturation curves of PP-SSM in buffers of different pH in the absence of sodium chloride

Comparative plots for the determination of association molar ratios of PP-SSM at various pH in the absence of sodium chloride. Plot represents fluorescence enhancement ratio (I/I₀) of PP with increasing concentration of DSPE-PEG₂₀₀₀. PP concentration kept constant at 4 µM. All data are mean ± S.D. (n = 3). Filled circle represents pH 6.5, empty circle represents pH 7.4 and inverted filled triangle represents pH 8.0.
Figure 12. Saturation curves of PP-SSM in normal saline or buffers of different pH in the presence of sodium chloride

Comparative plots for the determination of association molar ratios of PP-SSM at various pH in the presence of sodium chloride (0.9% w/v). Plot represents fluorescence enhancement ratio (I/I₀) of PP with increasing concentration of DSPE-PEG₂₀₀₀. PP concentration kept constant at 4 µM. All data are mean ± S.D. (n = 3). Filled circle represents normal saline (pH 4.5 – 7), empty circle represents pH 6.5, inverted filled triangle represents pH 7.4 and empty triangle represents pH 8.0.
**TABLE III.** Effect of pH and presence or absence of sodium chloride in the aqueous media on the association of PP with SSM

<table>
<thead>
<tr>
<th>pH</th>
<th>Absence of sodium chloride</th>
<th>Presence of sodium chloride</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Molar ratio of association</td>
<td>Molar ratio of association</td>
</tr>
<tr>
<td></td>
<td>(Lipid: PP)</td>
<td>(Lipid: PP)</td>
</tr>
<tr>
<td></td>
<td># PP molecules per SSM</td>
<td># PP molecules per SSM</td>
</tr>
<tr>
<td>Normal saline (pH 4.5 – 7.0)</td>
<td>-</td>
<td>41: 1</td>
</tr>
<tr>
<td>6.5</td>
<td>8: 1</td>
<td>3</td>
</tr>
<tr>
<td>7.4</td>
<td>9: 1</td>
<td>2</td>
</tr>
<tr>
<td>8.0</td>
<td>11: 1</td>
<td>2</td>
</tr>
</tbody>
</table>
**Figure 13.** Secondary structure of PP at different pHs in presence or absence of SSM prepared in phosphate buffer (PB) A) pH 6.5; B) pH 7.4; and C) pH 8.0
3. **Freeze-drying of PP-SSM**

For effective translation of a drug product to the clinics, it is essential to formulate products in such a way that they have acceptable shelf-life. Peptide and phospholipid solutions are prone to oxidative and hydrolytic degradation and are not stable for the stipulated period of time required for pharmaceutical products and therefore need to be stored in the dry form. Since we had observed a clear difference in peptide-micelle association based on the presence or absence of sodium chloride in the aqueous media of the formulation, we decided to test PP-SSM prepared and reconstituted after freeze-drying in different aqueous media to evaluate whether such differences brought about any change in the peptide/micelle association of the final product and to identify the formulation that is most suitable for lyophilization. To this end, one sample was prepared in pH 7.4 phosphate buffered saline and reconstituted using sterile water [A], while the other was prepared in pH 7.4 phosphate buffer and reconstituted using normal saline [B]. A lipid: peptide molar ratio of 35: 1 was used for both samples to ensure maximum peptide loading in each micelle. Elegant freeze dried cakes of both samples were obtained after lyophilization (Figure 14). Analysis of particle size distribution before and after lyophilization revealed no significant change in the size distribution before and after lyophilization for samples prepared in phosphate buffered saline and reconstituted in sterile water (Figure 15A) with mean hydrodynamic diameters of 13.4 ± 0.8 nm before lyophilization and 14.2 ± 0.1 nm after lyophilization. However, in samples prepared in the absence of sodium chloride, smaller sized micelles were observed with a mean hydrodynamic diameter of 7.7 ±
0.72 nm; which after reconstitution with normal saline, had size in the normal range of 14.2 ± 0.47 nm (Figure 15B; Table IV).

The fluorescence emission of the samples prepared in the phosphate buffered saline decreased significantly after lyophilization which maybe due to dissociation of some peptide molecules from micelles during lyophilization. However, the fluorescence emission after lyophilization from PP-SSM samples that were prepared in phosphate buffer and reconstituted in normal saline did not decrease significantly indicating that the peptide was retained more effectively in this formulation (Figure 16). Circular dichroism studies before and after lyophilization did not demonstrate any change in the alpha-helicity of the peptide in samples prepared with or without sodium chloride and reconstituted using sterile water or normal saline respectively (Figure 17).
**Figure 14.** Representative photograph of lyophilized cake of PP-SSM

**TABLE IV.**
Particle size distribution of PP-SSM before and after freeze-drying

<table>
<thead>
<tr>
<th>Sample</th>
<th>Particle size (nm)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before FD</td>
<td>After FD</td>
<td></td>
</tr>
<tr>
<td>PP-SSM [A]</td>
<td>13.4±0.8</td>
<td>14.2±0.1</td>
<td></td>
</tr>
<tr>
<td>PP-SSM [B]</td>
<td>7.7±0.7</td>
<td>14.2±0.5</td>
<td></td>
</tr>
</tbody>
</table>
Figure 15. Particle size distribution of PP-SSM before and after lyophilization

