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# ABSTRACT

Title of Dissertation: Water Soluble Polymer Drug Therapies for Targeted  
Delivery to Pancreatic Cancer

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Current therapies of advanced staged pancreatic cancer are limited by poor response and high toxicity of chemotherapeutics. As the molecular basis of pancreatic cancer has become better understood the need for a targeted therapy could help provide an increase in therapeutic response while also limiting side effects. N-(2-hydroxypropyl) methacrylamide (HPMA) copolymer drug conjugates have demonstrated their potential use as carriers of small molecule drugs to improve cancer therapies. The overall goal of this research was to develop a polymer peptide drug conjugate based on HPMA copolymers, which can increase the therapeutic index of pancreatic cancer chemotherapy. Previous investigation of the uMUC1 receptor, which is a glycoprotein overexpressed on the surface of pancreatic tumors, has lead to the development of EPPT1, a small peptide has been found to have a strong binding affinity to uMUC1, ( $K_d=20\mu\text{M}$ ). Our hypothesis HPMA copolymer with an active target EPPT1 to the uMUC1 receptor will enhance

therapeutic action of a cancer chemotherapeutic drug such as gemcitabine. In this study, we were successful in the synthesis and characterization of a series of HPMA copolymer-EPPT1-Gemcitabine conjugates. Using model pancreatic cancer cell lines, the binding efficiency, internalization and mechanisms of cellular uptake were evaluated with polymer EPPT1 conjugates. Polymer gemcitabine conjugates were evaluated for efficacy against free gemcitabine. The optimized polymer peptide drug conjugates were evaluated for efficacy and drug release. Results during synthesis and characterization indicated that copolymer yield, solubility and performance were influenced by each incorporation of peptide and drug. Flow cytometry determined that polymer peptide conjugates were able to bind with Capan-2 and Panc-1 cell lines. Confocal microscopy verified that polymer peptide conjugates were not only getting internalized into the cytoplasm but also routing to the lysosome. Using endocytosis inhibitors, confirmed that polymer peptide conjugates use clathrin mediated endocytosis pathways when getting internalized into the cell. Drug release studies revealed that gemcitabine will detached from the polymer in lysosomal conditions. Polymer drug conjugates compared to free gemcitabine alone against pancreatic cancer cells in MTT assay had equal efficacy. Attachment of the active targeting moiety EPPT1, exhibited that polymer peptide drug conjugates were superior in killing cells to free gemcitabine alone.

Water Soluble Polymer Drug Therapies for Targeted Delivery to Pancreatic Cancer

by  
Joseph Dawson Stanton

Dissertation submitted to the faculty of the Graduate School  
of the University of Maryland Baltimore in partial fulfillment  
of the requirements for the degree of  
Doctor of Philosophy  
2014

To my Grandfathers, Joseph Donald Stanton Sr. WWII veteran, and Victor Charles  
Douglas Dawson, PhD.

## ACKNOWLEDGEMENTS

It truly has been an honor to meet and work with talented, inspiring and successful individuals throughout my stay in University of Maryland Baltimore. There are many people to thank as each person has helped me reach this point in my life.

I owe a huge debt of gratitude to **Dr. Peter Swaan**, without him I would not be where I am today. His enthusiastic commitment and supervision urged me to complete this dissertation even in the darkest hour. I am eternally indebted to him for taking me on as a student when I had nowhere to go. His expertise and support saved this project and I cannot thank him enough for the countless hours he spent with me teaching and mentoring. It truly has made me a better scientist.

I would like to offer a warm acknowledgement to the chair of the department of pharmaceutical sciences, and committee member **Dr. Andrew Coop**. His support and motivation has pushed my limits in learning I never knew I had. His interest in my graduate education has never waned since my arrival at UMB and under his careful assistance, my life and graduate research in the PhD program continued without interruption. For this I cannot not thank him enough.

My sincerest thanks to all my committee members, **Dr. Yan Shu**, and **Dr. Anthony Kim**, who both graciously agreed to serve on my committee and for their helpful comments. A special thanks to **Dr. Paul Shapiro** for joining the committee on short notice. Life truly has come full circle. It was Dr. Shapiro who came to Towson University in 2006 and recruited me to join the School of Pharmacy and now almost 8 years later he will be apart of my committee that sees me graduate.

I remain indebted to **Dr. Ahbay Andar** and **Dr. Mark Taraban**. Both have served as “junior” advisors and have had a tremendous impact on teaching me lab techniques and also for help with many experiments throughout this project. I wish them continued success in their young careers. They truly deserve it. I thank all my past and present lab mates for their friendship and untiring help during this project. I would particularly like to thank **Aaron Smith** and **Brittany Avaritt** as the last remaining classmates and now my friends. It was wonderful to work with all these bright individuals and in a vibrant work environment.

I owe a debt of gratitude to all my friends, faculty members, **Dean Natalie Eddington** and **Dr. Angela Wilks**, graduate students, and the staffs of the Department of Pharmaceutical Sciences for their unselfish assistance, advice and support.

Words alone cannot express the thanks I owe to my parents and family. **Joseph Donald Stanton Jr. and Maria Stanton**, I have always been able to rely on you to take care of me. It was both of you that inspired me to continue my education beyond college and for that I am truly thankful. Also a special shout-out to my siblings, **Anglea Grossfeld, Josh Grossfeld, Monica Scheuerman. Chris Scheuerman and Greg Stanton**. I truly have the best family in the world. Finally, I want to thank my beautiful and inspiring girlfriend, **Lindsay**. Her love and continuous support to push me to finish has been such a blessing. I couldn't have made it with you. She is the hardest working person I know and inspired me to work harder. I love you so much and I look forward to our next adventure.

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## List of Abbreviations

<u>ABBREVIATIONS</u>	<u>FULL NAME</u>
5-FU	5-fluorouracil
AIBN	N,N'-azobisisobutyronitrile
Alexa Fluor 488 5-TFP	Alexa Fluor 488 carboxylic acid, 2,3,5,6-tetrafluorophenyl ester
CA-19-9	Carbohydrate antigen 19-9
CDA	Cytidine deaminase
CPB	Bovine spleen cathepsin B
CLIO	Cross linked iron oxide nanoparticle
DCC	N,N'-Dicyclohexylcarbodiimide
DCU	Dicyclohexylurea
DCK	Deoxycytidine kinase
DMEM	Dulbecco's Modified Eagle Medium
DMF	Dimethylformaldehyde
DMSO	Dimethyl sulfoxide
DYN	Dynasore
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ENT1	Equilibrative nucleoside transporter 1
EPR	Enhanced permeability and retention
EPPT1	(YCAREPPTRTFAYWG)
ER	Estrogen receptor

FAC or CAF	Doxorubicin, cyclophosphamide and fluorouracil
FBS	Fetal bovine serum
FIL	Filipin
FITC	Fluorescein isothiocyanate
FPLC	Fast Protein Liquid Chromatography
Gem	Gemcitabine
GFLG	Gly-Phe-Leu-Gly
HBSS	Hank's Balanced Salt Solution
HPMA	<i>N</i> -(2-hydroxypropyl) methacrylamide
HSP90	Heat shock protein 90
IC <sub>50</sub>	Half maximal inhibitory concentrations
mAb	Monoclonal antibody
MAGFLG-OH	Methacryloyl-glycylphenylalanylleucylglycine
MAGGONp	Methacryloylglycylglycyl-paranitro phenyl ester;
MDC	Monodansyl cadaverine
MDR	Multidrug resistance
M <sub>n</sub>	Number-average molecular weight
MPA	3-mercaptopropionic acid
MRP	Multidrug resistance-associated protein
MTT	3-(4,5-dimethyl-2-tetrazolyl)-2,5-diphenyltetrazolium bromide
M <sub>w</sub>	Weight-average molecular weight
MUC1	Mucin 1
NO	Nitric oxide

$N_p$	Average number of polymer chains
ONp	Paranitro phenyl group
PAMAM	Poly (amidoamine)
PARP	Poly (adenosine diphosphate-ribose) polymerase
PBS	Phosphate buffered saline
PEG	Polyethyleneglycol
PFS	Progression free survival
PGA	Polyglutamic acid
P-gp	P-glycoprotein
Pt	Cisplatin
PVDF	Polyvinylidene difluoride
RAF	Receptor activation factor
RES	Reticuloendothelial system
RFS	Relapse free survival
RR	Responsive rate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size-exclusion chromatography
TBS-T	Tris-Buffered Saline with Tween
TEA	Triethylamine
uMUC1	Underglycosylated Mucin 1

## **Chapter 1. Introduction**

### **1.1 Introduction**

Pancreatic cancer is the 4<sup>th</sup> leading cause of cancer-related death in the United States <sup>1</sup>. New disease cases for 2014 were projected to be about 46,000 cases with a probable 39,000 patients dying from the disease<sup>1</sup>. The overall 5 year survival rate among patients with pancreatic cancer is <5%<sup>1</sup>. Pancreatic cancers cause is yet to be determined however, several environmental factors and genetic mutations have been implicated. Approximately 5 to 10% of patients have a family history of the disease<sup>1</sup>. The main types of treatment for pancreatic cancer are surgery, radiation and chemotherapy<sup>2</sup>. Surgical removal is often deemed too difficult due to the location of the pancreas and will only be used if the cancer is localized. Radiation therapy is often used in combination with surgical removal and/or chemotherapy <sup>2,3</sup>.

The chemotherapeutic Gemzar ® (gemcitabine HCl), is a nucleoside analogue that exhibits antitumor activity and has been extensively reviewed for antitumor efficacy<sup>4</sup>. Its mechanism of action is to inhibit DNA synthesis of cells in the G1/S-phase boundary of the cell cycle<sup>4</sup>. Although our focus for this drug is primarily for pancreatic cancer,

gemcitabine is used as a chemotherapy for several other cancers as well<sup>4</sup>. Gemcitabine (Gem) is considered to be the frontline therapy for pancreatic cancer<sup>4</sup>. In 2010 clinical trials i.e. gem was compared against the previous frontier therapy for pancreatic cancer, 5 fluoruracil (5-FU). Patients treated with gem showed statistically significant increases in clinical benefit response which involves survival time and time to disease progression<sup>4</sup>. However, clinical investigations with the drug have shown that long-term treatment may lead to several adverse side effects and toxicities<sup>4</sup>. Gem treatment is associated with non-specific toxicity such as suppression of bone marrow function as manifested by leucopenia, thrombocytopenia and anemia. Pulmonary toxicities and renal failure have also been reported<sup>5</sup>. To effectively reduce gemcitabine's toxicity profile, the rationale of this project is to use a novel drug delivery system made of HPMA copolymer backbone containing gem covalently attached to the polymer's side-chains. This system could enhance efficacy and reduce non-specific toxicity by further attaching an active targeting moiety.

HPMA copolymers are widely used as drug carriers<sup>6-15</sup>. Favorable properties of HPMA polymers are: they are highly water soluble, non-immunogenic, and are amenable to large-scale production. HPMA copolymers have been employed as drug carriers to modify in vivo biodistribution of several chemotherapeutic agents and enzymes.<sup>16-22</sup> They are not concentrated by any body tissue and are eliminated primarily through the kidneys. HPMA copolymers have also been proven to be safe and effective in cancer chemotherapies. HPMA copolymers can be tailor-made with simple chemical modifications to regulate drug and targeting moiety content for optimum biorecognition, internalization or subcellular trafficking depending on specific therapeutic needs<sup>13, 23-27</sup>.

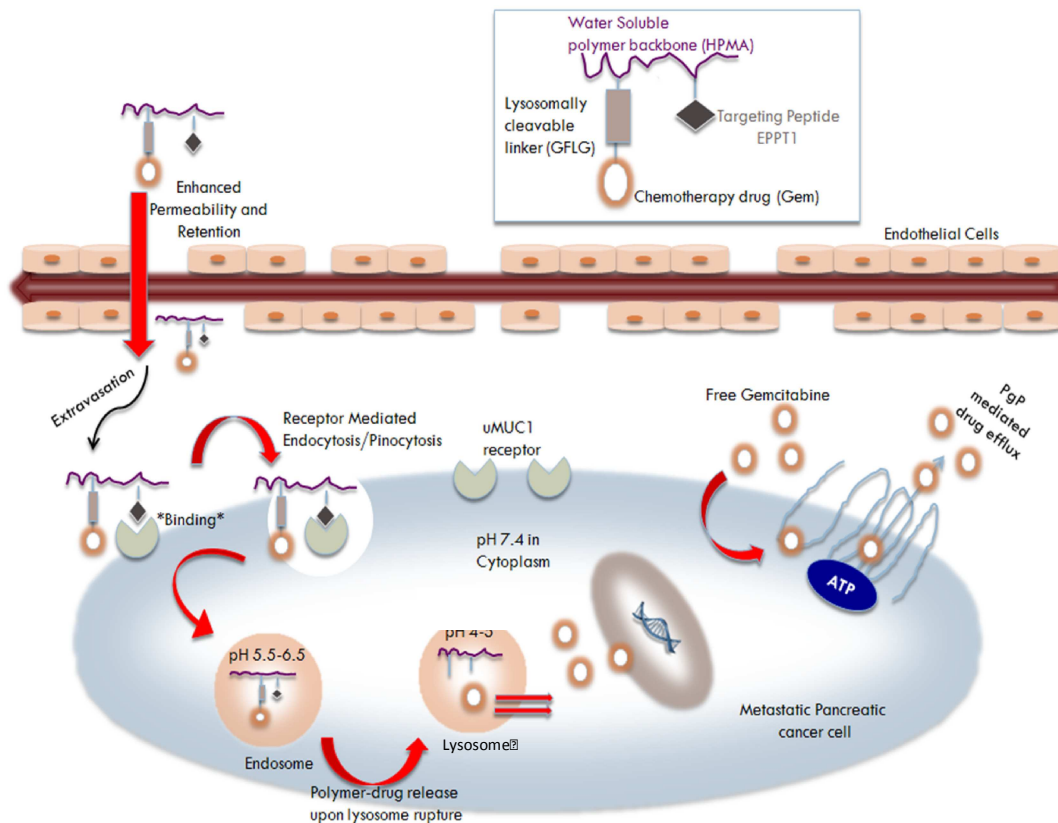
Gem will be covalently linked to the HPMA backbone via a glycine-phenylalanine-leucine-glycine (GFLG) side chain. This degradable peptide linker is used because it is stable in circulation but cleaved by lysosomal proteases such as cathepsin B once internalized by endocytosis. This linker is essential for polymeric drug delivery because the drug remains inactive while bound to the linker but once cleaved while in the lysosome it becomes active. Moreover, by linking the drug to the polymer, its route of entry into cells is limited to endocytosis, which potentially can bypass mechanisms of drug resistance such as efflux pumps<sup>17, 21, 28</sup>.

HPMA copolymer based drug delivery systems utilize the enhanced permeability and retention effect (EPR) taking advantage of the physiological disturbances caused due to cancer. The most significant physiological characteristic of tumor tissue is its leaky vasculature, which in this case allows drugs to passively localize near the tumor. Tumors are also associated with a decreased lymphatic drainage, which allows macromolecular drug delivery systems to be retained for a longer time<sup>29, 30</sup>. However, heterogeneity of clinical presenting cancers makes it difficult to achieve uniform drug distribution at the tumor site. Also the EPR effect is very tumor specific and provides only a passive form of targeting; thus, a targeting moiety is necessary to further increase the amount of drug specifically endocytosed by the cancer cells<sup>30</sup>.

Active targeting is the key to utilizing the HPMA polymeric drug delivery system. Mucin 1 (MUC1) is an ideal candidate for targeting because of its large over expressed mucin granules that are exposed on the apical surface of almost all human epithelial cell adenocarcinomas including pancreatic cancer<sup>31-34</sup>. The mucin molecule has been found to extend about 100-200nm above the surface, which is 5-10 fold higher than the length of

most membrane molecules. In normal tissue, MUC1 is heavily glycosylated however, in neoplastic tissue MUC1 is underglycosylated (uMUC1). This reduced glycosylation permits access to the peptide core and reveals epitopes that are normally masked. The extracellular domain of uMUC1 is defined by the presence of the amino acid sequence PDTRP, which is recognized by many targeting moieties such as antibodies and peptides. A small peptide EPPT1 (YCAREPPTRTFAYWG) is the focus for our project because it can overcome the shortcomings of the monoclonal antibodies, which include immunogenic response and long plasma half-life. EPPT1 is derived from the CDR3 antibody and has a beta strand type conformation as its active binding site. Analysis of the peptide had shown that it has a significant affinity for the uMUC1 derived peptide PDTRP with a  $K_d = 20\mu\text{M}$  making it an ideal targeting moiety for our polymer delivery system<sup>31-34</sup>.

The rationale of the proposed research is represented schematically in Figure 1 An HPMA copolymer-EPPT1-(GFLG)-Gem conjugate has been preliminarily developed as a novel drug delivery system for targeting to pancreatic cancer. The **overall hypothesis** is that when delivered systemically this novel polymer-peptide-drug conjugate will utilize the enhanced permeability and retention effect, to preferentially accumulate at the tumor site. The presence of the targeting moiety will further permit the polymer to actively target the uMUC1 receptor on pancreatic cancer cells. This will ensure that the anticancer drug Gem will be more effectively delivered by endocytosis therefore increasing its efficacy while also reducing toxicity (by minimizing accumulation at non-target sites).



**Figure 1: Project Rationale**

Scheme depicting the rationale for targeted drug delivery to pancreatic cancer using HPMA copolymer-drug conjugates. Upon systemic administration, polymer conjugates will utilize the EPR effect and be able to target the uMUC1 receptor. Upon binding to the cancer cells, the polymer conjugate will be internalized by receptor-mediated endocytosis and traffic to the lysosomal compartment. In the lysosome, proteases (e.g. Cathepsin B) will cleave the tetrapeptide (GFLG) to allow controlled release the drug. The released drug will subsequently escape the lysosomes and reach its nuclear target, causing a stop to DNA synthesis and eventual death. Since polymer-drug conjugates are specifically delivered by endocytosis, this process will bypass resistant machineries in cancer cells such as p-glycoprotein (PgP) mediated efflux pumps.

## 1.2 Specific aims

The overall aim of this dissertation was to investigate the utility of HPMA copolymer tethered to EPPT1 peptide for targeted delivery of gemcitabine to pancreatic cancer cells.

The specific Aims of this research are as follows:

1. To synthesize and characterize the monomer building blocks and HPMA copolymer conjugates for targeted delivery to pancreatic cancer cell lines.
2. Systemically evaluate polymer peptide conjugates for targeted delivery and polymer drug conjugates for cytotoxicity to pancreatic cancer cell lines. HPMA copolymer EPPT1 conjugates will be evaluated *in vitro* for binding and internalization. HPMA copolymer Gem conjugates will be evaluated *in vitro* for efficacy.
3. From the evaluation of optimized polymer peptide conjugates and polymer drug conjugates in aim 2, synthesis of polymer peptide drug conjugates for *in vitro* binding, internalization, drug release and efficacy will be completed. HPMA-EPPT1-Gem conjugates will also be tested for drug release and efficacy.

These specific aims were formulated to test the following hypotheses: Through means of free-radical polymerization HPMA copolymer peptide drug conjugates containing an optimized amount of the stable EPPT1 peptide and gemcitabine can be synthesized.

Polymer conjugates with optimum EPPT1 content will demonstrate comparable or enhanced activities namely binding to the uMUC1 receptor, cellular uptake by uMUC1 overexpressing cancer cells and improved or equal anticancer efficacy, when compared to free drug alone or non targeted conjugates.

Polymer conjugates with active targeting and drug will demonstration enhanced therapeutic efficacy in pancreatic cancer cell lines compared to free drug. Release of gem from the polymer will occur in lysosomal conditions.

### **1.3 Scope and Organization**

Chapter 2 of this dissertation presents a mini-review of the literature on pancreatic cancer and the approaches for treatment of pancreatic cancer. In this chapter, the development of gemcitabine as an anticancer agent and its involvement as chemotherapy for several cancers but specifically pancreatic cancer are presented. Additionally, the role of MUC1 is explored as an ideal target for drug delivery vehicles, specifically when using the novel peptide EPPT1. The function of polymer conjugates, particularly HPMA copolymers are examined as drug delivery vehicles.

Chapter 3 describes the synthesis and characterization of the comonomer building blocks, which make up different HPMA copolymer conjugates. *In vitro* evaluations of HPMA copolymers are done to look at the binding, internalization and cytotoxicity of different conjugates. This chapter will serve as a preliminary platform for the development of the final HPMA-EPPT1-(GFLG)-Gem copolymer. This chapter will cover the development of several “control” copolymer conjugates that have different amounts of EPPT1 and Gem added to the backbone but never both together.

In chapter 4 the synthesis and characterization of 4 conjugates are explored for further evaluation to determine the optimal candidate for future studies. HPMA-EPPT1 conjugates are investigated to determine the subcellular fate and to observe the mechanism of which endocytosis pathway the conjugate will windup. HPMA-(GFLG)-Gem conjugates will be incubated with and without EPPT1 peptide in lysosomal

conditions to monitor drug release. Finally, HPMA-EPPT1-(GFLG)-Gem conjugates are evaluated for cytotoxicity to compare against free gemcitabine.

In the 5<sup>th</sup> chapter we summarize the finding of the chapters and propose future studies for this project.

## **Chapter 2. Background**

### **2.1 Introduction**

Recently, there has been a large emphasis and advancement of understanding the molecular biology of pancreatic cancer as well as its diagnosis, staging and treatment in patients with early stage tumors. However, insignificant progress has been in the early diagnosis, prevention and treatments for patients with advanced disease<sup>1</sup>. Metastatic pancreatic cancer is a terminal disease because effective clinical treatments are currently not available. While new drug treatments show potential in preclinical models, pancreatic cancer deaths are still on the rise. In general, this has been attributed to several factors: i) clinical response is not correlated typically with preclinical model, ii) chemotherapeutics are dose-limited causing major side effects and lack specificity to their tumor target, iii) limitations on cellular entry and intracellular delivery, iv) tumor development of resistances to therapies. Although the development and design of a variety of novel drug delivery systems show some promise in other cancers<sup>35</sup>, the need for a novel delivery system that can overcome pancreatic cancer's clinical challenges is still not around. Development of an effective drug delivery system for pancreatic cancer treatment will be discussed.

## **2.2 Pancreas**

The pancreas is an organ deep within the body, residing behind the stomach. It stretches across behind the stomach horizontally and is connected to the duodenum via the pancreatic duct. It is a dual function glandular organ in the digestive and endocrine system<sup>36</sup>. Within those systems there are two different types of glands, exocrine and endocrine. The exocrine gland assists in the digestive system from cells secreting pancreatic fluid that contains digestive enzymes, which break down fats and proteins in our diet<sup>36</sup>. The duct drains the fluid directly into duodenum. A small number of cells in the pancreas are endocrine. These  $\beta$  cell clusters are called islets of Langerhans. This function of the pancreas produces important glucose regulating hormones like insulin that help balance the amount of sugar in the blood.

## **2.3 Pancreatic Cancer**

Pancreatic cancer is an adenocarcinoma of the pancreas which predominately involves a mutation in the ductal epithelium cells and evolves from pre malignant lesions to fully invasive adenocarcinoma<sup>37</sup>. The cause of pancreatic cancer remains unknown, although there are some indications that several environmental factors may be implicated, along with hormonal factors, diet and genetics. K-Ras gene mutations have been found in over 90% of pancreatic carcinomas, but it is unknown whether this occurs before the formation of the actual cancer<sup>37</sup>. It also is believe that family history may also have an indirect influence<sup>1, 37</sup>. This disease continues to be a major health problem in the United States which, in 2014 roughly 46,000 people (24,000 men and 22,000 women) will be diagnosed with pancreatic cancer<sup>2, 37, 38</sup>. In addition to this high prevalence that has been

slowly increasing over the past 10 years (in 2008 34,000 people diagnosed) is pancreatic cancer is associated with high rates of mortality<sup>38</sup>. An estimated 40,000 deaths in 2014 make this disease one of the most deadly cancers<sup>38</sup>. The overall 5 year survival rate among patients with pancreatic cancer is >5%, making this the 4<sup>th</sup> leading cause of cancer related deaths<sup>38</sup>. Incidence of pancreatic cancer is related to age, most commonly found in the elderly, and less than 20% of patients present a localized, potentially curable tumor<sup>1, 37, 38</sup>.

### **2.3.1 Pancreatic Cancer detection and diagnosis.**

Having a reliable screening practice for the assessment of cancers has been controversial because current supportive evidence does not exist<sup>39</sup>. Death rates for other cancers such as, prostate, breast and colorectal cancers have been slowly declining over the past decade<sup>3, 39</sup>. Widespread improvement in screening for these cancers has been attributed to this decline<sup>3, 39</sup>. However, with all these screening practices getting better it seems to have minimal effect for pancreatic cancer. Techniques used for pancreatic cancer have been limited in abilities to identify, stage and locate tumors<sup>39</sup>. The ideal goal for screening of pancreatic cancer is to detect the disease at an early phase, (pre invasive or early invasive)<sup>3</sup>. Unfortunately, the critical factor of the deadly nature of pancreatic cancer is due to its relatively quiet signs and symptoms<sup>3, 39</sup>. Obstructive jaundice, abdominal pain and weight loss are among the common indications of patients with pancreatic cancer. Despite advancements for other cancers, today the most common tools used are biopsy, CT scans, magnetic resonance imaging (MRI) and endoscopic ultrasound (EUS), no ideal single test exist<sup>1, 3, 39</sup>. These imaging studies are used for the diagnoses of pancreatic cancer once the tumor is larger enough to cause symptoms<sup>3</sup>. Most

patients would benefit from an improved screening and diagnosis but unfortunately, it is very difficult to find pancreatic cancer early<sup>1, 3, 37</sup>. Although there are many potential serum biomarkers for diagnosis, an elevated level of carbohydrate antigen 19-9 (CA19-9) is the only one that has demonstrated clinical usefulness<sup>1, 39</sup>. However, use of CA 19-9 as a biomarker as a screening tool is generally not accepted because of its low sensitivity and specificity<sup>39</sup>. The signs and symptoms are not only relatively everyday problems but, the pancreas is located deep within the body so doctors cannot see or feel tumors during routine physical exam<sup>39</sup>. They must rely 100% on diagnostic/imaging tools.

### **2.3.2 Types of pancreatic cancer**

Pancreatic tumors are classified similar to that of cancers arising from other organs. Classification is done according to cell type origin, structure and behavior<sup>40-42</sup>. As described earlier, the pancreas has two glands, exocrine and endocrine, both of which have different roles in digestion and production of glucose and insulin<sup>40-42</sup>. Nearly 90% of all pancreatic cancers are exocrine in that they originate from the epithelial cells line forming gland-like structures on the pancreatic duct<sup>40-42</sup>. The second most common type of exocrine pancreatic cancer is mucinous tumors, which account for <10% of all tumors<sup>40, 43</sup>. This type of tumor forms sac-like structures and secrete mucin<sup>43</sup>. They form from the pancreatic ductal epithelium and are typically less invasive than other types of exocrine pancreatic cancers<sup>40, 43</sup>. Far less common cancers arise from islet cells in the endocrine duct which only account for <5% of all pancreatic cancers. These types of tumors include insulinomas and gastrinomas<sup>40, 44</sup>. However, rather than preventing endocrine functions, they are often detected due to symptoms caused by excessive

hormone levels because the cancerous cells start producing hormones at a high levels<sup>40</sup>,

45

### **2.3.3 Pancreatic cancer metastasis/staging**

The advancement of pancreatic cancer has been suggested to be the result of a successive accumulation of gene mutations in which the cancer begins formation in the ductal epithelium and progresses from premalignant lesion to fully invasive cancer<sup>46</sup>. In pancreatic cancer, metastasis or spreading of the cancer beyond its point of origin is similar to that of other cancers, however pancreatic cancer seems to be a less vascularized tumor<sup>47</sup>. Data suggests that the mechanism of invasion and metastasis of pancreatic cancer as a continuous process by which, a reduced cell-cell adhesion, alteration in the interactions of the tumor cell with the extra cellular matrix, and an invasion into surrounding tissues including the blood stream and lymph duct<sup>48, 49</sup>. The E-cadherin-catenin complex (part of the extra cellular matrix or ECM) which plays a role in the mediating of homotypic cell-cell adhesion and controls epithelial cells architecture and differentiation is present in almost all epithelial cells<sup>50-53</sup>. It is recognized as part of the metastasis process<sup>50-53</sup>. An abnormal structure and dysfunction of any of the molecules in E-cadherin-catenin complex has shown to correlate with an increase of invasion and metastatic ability in which cells will spread to adjacent microenvironment, which includes bordering organs, blood vessels and lymph nodes<sup>47, 54-57</sup>. Studies and years of research have determined that metastasis occurs mainly in liver and peritoneal cavity<sup>1</sup>.

The staging of pancreatic cancer is based on most recent edition of the American Joint Committee on Cancer (AJCC) tumor-node-metastasis classification. From this table the assessment of resectability of a tumor is determined. T1, T2 and T3 tumors are

potentially resectable where as T4 tumors are nonresectable. T4 tumor involvement with the superior mesenteric artery and celiac arteries, both of which carry large amounts of blood and cannot be disrupted. Table 1 denotes the actual staging of pancreatic cancer: Resectable; meaning the cancer is confined to the pancreas and all of it can be removed. Locally advanced (unresectable); The tumor has spread to tissue and blood

Staging of Pancreatic Cancer					
Stage	Tumor Grade	Nodal Status	Distant Metastases	Median Survival mo	Charatceristics
IA	T1	N0	M0	24.1	Tumor limited to pancreas, ≤2cm in longest dimension
IB	T2	N0	M0	20.6	Tumor limited to pancreas, >2cm in longest dimension
IIA	T3	N0	M0	15.4	Tumor extends beyond the pancreas but does not involve the celiac axis or superior mesenteric artery
IIB	T1, T2, or T3	N1	M0	12.7	Regional lymph-node metastasis
III	T4	N0 or N1	M0	10.6	Tumor involves the celiac axis or the superior mesenteric artery (unresectable disease)
IV	T1, T2, T3, T4	N0 or N1	M1	4.5	Distant metastasis

**Table 1: Staging of Pancreatic Cancer**

'N' denotes regional lymph nodes, 'M' distant metastases, and 'T' for primary tumors. Data are Hildago et al. [1].

vessels around the pancreas but not to other distant organs and metastatic; tumor has spread to distant organs.

## **2.4 Treatments for pancreatic cancer**

There are several treatment options available for patients with pancreatic cancer some of which will not be explored in this paper. Most experts would agree that patients with pancreatic cancer get the best treatment opinions when multidisciplinary teams are involved<sup>1</sup>. This would include, surgeons, medical and radiation oncologists, radiologists, gastroenterologist, nutritionist and pain specialist<sup>1</sup>. As discussed earlier, there are two types of pancreatic cancer, exocrine and endocrine. While only 2-4% of patients have endocrine pancreatic cancer, standard treatments for these patients include surgery, chemotherapy and hormone therapy<sup>58</sup>. Prognosis for the type of cancer is strictly dependent of the type of islet cell cancer and the median survival time is between 2-3 years<sup>58, 59</sup>. Pancreatic cancer originates in the exocrine for about 95% of patients<sup>58</sup>. Due to metastasis into adjacent or distant organs, more than 80% of these carcinomas are unresectable<sup>60</sup>. This means that only 20% of pancreatic cancers are detected early enough for removal. These patients would be eligible for a pancreaticoduodenectomy (surgical removal of the pancreatic head, duodenum, gallbladder and bile duct), with or with the gastric antrum, this is also known as The Whipple operation<sup>61</sup>. However, even after surgical removal of the tumor, the recurrence rate of the tumor is high<sup>62</sup>. Chemoradiotherapy followed by chemotherapy is considered to be the best treatment for postoperative adjuvant therapy<sup>62</sup>. Clinical trials were done and shown that with over 1,600 patients treatments with 5-fluorouracil (5-Fu) or the gemcitabine (gem) with

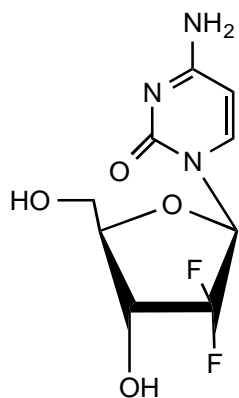
radiation prolonged the overall survival interval of a 2 year survival rate from less than 20% to 30-40%<sup>63</sup>. Therapies for locally advanced pancreatic cancer would include tumors that have locally spread or have encased critical vascular structures and therefore cannot be surgically removed. Due to inconsistent results of clinical trials, the common practice is to use 5-fu based chemoradation followed by gem or 5-Fu based chemotherapies<sup>64</sup>. Symptomatic relief and prolonging survival is the goal of systemic chemotherapy. For patients with metastatic advanced disease, tumors tend to be highly chemoresistant; response rates to multiple classes or agents (i.e., antimetabolites, alkylating agents, antibiotics and anthracyclines), used as a single agent and in combination therapy are less than 20%<sup>58</sup>. Recently, gemcitabine has replaced 5-Fu as the standard treatment for metastatic pancreatic cancer. In other recent studies, gem has been combined with many different types of chemotherapeutic agents ie., antimetabolite(fluorouracil, pemetrexed), topoisomerase I inhibitors (irinotecan, exatecan), platinumums (cisplatin, oxaliplatin), and taxanes (paclitaxel, docetaxel)<sup>65</sup>. However, despite the recent advances, metastatic pancreatic cancer remains to have a dreary prognosis with median survival of less than 12 months. This lack of effective treatment remains to be one of the greatest challenges in clinical oncology.

#### 2.4.1 Gemcitabine

Gemzar® (gemcitabine HCl) (Gem) is a nucleoside analogue that exhibits anti-tumor activity and is marketed by Eli Lilly and Company©<sup>4</sup> (Figure 2). The first synthesis of this compound was done in the early 1980s by Larry Hertel<sup>66</sup>. Its original purpose was to be used as an antiviral drug but it proved to be effective against leukemia cells *in vitro*<sup>66</sup>. The empirical formula for gemcitabine HCl is C<sub>9</sub>H<sub>11</sub>F<sub>2</sub>N<sub>3</sub>O<sub>4</sub> • HCl. It has a



Gemcitabine



**Figure 2: Structure of Gemcitabine**

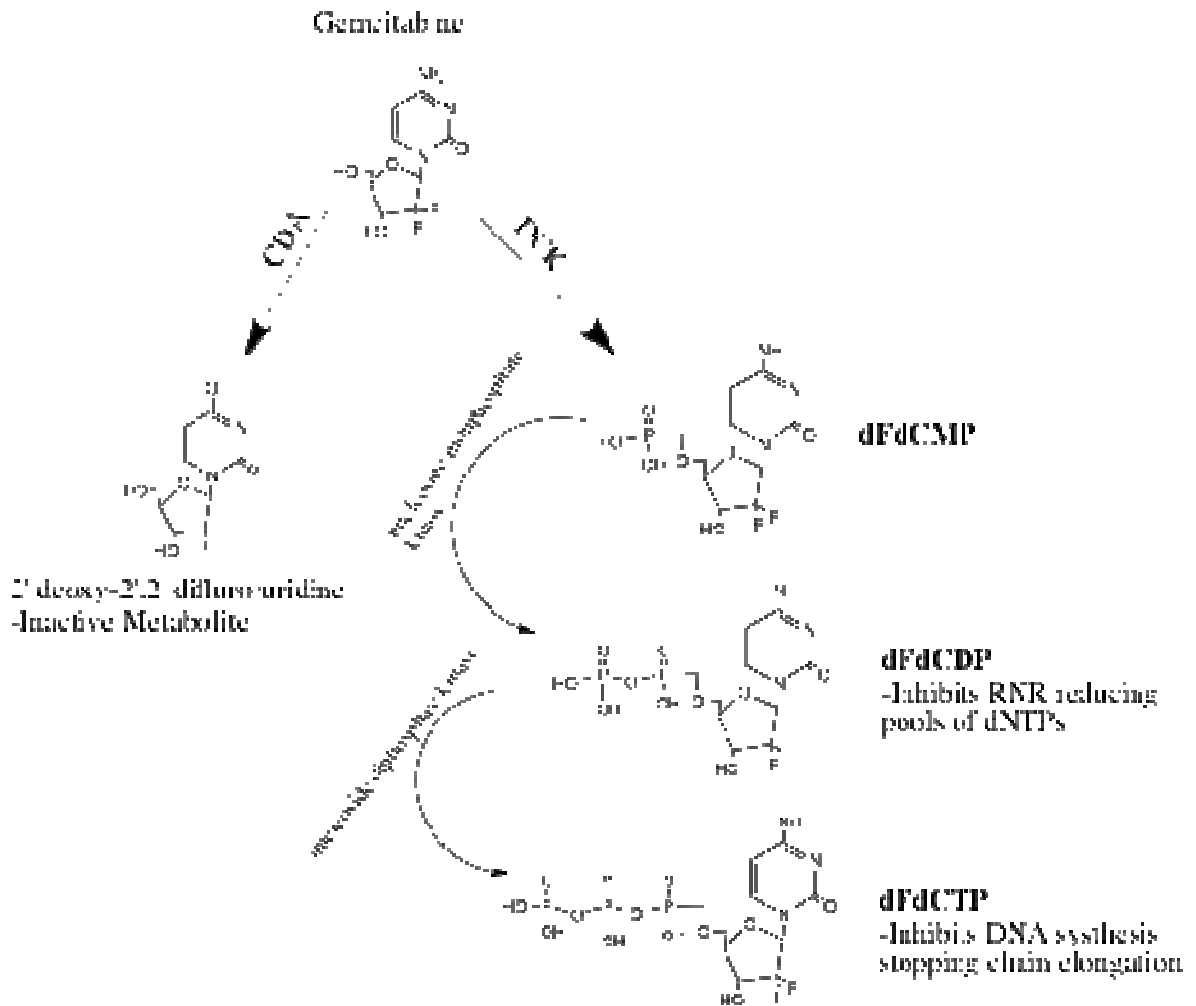
molecular weight of 299.66<sup>4</sup>. Gemcitabine HCl a white powder is soluble in water, marginally soluble in methanol and is almost completely insoluble in ethanol and other polar solvents<sup>4</sup>. Supplied in a sterile form, the clinical formulation is for intravenous use only<sup>4</sup>. Gem is used in a variety of clinical assays as a lone agent or in combination with other drugs. Positive reports of its use in treatment for patients with non-small cell lung cancer, breast cancer, pancreatic cancer and ovarian cancer prompted gem to be approved by the Food and Drug administration (FDA) and European Agency for the evaluation of Medicinal Products (EMA) in July of 2006<sup>67, 68</sup>. Gem is considered a pro-drug because of the fact that it must be converted to a different compound in order to exert its effect<sup>67</sup>. Gem's pharmacokinetics are linear and described in a 2 compartment model<sup>4, 69, 70</sup>. It was shown that volume of distribution of gemcitabine was significantly influenced by the duration of its infusion and gender<sup>4, 69, 70</sup>. The half-life for gemcitabine is relatively short. During short term infusions the clearance was measured to be 32-94 minutes<sup>4, 69, 70</sup>. However during longer infusions the clearance varied from 245-638 minutes<sup>4, 69, 70</sup>. Dosages of Gem are varied between 500-3600 mg/m<sup>2</sup><sup>4</sup>. Due to its rapid body clearance, efficacy has shown to be limited, due to kidney excretion and metabolism by certain enzymes. Gem is easily phosphorylated into an inactive metabolite 2', 2'-difluorodeoxyuridine (dFdU)<sup>4, 69, 70</sup>. Thus longer infusion at high doses is required which causes significant side effects<sup>4, 69, 70</sup>. Unfortunately, infusion times longer than 60mins and more than once a week treatment have shown very adverse reactions in patients<sup>4</sup>. In hematology, suppression of bone marrow function can spur leukopenia, thrombocytopenia, and anemia<sup>4</sup>. Gem has also shown pulmonary toxicity along with renal failure, hemolytic uremic syndrome, potentially leading to death or requiring

dialysis<sup>4</sup>. Although it is rare, serious hepatotoxicity including liver failure and death have also been reported<sup>4</sup>. Generally, a medical professional that will use gem as a cancer chemotherapy will have to monitor patients very closely who receive gem<sup>4</sup>.

#### ***2.4.1.1 Gemcitabine mechanism of action***

As mentioned before, Gemcitabine (Gem) is a nucleoside analog and prodrug, which needs to be phosphorylated in order to exert its mechanism of action<sup>4, 71-73</sup> (Figure 3). The chemical structure of gem is very similar to that of deoxycytidine triphosphate (dCTP) except that it has two fluorines at the 2' position<sup>4, 71-73</sup>. When Gem is brought into a cell (whether it is cancerous or not) it is facilitated by 5 different nucleoside transporters, however the main transporter is equilibrative nucleoside transporter 1 (ENT1)<sup>4, 71-73</sup>. When Gem is phosphorylated and subsequently phosphorylated two more times, it can exert its cytotoxic effect by destroying cells that are undergoing DNA synthesis<sup>4, 71-73</sup>. During the cell cycle DNA replication occurs during the S (synthesis) phase, which is where gemcitabine exerts its cytotoxic action. Gem is phosphorylated by deoxycytidine kinase (DCK)<sup>4, 71-73</sup>. Gem once phosphorylated, becomes difluoro deoxycytidine monophosphate (dFdCMP), diphosphate (dFdCDP), or triphosphate (dFdCTP)<sup>4, 71-73</sup>. There are several sites of action in cells by which dFdC's work to stop DNA synthesis: DNA polymerases are proposed as the major target<sup>4, 71-73</sup>. dFdCTP is a weak inhibitor of dCTP for direction inhibition of DNA polymerase<sup>4, 71-73</sup>. The incorporation of dFdCMP into the C sites of DNA strands causes a major pause in polymerization and, once incorporated, dFdCMP stops the editing function at the 3'-5' exonuclease<sup>4, 71-73</sup>. dFdCDP inhibits ribonucleotide reductase and cause a substantial decrease in DNA dNTPs synthesis<sup>4, 71-73</sup>. This reduction in dCTPs would then also increase the potential for

incorporation of dFdCTP into the DNA<sup>4, 71-73</sup>. Finally reduction of the dCTP may release deoxycytidine kinase from feedback inhibition by dCTP and therefore increase the dFdc's phosphorylation process<sup>4, 71-73</sup>. Prevention of the growing DNA chain induces internucleosomal DNA fragmentation, which is a characteristic of apoptosis<sup>4, 71-73</sup>. 90% of Gem is converted by cytidine deaminase (CDA) to an inactive metabolite, which gets excreted out of the cell<sup>4, 71-73</sup>.



**Figure 3: Gemcitabine's mechanism of action**

CDA= Cytidine deaminase, DCK= deoxycytidine kinase, **dFdCMP**= difluoro deoxycytidine monophosphate, **dFdCDP**= difluoro deoxycytidine diphosphate, **dFdCTP**= difluoro deoxycytidine triphosphate

### ***2.4.1.2 Gemcitabine clinical trials for pancreatic cancer***

Gemcitabine (Gem) is considered a first line treatment for cancer patients with a locally advanced (nonresectable Stage II or Stage III) or metastatic (Stage IV) adenocarcinoma of the pancreas<sup>4</sup>. However, it also can be used in combination with cisplatin and 5-Fu<sup>4,69</sup>. In 2 original clinical trials, data was evaluated in patients who were treated with gem that had locally advanced or metastatic pancreatic cancer. 5-Fu was compared to gem in patients who had no prior chemotherapy treatments<sup>4</sup>. In the second trial, gem was studied in pancreatic cancer patients who already were on a 5-fu treatment or had previous treatments<sup>4</sup>. In both studies, the primary efficacy parameter measured was “clinical benefit response,” a measure of clinical improvement which involves parameters of analgesic consumption, pain intensity, performance status and weight change. To be considered a clinically benefited responder, patients must be able to:

Patient shows a  $\geq 50\%$  reduction in pain or consumption of pain medication for a period of at least 4 weeks.