Representative Nicomp figures showing particle size distribution of PP-SSM at lipid: peptide molar ratio of 35:1 (i) before and (ii) after freeze drying. (A) Samples prepared in phosphate buffered saline and reconstituted using sterile water; (B) samples prepared in phosphate buffer and reconstituted using normal saline. Lipid and peptide concentration kept constant at 5 mM and 143 μM for all samples.
**Figure 16.** Fluorescence emission of PP-SSM before and after lyophilization

Fluorescence emission of PP-SSM before and after freeze drying (FD) at 35:1 lipid: peptide molar ratios; [A] samples prepared in phosphate buffered saline (pH 7.4) and reconstituted in sterile water; [B] samples prepared in phosphate buffer (pH 7.4) and reconstituted using 0.9% w/v normal saline (n=3). Lipid and peptide concentration kept constant at 5 mM and 143 μM respectively for all samples. All results are mean ± S.D. * represents statistical significance at p<0.05 in comparison to fluorescence emission of PP-SSM before lyophilization (n = 3/group; determined using ANOVA followed by Tukey post-hoc analysis).
Figure 17. Secondary structure of PP before and after lyophilization

Representative CD spectra of PP in buffer and in SSM for samples prepared in [A] pH 7.4 PBS and reconstituted using sterile water and [B] prepared in pH 7.4 phosphate buffer without sodium chloride and reconstituted using normal saline. Dotted line represents spectra before lyophilization while solid line represents spectra after lyophilization.
4. **Assessment of stability of PP-SSM against proteolytic degradation**

The study was conducted to determine whether association of PP with SSM affords any stability to the peptide against proteolytic degradation. To conduct the test, PP in buffer or in SSM were incubated with trypsin for different periods of time and the percent of PP remaining was determined based on the original amount of PP in the sample. PP amounts were determined from areas under the curve of the peaks for PP. Results of the study indicated that SSM significantly improved the stability of PP against proteolytic degradation at all the time points studied. This protective effect was more prominent during the first 15 minutes where the percent PP remaining in the solution was ~95% whereas only about 55% of PP remained undegraded in PP in buffer samples (Figures 18 and 19). The degradation rate for PP and PP-SSM were calculated to be 0.028 and 0.011 min\(^{-1}\) respectively and the corresponding half-life of the peptide was calculated to be 24.8 and 63.0 minutes respectively (Table V). Therefore, in the presence of micelles, the stability of PP improved by approximately 2.5 fold.

<table>
<thead>
<tr>
<th>Sample</th>
<th>(k) (min(^{-1}))</th>
<th>(\tau^{1/2}) (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP</td>
<td>0.028</td>
<td>24.8</td>
</tr>
<tr>
<td>PP-SSM</td>
<td>0.011</td>
<td>63.0</td>
</tr>
</tbody>
</table>
Figure 18. Tryptic digestion kinetics of PP and PP-SSM

Degradation kinetics of PP in presence or absence of SSM upon incubation with trypsin (n=3). * represents statistical significance at p < 0.05 in comparison to PP in buffer for the same time point (determined using student T-test).
Figure 19. Semi log plot of tryptic digestion kinetics of PP and PP-SSM

Degradation kinetics of PP in presence or absence of SSM upon incubation with trypsin (n=3) represented in semi-log graph.
5. **In vitro bioactivity assessment of PP-SSM**

Pancreatic polypeptide is known to inhibit cAMP production in cells. In this study, Y4 receptor binding and activation of PP in SSM was assessed and compared to peptide alone to determine whether bioactivity of the peptide is retained when it is associated with micelles. PP in buffer and PP-SSM significantly decreased forskolin stimulated cAMP production in human neuroblastoma SK-N-MC cells to a similar extent as compared to positive control (Figure 20). Approximately 40 - 50% reduction in cAMP concentration was observed for PP and PP-SSM at the doses tested compared to positive control. A peptide dose dependent inhibition of cAMP production was not observed. Treatment of SK-N-MC cells with empty micelles and PP-SSM corresponding to the highest respective concentration of lipid and peptide tested in the study, did not significantly change cAMP level as compared to the buffer treated group.
Figure 20. Inhibition of forskolin stimulated cyclic AMP production by PP and PP-SSM.

Percent inhibition of forskolin stimulated cAMP response in SK-N-MC cells by PP in buffer and PP-SSM at peptide doses of 1, 10 and 100 nM (Normalized to forskolin treated group). * indicates statistical significance at $p < 0.05$ (determined using student T-test) compared to forskolin treated group (positive control; PP = 0 nM). Inset represents normalized cAMP concentration in SK-N-MC cells treated with PBS, empty SSM, PP-SSM and forskolin.
B. **IN VIVO EXPERIMENTS**

The purpose of this study was to evaluate the effectiveness of PP nanomedicine (PP-SSM) in treating pancreatogenic diabetes. This was evaluated through assessment of glucose tolerance and insulin resistance (the two hallmarks of pancreatogenic diabetes). The antidiabetic efficacy of PP/PP-SSM and controls was evaluated by the following tests:

- Glucose tolerance test on normal, diseased and treated rats
- Insulin sensitivity test on normal, diseased and treated rats
- Hepatic glycogen content analysis on all rats at the end of treatment period

Glucose tolerance test was conducted to determine whether treatment with PP/PP-SSM and controls is able to overcome impaired glucose tolerance developed in the animals as a result of high dose of arginine injections. Insulin sensitivity test and quantification of hepatic glycogen was performed to determine whether administration of PP-SSM overcomes hepatic insulin resistance. Insulin and metformin (drugs that are utilized for treatment of PD) were used as positive controls to compare the efficacy of PP-SSM nanomedicine with the present standards of care.
1. **Glucose tolerance test**

Glucose tolerance test was determined by fasting the animals for a period of 8h followed by intraperitoneal injection of 30% w/v glucose (1 ml/100g body weight). Blood glucose levels were measured before the injection and 30 and 60 minutes after glucose injection. Impaired glucose tolerance was defined as significant difference in blood glucose concentration after 1h of glucose injection as compared to normal animals (animals before disease induction).