OR:

Patient is stable on all of the parameters listed and shows a marked weight gain ( $\geq 7\%$  increase maintained for up to 4 weeks) not due to fluid accumulation.

In summary, the first clinical trial where gem was compared to 5-Fu with patients who had no prior chemotherapy treatment, 22% of patients had a clinical benefit response when treated with gem, as opposed to 5-Fu, which only had 4.8% of patients with a clinical benefit response<sup>4</sup>. In the second study in which they studied gem treatment on

patients who had already had prior chemo treatments found that 27% of the patient taking gem showed a clinical benefit response with a median survival of only 3.9 months<sup>4</sup>.

#### **2.4.1.3 Gemcitabine resistances**

Recent results have attributed cellular resistance to gemcitabine treatment as an intrinsic resistance. However it is always been believed to be developed during gem treatments<sup>74</sup>. *In vitro* studies have shown that limited intracellular uptake of gem can be credited to a decrease in hENT1 expression<sup>75</sup>. In a clinical study, with 102 patients who were treated with gem and had an unresectable pancreatic adenocarcinoma, transcription analysis of their genes confirmed hENT1 levels had a significant effect on median survival<sup>76</sup>. Patients whose hENT1 levels that were measured to be higher were found to have a significantly longer median survival compared to patients with lower hENT1 levels<sup>76</sup>. Immunohistochemistry for hENT1 has been advocated as a potential predictive tool for selecting patients who may benefit most from gem treatments<sup>77</sup>. Deficiency in DCK activity has also been rooted as a possible resistance pathway<sup>75, 78</sup>. Although clinically it is unclear, overexpression of RR is an important part of the mechanism of gem resistance since RR is a responsible for the conversion of ribonucleoside diphosphates to deoxyribonucleoside diphosphates, which are essential for DNA synthesis and repair<sup>79</sup>. In more recent study, hENT1, DCK, and RR subunits M1 and M2 (RRM1, RRM2) were shown to be associated with gem resistance and that the expression of several other genes played a significant role in gem sensitivity on eight different cell lines<sup>79</sup>. Another potential key player in gem resistance is the HGMA1 protein complex. Composed of transcription factors that regulate the transcription of a number of genes promoting tumor progression, it has been reported to be overexpressed in patients with pancreatic cancer<sup>80-</sup>

<sup>82</sup>. This over expression of HGMA1 is associated with increased resistance to apoptosis and increased expression of growth factors and cytokines<sup>80-82</sup>. However, gem resistance is still under heavy review and seems to have multiple influences on its resistance pathway.

## **2.5 Mucin 1-membrane cell surface protein**

The mucin 1 membrane cell surface receptor protein (MUC1) was first identified as a molecule recognized by mouse monoclonal antibodies directed at malignant mammary epithelial cells<sup>83</sup>. Mucins are high molecular weight glycoproteins that are heavily glycosylated<sup>84</sup>. Side chains of many oligosaccharides also make up mucins in which they link to a protein backbone called apomucin<sup>85</sup>. At least 21 mucins genes have been identified and divided into two groups: Those that encode for membranous mucins and those that secrete mucins. While membrane-associated mucins are involved in cell-cell interactions, secreting mucins are glycoproteins that are considered to be the major component of mucus<sup>86</sup>. Almost all epithelial cells have mucins on the surface because of its involvement of protection and lubrication. Although as mentioned there are many mucins, MUC1 has been the most researched. MUC1 is large, rigid, negatively charged and heavily glycosylated structure extruding from the apical surface of many types of cellular membranes<sup>87</sup>. The heavy glycosylation of MUC1 is due to the fact that 50-90% of its molar mass is made up of O-linked carbohydrates<sup>83</sup>. Different types of epithelia cells and in different physiologic states, heterogeneously express MUC1, meaning that the glycosylation quantities can range for 50% in normal mammary gland to 80% in cancer cells<sup>88</sup>. The glycoprotein MUC1 is a complex molecule, within its core, a large domain of variable number tandem repeats represents the extracellular protein<sup>86</sup>. This extracellular domain has been found to be made up 20 amino acid tandem repeats, each

of which have 5 potential O-glycosylation sites<sup>89</sup>. Those O-glycosylation sites are flanked by degenerate tandem repeats, which also contain 5 potential N-glycosylation sites closer to the transmembrane domain<sup>90</sup>. Epitopes of 3, 4, or 5 amino acids within the hydrophilic regions, APDTRPAP, is reactive with many anti-MUC1 antibodies and peptides<sup>89, 90</sup>.

### **2.5.1 Roles in cancer**

MUC1 has been reported to facilitate anti-adhesion and adhesion effects<sup>33</sup>. Glycosylated tandem repeats of MUC1 confers a protruding rod like structure that can extend 200-500nm above the cell surface<sup>91</sup>. This implies that the extracellular domain of MUC1 extends more than 150-450nm above estimated length of other cell surface adhesion molecules. However, in cancer cells MUC1 can become altered<sup>83</sup>. As mentioned before, MUC1 is heavily glycosylated however, in neoplastic tissue MUC1 is underglycosylated<sup>83</sup>. A reduction in glycosylation permits access of targeting ligands to reach the peptide core of the tumor associated underglycosylated MUC1 antigen (uMUC1). The reduction in glycosylation also can reveal epitopes that are normally concealed. Novel regions of the protein core in MUC1 are shown to consistently be exposed. MUC1 in cancer cells is thereby made antigenically distant from MUC1 found on normal epithelial tissue. MUC1 is also been reported to be ubiquitously expressed all over the cell surface. These features of MUC1 in cancer cells have encouraged the development of targeting moieties for imaging and targeted cancer therapy.

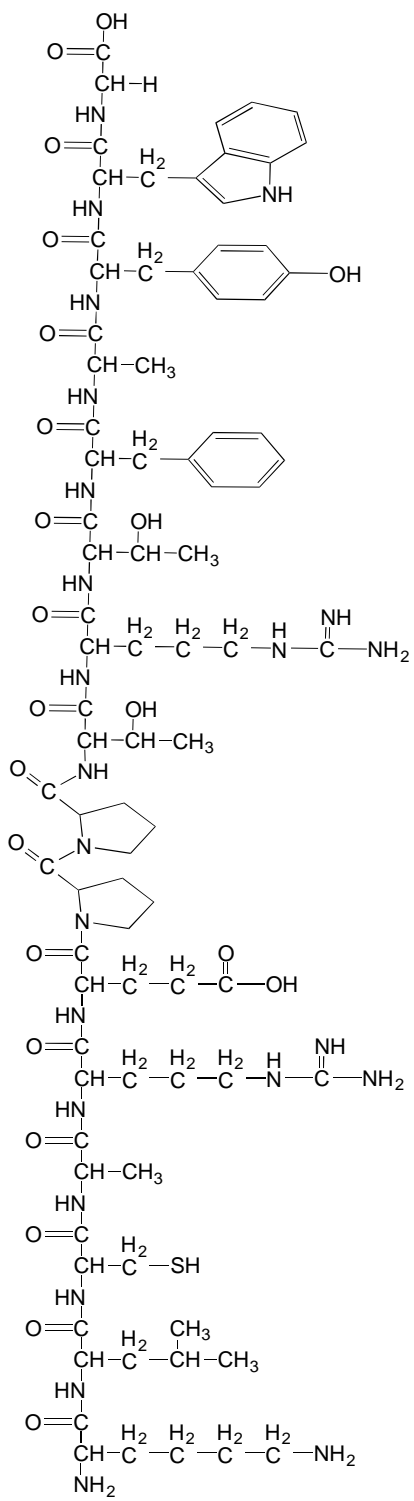
## **2.5.2 Glycosylation**

Glycosylation is defined as the enzyme-catalyzed covalent attachment of a carbohydrate to a polypeptide, lipid, polynucleotide, carbohydrate, or other organic compound, generally catalyzed by glycosyltransferases, utilizing specific glycan nucleotide donor substrates. Although most glycosylation reactions occur in the Golgi, precursor activation and interconversions occur mostly in the cytoplasm. Nucleotide sugar-specific transporters carry the activated donors into the Golgi. Glycan's, which are built up and stacked during glycosylation, have varied biological roles, which include any protective, stabilizing, organizational, and barrier functions. It may also have important regulatory roles as well. Every free-living cell and every cell type within multicellular organisms is covered with a dense and complex layer of glycan's due to the process of glycosylation. However, the point of explaining glycosylation is that in malignant cells an altered mechanism of glycosylation is occurring. The process and function of that alteration in which glycosylation slowed or reduced at the molecular level is beyond the scope of this project.

## **2.6 EPPT1 peptide**

Many recent new studies have been dedicated to the prospective use of uMUC1 as a target for immunotherapy<sup>87, 92-94</sup>. The APDTRP sequence tandem repeat that was mentioned before has been the main target for the development of multiple monoclonal antibodies. However, antibodies were found to be unsuccessful. Immunogenicity and a long plasma half life proved to be detrimental for imaging and targeting<sup>95</sup>. The use of small peptides has proved thus far to be a better option for targeting uMUC1 because of the fact that they can overcome the limitations of antibodies<sup>87, 92</sup>. A synthetic peptide

EPPT1 (YCAREPPTRTFAYWG), was developed from the CDR3 Vh region of the monoclonal antibody (ASM2) that was raised against human epithelial cells<sup>87, 92</sup>. The structure of the peptide has been shown to have a  $\beta$  strand type conformation as the active binding site<sup>87</sup>. It also has been revealed that the EPPT1 peptide has a significant binding affinity ( $K_d = 20 \mu\text{M}$ ) for the uMUC1-derived peptide PDTRP<sup>87, 92</sup>. The idea for EPPT1 to be used as a target for cancer was first developed as an imaging agent for early tumor detection<sup>87</sup>. Since at the time of the peptides development, uMUC1 was being reported as a highly expressed protein on 72% of new cases and 66% of deaths in several cancers<sup>87, 96</sup>. Therefore researchers surmised to look into using two imaging modalities, cross-linked iron oxide nanoparticles (CLIO) as MR-imaging contrast agent and Cy5.5 dye as near infrared fluorescence (NIRF) optical probe to explore early tumor detection<sup>87</sup>. The imaging probe was made up of a CLIO, which was modified with the Cy5.5 dye and carrying EPPT1 peptide attached to the dextran coat of the nanoparticle<sup>87</sup>. Using different characterization and imaging tests, the probe was found to successfully recognize tumors in vivo, produce high resolution signal on MR-imaging, and allowed for real time data acquisition by NIRF<sup>87</sup>. However, the goal of this dissertation is to utilize the targetability of the EPPT1 peptide, which proved to be successful for targeted in vivo imaging and apply it to a drug delivery vehicle for cancer therapy.



**Figure 4: Structure of EPPT1**

## **2.7 Polymer Conjugates**

Polymer conjugates have a distinguished record in clinical relevance and is an advancing field. They have been used in clinics and/or clinical trials with proteins, small molecular weight drugs, modification of liposomes, surface modifications of bio materials and carriers of genes and oligonucleotides<sup>97</sup>. Although over the last century water soluble macromolecular carrier has evolved, the term coined in 1906 by Ehrlich “magic bullet” is still recognized the main concept of biorecognition for successful drug delivery<sup>97</sup>. Polymer conjugates have been able to be successful enhancing therapeutic efficacy of drug by altering the biodistribution of proteins and drugs to increase plasma half life by improving cellular localization and improving solubility of poorly soluble drugs<sup>28</sup>. Several polymer with linear, random coiled structures have been used including, polyethylene glycol (PEG), N-(2-hydroxypropyl)methacrylamide (HPMA), dextran and poly(glutamic acid) (PGA)<sup>98</sup>. These systems are advantageous for drug delivery because they allow for many options for chemical modifications and alterations in molecular weight. However, this advantage creates a challenge because by nature the polymer exists in a random orientation of molecular weight that can influence the variability of in vivo disposition and biological activity<sup>28, 99</sup>. Polymers characteristics in terms of molecular weight are in general estimated and expressed as weight average molecular weight (Mw) or number average molecular weight (Mn)<sup>100, 101</sup>. The ratio between Mw and Mn gives the polydispersity and a sense of size distribution of a given polymer. Beyond the scope of this dissertation, later discussions of important polymer conjugates will be applicably

concentrated on to those that have advanced onto clinical practice, as they have demonstrated successful pathways to achieving human efficacy.

### **2.7.1 Polymer-protein conjugates**

Protein based drugs as a therapeutic is a promising field for many diseases, but there are many flaws in its design. However, short plasma half-life, undesirable stability and immunogenicity limit its effectiveness<sup>102</sup>. As development of polymer conjugates became more readily available, linear PEG polymers were conjugated with therapeutic proteins (termed PEGylation) to increase protein solubility and stability and reduce immunogenicity<sup>103</sup>. By using PEG, it helps lengthen the plasma half-life because it prevents rapid renal clearance. It also prevents protein uptake in the reticuloendothelial system, which also helps in prolonging protein plasma half-life. It was also found that by varying the molecular weights of the PEGs from 5 to 40kDa, the pharmacokinetics can be altered<sup>103</sup>. PEG polymers that increase the ratio beyond the common one polymer per protein have been found to add to the variability in biodistribution. Polymers with only one reactive site per strand, named semi-telechelic polymer, are required for the synthesis of polymer protein conjugates to prevent crosslinking<sup>103</sup>. In recent studies, polymer protein conjugation reactions with PEG have advanced to include enzymatic reactions, which improve precision and ease of conjugation and decrease protein degradation<sup>104</sup>.

The PEGylated protein PEG-L-asparaginase was the first of its kind to be treated for cancer<sup>105</sup>. Used for patients with acute lymphoblastic leukemia, the conjugate demonstrated greatly reduced hypersensitivity reactions, longer plasma half-life and an overall slower total clearance. Furthermore, patients later were found to need less frequent administration. Another conjugate, which is used in cancer therapy for the

prevention of serious chemotherapy-induced neutropenia, is the PEG-recombinant methionyl human granulocyte colony stimulated factor. This polymer conjugate was found to be as beneficial as the first PEGylated polymer<sup>106</sup>. Lastly, improvements in toxicity and plasma half-life were achieved when PEG-interferon- $\alpha$  conjugates were tested in renal cell carcinoma patient<sup>107</sup>.

### **2.7.2 Polymer drug conjugates**

The objective of polymer drug conjugates designed for cancer therapy are to improve drug targeting to the site of action while reducing systemic toxicity by restricting its biodistribution<sup>108</sup>. By attaching the drug to a carrier rendering it inactive, there is a potential to avoid drug resistance mechanisms such as P-gp mediated efflux. It has also been found that water-soluble polymer can help in improving the solubility of hydrophobic drugs allowing for a simpler formulation and easier patient administration. However, as the advancement of polymer-drug carriers have come about in the clinical setting, polymer induced toxicities and immunogenicity have been seen<sup>109</sup>. One of the biggest limitations of polymer-drug conjugates is the carrying capacity of the single polymer chain. This is why there are a small amount of examples in the clinical setting with PEG-drug conjugates. PEGs limitation is due to the fact that drugs can only be conjugated at the ends of polymer chains, low drug loading. However, one example of a PEG-camptothecin conjugate containing 1.7 wt% of drug showed response in patients with gastric cancer<sup>110</sup>. In contrast, PGA-paclitaxel conjugate containing 37 wt% of the drug a polymer therapeutic is an example of high drug loading<sup>111</sup>. By linking the drug to the 2' position of the PGA researchers were able to achieve a higher drug loading. Clinical trials of this polymer drug conjugate were able to result in a partial response or

stable disease in cancer patients with patients with mesothelioma, renal cell carcinoma and ovarian cancer<sup>112, 113</sup> A more recent phase III clinical trial with the drug exhibited the effectiveness of this conjugate in a therapy for non small cell lung cancer<sup>35</sup>. The polymers attributed success is due to the fact that it is biodegradable by hydrolytic mechanism, but also discharges the drug in greater quantities under lysosomal enzymatic conditions following endocytosis. Furthermore, the high drug loading capacity of the carrier developed a larger Mw of 49 kDa. This could allow for greater tumor localization with the attempts of size related renal clearance<sup>111</sup>.

HPMA polymer conjugates will be discussed in later sections as one of the most widely studied polymer-drug conjugates. The important considerations of polymer-drug mechanism of action and biological fate will be covered next.

### ***2.7.2.1 Biological fate***

One of the greatest advantages of polymer-drug conjugates is when administered intravenously, the drug is restricted to the blood stream and has limited access to potential sites of toxicity<sup>108</sup>. Due to the drug being connected to its carrier, the typical route of elimination of the conjugate is renal, which helps prevent sequestration of the free drug in potentially sensitive organs such as the liver and brain<sup>114</sup>. Although an important characteristic of these conjugates is renal elimination, the underlying issue to be protected is that these conjugates do not undergo fast plasma clearance via rapid renal filtration or organ capture<sup>108</sup>. Polymer-drug conjugates in the blood stream can achieve tumor localization because of the process known as enhanced permeability and retention effect (EPR)<sup>115</sup>. While developing polymer conjugates of styrene maleic anhydride polymer and the antitumor protein nocarzinostatin (SMANCS), EPR was discovered by

Maeda and coworkers. They found higher tumor concentrations of their conjugate and determined that the discontinuous nature of the tumor endothelium results in a “leaky” vasculature, providing a route for macromolecules to pass the blood barrier and accumulate in tumor tissue. The lack of an effective lymphatic drainage in tumors also fosters the retention of polymer. EPR-mediated tumor accumulation seems to be managed by the circulating plasma concentrations which has direct relations to polymer molecular weight and renal excretion<sup>116</sup>.

The ideal polymer-drug carrier would have a linker, which connects the drug to the carrier, which allows for stable transport to the tumor but releases the drug upon reaching the site of action. Although some transcytotic mechanisms of endocytosis are observed<sup>117</sup>, most drugs conjugated to water soluble polymers have limited cellular uptake<sup>98</sup>. Cellular internalization of conjugates depends on the nature of the polymer and whether it binds to the cell surface through non-specific interactions or specific binding<sup>108</sup>. Most polymer-conjugates route of internalization is either through fluid-phase pinocytosis or receptor mediated endocytosis<sup>108</sup>. Once internalized, drug release from the carrier would occur in the lysosomal compartment through exposure to lysosomal enzymes if the drug were connected via degradable peptide spacer<sup>118, 119</sup>. It also can attribute to pH-dependent hydrolysis of drug from polymers resulting from acidification of drugs<sup>119</sup>. Drug delivery via intracellular compartments supports a bypass of multidrug resistance proteins such as the P-glycoprotein efflux transporter that can rapidly remove drugs molecules from the cell once they have diffused through the phospholipid bilayer<sup>119</sup>.

### 2.7.2.2 *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers

In the late 1970s, Dr. Kopecek developed HPMA copolymer by systematic research on hydrophilic biomedical polymer<sup>120</sup>. The copolymer synthesized via free radical polymerization of comonomer units which contain acrylamide groups<sup>121</sup>. HPMA copolymer are highly water soluble, stable and biodegradable<sup>122</sup>. These random copolymers can be functionalized along the copolymer chain and create a versatile nature of these systems to incorporate different, drug, targeting moieties, imaging agents and radioisotopes. See Figure 5. Early studies with HPMA copolymers demonstrated that they were non-toxic and posed no immunogenic risk as established by low and non-significant levels of specific serum antibodies present in mice following treatment<sup>27</sup>. As research progressed the development of degradable drug linker side chains for the HPMA copolymer became fundamental for the effectiveness of these systems as macromolecular molecules<sup>122</sup>. HPMA copolymers typically have the most effectiveness when using either a tetra-peptide linker Gly-Phe-Leu-Gly (GFLG) and/or hydrazine linkers. GFLG tetrapeptide linker is very stable in plasma during copolymer circulation and trafficking<sup>123</sup>. Upon internalization and subsequent endocytosis into the lysosomal compartment, the tetrapeptide is cleaved by lysosomal protease cathepsin B<sup>124</sup>. The hydrazine linkers are pH dependent and drug release from the carrier in the lysosomal compartment is in response to lower pH (pH 5.5) while being stable at pH 7.4<sup>125</sup>. In vivo studies with HPMA copolymer doxorubicin conjugates containing a hydrazone linker showed better anti-tumor activity when compared to conjugates with the tetrapeptide



lysosomal membrane but the free drug is small enough to diffuse<sup>27</sup>. However, in some case it has been shown in vitro that drug release is not required to have an effect and that the presence of the GFLG spacer without drug can be cytotoxic<sup>127</sup>.

The enhanced permeability and retention effect has made it possible for not only other polymer but also HPMA copolymers to passively target tumors. Since molecular weight has such a substantial influence on the extent of how much polymer can accumulate near the tumor with only passive targeting, researchers set out to test those limits<sup>116</sup>. Tumor accumulation of larger copolymer fractions greater than 74kDa had increased activity over a 24h period however, it was also found that smaller copolymers less than 40kDa were rapidly cleared by the kidneys<sup>116, 128</sup>. Copolymers found to have lower tumor accumulation, were also found to have lower blood concentrations. This is attributed to the fact that smaller polymers will experience fast glomerular filtration, which agrees with earlier findings that molecular copolymers that were below the 45kDa glomerular filtration limit were rapidly cleared from blood circulation<sup>128</sup>.