The mean blood glucose level of animals (before disease induction through daily intraperitoneal arginine injections) was found to be 113.1 ± 14.2 mg/dl across all groups after 1h of glucose injection. Upon daily injection of L-arginine, the mean blood glucose level after 1h of glucose injection in all animals that had been injected with arginine for 2-3 weeks was significantly increased to 160.1 ± 10.6 mg/dl. This clearly demonstrated that the animals were not able to clear glucose from the blood stream as effectively as before arginine injections were started and therefore they were considered to have developed impaired glucose tolerance.

Treatment with PP and PP-SSM for five consecutive days led to a non-significant drop in blood glucose levels after 1h of glucose challenge in PP treated animals (145.8 ± 44.8 mg/dl) while a significant drop in blood glucose levels was observed for animals treated with PP-SSM (114.6 ± 36.9 mg/dl) (Figure 21). A similar significant improvement in glucose tolerance was also observed in group of animals treated with metformin (113.2 ± 25.2 mg/dl). It must be noted that glucose tolerance in both PP-SSM and metformin treated groups were brought to normal levels, *i.e.* levels observed in pre-arginine injected rats. All other control groups did not show
any significant improvement in glucose tolerance at the end of treatment period (Figure 22). Blood glucose levels of animals after 1h of glucose challenge was noted to be $156.2 \pm 10.3$ mg/dl for buffer treated group; $157.4 \pm 35.9$ mg/dl for SSM treated group and $122.4 \pm 45.1$ mg/dl for Insulin treated group (Table VI).

### TABLE VI.
Glucose tolerance in normal, diseased and drug treated animals

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Blood glucose concentration at 1h after glucose injection (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal animals</td>
<td>113.1 ± 14.2</td>
</tr>
<tr>
<td>Diseased animals</td>
<td>160.1 ± 10.6</td>
</tr>
<tr>
<td>Buffer treated animals</td>
<td>156.2 ± 10.3</td>
</tr>
<tr>
<td>SSM treated animals</td>
<td>157.4 ± 35.9</td>
</tr>
<tr>
<td>PP treated animals</td>
<td>145.8 ± 44.8</td>
</tr>
<tr>
<td>PP-SSM treated animals</td>
<td>114.6 ± 36.9</td>
</tr>
<tr>
<td>Insulin treated animals</td>
<td>122.4 ± 45.1</td>
</tr>
<tr>
<td>Metformin treated animals</td>
<td>113.2 ± 25.2</td>
</tr>
</tbody>
</table>
Figure 21. Glucose tolerance in normal animals, diseased animals and animals subjected to various treatments.

Blood glucose levels at 60 minutes after glucose injection in normal, diseased and drug treated animals. Data represented as mean ± S.D. ‡ indicates statistically significant difference in blood glucose levels between normal and diseased animals while * represents statistically significant improvement in disposal of glucose from blood, after injection in drug treated animals as compared to diseased animals. (p < 0.05; n=5/group; determined using student T-test).
**Figure 22.** Glycemic excursions in animals subjected to different treatments after glucose injection

Blood glucose levels in animals at the end of treatment period through 60 minutes after glucose injection. Data represented as mean ± S.D.
2. **Insulin sensitivity test**

Insulin sensitivity test was performed to assess the extent of insulin resistance in animals. The test was conducted by fasting the animals for a period of 6h followed by intraperitoneal injection of insulin (1 U/kg body weight) and determination of blood glucose levels before injection and 30 minutes after injection.

The insulin sensitivity in the animals was evaluated as percent drop in blood glucose from basal level after 30 minutes of insulin injection. Normal animals (before disease induction) showed a drop of approximately 52.2 ± 6.1% in blood glucose level upon insulin injection. However, a significant loss in insulin sensitivity was observed in the animals after 2-3 weeks of arginine injection. The mean percent drop in blood glucose level was only 27.13 ± 9.6 in all animals that had been injected with arginine. This significant loss in insulin sensitivity indicated the development of insulin resistance in the animals. At the point when animals displayed both impaired glucose tolerance as well insulin resistance (the two hallmarks of PD); they were considered to have successfully developed pancreatogenic diabetes and treatment with PP/PP-SSM and controls were thereafter started.

The animals that were treated with PP-SSM or metformin were found to significantly improve insulin sensitivity. PP-SSM treated group demonstrated a 52.6 ± 9.6% drop in blood glucose level within 30 minutes of insulin injection while metformin treated group showed a drop of 49.2 ± 8.7% (Figure 23). As observed with glucose tolerance test, both PP-SSM and metformin treatment restored insulin sensitivity in animals to the extent observed in normal animals, *i.e.* animals before disease induction. All other treatment groups did not bring about
any significant improvement in insulin sensitivity. The percent drop in blood glucose level was found to be 21.8 ± 6.4 for buffer treated group; 28.9 ± 7.6 for SSM treated group; 39.8 ± 7.2 for PP treated group and 31.9 ± 11.5 for insulin treated group (Table VII). A significant difference in the PP and PP-SSM treated group was also observed. The study demonstrated significantly higher efficacy of PP-SSM in overcoming insulin resistance in animals with PD as compared to PP in buffer.

**TABLE VII.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent drop in blood glucose concentration at 30 minutes after insulin injection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal animals</td>
<td>52.2 ± 6.1</td>
</tr>
<tr>
<td>Diseased animals</td>
<td>27.13 ± 9.6</td>
</tr>
<tr>
<td>Buffer treated animals</td>
<td>21.8 ± 6.4</td>
</tr>
<tr>
<td>SSM treated animals</td>
<td>28.9 ± 7.6</td>
</tr>
<tr>
<td>PP treated animals</td>
<td>39.8 ± 7.2</td>
</tr>
<tr>
<td>PP-SSM treated animals</td>
<td>52.6 ± 9.6</td>
</tr>
<tr>
<td>Insulin treated animals</td>
<td>31.9 ± 11.5</td>
</tr>
<tr>
<td>Metformin treated animals</td>
<td>49.2 ± 8.7</td>
</tr>
</tbody>
</table>
**Figure 23.** Insulin sensitivity in normal animals, diseased animals and animals subjected to various treatments.

Insulin sensitivity represented as percent change in blood glucose level from baseline after 30 minutes of insulin injection for normal, diseased and treated animals. Data represented as mean ± S.D. ‡ represents statistically significant loss in insulin sensitivity between normal and diseased animals while * indicates significant improvement in insulin sensitivity in treated animals compared to diseased animals (p<0.05, n=5/group). # represents statistically significant enhancement of activity in PP-SSM treated group compared to PP in buffer treated group (p<0.05; n=5/group; determined using student T-test).
3. **Assessment of hepatic glycogen content**

This study was performed at the end of treatment with PP/PP-SSM and controls by injecting 30% w/v glucose solution (1 ml/100g body weight) into animals at fed state. The rats were sacrificed after 2h of glucose injection, their livers were harvested and the hepatic glycogen content was quantified.

Data obtained from this study revealed significantly higher glycogen content in livers of rats treated with PP-SSM (96.4 ± 15.2 mg/dl) compared to PP (57.4 ± 7.4 mg/dl). Animals treated with metformin also demonstrated higher hepatic glycogen content (91.6 ± 11.6 mg/dl) as compared to other treatment groups. Glycogen content in the livers were 28.2 ± 3.8 mg/dl for buffer treated group; 34.2 ± 7.6 mg/dl for SSM treated group and 48.6 ± 3.7 for insulin treated group (Figure 24; Table VIII). This study further corroborated the fact that PP-SSM is more effective in overcoming insulin resistance in animals with pancreatogenic diabetes than peptide in aqueous media.
TABLE VIII.
Hepatic glycogen content in drug treated animals

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hepatic glycogen content in treated animals (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer treated animals</td>
<td>28.2 ± 3.8</td>
</tr>
<tr>
<td>SSM treated animals</td>
<td>34.2 ± 7.6</td>
</tr>
<tr>
<td>PP treated animals</td>
<td>57.4 ± 7.4</td>
</tr>
<tr>
<td>PP-SSM treated animals</td>
<td>96.4 ± 15.2</td>
</tr>
<tr>
<td>Insulin treated animals</td>
<td>48.6 ± 3.7</td>
</tr>
<tr>
<td>Metformin treated animals</td>
<td>91.6 ± 11.6</td>
</tr>
</tbody>
</table>
**Figure 24.** Glycogen content in livers of animals subjected to different treatments.

Hepatic glycogen content represented in mg/dl from animals treated with PP/PP-SSM and controls obtained at the end of study. * indicates statistically significant higher hepatic glycogen content in PP-SSM treated group compared to PP in buffer treated group (p<0.05 n=5/group; determined using student T-test).
IV. DISCUSSION

A. IN VITRO EXPERIMENTS

1. Preparation and characterization of PP-SSM

Peptide micelle interaction was characterized using particle size distribution and fluorescence emission study. Results from this study revealed that pancreatic polypeptide spontaneously associated with phospholipid micelles. The peptide formed heterogeneous aggregates in aqueous media. However, when incubated with micelles, formation of such peptide aggregates was abated and particles around the normal size of micelles were observed, indicating that peptide molecules preferentially interacted with phospholipid micelles and did not self-associate to form its own aggregates. This notion was corroborated through fluorescence studies that exploited the intrinsic fluorescence of the tyrosine residues in the peptide to determine peptide-micelle association. We observed that the fluorescence emission of the peptide increased approximately three fold in presence of phospholipid micelles. The increase in fluorescence is postulated to be due to association of individual peptide molecules with the micelles that reduces fluorescence quenching as observed in PP aggregates.

Circular dichroism study revealed that the alpha helical secondary conformation of the peptide was enhanced when associated with micelles. This was evident from the increased negative bands corresponding to those for alpha-helical peaks and was observed for all pHs and in the presence or absence of sodium chloride in the aqueous media. These findings are in concordance with the observations of other researchers. Lerch et. al. have observed that alpha-helical secondary structure of PP was stabilized when bound to dodecylphosphocholine.
micelles as compared to peptide in solution (Lerch et al., 2002). With respect to effect of pH on change in peptide conformation, Tonan et. al. showed that ellipticity of PP was not pH dependent above pH 5.0 (Tonan et al., 1990) which is in agreement with our findings. Increased conformational stability of the peptide within the micelle offers the advantage of enhanced receptor interaction therefore enhanced bioactivity.