In addition to molecular weight having an influence on tumor accumulation, HPMA copolymer were also found to be influenced by their charge content which could further impact its biodistribution and organ accumulation when analyzed in vivo. Studies were done to evaluate the charge and functionality of incorporated side chains<sup>129</sup>. In vivo studies showed that polymers of higher molecular weight 65kDa had higher tumor accumulation than polymers of lower molecular weight 23kDa. Copolymer synthesized with negatively charged carboxyl groups and positively charged hydrazine amino groups were found to cause the most rapid blood elimination which cause the lowest tumor accumulation. Adding a drug or protein/peptide targeting moiety also showed to

cause a decrease in tumor accumulation as opposed to just free unconjugated HPMA copolymer<sup>129</sup>. It also have been shown that chemically modified HPMA copolymer also have altered kidney accumulation which would result in decreased tumor accumulation. Despite the kidney accumulation of chemically modified HPMA copolymers, concentrations evaluated of tumor-to-organ ratios remained unchanged even if tumor accumulation was decreased<sup>129</sup>.

Understanding the subcellular fate of HPMA copolymer is also important in evaluating conjugates once the accumulated near the tumor. The fluorescent drug compound Adriamycin and either galactosamine or monoclonal antibodies were conjugated to a HPMA copolymer and evaluated for subcellular fate in cancer cells<sup>130</sup>. The study confirmed two fates for HPMA copolymer conjugates. The first is that that HPMA copolymer Adriamycin conjugates demonstrated receptor mediated endocytosis and that fluorescence patterns of the drug in the nucleus were similar to that a free drug. The second is that in adriamycin-resistant cancer cells, HPMA copolymer adriamycin conjugates had internalization that was greater than that of the free drug. This would suggest the route of cellular entry for copolymers can bypassed efflux transporters, which contribute to drug resistance<sup>130</sup>.

### ***2.7.2.3 Clinical evaluation of HPMA copolymers***

Passive targeting utilizing the EPR effect, in vivo studies showed improved antitumor activity of HPMA copolymer-doxorubicin conjugates compared to free drug<sup>131</sup>. These results correlated with other similar studies lead to the development of conjugates for clinical evaluation. Titled PK1, this HPMA copolymer with doxorubicin bound via GFLG peptide sequence was the first of several conjugates to be evaluated clinically<sup>132</sup>.

Characteristics of PK1 were evaluated and found that its approximate molecular weight was 30 kDa, containing 8 wt% of doxorubicin and was administered to patients up to the maximum tolerated dose of 320 mg/m<sup>2</sup>. That dose level increased the maximum tolerated dose in patients four to five fold compared to the free drug alone. Antitumor activity was observed in lung, colorectal, and breast cancers patients. Studies in phase II clinical trials showed only limited activity in lung and breast cancer patients, however researchers were able to demonstrate safe administration even with high doses of polymer with drug<sup>133</sup>.

A second clinical trial was also done with HPMA copolymer conjugate of doxorubicin and a targeting ligand galactosamine. The first of its kind, this HPMA copolymer conjugate had both a drug attached and a targeting moiety and was named PK2<sup>134</sup>. The conjugate was designed for hepatocyte asialoglycoprotein receptor for liver cancer treatment. Dose limiting toxicities were consistent with those seen with the free drug which included febrile neutropenia and mucositis. Maximum tolerated dose was 160 mg/m<sup>2</sup>. Out of the 23 patients enrolled in the study all of which had liver cancer, 3 saw favorable response in decreased tumor volumes while 11 patients had stable conditions<sup>134</sup>.

Although there have been a total of 5 HPMA copolymers drug conjugates which were evaluated clinically they were deemed much less successful. HPMA copolymers bearing chemotherapies paclitaxel and/or camptothecin did not cause a tumor response and were hampered by severe dose limiting toxicity. Ester drug linkage, which caused early release of the drug, were deemed as the cause for such poor results. Thus the need for a better conjugate that has improved drug linker stability and drug loading was the emphasis for future conjugates<sup>108</sup>.

## **Chapter 3. Water Soluble Polymers for Targeted Therapy to Pancreatic Cancer**

### **3.1 Abstract**

Current therapies of pancreatic cancer are limited by poor response, lack of selectivity and high toxicity of chemotherapeutics. Targeted drug delivery can provide an increased response while limiting side effects. The goal of the present study was to evaluate the targetability and efficacy of N-(2-hydroxypropyl) methacrylamide (HPMA) copolymer-(EPPT1)-Gemcitabine conjugates as novel macromolecular drug delivery systems against pancreatic cancers. The synthetic peptide ligand EPPT1 (YCAREPPTRTFAYWG) has appreciable affinity ( $K_d = 20 \mu\text{M}$ ) for the underglycosylated glycoprotein mucin receptor 1 (uMUC1) which is overexpressed on the surface of pancreatic cancer cells making it a favorable candidate for targeted delivery of anticancer drugs like Gemcitabine (Gem). Polymer conjugates with drug and peptide ligand were synthesized by radical precipitation copolymerization using corresponding comonomers. Gem was attached to the polymer via an intracellularly degradable tetrapeptide (GFLG) spacer. Size exclusion chromatography (SEC) was used to purify the polymers and to determine their

hydrodynamic molecular weight and polydispersity. Drug and peptide content were determined by amino acid analysis and UV spectrophotometry. The binding efficiency (flow cytometry) and anticancer efficacy of the conjugates were evaluated in uMUC1 positive CaPan-2 and Panc-1 pancreatic cancer cells. HPMA-EPPT1 peptide conjugates (Mw=41-44 kDa; polydispersity=1.7-2.1) showed active targeting to both pancreatic cancer cells in a concentration- and peptide content-dependent manner. HPMA-Gem drug conjugates inhibited cancer cell growth with a potency that was comparable to free Gem ( $IC_{50}$ =0.004-0.01  $\mu$ M). These results suggest that the polymer conjugated Gem retained its bioactivity in vitro which could be further enhanced in conjunction with the EPPT1 targeting peptide. Together these studies support the hypothesis that HPMA copolymers with optimized peptide content could afford a novel carrier that can deliver Gem and improve its therapeutic effect to combat pancreatic cancer.

### **3.2 INTRODUCTION**

Pancreatic cancer is the fourth leading cause of cancer related death in the United States<sup>135</sup>. It is one of the most deadly forms of cancer due to the fact that there are no early signs or symptoms; therefore, the majority of patients have an advanced metastatic form. Unfortunately, the incidence of pancreatic cancer almost equals its mortality rate<sup>135</sup>. Treatment for pancreatic cancer traditionally had been 5-fluoruracil (5-Fu) but because of its side effects and less than mediocre success rate, gemcitabine (Gem) has become a frontline drug of choice for adjuvant and neoadjuvant therapies<sup>1, 2, 136</sup>. Gem is a nucleoside analogue that exhibits S cell phase specificity by predominantly destroying cells undergoing DNA synthesis. In clinical trials, treatment with Gem alone showed clinical response in 22% of patients and a median survival rate of 5.7 months, much

longer than traditional 5-fluoruracil <sup>4</sup>. However, Gem treatment(s) is associated with non-specific toxicity such as suppression of bone marrow function as manifested by leucopenia, thrombocytopenia and anemia. Pulmonary toxicities and renal failure have also been reported <sup>2, 4, 136</sup>. The median survival of patients with advanced diseases ranges around 6 months because pancreatic cancer becomes resistant to gemcitabine treatment, possibly mediated by MDR efflux pumps <sup>80</sup>. The objective of the present study is to design a novel drug delivery system to overcome the pitfalls of gemcitabine treatment alone. Using N-(2-hydroxypropyl) methacrylamide HPMA copolymer backbone containing Gem covalently attached to the polymer's side-chains this system could enhance efficacy and reduce non-specific toxicity by further attaching an active targeting moiety. HPMA copolymers are widely used as drug carriers <sup>97, 99, 109, 130, 137-143</sup>. This is due to their high water solubility, non-immunogenicity, and amenability to large-scale production. HPMA copolymers have been employed as drug carriers to modify in vivo biodistribution of several chemotherapeutic agents and enzymes <sup>120, 122, 144-148</sup>. Drugs can be covalently linked to the HPMA backbone via a glycine-phenylalanine-leucine-glycine (GFLG) side chain, which is stable in circulation but cleaved by lysosomal proteases such as cathepsin B once internalized by endocytosis <sup>149</sup>. This linker is essential for polymeric drug delivery because the drug remains inactive while bound to the linker but activates upon cleavage within the lysosomes. Moreover by linking the drug to the polymer, its route of entry into cells is limited to endocytosis, which potentially can bypass mechanisms of drug resistance such as efflux pumps <sup>118, 145, 147</sup>. Active targeting is a major advantage to utilizing HPMA polymeric drug delivery systems. MUC1 is an ideal candidate for targeting because it is a large over expressed mucin molecules that are

exposed on the apical surface of almost all human epithelial cell adenocarcinomas including pancreatic cancer<sup>83, 86, 87, 92</sup>. The mucin molecule has been found to extend about 100-200nm above the surface, which is 5-10 fold higher than the length of most membrane molecules<sup>83, 86</sup>. While MUC1 is heavily glycosylated in normal tissue, it is underglycosylated in neoplastic tissue (uMUC1), revealing epitopes that are normally masked<sup>83, 86, 150, 151</sup>, such as PDTRP, which is recognized by many targeting moieties such as antibodies and peptides<sup>83</sup>. Anti-MUC1 is associated with immunogenicity and long plasma half-lives, which prompted us to focus on EPPT1 (YCAREPPTRTFAYWG), the small peptide derived from the CDR3 antibody that has a beta strand type conformation as its active binding site [29-32]. Analysis of the peptide had shown that it has a significant affinity for the uMUC1-derived peptide PDTRP ( $K_d \sim 20\mu\text{M}$ ) making it an ideal targeting moiety for polymer delivery systems [29-32]. In this study we explore the potential for a HEMA-copolymer which utilizes the EPPT1 peptide binding affinity to the MUC1 receptor on 2 different pancreatic cell lines while also investigating the therapeutic effect of a non-targeted HEMA-copolymer Gemcitabine conjugate compared to gemcitabine alone.

### **3.3 EXPERIMENTAL SECTION**

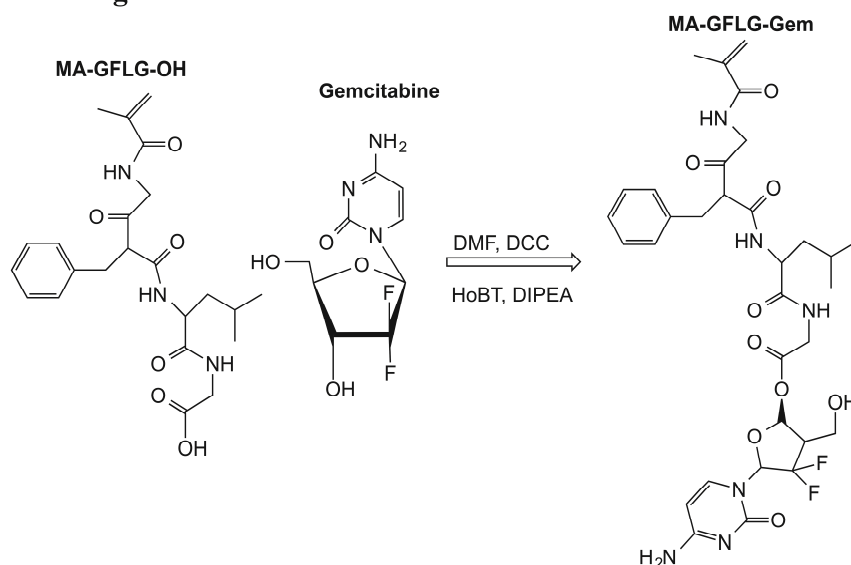
#### **3.3.1 Materials.**

Gemcitabine HCl was obtained from Netqem, LLC (Triangle Park, NC). All other reagents were of reagent grade from Sigma-Aldrich (St. Louis, MO).

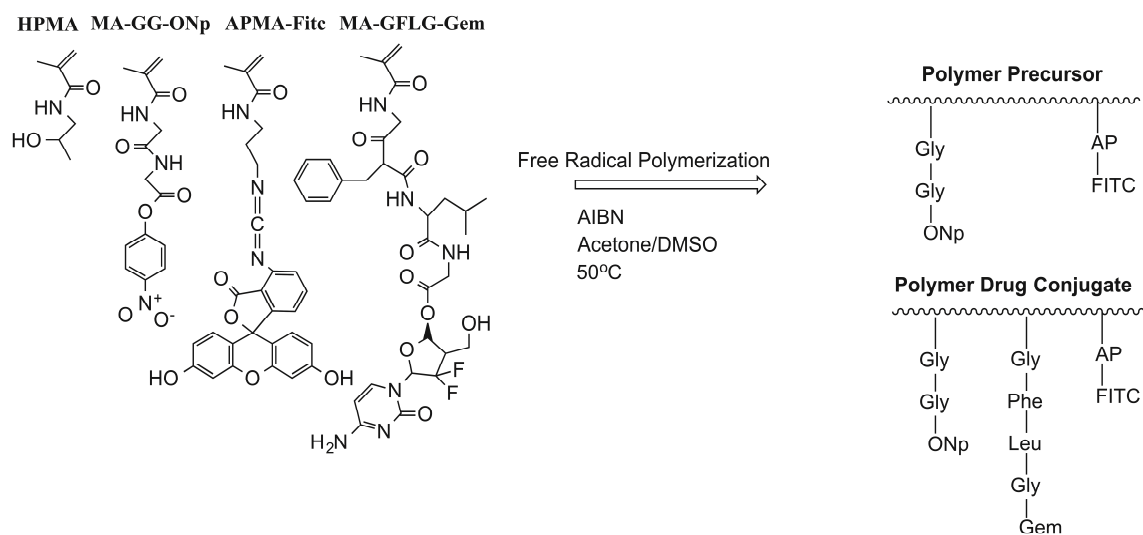
### 3.3.2 Synthesis of Comonomers.

The following monomer building blocks were synthesized according to published methods: (i) N-(2-hydroxypropyl)methacrylamide (**HPMA**, m.p. 66-68°C, MW 143.8), a comonomer that forms the polymer backbone and renders aqueous solubility<sup>150</sup>; (ii) methacryloyl-glycylglycyl-p-nitrophenyl ester (**MA-GG-ONp**, m.p. 160-163°C, MW 321.7,  $\epsilon_{273} = 9500 \text{ M}^{-1}\text{cm}^{-1}$ ), a non-degradable dipeptide linker containing reactive comonomer to which the EPPT1 peptide is attached following polymerization; (iii) 5-(3-(methacryloyl-aminopropyl) fluorescein isothiocyanate (**APMA-FITC**, MW 533.60,  $\epsilon_{495} = 8200 \text{ M}^{-1}\text{cm}^{-1}$ ), a fluorescent comonomer to monitor in vitro cellular binding and internalization<sup>150</sup>; (iv) methacryloyl-glycylphenylalanylleucylglycine (MA-GFLG-OH, MW 460.52), a lysosomally degradable tetrapeptide comonomer intermediate to which Gem is covalently bound prior to polymerization. The drug containing comonomer (v) **MA-GFLG-Gem** was synthesized by reacting Gem HCl with MAGFLGOH (1:1 molar) in anhydrous dimethylformamide (DMF) using N,N-diisopropylethylamine (DIPEA) (1.5x), hydroxybenzotriazole (HOBT) (1.1x), N,N'-dicyclohexylcarbodiimide (DCC) (1.1x) and 4-dimethylaminopyridine (DMAP) (.15x) at room temperature for 16 hours. The final product was isolated and purified by column chromatography (silica gel, mobile phase: dichloromethane 83%/Methanol 15%/Acetic Acid 1%) and analyzed by thin layer chromatography ( $R_f$  0.48) and mass spectrometry (MW 706.3). See Figure 6

## 1. Drug containing comonomer



## 2. Polymer conjugates



**Figure 6: Schematic of Reactions**

1. MAGFLG-OH reacted with Gemcitabine to form lysosomal drug linker. 2. Free radical polymerization of Polymer precursor and polymer drug conjugates

### 3.3.3 Synthesis and characterization of HPMA copolymer-EPPT1 peptide conjugates.

In the first step, the HPMA, MA-GG-ONp and APMA-FITC comonomers were dissolved in acetone (with 0-10% DMSO as needed to ensure complete solubilization) and combined with the initiator N, N'-azobisisobutyronitrile (AIBN) and sealed in ampules under nitrogen. Free radical precipitation copolymerization of monomers was performed by reacting at 50°C. The polymer precursor as synthesized was isolated by precipitation in ether/acetone (1:1) mixture. The final polymer-peptide conjugates were synthesized by aminolysis of the pendent ONp groups in the polymer precursor with EPPT1 peptide (ONp: EPPT1=1:1 molar). The reaction was carried out under nitrogen and anhydrous DMF. The reaction was terminated by 0.1N sodium hydroxide (NaOH) and then purified on a Sephadex G-25 PD10 column (GE Healthcare, Piscataway, NJ) and finally lyophilized. The weight average ( $M_w$ ) and number average ( $M_n$ ) molecular weights of HPMA copolymer precursors and polymer-peptide conjugates were estimated by size exclusion chromatography (SEC) on an AKTA fast protein liquid chromatography (FPLC) system using a Superose 12 column calibrated with a HPMA copolymer standards UV detection at 280 nm, mobile phase: phosphate buffered saline ((PBS), pH=7.4). The polydispersity index ( $M_w/M_n$ ) was calculated to estimate the molecular weight distribution. EPPT1 content of the conjugates was determined by amino acid analysis (AIBiotech, Richmond, VA). FITC content of the polymers was determined by UV spectrophotometry (DMSO/1% acetic acid,  $\epsilon_{495}=81000 \text{ M}^{-1} \text{ cm}^{-1}$ ).

### **3.3.4 Synthesis and characterization of HPMA copolymer-Gem drug conjugates.**

Polymer-drug conjugates were synthesized in one step by free radical precipitation polymerization similarly as described for polymer-peptide conjugates using the comonomers: HPMA, MA-GG-ONp, MA-GFLG-Gem and APMA-FITC. The content of pendent ONp, Gem and FITC were estimated by UV spectrophotometry. Gem and FITC were measured in 0.1N NaOH at 263 nm ( $\epsilon_{263} =$  and 495 nm ( $\epsilon_{495} = 79000\text{M}^{-1}\text{cm}^{-1}$ ) respectively. Total ONp in the polymer was measured in DMSO/1% acetic acid ( $\epsilon_{272} = 9100\text{M}^{-1}\text{cm}^{-1}$ ). To measure ONp content that is accessible/available for further conjugation to peptide, the ONp groups were hydrolyzed and released p-nitrophenol measured in 0.1N NaOH at 400nm ( $\epsilon_{400} = 9000\text{M}^{-1}\text{cm}^{-1}$ ) The molecular weight and weight distributions of the polymer-drug conjugates were estimated by SEC as described earlier.

### **3.3.5 Cell Culture.**

CaPan-2 and Panc-1 (ATCC, Manassas, VA) (passages 10-35) were grown at 37°C in an humidified atmosphere of 5% CO<sub>2</sub>. Cells were maintained in T-75 flasks using Dulbecco's Modified Eagle's Medium (DMEM) and McCoy's 5A respectively, supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 10,000 units/mL penicillin, 10,000 µg/mL streptomycin and 25 µg/mL amphotericin B. Media was changed every other day and cells were passaged at 80–90% confluence using a 0.25% trypsin/ethylenediamine tetraacetic acid (EDTA) solution. Incubation buffer used in the assays consisted of Phosphate Buffered Saline (PBS) without calcium or magnesium chloride. For all experimental procedures, the confluent cultures were harvested by treatment with trypsin/EDTA.

### **3.3.6 Cellular binding of polymer-peptide conjugates.**

The binding affinity of HPMA copolymer-EPPT1 peptide conjugates was quantified using a flow cytometry assay. Active binding of the EPPT1 peptide to the uMUC1 receptor on pancreatic cancer cells was measured when linked to an HPMA copolymer backbone. Binding studies were carried out against two model primary pancreatic cancer cell lines CaPan-2 (high MUC1 levels) and Panc-1 cells (lower MUC1 levels)<sup>151, 152</sup>. The cells were seeded in 12 well plates (CaPan-2: 150,000 cells/ well, Panc-1 65,000 cells/well) and grown until they were 90% confluent (CaPan-2: 3-4 days, Panc-1: 48 h). The cells were then treated with varying concentrations (10-40uM) of FITC labeled HPMA-EPPT1 conjugates for 30 minutes at 37 °C. To determine competitive (active) binding of the polymer-peptide conjugates, cell lines were pretreated with free EPPT1 peptide at a 10-fold higher (200uM) concentration than its  $K_d$  value. After incubation, cells were washed and harvested in PBS with 1% fetal bovine serum (FBS) and analyzed by flow cytometry (FACScan BD Newark, New Jersey), The cell count and mean fluorescence intensity was recorded to measure the bound and internalized polymer-peptide conjugates.