2. Effect of electrolytes on peptide loading in micelles

The peptide-micelle interaction did not vary according to aqueous media pH conditions but was influenced by the presence or absence of sodium chloride in the formulation. In the absence of sodium chloride, a lower saturation molar ratio of association was observed compared to formulations prepared in the presence of sodium chloride; however the number of peptide molecules associated with each micelle at saturation remained similar in both formulations. This can be explained on the basis of difference in properties of micelles prepared in the presence or absence of sodium chloride in the formulation. In the presence of sodium chloride, the phospholipid head groups of micelles are enveloped in a cloud of counterions, that decrease repulsion between negatively charged phosphate groups, increases aggregation number that results in increase in hydrodynamic size of micelles. Conversely, in the absence of strong counterions in the aqueous media, the negative charges on the phospholipids are not neutralized that leads to increased repulsion between phospholipids, decrease in aggregation number, thereby decrease in micelle size. This view has been substantiated through our recent experimental as well as molecular dynamics simulation studies, where we observed a decrease
in micelle size and aggregation number of micelles prepared in the absence of strong counterions (Vukovic et al., 2011). Based on this rationale, formulations prepared in the presence of sodium chloride will contain large sized micelles but fewer in number while those prepared in absence of sodium chloride will contain small sized micelles but more in number, given the same amount of lipid is used in both cases. The abundance of micelles in the absence of sodium chloride increases the likelihood of interaction between peptide molecules and micelles that subsequently lowers the saturation molar ratio of association between SSM and PP. However, the number of peptide molecules associated with each micelle remains similar in both formulations because tighter stacking of lipids in large micelles decreases the available volume for peptide to reside whereas increased repulsion between phosphate headgroups in small sized micelles increases the available volume for peptide-micelle association. In addition to this, charge interaction between peptide and micelles is also deliberated to play a role in the association between SSM and PP. In the absence of sodium chloride, higher peptide-micelle interaction is observed due to electrostatic affinity between phospholipid head groups of micelles and charged amino acid residues on the peptide. However, in the presence of sodium chloride, charges on both entities are neutralized by counterions that abrogate charge based interaction between peptide and micelles and decrease association. Furthermore, sodium chloride is known to stabilize the alpha-helix of amphipathic peptides (Lazo and Downing 1998) that can decrease the driving hydrophobic force in peptide-micelle interaction.
3. **Freeze drying stability of PP-SSM**

Lyophilization of PP-SSM prepared in pH 7.4 phosphate buffered saline resulted in a significant decrease in fluorescence emission after lyophilization. This was most likely due to dissociation of peptide molecules from the micelles upon freeze-drying that may result in their aggregation and subsequent fluorescence quenching. For formulations prepared in phosphate buffer and reconstituted in normal saline, no significant decrease in fluorescence emission was observed after lyophilization suggesting that peptide-micelle association is retained to a greater extent after lyophilization when PP-SSM is prepared in formulations not containing sodium chloride. A plausible reason for this observation is that PP-SSM prepared in the absence of sodium chloride interacts with each other through both electrostatic as well as hydrophobic interactions that strengthen peptide-micelle association and lead to reduced dissociation of peptide molecules from SSM during lyophilization. Based on this result, it is evident that only the formulation of PP-SSM prepared in the absence of sodium chloride and reconstituted after freeze-drying in normal saline is robust to the process of lyophilization. Therefore we propose that during development of final PP-SSM product, the formulation should be prepared in aqueous media not containing sodium chloride and reconstituted after lyophilization in normal saline as this will ensure the stability of the peptide in micelles during freeze-drying.

4. **Assessment of stability of PP-SSM against proteolytic degradation**

In the presence of SSM, the stability of PP was significantly improved against proteolytic degradation. An approximate 2.5 fold enhancement in stability of PP was observed compared
to peptide alone in buffer when the samples were incubated with trypsin. The peptide is postulated to reside in the PEG corona of the micelles, which provide a steric barrier and prevent recognition and degradation of the peptide by proteolytic enzymes, thus improve half-life of the peptide. Based on this result we anticipated that in an in vivo setting, these phospholipid micelles will protect PP against rapid proteolytic degradation and make them long-circulating that will lead to enhanced activity and requirement of less frequent dosing to achieve therapeutic efficacy.

5. **In vitro bioactivity assessment of PP-SSM**

*In vitro* bioactivity studies of PP and PP-SSM indicated that the bioactivity of PP was retained when associated with micelles. A significant inhibition in cAMP levels were observed at all the doses tested for the peptide. The extent of inhibition of cAMP was similar for PP in buffer and PP in SSM which is expected as the studies were conducted in serum free media, thus in an environment lacking proteolytic activity. A dose dependent inhibition of cAMP production was not observed. This is postulated to be due to weak expression of Y4 receptors in SK-N-MC cells which may have resulted in saturation of the receptors available for binding by PP at the concentrations tested. Overall, the study exhibited that association of PP with SSM does not hamper interaction of the peptide with its receptors.
B. **IN VIVO EXPERIMENTS**

The objective of the *in vivo* studies was to test the anti-diabetic efficacy of PP-SSM in a rat model of pancreatogenic diabetes. PD is characterized by impaired glucose tolerance and loss of hepatic insulin sensitivity. Therefore to establish the anti-diabetic efficacy of PP-SSM for PD, assessment of glucose tolerance, insulin sensitivity as well as hepatic glycogen content was performed in diabetic animals treated with PP-SSM and controls.