### **3.3.7 Confocal internalization studies.**

Intracellular internalization of polymer peptide conjugates of varying EPPT1 content within Capan-2 and Panc-1 cells was also investigated in addition to their uptake mechanism. Panc-1 cells were seeded at 4000 cell/cm<sup>2</sup> on 8-chamber slides. CaPan-2 cells were seeded at 10000 cells/cm<sup>2</sup>. Slides were used 3 days to 5 days after incubation

at 37 °C, 95 % relative humidity and 5% CO<sub>2</sub>. Cells were washed with warm HBSS and then were treated with a concentration (40uM) of FITC labeled HPMA-EPPT1 conjugates for 30 minutes at 37 °C. After which cells were washed with ice-cold DPBS, and fixed with 4 % (wt %) paraformaldehyde, 4 % (wt %) sucrose in DPBS for 15 min. Cells were washed with permeabilization buffer (PBS containing 300 mM sucrose, 50 mM NaCl, 3 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 20 mM HEPES, 0.5 % Triton X 100 (vol %), pH 7.2), which was added to the samples and kept at 4 °C for 5 minutes. The permeabilization buffer washed out twice with DPBS and later incubated at 37 °C with blocking buffer (3 % bovine serum albumin (BSA) (wt/vol) in DPBS) for 5 minutes. Cells were washed with DPBS. Cells were then incubated with 300 nM 4',6-diamidino-2-phenylindole (DAPI) for 10 min to stain the nuclei. The cells were then washed twice with DPBS and the chambers were removed. The slides were mounted and covered with glass coverslips. They were allowed to dry for a few hours before they were sealed with nail varnish and stored at 4 °C. Images were acquired using a Nikon A1 Inverted confocal laser-scanning microscope (Nikon Instruments, Melville, NY).

### **3.3.8 Cytotoxicity of polymer-drug conjugates.**

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to determine in vitro cell growth inhibition of Gem and HPMA copolymer-Gem drug conjugates against the model pancreatic cancer cell lines, CaPan-2 and Panc-1. Briefly, CaPan-2 and Panc-1 cells were seeded into 96-well plates at an initial density of 5,000 and 2,000 cells/well respectively and allowed to attach for 24 h. Cells were treated with increasing concentrations of gemcitabine or copolymers in fresh media and incubated for

72 h. The efficacy of the polymer drug conjugates were compared to the free drug by calculating the growth inhibitory coefficient ( $IC_{50}$ ) based on the viability vs. concentration curves. All experiments were performed in triplicate.

## **3.4 RESULTS**

### **3.4.1 Characteristics of polymer precursors and polymer peptide conjugates.**

Initial studies were performed leading to synthesis and characterization of a series of polymer precursors (HPMA-(MA-GG-ONp)-FITC) and their corresponding polymer peptide conjugates (HPMA-(MA-GG-EPPT1)-FITC). (Table 3.1) All precursors contained 2% APMA-FITC while the reactive MA-GG-ONp comonomer content was varied at 0, 5 and 10 mol%. Based on spectrophotometric measurements it was observed that the amount of incorporated MA-GG-ONp in the precursor increased with increase in its feed comonomer content (Table 3.1) which is consistent with literature reports<sup>153, 154</sup>. However, with increasing MA-GG-ONp incorporation, the yield of the precursor (PP2, PP3) decreased. This can be explained by the use of more DMSO (up to 10% to solubilize MA-GG-ONp) during polymerization, which also solubilizes some of the precipitated polymers.<sup>128</sup> FITC content was observed to be constant and consistent with feed composition. See Table 2. Based on SEC data (Table 3) the hydrodynamic volume based molecular weight of the polymer precursors decreased as MA-GG-ONp content increased. This observation is attributed to the known chain-terminating effect of MA-GG-ONp when used in radical polymerization.<sup>155</sup> Upon aminolytic conjugation of EPPT1 peptide to the ONp groups of the polymer precursor, the incorporated peptide content was

found to increase with increase in feed ONp content. The molecular weight of the polymer peptide conjugates also increased relative to the corresponding precursors although the polydispersity values increased suggesting altered hydrodynamic conformations of the polymer due to peptide attachment (Table 3).

***Physicochemical Characterization of HPMA-MAGGPeptide-FITC Conjugates***

Sample	Feed Composition		Wt-%	Monomer content		
	HPMA	MAGGONp		ONp Content <sup>a)</sup>	FITC Content <sup>b)</sup>	Peptide Content <sup>c)</sup>
	mmol/g of polymer					
PP1	98	0	90.74	.0085 ± .06	.132 ± .06	-
PP2	93	5	69.12	.351 ± .03	.125 ± .06	0.0272
PP3	88	10	62.5	.543 ± .05	.119 ± .03	0.066
PP4	83	15	53	.676 ± .04	.113 ± .02	0.1207

**Table 2: Physicochemical Characterization of HPMA-MAGGPeptide-FITC Conjugates**  
Determined by a,b) UV spectroscopy ONp  $\epsilon_{278\text{nm}} = 9500 \text{ M}^{-1} \cdot \text{cm}^{-1}$  FITC  $\epsilon_{495\text{nm}} = 82000 \text{ M}^{-1} \cdot \text{cm}^{-1}$  , c) AA analysis

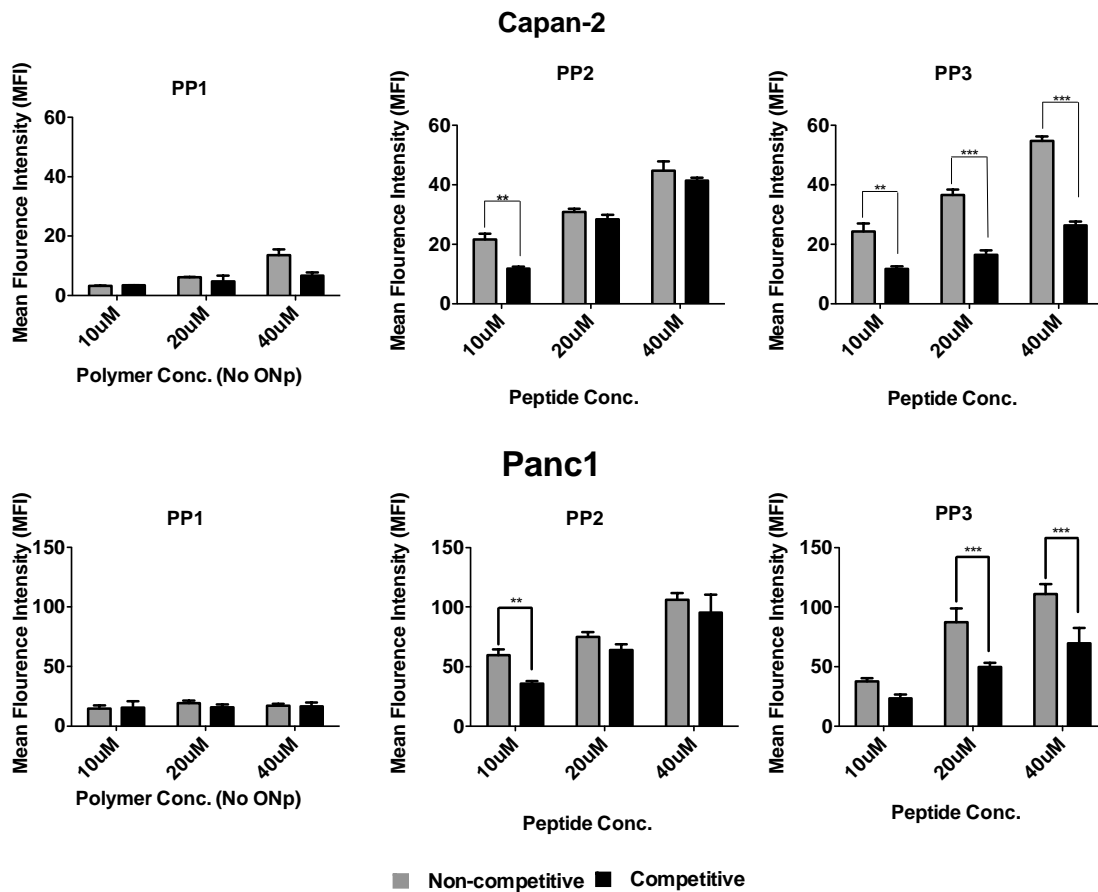
Size Exclusion Data						
Sample	Polymer Precursor			Polymer Peptide Conjugate		
	$M_w$	$M_n$	Polydispersity	$M_w$	$M_n$	Polydispersity
	$\frac{g}{mol}$	$\frac{g}{mol}$	(n)	$\frac{g}{mol}$	$\frac{g}{mol}$	(n)
PP1	97744	65800	1.5	-	-	-
PP2	29074	19846	1.5	44242	25620	1.7
PP3	22052	16693	1.3	43387	24597	1.8
PP4	20182	15767	1.3	41215	19338	2.1

**Table 3: Size Exclusion Data**

### 3.4.2 Cellular Binding of polymer peptide conjugates.

The ability of the EPPT1 peptide to augment the binding and/or uptake of HPMA-EPP1 peptide conjugates to uMUC1 expressing pancreatic cancer cells was substantiated using flow cytometry. In CaPan-2 cell line, overall binding increased as a function of the final peptide concentration (10-40  $\mu\text{M}$ ) for the polymer peptide conjugates PP1 and PP2 (Figure 7). In contrast, control polymer with no peptide (PP1) exhibited only marginal association with the cells. Interestingly, at equivalent peptide concentrations (20 or 40  $\mu\text{M}$ ), an increase in the peptide content of the polymers (PP2 vs. PP3) resulted in a corresponding increase in mean fluorescence intensity, which would indicate an increase in binding affinity. To demonstrate whether binding was uMUC1-mediated, we conducted competitive studies where the binding sites were saturated with pre-treatment with free peptide. This generally resulted in a reduced level of fluorescence intensity for both polymer-peptide conjugates at 20 and 40  $\mu\text{M}$  concentrations compared to studies with no-pretreatment. This further confirms receptor mediated binding of polymer-peptide conjugates with possibly greater affinity than the free peptide. PP3 showed greater binding when compared to PP2 suggesting that by optimizing the number of peptide chains on the polymer backbone it is possible to promote multivalent binding (several pendent peptides on a polymer backbone). In Panc-1 cells, similar observations were made as CaPan-2 cells. However Panc-1 cells overall showed higher mean fluorescence intensity equating to higher binding and internalization (Figure 7). This could be generally attributed to the differential expression of uMUC1 in these two cell lines. CaPan-2 cells are known to produce high levels of mucin<sup>152, 156</sup>, which may act as a barrier to efficient polymer binding or uptake<sup>92</sup>. Additionally Panc-1 cells have a faster

doubling time (52 h) than CaPan-2 (96 h) <sup>115, 156-158</sup> which would indicate a more aggressive cell line with faster rates of internalization.

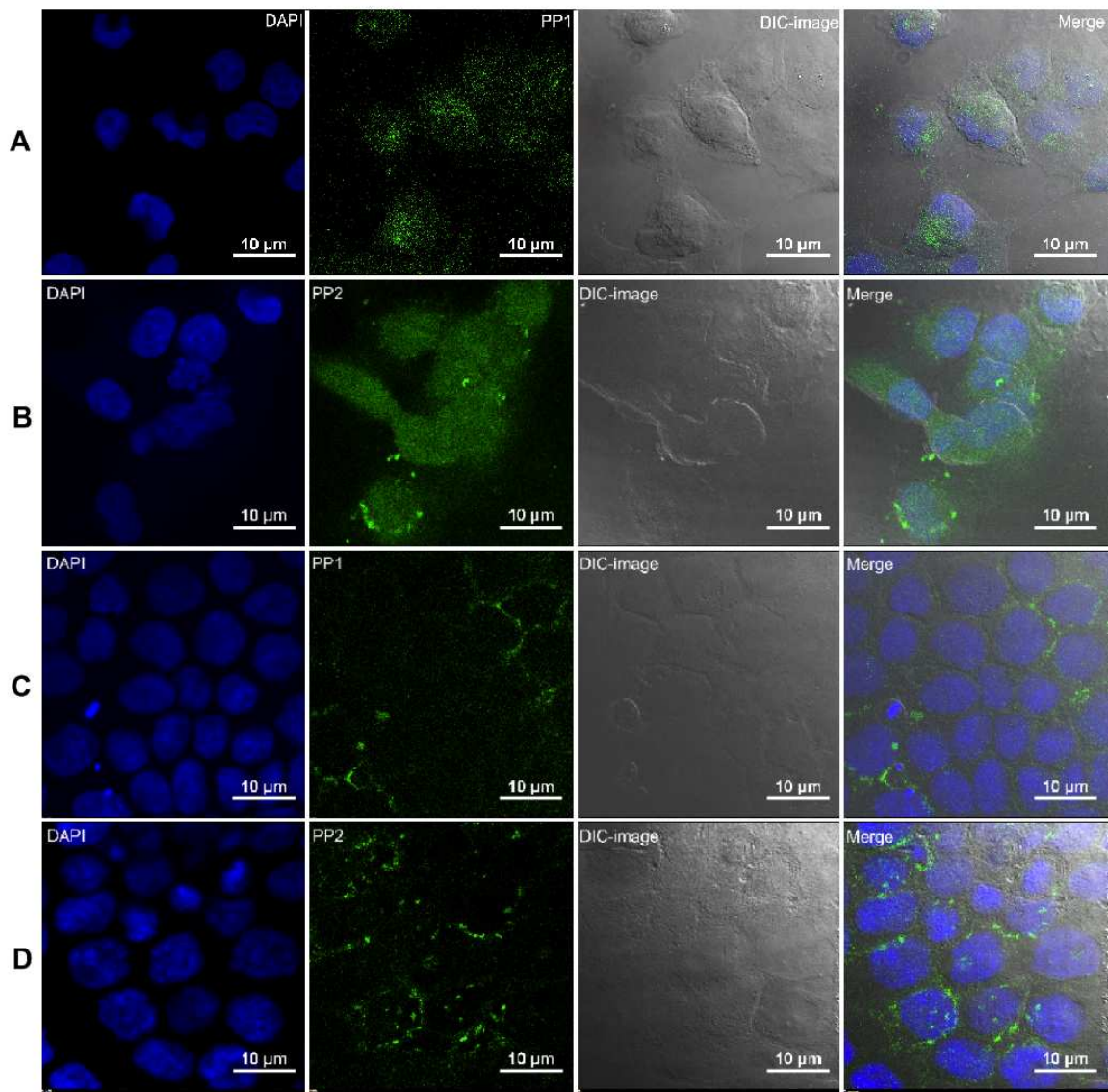


**Figure 7: Flow cytometry studies.**

In graphs PP1 polymers were incubated with a polymer without a peptide (no active targeting). The bottom 3 graphs represents incubation in Panc1 cells. The top 3 graphs represents incubation with CaPan-2 cells. In graphs PP2 was incubated for 30mins with varying concentrations (10uM, 20uM and 40uM) of EPPT1 peptide. In graphs PP3 was incubated for 30mins with varying concentrations (10uM, 20uM and 40uM) of EPPT1 peptide. Black bar graphs represent blocking of the uMUC1 receptor competitively with the free peptide EPPT1 for 30 mins prior to polymer conjugates that have the EPPT1 peptide attached. White bar graphs represent polymer peptide conjugates treated to cells without pre-treatment of EPPT1 peptide. \*\* p < 0.001, \*\*\* p < .0001

### **3.4.3 Confocal internalization studies.**

Internalizations studies (Figure 8) further confirmed that polymer peptide conjugates were getting internalized in the cells. The purpose of this qualitative study was to visualize that polymer-peptide conjugates effectively entered the cellular cytosol, which further corroborated flow cytometry data. Each slide was treated with PP2 and PP3 with 40 $\mu$ M of the EPPT1 peptide for 30mins. Confocal visualization suggests that Panc1 cells had a higher intracellular green fluorescence levels, in line with flow cytometry observations. PP3 shows more green fluorescence than PP2. CaPan-2 cells still showed internalization of the polymer peptide conjugate, but at a strongly reduced level compared to Panc1 cells.



**Figure 8: Internalization studies.**

Polymer peptide conjugates were treated with PP2 and PP3 at 40μM EPPT1 concentration for 30mins. (A) and (B) represent internalization of PP2 and PP3 respectively in Panc1 cells. (C) and (D) represent internalization of PP2 and PP3 respectively in CaPan-2 cells. Starting from left images are of DAPI, FITC, DIC and finally a merger of all 3 images.

### 3.4.4 Characteristics of polymer-drug conjugates.

After completing polymer-*peptide* conjugate characterization studies, the next series of experiments involved the synthesis, characterization and in vitro evaluation of a series of polymer-*drug* conjugates (without EPPT1 peptide) (See Table 4). These polymers had a general structure of HPMA-(MA-GG-ONp)-(MA-GFLG-Gem)-FITC. In an effort to optimize the side chain composition, the content of MA-GG-ONp (0, 5, 10 mol%) and MA-GFLG-Gem (0, 5, 10 mol%) were varied. FITC content was kept constant at 2 mol% as before. Upon measuring the content of the various comonomers in the polymer-drug conjugates (PD1-PD5, Table 4) it was observed that Gem incorporation is influenced by the content of MAGGONp. When Gem feed composition was kept at 5 mol%, an increase in the feed ratio of MA-GG-ONp from 5-10 mol% resulted in an increase in incorporated ONp content. However, it was observed that upon increasing the ONp content in the feed to 10 mol% a threshold was reached where Gem incorporation remained the same independent of feed mol%. It was interesting to observe that with 10 mol% feed of Gem, the incorporated reactive ONp groups were not accessible to alkaline hydrolysis (which is critical for subsequent peptide conjugation). In all the polymers, the incorporated FITC content was constant as expected but was generally lower than the polymer-peptide conjugates as discussed earlier. Overall, we concluded that incorporation of multiple comonomers in a polymer chain by random copolymerization will influence the content of each monomer making it extremely challenging for optimization of individual monomer content. SEC data in Table 4 revealed that polymer drug conjugates with ONp-containing side chains (PD2-PD5) were generally smaller than

the control polymer without ONp (PD1), possibly due to the chain-terminating effect of ONp groups.<sup>159</sup>

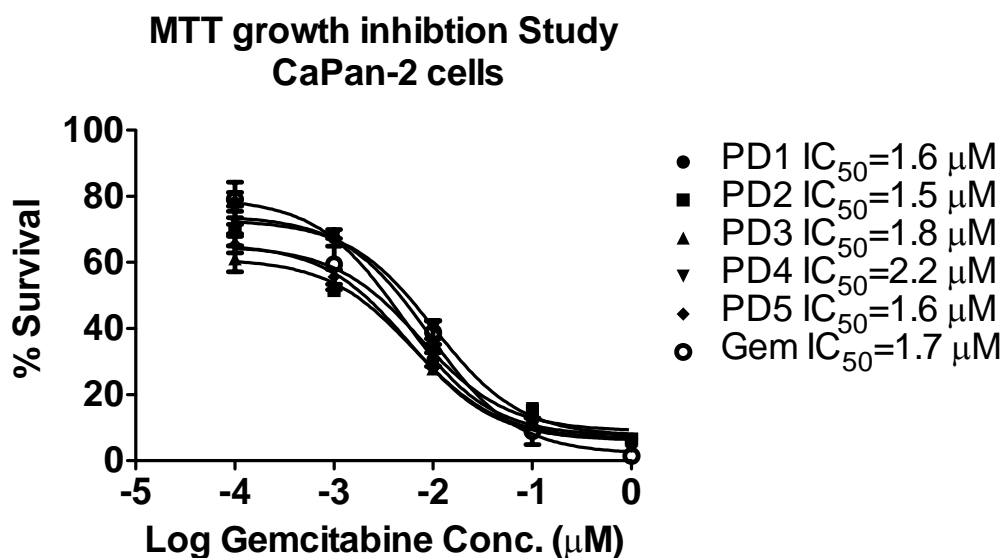
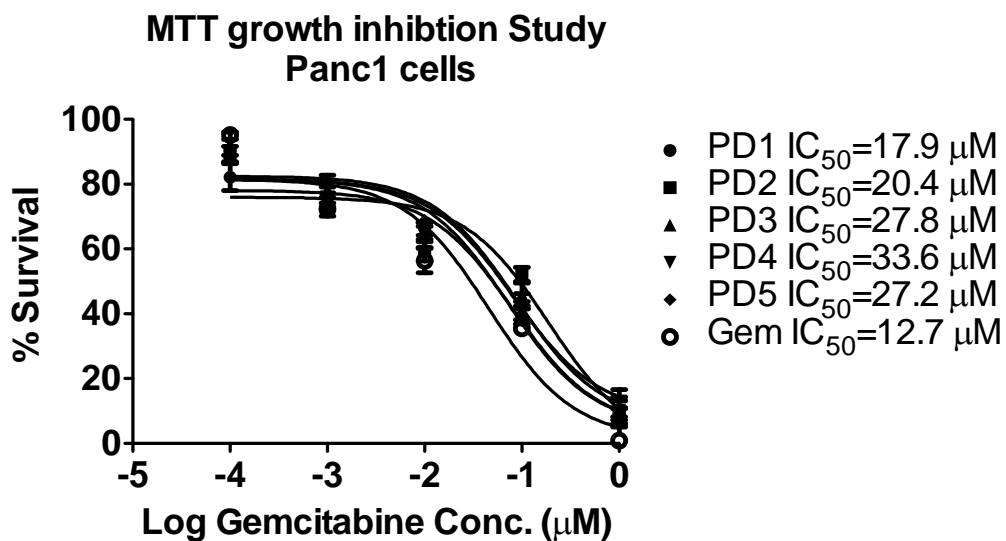
Sample	Feed composition of monomers (mol%)				Polymer characteristics				Size Exclusion Data		
	HPMA	MaGem	MAGGONp	APMA-FITC	MaGem content <sup>(a)</sup>	Total ONp content <sup>(b)</sup>	Accessible ONp content <sup>(c)</sup>	FITC content <sup>(d)</sup>	M <sub>w</sub>	M <sub>n</sub>	Polydispersity
					(mmol/g polymer)				g/mol	g/mol	n
PD1	93	5	0	2	0.36 ± .04	-	-	0.06 ± .07	90129	60320	1.8
PD2	88	5	5	2	0.22 ± .05	0.64 ± .03	0.24 ± .03	0.04 ± .02	33236	24345	1.4
PD3	83	5	10	2	0.31 ± .12	0.74 ± .03	0.00 ± .02	0.08 ± .06	38133	26061	1.5
PD4	83	10	5	2	0.46 ± .03	0.67 ± .03	0.46 ± .06	0.06 ± .03	24389	60320	1.3
PD5	78	10	10	2	0.31 ± .14	0.55 ± .06	0.00 ± .06	0.06 ± .03	39146	28142	1.4

**Table 4: Physicochemical Characterization of HPMA-MGGONp-MaGem Copolymer Conjugates**

Determined by: <sup>a,b,c,d</sup> UV spectroscopy. MaGem  $\epsilon_{268\text{nm}} = 9360 \text{ M}^{-1} \cdot \text{cm}^{-1}$  Total ONp  $\epsilon_{278\text{nm}} = 9500 \text{ M}^{-1} \cdot \text{cm}^{-1}$   
 Accessible ONp  $\epsilon_{400} = 9000 \text{ M}^{-1} \cdot \text{cm}^{-1}$  FITC  $\epsilon_{495\text{nm}} = 82000 \text{ M}^{-1} \cdot \text{cm}^{-1}$

### **3.4.5 Cytotoxicity of polymer drug conjugates.**

All polymer drug conjugates were tested for cellular toxicity on CaPan-2 and Panc-1 cell lines and compared to free Gem by employing the MTT assay. A range of concentrations from (0.0004-0.464  $\mu\text{M}$ ) of Gem equivalent was studied. Results in Figure 9 indicated that all polymer-drug conjugates irrespective of their Gem incorporation caused growth inhibition of both cell lines in a concentration-dependent manner. The measured  $\text{IC}_{50}$  values were similar and comparable to that of free Gem as indicated by previous research on both cell lines <sup>160</sup> and also show that Gem retained its bioactivity upon polymeric conjugation.



**Figure 9: Cytotoxicity of polymer drug conjugates.**

All polymer drug conjugates for both cell lines were treated with a range of concentrations (0.4-464 μM) Gem equivalent. Both cell lines were treated with free gem for negative control. In both cell lines, all polymer-drug conjugates showed concentration dependent anticancer effect that was comparable to free drug. IC<sub>50</sub> of free drug was comparable to research<sup>160</sup>.

### 3.5 DISCUSSION

Drug delivery via HPMA copolymers has been successful for enhancing the locoregional delivery of conventional anticancer agents since they were first introduced back in the 1970s<sup>120</sup>. The nanocarrier's ability to conjugate different drugs and targeting moieties, prevent the random diffusion of cytotoxic compounds into healthy tissues, consequently prolong the residence of the drug in the blood circulation and access tumors via the EPR effect of the neo-vasculature supporting solid tumors<sup>115, 161</sup>. Here, we aim to develop a targetable delivery system for trafficking therapeutic cargo to pancreatic cancer cells. MUC1 was chosen as an ideal pharmacological target because it is heavily glycosylated in normal pancreatic tissue and under-glycosylated (uMUC1) only in neoplastic tissue. This reduced glycosylation permits access to the peptide core and reveals epitopes that are normally hidden.<sup>86</sup> In order to develop a successful targetable polymer conjugate drug delivery system, our first step was to optimize the peptide incorporation in the polymers that will provide most favorable binding properties. As previously discussed in results, we were able to develop consistent nanocarriers. The MAGGONp content in the polymer precursor conjugates increased as we increased the amount of MAGGONp feed comonomer content Table 2 and Table 3 consistent with literature data<sup>153, 155</sup>. Size exclusion data Table 3 confirmed that higher precursor MAGGONp content increased the amount of peptide that was subsequently conjugated to the polymer<sup>153, 154</sup>. It was also observed that polymer-peptide conjugates were hydrodynamically larger than their precursors, which suggests that peptide is covalently bound to the polymer. Additionally, peptide bound to the polymer may have increased the polydispersity (n) of conjugates. Next, the binding and internalization of the polymer peptide conjugates were tested on

CaPan-2 and Panc-1 cell lines both of which are model pancreatic cell lines<sup>35-38</sup>. CaPan-2 is a good candidate for binding studies due to the fact that it features the highest level of MUC1 mucin granules amongst pancreatic cell lines. Panc-1 serves as a control cell line with lower overall expression of MUC1 mucin granules<sup>35-38</sup>. Using two cell lines with differential MUC1 expression might give a better perspective on general drug targeting efficiency. Flow cytometry data Figure 7 demonstrates that binding and internalization occurs in both cell lines, under both competitive and non-competitive conditions. As expected, binding/internalization was higher under non-competitive conditions compared with a competitive challenge using free peptide. It was also observed that as the amount of peptide bound to the polymer increased, the net fluorescence intensity increased correspondingly, suggesting higher intracellular delivery due to receptor-mediated internalization.

Total cellular binding/uptake of polymer-peptide conjugates in Panc-1 cells was higher compared to CaPan-2 cell lines (Figure 7). We hypothesize that even though Panc-1 has a lower MUC1 receptor expression, this cell line has a growth rate that is about twice as fast as the CaPan-2 cell line.<sup>156, 162</sup> supporting the observation that polymer-peptide conjugates internalization is higher and occurs at a higher rate in this cell line compared to Capan-2. The clinical significance of this finding is that both cells types show binding/internalization of the polymer-peptide conjugate demonstrating that MUC1 receptors can be effectively used for targeting and delivery to a variety of MUC1 receptor-expressing cancerous cells<sup>83</sup>. Confocal microscopy imaging (Figure 7) further confirmed internalization of polymer-peptide conjugates observed via flow cytometry.

Qualitatively, Panc1 cells clearly demonstrate increased intracellular fluorescence compared to CaPan-2 cells.

Once a peptide composition with efficient cellular binding was identified, we continued development of a second series of conjugates containing Gem (but no peptide) to allow optimization of drug content for therapeutic efficacy. Gem incorporation was influenced by the presence of other reactive comonomer (MA-GG-ONp) in the same polymer backbone. Importantly, this would provide challenges to the subsequent incorporation of peptides to drug-containing polymer backbone. It was specifically observed that a 10 mol% feed composition of MA-GG-ONp resulted in a polymer-containing pendent ONp group but none of those groups were hydrolysable under alkaline conditions. This observation suggests a significant change in the conformation of the final polymer-drug precursor possibly entrapping the ONp groups in its core in a micellar conformation. This would prevent polymer-precursors with 10% or higher content of ONp to be subsequently used to attach a peptide via ONp aminolysis. The conjugates however maintained bioactivity of incorporated Gem as evidenced by growth inhibition (Figure 9). Although polymer-drug conjugate cytotoxicity is not superior to free drug, their relative comparable efficacy is encouraging since these conjugates will display altered biodistribution in vivo that will favor tumor disposition (due to EPR and active targeting). Moreover, when such conjugates are administered in vivo they may feature reduced exposure to normal cells, thereby reducing side effects while maintaining their chemotherapeutic effects on pancreatic cancer cells. Finally, systemic toxicity can be expected to be minimal since Gem would be inactive until its release in the lysosome of the cellular cytoplasm.

In this report, we systematically obtained optimization criteria for a prospective polymer-peptide-drug conjugate containing HPMA copolymer, EPPT1, and Gem. Peptide and drug optimization parameters were investigated independently. We identified the threshold feed content of Gem (less than 5 mol%), FITC (2 mol%) and reactive precursor MA-GG-ONp (less than 10 mol%) (Table 4: Physicochemical Characterization of HPMA-MGGONp-MaGem Copolymer Conjugates). We further determined that 5-10 mol% peptide incorporation would result in active targeting to pancreatic cancer cells via uMUC1 binding. Future studies would combine the advantages of both an active targeting polymer and a potent chemotherapeutic drug. We hypothesize that adding gemcitabine to an already optimized active targeting polymer could significantly increase the potency of gemcitabine and thereby improve its chemotherapeutic effect. Targeted drug delivery for improving chemotherapy has been employed in a number of phase 2 and phase 3 clinical trials<sup>99, 148</sup>; however, these trials are limited by the availability of unique pharmacological targets. Our study for the first time uses the potential targeting ability of the EPPT1 peptide as a targeting agent for pancreatic cancer-directed therapeutics. By utilizing the MUC1 receptor we demonstrate the possible exploitation of a novel overexpressed ligand on pancreatic tumors, by revealing that the present drug-polymer conjugate is comparably effective to free drug.

## **Chapter 4. HPMA based Gemcitabine delivery to pancreatic cell**

### **MUC1 receptors**

#### **4.1 Abstract**

Pancreatic cancer therapies are inadequately successful due to their major side effects and lack of sufficient therapeutic response. An active targeting drug delivery system could be employed to overcome the shortfalls of these therapies. The goal of the present study was to evaluate the targetability and efficacy of N-(2-hydroxypropyl) methacrylamide (HPMA) copolymer-(EPPT1)-Gemcitabine conjugates as novel macromolecular drug delivery systems against pancreatic cancers. In previous studies, we developed a polymer-peptide conjugate which can actively target pancreatic cancer cell lines and also developed a polymer drug conjugate which showed equally effective cytotoxicity compared to free drug. In this study, we continue our development of a polymer peptide drug conjugate that confirms a 5%–ONp incorporation which EPPT1 peptide is linked too is sufficient to reach the intracellularly lysosome compartments. We also verify that in lysosomal conditions, where the polymer conjugate is incubated with a known lysosomal enzyme cathepsin B and at a pH of 5.5, gemcitabine is released regardless of whether the peptide EPPT1 is bound or not. Mechanistic studies using endocytosis inhibitors were

done to determine that clathrin-mediated endocytosis is one potential pathway our polymer peptide conjugates are being internalized. Cytotoxicity studies were performed with three different endocytosis inhibitors to establish the optimal concentration for internalization studies. Two finalized polymer peptide drug conjugates were used in cytotoxicity studies compared to free drug and found to improve therapeutic effect. The study concludes for in vitro studies having found two potential polymer peptide drug conjugates which could be used for further studies in vivo to help improve pancreatic cancer treatment.

## **4.2 Introduction**

Although there have been many new developments in the fight towards pancreatic cancer, new statistics have shown no decrease in the survival rate<sup>1, 38, 135</sup>. Neoplasms of the pancreas are one of the most deadly forms of cancer<sup>1, 135, 163</sup>. Treatments have been slow to change and yet incidence of this disease almost equals in mortality rate<sup>135, 163</sup>. Gemcitabine (Gem) is considered the leading chemotherapy for pancreatic cancers<sup>1, 136</sup>. Its mechanism of action is primarily as a cell phase specific target by disrupting cells undergoing DNA synthesis<sup>4</sup>. Previous clinical trials for treatment of pancreatic cancers with gem have shown a median survival rate of less than 6 months<sup>4, 136</sup>. However, gemcitabine has with many side effects associated with it. Side effects for cancer treatment bring about non-specific toxicity, pulmonary toxicities, and renal failure<sup>4, 136</sup>. Although, still under heavy investigation, tumor cell resistance to gemcitabine treatment have also been reported<sup>136</sup>. In this section we aim to overcome the many pitfalls of the free gemcitabine treatment. Therefore, we are using N-(2-hydroxypropyl) methacrylamide (HPMA), a water soluble polymer to assist in

gemcitabine treatment. As previously reported, HPMA copolymers are widely used as drug carriers due to their favorable properties of being highly water soluble, non-immunogenic and are adaptable for large scale production<sup>164</sup>. Previously we covalently linked gem via a glycine-phenylalanine-leucine-glycine (GFLG) side chain to HPMA backbone<sup>164</sup>. This degradable peptide linker is stable in normal circulation but is cleaved by lysosomal proteases such as cathepsin B, once internalized by endocytosis<sup>101, 149</sup>. This linker is vital for HPMA drug delivery because the drug remains non-active while attached to the polymer. Moreover, by keeping it inactive until it gets into the cell, it is also brought closer to the site of action and thereby increases its therapeutic effect<sup>100, 101, 149</sup>. The other part of getting gem into the cell is by actively targeting pancreatic cancer cells. As previously stated, the MUC1 receptor is an ideal candidate for targeting because of its large over-expressed mucin granules that are exposed on the apical surface of almost all human epithelial cell adenocarcinomas which includes pancreatic cancer<sup>86, 165</sup>. Being that the MUC1 receptor extends out further than most membrane molecules and in neoplastic tissue it becomes underglycosylated exposing its peptide core, it became an ideal target for our project<sup>92</sup>. EPPT1 is a peptide that has shown to have a significant affinity to the uMUC1 receptor with a  $K_d=20\mu\text{M}$ <sup>92</sup>. Our previous work has shown successful conjugation of free gem to the HPMA polymer<sup>164</sup>. In vitro studies with MTT assay also have proven that HPMA-Gem has equal toxic effect on 2 different types of pancreatic cell lines as free gem<sup>164</sup>. Other previous work has shown that when HPMA has the EPPT1 peptide bound to it via a non-degradable bond, there was an increase in cellular uptake. Our goal was to further narrow down the ideal polymer peptide drug conjugate that would be promoted for in vivo studies.

## 4.3 Materials and Methods

### 4.3.1 Materials

See section 3.3.1

### 4.3.2 Synthesis of Comonomers

Monomer building blocks were synthesized according to previously published methods:

(i) N-(2-hydroxypropyl)methacrylamide (HPMA, m.p. 66-68°C, MW 143.8), a comonomer that forms the polymer backbone and renders aqueous solubility<sup>150</sup>; (ii) methacryloyl-glycylglycyl-p-nitrophenyl ester (MA-GG-ONp, m.p. 160-163°C, MW 321.7,  $\epsilon_{273} = 9500 \text{ M}^{-1}\text{cm}^{-1}$ ), a non-degradable dipeptide linker containing reactive comonomer to which the EPPT1 peptide is attached following polymerization; (iii) 5-(3-(methacryloyl-aminopropyl) fluorescein isothiocyanate (APMA-FITC, MW 533.60,  $\epsilon_{495} = 8200 \text{ M}^{-1}\text{cm}^{-1}$ ), a fluorescent comonomer to monitor in vitro cellular binding and internalization<sup>150</sup>; (iv) methacryloyl-glycylphenylalanylleucylglycine (MA-GFLG-OH, MW 460.52), a lysosomally degradable tetrapeptide comonomer intermediate to which Gem is covalently bound prior to polymerization. The drug containing comonomer (v) MA-GFLG-Gem was synthesized by reacting Gem HCl with MAGFLGOH (1:1 molar) in anhydrous dimethylformamide (DMF) using N,N-diisopropylethylamine (DIPEA) (1.5x), hydroxybenzotriazole (HOBT) (1.1x), N,N'-dicyclohexylcarbodiimide (DCC) (1.1x) and 4-dimethylaminopyridine (DMAP) (.15x) at room temperature for 16 hours. The final product was isolated and purified by column chromatography (silica gel, mobile

phase: dichloromethane 83%/Methanol 15%/Acetic Acid 1%) and analyzed by thin layer chromatography ( $R_f$  0.48) and mass spectrometry (MW 706.3).

#### **4.3.3 Synthesis and Characterization of HPMA copolymer-Gem drug conjugates**

Polymer-drug conjugates were synthesized in one step by free radical precipitation polymerization as similar to polymer precursor as previously reported. HPMA, MA-GG-ONp, MA-GFLG-Gem and APMA-FITC. The content of pendent ONp, Gem and FITC were estimated by UV spectrophotometry. Gem and FITC were measured in 0.1N NaOH at 263 nm ( $\epsilon_{263}=9630M^{-1}cm^{-1}$  and 495 nm  $\epsilon_{495}=79000M^{-1}cm^{-1}$ ) respectively. Total ONp in the polymer was measured in DMSO/1% acetic acid ( $\epsilon_{272}=9100M^{-1}cm^{-1}$ ). To measure ONp content that is accessible/available for further conjugation to peptide, the ONp groups were hydrolyzed and released p-nitrophenol measured in 0.1N NaOH at 400nm ( $\epsilon_{400}=9000M^{-1}cm^{-1}$ ). ONp content for non-drug containing polymers was incorporated at 5 and 10%. ONp content for drug containing polymer were held at 5%. Gem content was varied at 5% and 2.5%. FITC incorporation was held to 2% for all polymers. The molecular weight and weight distributions of the polymer conjugates were estimated by SEC as previously reported<sup>164</sup>.

#### **4.3.4 Synthesis and characterization of HPMA-EPPT1-Gem conjugates**

EPPT1 conjugation to copolymers was synthesized in steps as previously reported(REF). The only difference is that conjugation of the EPPT1 peptide was also done to polymer that had Gem attached as well. Table 5 .

## **4.4 In vitro Evaluations**

### **4.4.1 Cell Culture**

CaPan-2 and Panc-1 (ATCC, Manassas, VA) (passages 10-35) were grown at 37°C in an humidified atmosphere of 5% CO<sub>2</sub>. Cells were maintained in T-75 flasks using Dulbecco's Modified Eagle's Medium (DMEM) and McCoy's 5A respectively, supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 10,000 units/mL penicillin, 10,000 µg/mL streptomycin and 25 µg/mL amphotericin B. Media was changed every other day and cells were passaged at 80–90% confluence using a 0.25% trypsin/ethylenediamine tetraacetic acid (EDTA) solution. Incubation buffer used in the assays consisted of Phosphate Buffered Saline (PBS) without calcium or magnesium chloride. For all experimental procedures, the confluent cultures were harvested by treatment with trypsin/EDTA.

### **4.4.2 Confocal Internalization**

Intracellular trafficking of polymer peptide conjugates of different ONp incorporation within CaPan-2 and Panc1 cells were examined for specific finalization of intracellular destination. CaPan-2 and Panc1 cells were seeded at 5,000 and 2,000 cell/cm<sup>2</sup> on 8-chamber slides respectively. Cell were kept for 3 days to 5 days of incubation at 37°C and 95% relative humidity and 5% CO<sub>2</sub>. After which cells were washed with warm

HBSS and then incubated with copolymer peptide conjugates in HBSS for 30 mins. After which cells were washed with ice-cold DPBS, and fixed with 4% (wt%) para-formaldehyde, 4% (wt%) sucrose in DPBS for 15 min. Cells were washed with permeabilization buffer (PBS containing 300 mM sucrose, 50 mM NaCl, 3 mM MgCl<sub>2</sub> · 6H<sub>2</sub>O, 20 mM HEPES, 0.5% Triton X 100 (vol%), pH 7.2), which was added to the samples and kept at 4°C for 5 min. The permeabilization buffer washed out twice with DPBS and then incubated at 37°C with blocking buffer (3% bovine serum albumin (BSA) (wt/vol) in DPBS) for 5 min. Cells were washed with DPBS and primary antibodies for lysosome-associated membrane protein 1 (rabbit polyclonal lysosome antigen – 1 (LAMP-1); Molecular Probes) (solution prepared at 1:500 vol.ratio in 3% BSA solution) were added to separate cell sample chambers and left for 30mins in the incubator at 37°C. The primary antibody was then removed and the cells were washed 3 times with blocking buffer and then the secondary antibody Alexa flour-488 goat anti-rabbit IgG (Molecular Probes, Carlsbad, CA) was added at 1:1000 in the blocking agent solution for 30 mins. The cells were then washed with 0.5 vol (vol%) Tween 20 in DPBS and finally three times with DPBS. Cells were then incubated with 300 nM 4',6-diamidino-2-phenylindole (DAPI) for 10 min to stain the nuclei. Finally, cells were then washed twice with DPBS and the chambers were detached. The slides were mounted and covered with glass coverslips. They were allowed to dry for a few hours before they were sealed with nail varnish and stored at 4°C. Images were acquired using a Nikon A1 Inverted confocal laser- scanning microscope (Nikon Instruments, Melville, NY). Co-localization between 5%ONp copolymer and 10%ONp copolymer in the lysosome was quantified using the Elements software supplied with the Nikon microscope. The extent

of co-localization between the overlapping channels was determined using the Mander's overlap coefficient ( $M_{x(oc)}$ ). The extent of co-localization between the red and green channels ( $M_{x(oc)}$ ) was calculated using the following equation:

$$M_{x(oc)} = \frac{\sum_i x_{icoloc}}{\sum_i X_i}$$

where  $x_{icoloc}$  is the value of voxels of the overlapped red with green components, and  $x_i$  is the value of the red component.  $M_{x(oc)}$  is reported for each treatment as an average of the 6 images where the threshold was adjusted to 5% for all images, in order to compensate for any background noise. This method of calculating the co-localization was adopted from a previously published journal articles<sup>166</sup>, however here we used Nikon Elements for acquisition and the Volocity 3D image analysis software version 6.3 (PerkinElmer Waltham, MA) to determine our co-localization values<sup>166, 167</sup>.

#### 4.4.3 Drug Release Studies

The *in vitro* release of Gemcitabine from HPMA copolymers was evaluated using the model lysosomal enzyme cathepsin B (CPB) according to previously described procedures with minor modifications<sup>101</sup>. Enzyme incubation mixture consisted of CPB stock solution (0.98 ml, 0.572 mg/ml (12.2 units/ml) in 0.1 M ammonium acetate buffer pH=5.5, 1 mM EDTA) and cysteine solution (0.02 ml, 250 mM in acetate buffer pH=5.5, 1 mM EDTA). 5 mg of HPMA copolymer–Gem conjugates P1 and HPMA-EPPT1-Gem (see Table I) were dissolved in the incubation mixture (1 ml, 5 min preincubation at 37°C). At 15, 60 and 180 min, 100 µl samples were removed, drug extracted twice with 1 ml ethyl acetate, organic layer separated and dried under nitrogen. Resulting residue was

dissolved in 0.5 ml of mobile phase and evaluated for drug content using reverse-phase high performance liquid chromatography (HPLC). Mobile phase consisted of 0.05 M ammonium acetate buffer pH=4.7 and acetonitrile (65:35, v/v) at a flow rate of 1 ml/min (ADD REF). HPLC analyses were performed with a Waters 717 autosampler (Waters Corporation, Milford, MA) with Waters 2487 dual wavelength detector set at 260 nm using a Waters XBridge column (C18, 4.6×150 mm, 5 µm). The analytical column was protected with a Waters Xbridge guard column (C18, 4.6×20 mm, 5 µm). A calibration curve was generated by extracting and processing as noted above standard solutions of Gemcitabine dissolved in 0.1 M ammonium acetate buffer pH=5.5 using the corresponding peak area *versus* concentration. Direct injections of standard solutions without extraction were used to determine extraction efficiency. Percent drug release was calculated from the drug content of the copolymers measured by UV spectroscopy and the content of free drug quantified by HPLC. A stock solution of the standard substrate *N*- benzoyl-Phe-Val-Arg-p-nitroanilide hydrochloride (50 mM in DMSO) was used to verify enzyme activity at the given time intervals.