1. **Glucose tolerance test**

Glucose tolerance test was conducted to determine the response of the animals to a high dose of glucose in blood. This test measures both the capacity of the pancreas to secrete insulin and ability of the liver to respond to circulating insulin by clearing glucose from the blood stream. Therefore glucose tolerance test is used for the diagnosis of pancreatic endocrine function as well as insulin resistance in diabetes.

This study revealed that PP-SSM and metformin treated animals were able to significantly improve glucose tolerance when compared to diseased animals. The improvement in glucose tolerance observed in PP-SSM treated group can be attributed to the fact that PP in SSM is passively targeted to its site of action *i.e.*, to the liver via EPR effect observed through the liver fenestrations. In addition, it is postulated that PP in SSM is well-protected against proteolytic degradation *in vivo* as opposed to the formulation of peptide in buffer. These two
factors contribute toward greater accumulation of PP at the site of action and accounts for enhanced efficacy of PP when associated with SSM.

2. **Insulin sensitivity test**

Insulin sensitivity test is used to measure the extent of insulin resistance in the body. Insulin resistance occurs as a result of reduced responsiveness of the body to given insulin. The etiologies for insulin resistance are mutation in insulin, presence or antibodies against insulin, decreased expression of insulin receptors in hepatic, muscle and adipose tissues, decreased binding of insulin to insulin receptors, mutations in the receptors or antibodies against insulin receptors. Other causes of insulin resistance include post receptor failure such as defective signal transduction and mutations in glucose transporter GLUT4 (Olatunbosun and Dagogo-Jack 2011). Insulin resistance observed in pancreatogenic diabetes has been generally attributed to deficiency of PP which leads to decreased expression of insulin receptors in the liver (Cui and Andersen 2011). Therefore, exogenous administration of PP should be able to restore hepatic insulin sensitivity.

In this study, we observed that animals treated with PP-SSM or metformin had significantly higher sensitivity to insulin compared to other treatment groups. Furthermore, in both groups, insulin sensitivity was restored to almost normal levels (as observed in animals before induction of the disease). A significant increase in efficacy was also observed in PP-SSM treated group compared to peptide alone group which further corroborated the fact that SSM
is able to effectively protect PP from enzymatic degradation \textit{in vivo} and deliver it in its active form to the target site (liver), thereby eventually bringing about a better therapeutic effect than peptide alone.

3. \textbf{Assessment of hepatic glycogen content}

Hepatic glycogen content assay was conducted to quantitate glycogen content in the liver after glucose challenge. This study was performed to assess the ability of liver to uptake glucose from the blood and store as glycogen in the liver. Our results indicated that livers of rats treated with PP-SSM had significantly higher glycogen content as compared to that of PP in buffer treated group. Animals treated with metformin also showed equivalent glycogen content in the liver as PP-SSM. This result is expected since metformin is known to increase the activity of glycogen synthase in the liver (Hundal et al., 2000; Reddi and Jyothirmayi 1992). In all the animal studies, metformin demonstrated equivalent therapeutic efficacy as PP-SSM. However, it must be noted that metformin causes lactic acidosis in many patients which is a clinical condition wherein glucose is broken down anerobically leading to accumulation of lactic acid in the blood and a drop in blood pH which can be fatal. In addition, many patients with CP induced PD cannot be treated with metformin since the drug is contraindicated in alcoholism which is the major reason for development of PD in North America. Apart from this, the drug increases the levels of insulin secretagogue GLP-1 in the body. Increased levels of GLP-1 will lead to increased levels of insulin in the blood that can exacerbate hyperinsulinemic conditions due pre-existing insulin resistance in PD. These points draw attention to the limitations of
metformin therapy for management of PD and should be considered by physicians before recommending the drug for PD therapy.

In vivo studies asserted the fact that efficacy of PP is significantly improved when it is delivered in SSM. PP-SSM due to its small size extravasates out of circulation only at the leaky vasculature present in the liver capillaries. This not only serves to increase the dose of the peptide reaching the target site but also reduces any adverse effect generated due to interaction of the peptide with receptors present in other parts of the body. The enhancement in therapeutic effect is also attributed to the fact that PEGylated micelles protect peptides from enzymatic degradation in vivo thus making them long-circulating and ultimately leading to greater accumulation of the peptide in the target organ. In addition, SSM prevents self-aggregation of PP, therefore prevents loss of peptide dose that is intended for therapy. Aggregated peptides (PP in buffer formulations), do not elicit much therapeutic effect as monomeric form of the peptide is required for interaction with receptors and aggregated peptides are rapidly cleared from the circulation. Association of PP with SSM not only provides PP in monomeric form but also presents it to the receptor in its most favorable conformation for receptor interaction. All these factors, we believe, together contribute toward improving the therapeutic efficacy of PP when it is delivered in SSM. With regards to safety concern of PP-SSM, it must be pointed out that unlike small molecule drugs or free peptide in aqueous media, PP in SSM is not likely to show adverse effects as the peptide is targeted to its site of action and does not extravasate out of the circulation. Moreover, the PEGylated phospholipids used for the preparation of SSM has been approved by FDA for use in other parenteral products.
Therefore adverse effects as a result of long-term use of PP-SSM nanomedicine for the management of PD should not be a major concern.