#### **4.4.4 Cytotoxicity of Endocytosis Inhibitors**

Cytotoxicity of endocytosis inhibitors was assessed in both Panc1 and CaPan-2 cell lines to ensure the cell viability over short-term exposure to these chemicals during uptake experiments. Endocytosis inhibitors were prepared at a range of concentrations known to reduce the different pathways (purchased from Sigma Aldrich, St. Louis, MO). Inhibitors used for their respective pathways were as follows: monodansyl cadaverine (MDC) (150 to 600 µM) for clathrin mediated endocytosis; filipin (FIL) (2 to 8 µM) for reduction of

caveolin-mediated endocytosis; and dynasore (DYN) (25  $\mu$ M to 100  $\mu$ M) for dynamin-dependent endocytosis. (The range of all the inhibitors was determined through literature<sup>166, 167</sup>. Cytotoxicity of the inhibitors was assessed by the water-soluble tetrazolium salt (MTT assay) assay. Panc1 cells were seeded at 25,000 cells per well and CaPan-2 cells were seeded at 35,000 cells per well in a 96-well plates(Corning, NY). Cells were incubated at 37°C, 95% relative humidity and 5% CO<sub>2</sub> for 48 h. Cells were washed with warm Hank's balanced salt solution (HBSS) buffer and incubated for 2 h with 100  $\mu$ L solutions containing a varied concentration of endocytosis inhibitors. The solutions were removed after 2 h and cells were washed twice with HBSS buffer. MTT assay reagent solution was added to each well and incubated for 4 h at 37°C. Following the incubation time, MTT was dissolved in DMSO and the plate was mixed well and then measured for absorbance on a plate reader. Absorbance at 460 nm and background at 600 nm were measured using a SpectraMax plate reader (Molecular Devices, Sunnyvale, CA). Cells incubated in HBSS were used as the negative control for 100% viability and cells incubated in Triton-X were used as the positive control. Cell viability  $\geq$ 80% was classified as concentrations acceptable for uptake studies. As represented in Table II, MDC at 300 $\mu$ M, FIL 4  $\mu$ M and DYN at 50  $\mu$ M were used for the cellular uptake studies.

#### **4.4.5 Cellular Uptake**

Cellular uptake of the copolymers was determined in the presence and absence of endocytosis inhibitors. Inhibitors used were at concentrations that showed a minimum of 85% of viability during the 2 h period of the assay incubation period. Panc1 and Capan-2 cells were seeded at 150,000 cell/well and 300,000 cells/well in a 12-well plate (Corning, NY) and grown for 3 days - 4 days at 37°C, 95% relative humidity and 5% CO<sub>2</sub>. Culture

medium was then removed and cells were washed twice with warm HBSS. The required amount of HBSS and Polymer solution was added to each well such that the total volume was 400  $\mu$ L. Cells were incubated with the copolymer solution for 1 h at 37°C. The copolymer mixture was then removed and cells were washed twice with a 1 mL solution of ice-cold Dulbecco's phosphate buffered saline (DPBS). Cells were then incubated with trypsin for 5 min after which cell culture medium was added to halt this detachment process. Cells were removed from plates, transferred to microcentrifuge tubes, and centrifuged for 4 min at 1500 rpm. After removing the supernatant, cells were washed in DPBS and finally fixed in 1% (wt%) paraformaldehyde (in DPBS) solution. Flow cytometry was used to measure the cellular fluorescence using the BD LSR flow cytometer (Becton Dickenson, Franklin Lakes, NJ) with filters, Ex - 495 nm; Em - 519 nm. A 200  $\mu$ L solution of 0.4% (wt%) Trypan Blue was added to each sample and 10,000 events to 20,000 events were taken per sample for 3 repeat experimental samples. Percentage uptake was determined for different cell populations by the shift in mean fluorescence (region of interest was determined using FlowJo software) in the presence of endocytosis inhibitors compared to the controls in HBSS. Uptake analysis for the influence of liposome size on cell populations was determined by comparing the shift in means. The data for fluorescence intensity events for the polymer recorded by flow cytometry was normalized to the total uptake of liposomes at 37°C in the absence of any inhibitor (this gives the 100% value on the graph) and to the difference between the uptake in presence of inhibitors by its uptake value at 4°C (for the copolymer the 4°C uptake was different since this represents the passive uptake and hence highlights the

region of interest). Hence the values presented represent the percentage of copolymer not affected by the inhibitor

#### **4.4.6 Cytotoxicity of polymer peptide drug conjugates**

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to determine in vitro cell growth inhibition of Gem and HPMA copolymer-EPPT1-Gem drug conjugates against the model pancreatic cancer cell lines, CaPan-2 and Panc-1. Briefly, CaPan-2 and Panc-1 cells were seeded into 96-well plates at an initial density of 5,000 and 2,000 cells/well respectively and allowed to attach for 24 h. Cells were treated with increasing concentrations of gemcitabine or copolymers in fresh media and incubated for 72 h. The efficacy of the polymer peptide drug conjugates were compared to the free drug by calculating the growth inhibitory coefficient (IC<sub>50</sub>) based on the viability vs. concentration curves. All experiments were performed in triplicate.

### **4.5 Results**

#### **4.5.1 Characterization of HPMA Copolymer conjugates**

Polymer peptide conjugates and Polymer peptide drug conjugates that were synthesized and characterized retained similar properties as previously reported. The characteristics of HPMA copolymers with or without Gemcitabine and EPPT1 peptide are summarized in Table 5. The content of each monomer was determined to be nearly equivalent to theoretical amount and comparable to previously reports. As demonstrated previously, we measured the total ONp in the polymer and the accessible ONp along with Gemcitabine content and FITC content. Peptide content was measured by amino acid analysis. Estimated molecular weight of each polymer precursor and polymer with/without drug

were measured. Results showed similar size differences and polydispersity compared to previously reported in section 3.4.1.

*Physicochemical Characterization of HPMA-MAGGONp-MaGem Copolymer Conjugates*

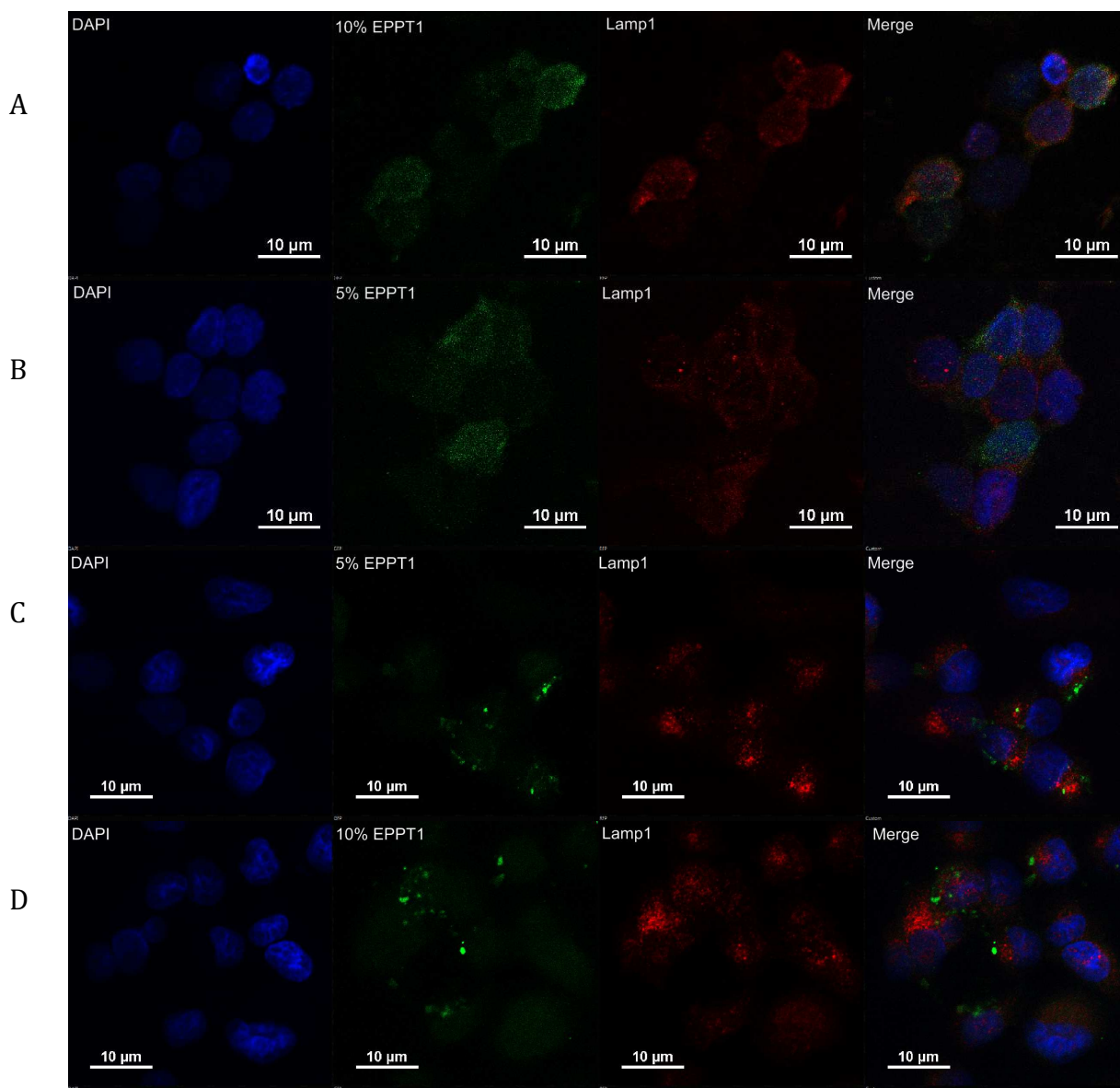
Sample	Feed composition of monomers			Polymer characteristics						Size Exclusion Data				
	HPMA	MaGem	MAGGONp	MaGem Content <sup>d)</sup>	Total ONp Content <sup>b)</sup>	Accessible ONp Content <sup>c)</sup>	Peptide Content <sup>b)</sup>	FITC Content <sup>d)</sup>	M <sub>w</sub> <sup>f)</sup>	M <sub>n</sub> <sup>f)</sup>	Polydispersity <sup>g)</sup>	M <sub>w</sub> <sup>f)</sup>	M <sub>n</sub> <sup>f)</sup>	Polydispersity <sup>g)</sup>
				mmol/g of polymer						Polymer Precursors			Polymer Peptide Drug Conjugates	
									<u>g/mol</u>	<u>g/mol</u>	<u>(n)</u>	<u>g/mol</u>	<u>g/mol</u>	<u>(n)</u>
A*	92	-	5	-	.31 ± .04	.25 ± .03	0.03	.13 ± .01	29,074	19,846	1.5	44,242	25,620	1.7
B*	88	-	10	-	.57 ± .02	.55 ± .03	0.07	.10 ± .01	22,052	16,693	1.3	43,387	24,597	1.8
C	88	5	5	.28 ± .03	.25 ± .04	.23 ± .02	0.016	.11 ± .01	33,092	23,319	1.4	54,798	51,968	1.1
D	90.5	2.5	5	.11 ± .05	.29 ± .09	.27 ± .01	0.022	.12 ± .01	28,506	20,692	1.4	46,525	26,109	1.8

**Table 5: Characterization of Polymers**

Determined by:<sup>a,b,c,e</sup> UV spectroscopy. <sup>e</sup> Amino acid analysis. <sup>f</sup> Size exclusion chromatography. <sup>g</sup> Polydispersity. \* for polymers A and B indicated previously mentioned result Table 2. Provided here for comparison only.

#### **4.5.2 Co-localization of Polymer Peptide Conjugates**

Intracellular trafficking of polymers A and B (without drug) was done to determine whether an HPMA copolymer with 5% ONp would be able to traffic to the lysosome. It was already determined that in previous studies that a 10% ONp copolymer with EPPT1 peptide attached had more uptake in flow cytometry than a 5% ONp copolymer. However, in this studies, the ultimate goal was to get the polymer with drug accumulate in lysosomal compartments where the molecule is digested ultimately releasing the drug and subsequently be transported to the nucleus to inhibit S-phase cell production. Polymers were colocalized at a concentration of 40uM of EPPT1 with lysosome (LAMP1) compartment after 30 min incubation at 37°C. CaPan-2 cells when tested against both copolymers 5% and 10%, they showed similar levels of co-localization with lysosomal regions independent of the co-polymer content. In Panc1 cells, however, the 5% co-polymer seemed to show slightly less accumulation within lysosomal compartments than that compared with the 10% co-polymer, as represented in Figure 10.



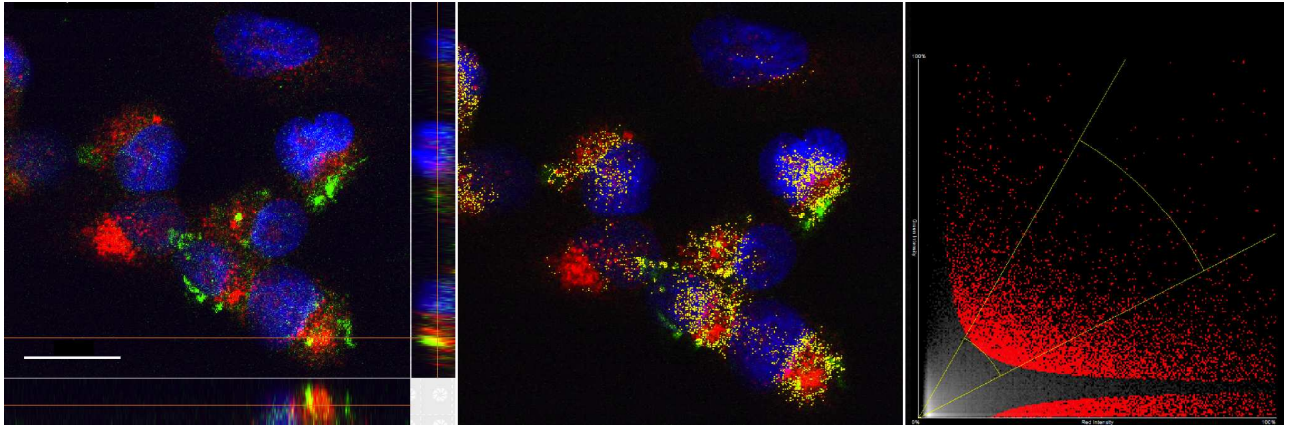
**Figure 10: Confocal imaging of colocalization of Polymers A and B with lysosomal regions.**

CaPan-2 (A and B) and Panc1 (C and D) cells were incubated for 30mins Polymer peptide conjugates (40uM EPPT1) A & C with 5% -ONp and B and D with 10% -ONp. Lysosomes were stained using Alexa Fluor 488 (green) for anti Lamp-1 antibody. Samples were examined by confocal microscope (Nikon A1). The nucleus was stained with Dapi.

5% ONp-Panc1

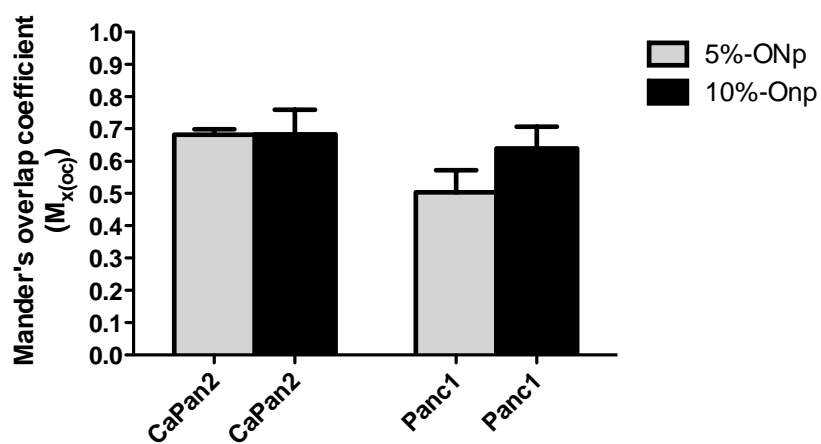
Areas of Co-localization

Co-localization ROI



**Figure 11: Measuring Co-localization using z-stacks**

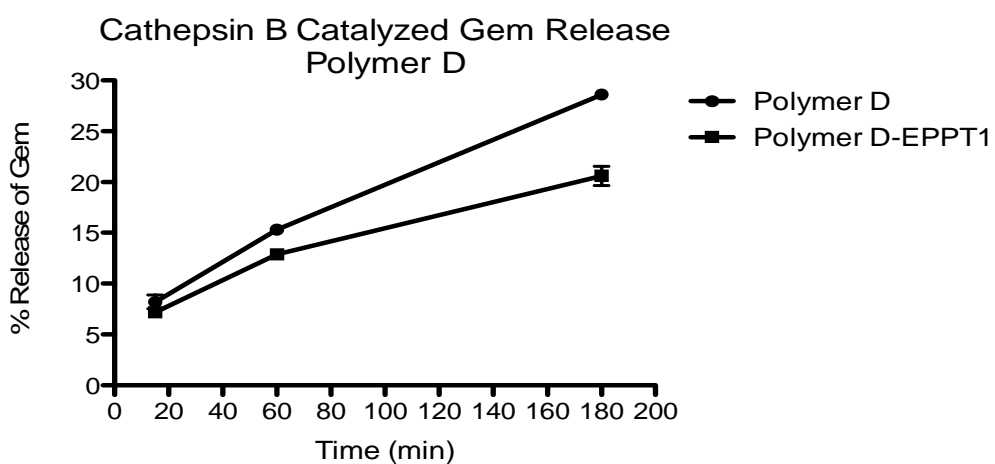
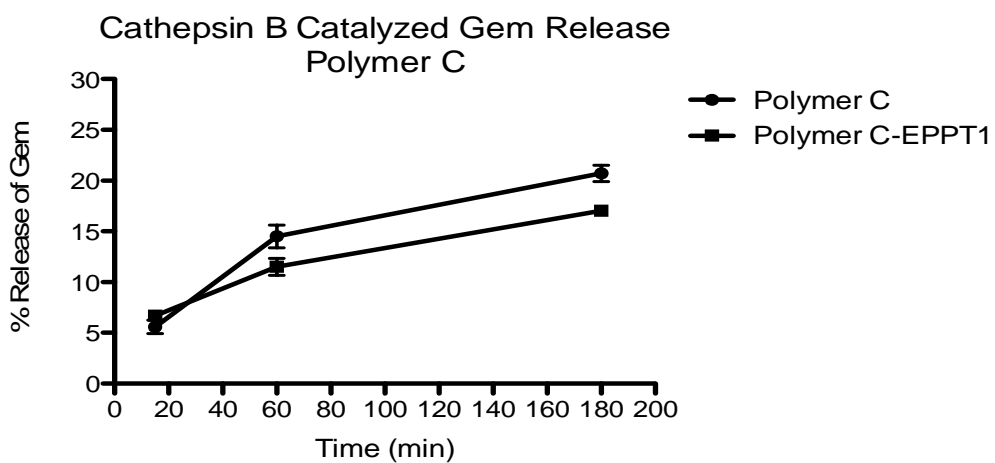
Images represent the measurement of co-localization for confocal z-stack for all samples used and explained in Figure 10. Here we have shown an example of the 5%ONp-Panc1 z stack, where the yellow color signifies the co-localized areas. The Co-localization Region of Interest represents the area of true co-localization and used to calculate the Mander's coefficient, when the threshold is set to a minimum of 5%.



**Figure 12: Co-localization of polymer A and B (gray and black) respectively with LAMP-1.** Intracellular trafficking was measured after 30min incubation. Colocalization between the different -ONp polymers and LAMP1 was measured using Mander's overlap coefficient ( $M_{x(oc)}$ ). Reported as Mean  $\pm$  Standard Deviation in 6 z-stack images.