Taken together, these studies demonstrate the feasibility of formulating PP-SSM nanomedicine that has enhanced therapeutic efficacy compared to peptide in buffer. PP in SSM remains in its active alpha-helical conformation and has improved stability against proteolytic degradation without any compromise to its bioactivity. The formulation when prepared in the absence of sodium chloride is robust to freeze-drying. The nanomedicine shows significant anti-diabetic efficacy in terms of improving glucose tolerance and overcoming insulin resistance and therefore should be further developed as a commercial product for use in clinics for PD therapy.
V. CONCLUSIONS

1. Pancreatic polypeptide underwent spontaneous association with SSM as indicated by the absence of PP aggregates in presence of SSM as well as enhancement in the fluorescence emission and alpha-helical secondary structure of the peptide when incubated with SSM when compared to PP in buffer.

2. Characterization of association between PP and SSM demonstrated an influence of electrolytes such as sodium chloride in the aqueous media on peptide-micelle association. In the presence of strong electrolytes, a higher lipid: peptide saturation molar ratio of association between PP and SSM was observed compared to PP-SSM prepared in the absence of sodium chloride. This indicated that in the presence of electrolytes, the association between peptide and micelles is decreased. However, the number of peptide molecules associated per micelle was similar in both formulations (~2-3 molecules of PP associated with each micelle) due to differences in micelle property in presence/absence of electrolytes.

3. The peptide micelle association did not vary with change in pH of the aqueous media. Our results showed that PP-SSM prepared in pH 6.5, 7.4 and 8.0 phosphate buffers had similar lipid: peptide association molar ratios. The secondary structure of the peptide was enhanced in the presence of SSM in all the formulations but did not vary significantly from each other.
4. The formulation was effectively freeze-dried in the absence of any cryo/lyoprotectant. PP-SSM prepared in the absence of sodium chloride and reconstituted following lyophilization in normal saline did not show any significant loss in fluorescence emission of PP upon freeze-drying. However, difference in the hydrodynamic particle size of the micelles was observed before and after reconstitution for PP-SSM prepared in the absence of sodium chloride and reconstituted after lyophilization in normal saline. Smaller sized micelles were observed before lyophilization while larger sized micelles were observed after lyophilization in this formulation. Lyophilized products showed minimal shrinkage in cake height. The peptide also retained its alpha helical secondary conformation after freeze-drying. The study demonstrated the stability of the peptide in the micelles during freeze-drying. This is a major step toward development of PP-SSM nanomedicine as a commercial product for use in the clinics in the near future.

5. PP in SSM was significantly more stable to enzymatic degradation in vitro as compared to peptide in buffer. When incubated with trypsin for a period of 2h, the rate of degradation of PP in SSM was decreased by approximately 2.5 fold as compared to peptide alone in aqueous media. The study demonstrated the initial proof-of-concept that SSM protects PP against proteolytic degradation and makes them long-circulating that is postulated to
contribute toward better therapeutic efficacy compared to formulation of peptide in aqueous media.

6. The bioactivity of PP in SSM was retained *in vitro* in terms of its receptor binding and activation capability. PP is known to inhibit cAMP levels in cells. The cAMP levels in both PP and PP-SSM treated cells were significantly decreased as compared to untreated cells and no statistically significant difference in cAMP levels was observed between PP and PP-SSM treated cells. Based on the study, it was concluded that association of PP with SSM did not alter the bioactivity of the peptide.

7. PP-SSM formulation was tested for its anti-diabetic efficacy in a rat model of pancreatogenic diabetes. Treatment efficacy was assessed in terms of improvement in glucose tolerance, restoration of insulin sensitivity and hepatic glycogen content after glucose challenge. PP-SSM nanomedicine significantly improved glucose tolerance in PD rats. The insulin sensitivity was also significantly improved in the group of animals treated with PP-SSM as compared to PP treated group. Better therapeutic efficacy of PP-SSM over PP was again demonstrated when hepatic glycogen content in the rats was assayed. Glycogen content in livers of rats that were treated with PP-SSM was significantly higher than that of rats treated with PP. The only other drug that showed similar extent of anti-diabetic efficacy was metformin. However metformin may not be very suitable for the management of pancreatogenic diabetes due to
adverse effects such as lactic acidosis observed with the drug. This study provided the final proof-of-concept of our research hypothesis and we propose further development of PP-SSM nanomedicine for management of pancreatogenic diabetes with the goal of improving the quality of life of people suffering from this disease.
VI. FUTURE DIRECTIONS

1. The efficacy of treatment of PD by PP-SSM needs to be tested using different doses of PP. In this research project, the in vivo studies were performed using only one PP dose (200 µg/kg body weight). The dose tested restored insulin sensitivity and improved glucose tolerance to almost the pre-disease level. Therefore higher doses of PP will most likely not provide any better therapeutic effect. Nevertheless, lower doses of PP need to be tested as they may provide a similar extent of anti-diabetic efficacy as observed with 200 µg/kg body weight dose that was tested. A dose-response study will be useful in determining appropriate low dosing regimen of PP-SSM nanomedicine for the management of PD.

2. Apart from dose-response studies, additional animal studies are required to study the pharmacokinetics and biodistribution of PP-SSM. In this research project, we have demonstrated the stability kinetics of PP-SSM in vitro, but it does not exactly simulate in vivo conditions. Determination of the elimination rate constant, half-life of the peptide in plasma, the maximum drug concentration reached in plasma (Cmax), the time to reach maximum drug concentration in plasma (tmax) as well as clearance of the peptide administered with or without SSM will help in determining the percent of drug that reaches the target organ and kinetics of elimination of the nanomedicine from the body. Comparison of these parameters with those obtained for PP in buffer will further demonstrate the effect of SSM on the improvement of PP efficacy due to delivery. The
study can be used to determine the dose and dosing frequency of PP-SSM to obtain maximum therapeutic effect.

3. Toxicity studies of PP-SSM on a rodent and non-rodent animal model will be helpful to fulfill the requirements by FDA for filing for investigational new drug applications. For this purpose, the animals will need to be treated with escalating dose of PP-SSM to determine the maximum allowable dose beyond which toxicity due to peptide and vehicle begins to appear.

4. Apart from toxicity studies, PP-SSM may be tested as a preventive medicine for pancreatic cancer. PP-SSM can be used to effectively overcome hepatic insulin resistance thereby prevent development of hyperinsulinemia (a causal factor for development of cancer). Thus, treatment with PP-SSM may prevent development of cancer in PD patients and this point of view should be further investigated. For the study, the incidence of cancer that occur as a result of long-term PP-SSM therapy can be recorded and compared with the incidence that occur using the current standard of care. This study will help determine whether PP-SSM has a beneficial role in the prevention of pancreatic cancer when used for management of PD.
5. The long term shelf-life stability of lyophilized PP-SSM formulation needs to be tested. That will help determine the appropriate shelf-life of the product when it is being developed for commercial use. For this purpose, the lyophilized PP-SSM formulation can be stored for different periods of time and at different temperatures. At the end of the study, the samples can be reconstituted and their physico-chemical properties can be evaluated and compared to freshly prepared samples. Another aspect that needs to be investigated for commercial development of PP-SSM formulation is scale-up feasibility for large scale production. The physico-chemical properties of PP-SSM prepared in a large batch can be tested and compared with that obtained from PP-SSM prepared in a laboratory scale.

6. The anti-diabetic efficacy of PP-SSM formulation can also be tested in other diseases with PP deficiencies such as in maturity onset diabetes of the young type 1 (MODY1). This disease is a sub-type of type 1 and type 2 diabetes mellitus where impaired glucose tolerance and insulin resistance is observed. Since PP-SSM has been found to improve glucose tolerance and overcome insulin resistance in PD, the nanomedicine may show similar anti-diabetic efficacy in the treatment of MODY1.
CITED LITERATURE


APPENDIX

January 6, 2012

Hayat Onyukel
Biopharmaceutical Sciences
M/C 865

Dear Dr. Onyukel:

The protocol indicated below was reviewed at a convened ACC meeting in accordance with the Animal Care Policies of the University of Illinois at Chicago on 12/20/2011. The protocol was not initiated until final clarifications were reviewed and approved on 1/5/2012. The protocol is approved for a period of 3 years with annual continuation.

Title of Application: A Novel Nanomedicine for Treatment of Pancreatogenic Diabetes

ACC Number: 11-210

Initial Approval Period: 1/5/2012 to 12/20/2012

Current Funding: Currently protocol NOT matched to specific funding source. Modification will need to be submitted prior to Just in time or acceptance of award to match protocol to external funding source. All animal work proposed in the funding application must be covered by an approved protocol. UIC is the only performance site currently approved for this protocol.

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare (OLAW), NIH. This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the funding proposal are matched to this ACC protocol.

In addition, all investigators are responsible for ensuring compliance with all federal and institutional policies and regulations related to use of animals under this protocol and the funding sources listed on this protocol. Please use OLAW’s “What Investigators Need to Know about the Use of Animals” (http://grants.nih.gov/grants/olaw/InvestigatorsNeed2Know.pdf) as a reference guide. Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

Bradley Merrill, PhD
Chair, Animal Care Committee

cc: BRL, ACC File, Amrita Banerjee, Antonina Kuzmis

Office of Animal Care and Institutional Biosafety Committees (MC 673)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227
VITA

NAME Amrita Banerjee


M.S., Pharmaceutics, Birla Institute of Technology Mesra, Ranchi, India, 2007.

B.S., Pharmaceutical Sciences, Birla Institute of Technology Mesra, Ranchi, India, 2005.

PROFESSIONAL EXPERIENCE Research Assistant, Department of Biopharmaceutical Sciences, University of Illinois at Chicago, 2010-2012.

TEACHING Teaching Assistant, Department of Biopharmaceutical Sciences, University of Illinois at Chicago, 2007-2010.

Pharmacokinetics Lecture (Elimination of drugs through urine); Pharm D. course 2010-2012.

HONORS Van Doren Scholarship for Academic Excellence, 2012

University Grants Commission (UGC) Junior Research Fellowship, India, 2005-2007

Gold Medal for academic excellence, 2005

Graduate Aptitude Test in Engineering (GATE) - 92.99 percentile, India, 2005

J.H.Tarapore Medal for excellence in academics, 1999

PROFESSIONAL MEMBERSHIPS American Association of Pharmaceutical Scientists (AAPS)

Controlled Release Society (CRS)


ABSTRACTS

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