### **4.5.3 Cathepsin B Catalyzed Drug Release from HPMA copolymer Gemcitabine conjugates**

Extent of drug release from HPMA copolymer products was evaluated at 15, 60, 180 mins in model lysosomal conditions. Time points beyond 180 mins showed degraded products (data not shown). Cathepsin B (CPB) catalyzed drug release was quantified and identified using reverse phase HPLC and extraction efficacy was calculated to be 95%. Incubation with CPB resulted in the release of free gemcitabine. Drug was extracted twice with 1 ml ethyl acetate, organic layer separated and dried under nitrogen. Resulting residue was dissolved in 0.5 ml of mobile phase and evaluated for drug content. Total drug release was compared when peptide was bound or unbound to the different polymers C and D. See Figure 13

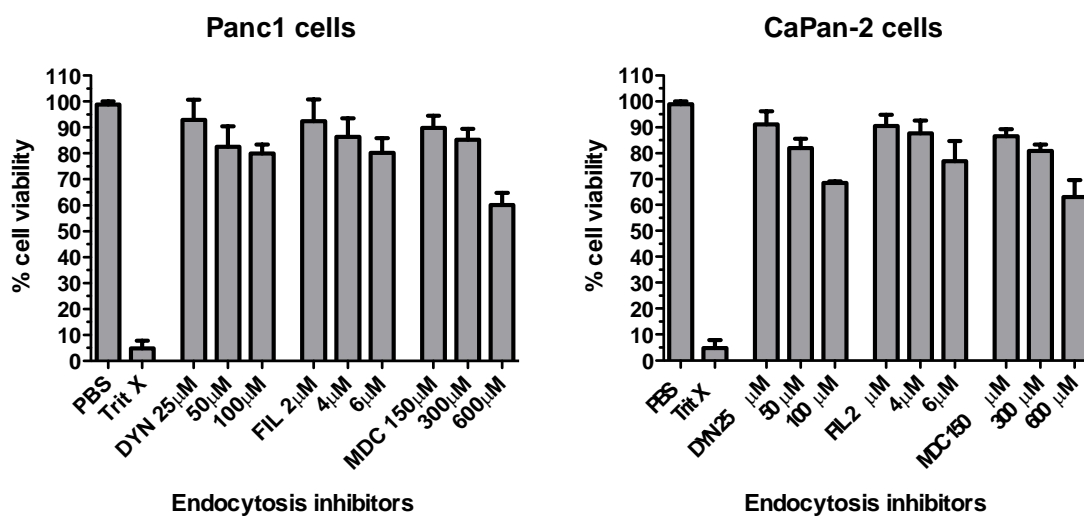


**Figure 13: Drug release of polymers C & D in model lysosomal conditions.** Comparison of copolymers with –EPPT1 peptide bound vs copolymers without –EPPT1 peptide bound. Time points were taken at 15, 60, and 180 mins. Time points beyond that degraded the polymers(Data not shown). %Release of Gem was measured. For sample characterization see Table. 4.1. Data represents mean  $\pm$  SD (n=3)

#### 4.5.4 Effect of Endocytosis Inhibition on Pancreatic Cancer cell lines

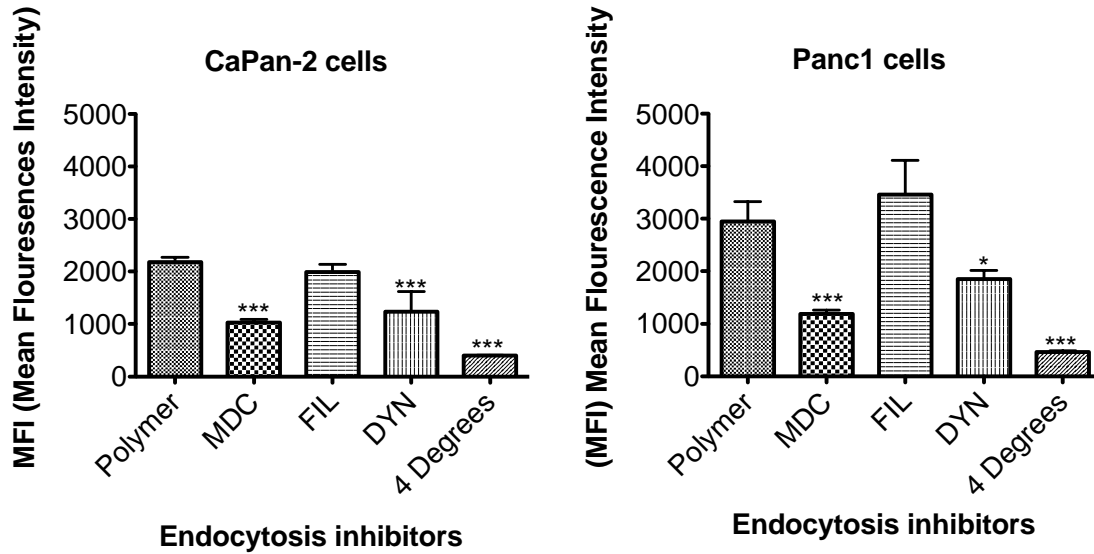
Three endocytosis inhibitors were used to examine the pathways of cellular uptake for polymer peptide conjugate A. The initial step of this process is to observe the Panc1 and CaPan-2 cell viability during a 2h treatment in the presence of endocytosis inhibitors. A range concentrations found in literature were used to not only test the viability of the cell lines but also to make sure the concentration was high enough to achieve endocytosis inhibition. The inhibition concentrations that showed cell viability above 80% were selected for the uptake assay. (Figure 14) The endocytosis inhibitor dynasore (DYN) (50uM), is a non-competitive inhibitor responsible for vesicle scission and GTPase activity of dynamin during both clathrin- and caveolin mediated pathways<sup>168</sup>. Monodansyl cadaverine (MDC) (300uM), was used to block clathrin mediated endocytosis. It is known to prevent formation of the clathrin coated pits, thereby preventing endocytosis<sup>166</sup>. Filipin (FIL) 4uM, was selected as an inhibitor of caveolin mediated endocytosis. It has been shown to disrupt this pathway by binding to cholesterol inside the caveolar pits, thereby disturbing the pathway. HPMA copolymers are strongly influenced by charge and molecular weight for their cellular uptake<sup>129</sup>. However, in our studies aimed to effectively distinguish whether our polymer uptake was dynamin dependent and either clathrin or caveolin mediated. The effect of each inhibitor was compared at 37°C and 4°C in the presence of each respective endocytosis pathway inhibitor at 37°C. At 4°C, passive cellular uptake is the only process functional. Low temperature suppresses all active endocytic pathways, thus copolymer uptake would be completely due to a passive internalization via the cellular membrane. In Figure 15, we

see that in the presence of 4°C, copolymer mean fluorescence intensity (MFI) was lowest. The uptake of the copolymer at 37°C without any endocytosis inhibitors represents a total cellular uptake. In Figure 15, represented the highest MFI for both cell lines. The highest inhibition we observed was in the presence of MDC (clathrin mediated pathway inhibitor). Both cell lines showed a statically significant reduction in MFI in the presence of MDC compared to positive control. FIL (caveolin mediated pathway inhibitor) showed to have the least effect on endocytosis uptake. MFIs for both cell lines were either equal or greater than positive control. In the presence of DYN (dynamin inhibitor), both cells saw a slight reduction in uptake but generally greater than the uptake the presence of MDC inhibition exerted.



**Figure 14: Cytotoxicity of endocytosis inhibitors.**

Both cell lines were incubated for 2 h at 37°C with various endocytosis inhibitors. The graph indicated the percentage of cell viability for the different concentrations for each endocytosis inhibitor and results are reported as mean  $\pm$  SD with n=6

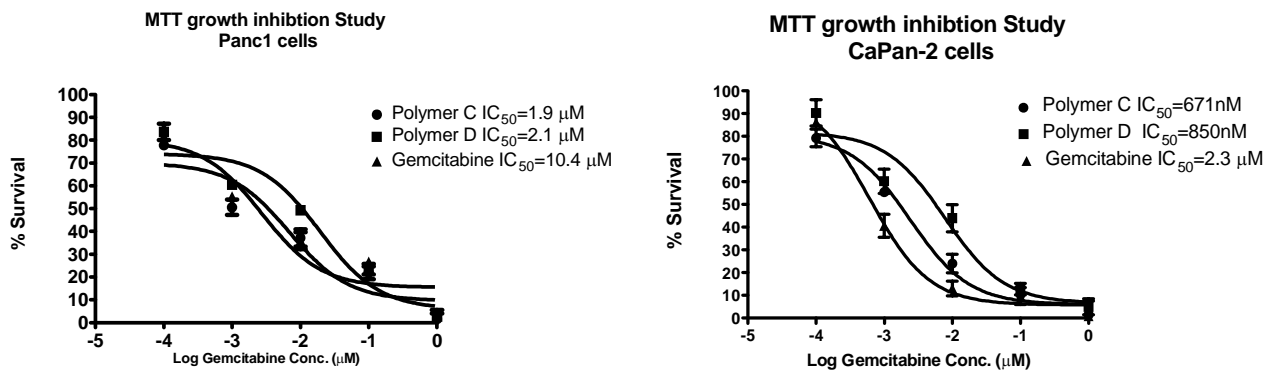


**Figure 15: Polymer uptake in presence of endocytosis inhibitors represented as mean fluorescence intensity (MFI).**

Polymer A was used for uptake studies at a concentration for EPPT1 at 40uM in presence of endocytosis inhibitors. Positive control represents no endocytosis inhibitors. Monodansyl cadaverine (MDC)(300uM), Filipin (FIL) (4uM) and Dynasore (DYN)(50uM) were used for inhibition. \*\*\* indicates a significant difference ( $p < 0.001$ ) by One-way ANOVA test when compared to positive control.

#### **4.5.5 Cytotoxicity of polymer peptide conjugates**

Polymer peptide drug conjugate C & D were tested for anticancer effects on CaPan-2 and Panc1 cell lines. These were then compared to free gemcitabine by using the MTT assay. A range of concentrations from (.0004-.464  $\mu\text{M}$ ) Gem equivalent was studied. Results indicated that  $\text{IC}_{50}$  values for the polymer peptide drug conjugates were lower compared to free gem. Thus polymer peptide drug conjugates provide a higher toxicity than free drug. For both cell lines polymer peptide drug conjugates improved the therapeutic effect of gemcitabine. See Figure 16.



**Figure 16: Cytotoxicity study of polymer peptide drug conjugates**

Polymer peptide drug conjugates C & D were treated in growth inhibition assay for 72 h. Treatment concentrations were kept to the same as previously reported Figure 9. In both cell lines polymer peptide drug conjugate showed more effective cytotoxicity than free gem. Gemcitabine IC<sub>50</sub> was comparable to previous reports Figure 9.

## 4.6 Discussion

The occurrence of pancreatic cancer have been on the rise in the past 10 year<sup>38</sup>. The need for therapeutics to evolve is essential in order to help the treatment and survival of this growing patient population. Drug delivery via HPMA copolymers have to shown improve the chemotherapeutics for certain types of cancers<sup>134</sup>. The delivery of a non-active drug payload has been proven that with the benefit of the EPR effect, the potential for an improved chemotherapy with reduced acute toxicities is a viable possibility<sup>118</sup>. This study continues the development of an HPMA copolymer with an active target EPPT1 peptide along with a chemotherapeutic drug, gemcitabine for pancreatic cancer as a possible improvement in therapeutic response. In Table 5, we characterize four polymers that were narrowed down from previous polymers conjugates. This characterization is for further develop the final optimal candidate for future in vivo studies. A and B polymers were studied again to make the final determination for the optimal ONp incorporation. It was also observed that A, C and D all were similar in the amount of MAGGONp content measured total or available. Gem content was measured in C and D polymers. Gem incorporation in the polymer lead to a increase in gem content which was measured using UV spectrometry. These measured contents of Gem were consistent with previously reported data See Table 4. FITC content was kept constant at 2% for all polymers and was shown to be consistent between polymers and previous reports Table 2. C and D are the newest polymers. Colocalization studies Figure 10, Figure 11 and Figure 12 confirmed that 5%ONp was successfully accumulate within lysosomal compartments. The final polymers were kept at 5%ONp since previous reports

showed that by increasing ONp causes a decrease in solubility and incorporation of other side chains. Amino acid analysis was done to provide the amount of EPPT1 peptide that was conjugated to the MAGGONp attached to the polymer. It was observed that the content remained consistent with the amount of ONp that was attached to the polymer backbone. Through size exclusion data, we observed that before the peptide is conjugated the size of all the polymers remains roughly the same. Polymer B is the exception because it has double the amount of MAGGONp. Once peptide is added to the polymers, the size of the polymers increase but again they stay relatively the same size. The only real difference is polymer C which has 5% ONp and peptide attached and 5% Gem attached was slightly larger. In Figure 10, Figure 11 and Figure 12 confocal studies were performed to observe the accumulation of polymer in the lysosomal compartment that has either 5% ONp or 10% ONp with peptide attached. Since our hypothesis is that the drug which is attached to the polymer via the lysosomally degradable linker, -GFLG we looked to confirm whether or not when peptide is attached to the polymer backbone does it colocalize with lamp1. Using Manders overlap coefficient we observed that all polymers were colocalizing into the lysosome at more than 50% when incubated for 30 mins in Panc1 and Capan-2 cell lines at 37°C. See Figure 12. This allowed us to confirm that even a 5% ONp polymer with EPPT1 conjugation would be sufficient to deliver a drug payload into the lysosome. Our hypothesis is that the rest of the non-colocalized polymer is being retained in the cellular matrix or endosome. For future studies it may be useful to look into whether if even more polymer can colocalize if incubated for more time. As a follow up to the colocalization studies, we provided evidence that the polymer with peptide was actually accumulating in the lysosome. However, the next step would be to

focus on the release of gem in the lysosomal conditions and therefore become a bioactive compound capable of disrupting DNA synthesis<sup>127, 149</sup>. Drug release from conjugates C and D was evaluated in vitro with model lysosomal enzyme CPB to understand the extent and differences in release profiles of polymers with or without peptide EPPT1. Figure 13 RP-HPLC was employed to accurately identify and quantify the release of gem in our drug delivery system. The time frame for the study was limited to 3 h because further incubation in acetate buffer (confirmed with previous reports) (pH 5.5) at 37°C caused degradation of the polymer<sup>153</sup>. We observed a slightly reduced enzymatically catalyzed drug release when EPPT1 targeting moiety was attached to the polymer Figure 13. We suspect that this reduction was due to the peptide limiting the accessibility of degradable peptide sequence –GFLG to the active site of CPB. The preliminary drug release studies were used to optimize the detection of release products using RP-HPLC following method development from free gem standards.

In our next study, we evaluated internalization of our polymer with 5% ONp by focusing on 3 main cellular uptake pathways, dynamin dependent, clathrin-mediated and caveolin mediated. These endocytosis pathways are distinctive in their internalization mechanisms by which pancreatic cancer cells respond to a nano-particle such a HPMA copolymer. Dynamin-dependent pathway plays an important role in the receptor mediated pathway by which the enzyme dynamin severs vesicles from the plasma membrane during endocytosis<sup>168</sup>. In clathrin mediated endocytosis, a formation of coated pits are assembled by the protein clathrin<sup>169</sup>. This triskelion-like shaped object composed of three clathrin heavy chains and three light chains forms the vesicle upon endocytosis of the particle. The caveolin-mediated pathway is regulated by the protein caveolin. This protein binds

cholesterol to sphingolipids in certain areas to form flask-like pits like a cave<sup>170</sup>. In our studies, we found that our polymer peptide conjugate uptake in both cell lines was inhibited when MDC was treated to the cells along with a slight inhibition of uptake from dynamin. This data would suggest that our internalization mechanism for receptor mediated endocytosis is heavily influenced by the clathrin mediated endocytosis pathway. Although this does not tell the whole mechanistic story, it is however one of the dominant pathways an HPMA copolymer is using. (Figure 15)

In our final study we looked to determine whether adding an active targeting moiety would improve cytotoxicity of gem Figure 16. In our previous section Figure 9, we found that when Gem was attached to our polymer without any active targeting was able to maintain the same cytotoxic effect as free drug<sup>164</sup>. In our most recent study, by adding the EPPT1 peptide we were able to improve the toxicity of gemcitabine in both cell lines. Free drug gem maintained a similar IC<sub>50</sub> in both cell lines compared to other published reports and our own previous results<sup>164</sup>. Although only a slight improvement was made, it is a further confirmation that this novel targeting peptide and drug combination needs to be promoted to in vivo studies for further evaluation.

In conclusion, our reports have shown that we can consistently make HPMA copolymers loaded with an active targeting moiety and conjugate drug to a degradable peptide linker. We confirmed that a polymer with 5% ONp-EPPT1 has enough targeting to reach the lysosomal compartment in both Panc1 and CaPan-2 cell lines. Further investigations into drug release profiles have proven that gemcitabine release is occurring, regardless of peptide being bound or not. Our mechanistic study into how our polymer is endocytosed has proven that caveolin mediated endocytosis may not be involved and that at the very

least clathrin mediated endocytosis seems to play a major role. Lastly, the combination of active targeting along with drug release was also shown to improve the potency of gem in cytotoxicity assay. The knowledge found here now can lead into further investigations of this unique drug delivery system for clinical procurement.

## CONCLUSION

The current state of pancreatic cancer treatment was described in Chapter 2. Advanced staged pancreatic cancer treatments are currently very limited and improvements in the therapeutic intervention for this disease could potentially have great effect on patient outcomes. Although gemcitabine has improved survival minimumally from previous treatments, the clinical use is still limited by toxicity and lack of efficacy. The use of a targeted drug delivery system for the delivery of gem and treatment of pancreatic cancer may be a possible promising pathway to improve the effect of this drug. As stated before, polymer conjugates have shown success for improving the *in vitro*, *in vivo* and clinical response of chemotherapeutic drugs. Furthermore, by adding a targeting moiety to such carriers like HPMA they now can specifically aim for more tumor accumulation instead of non-specific targets. This could help improve the delivery and efficacy aspects of chemotherapeutic drugs. This dissertation describes the development of an HPMA copolymer EPPT1 peptide conjugate bearing Gem for treatment of pancreatic cancer.

The synthesis of copolymers containing gem was achieved using free radical polymerization of HPMA, MAGGONp, MAGFLG-GEM and APMA-FITC. Each comonomer was added at different wt% determine the best incorporation ratios for future studies. Upon completion of each different conjugate synthesis, each polymer was put through several studies for analysis to understand the solubility, incorporation efficiency, molecular weight and molecular weight distribution, binding and internalization properties, drug release and finally cytotoxicity *in vitro* to model pancreatic cancer cell lines. In Chapter 3 development of “control polymer” was the main focus of that study. Every HPMA copolymer was synthesized with a 2% incorporation of APMA-FITC

because it served as the best incorporation % for imaging purposes. Building polymer precursors to attach on the targeting moiety EPPT1 was the first step in the process. Since MAGGONp was the non-degradable covalent linker, different wt% of that monomer was polymerized into the conjugate. Preliminary studies used 5, 10 and 15% of MAGGONp incorporation. Initial findings found that the total polymer HPMA-MAGGONp-FITC was stable only when MAGGONp was kept at or below 10% incorporation. Solubility of polymer with 15% MAGGONp was a major issue since more MAGGONp increase causes a decrease in its water solubility. The conjugation of EPPT1 to the MAGGONp was synthesized by an aminolysis reaction in a 1:1 ratio. Polymer precursors and polymer peptide conjugates were characterized using UV spectroscopy, size exclusion chromatography and amino acid analysis. Physicochemical characterizations were deemed successfully based on theoretical yield of reaction parameters and an increase molecular weight was also observed when EPPT1 peptide was conjugated to the polymer. *In vitro* studies were done using two model pancreatic cancer cell lines that had opposite MUC1 expression, CaPan-2 (High) and Panc-1 (Low). Based on flow cytometry competitive total uptake studies, polymer peptide conjugates could be taken up in both cell lines after 30min treatments. Polymers whose incorporation of MAGGONp was 10% were found to have higher mean fluorescence intensity signifying more total uptake to those of 5% MAGGONp. However, both still exerted statistically significant increase in mean fluorescence intensity than control. Confocal microscopy studies were done to visually confirm polymer uptake and further study their internalization after 30mins of incubation at 37°C in both cell lines. Both polymer peptide conjugates displayed encouraging visual internalization profiles. The next sets of

polymers were developed without the incorporation of EPPT1. Polymerization reactions with different amounts of HPMA, MAGGONp and MAGFLG-Gem were also tested. Using physiochemical characterization techniques we found that an increase in incorporation of either MAGGONp or MAGFLG-Gem negatively effected overall polymer solubility and the theatrically yields of the side chains incorporations. Therefore, 10% incorporation of either side chain is the solubility threshold. Through cytotoxicity study we were able to compare the polymer conjugates containing gem vs free gem. We found that polymer drug conjugates preformed equal to free drug in killing both pancreatic cell lines.

Based on chapter 3 results, HPMA copolymers with peptide were deemed successful at uptake and internalization to pancreatic cancer cell lines. HPMA copolymers with drug incorporation (no peptide) were deemed to be successful for cytotoxicity despite the fact that they were similar to free drug alone. Since, the ultimate goal is to deliver Gem to the nucleus where it exerts its cytotoxic effects, colocalization studies were preformed on two polymer peptide conjugates from previous studies to compare internalization of 5%ONp-EPPT1 vs 10%ONp-EPPT1 against a lysosomal marker Lamp-1. Results indicated that at least 50% of both conjugates colocalized in the lysosome compartment where drug release can occur. Therefore, 5%ONp polymers were deemed the best options for a targeting polymer drug conjugate based on the fact that 10%ONp polymers caused major solubility issues with combination of Gem incorporation. Mechanistic studies were done to examine the endocytosis route the polymer 5%ONp-EPPT1 conjugate may utilize in order to internalize into the lysosomal compartment. For the purpose of this mechanistic study we used 3 different endocytosis

inhibitors. Results indicated that when using the recommended concentrations of the inhibitors, cell viability was still at least 80% even after 2h exposure. When the polymer peptide conjugate was treated in conjunction with specific endocytosis inhibitors, results indicated that the polymers entry into the cell was hindered by clathrin-mediated inhibitors, which signifies that cellular entry might be clathrin mediated.

We also preformed a drug release study of polymer drug conjugates vs polymer peptide drug conjugates in lysosomal conditions, which we analyzed in RP-HPLC. Time points of 15, 60 and 180mins were analyzed to see if any drug release did occur in the lysosomal conditions and if the peptide affected any release of the drug. Results here indicated that 1) release of the drug does occur in the lysosomal conditions and 2) peptide incorporation may have an effect on the release of the drug. Finally, cytotoxicity studies were developed to compare 2 finalize HPMA-EPPT1-(GFLG)-Gem copolymers compared to free gem. The results showed that both polymer peptide drug conjugates had better therapeutic efficacy than free drug.

Overall, these studies were effective at a preliminary demonstration of the utility of HPMA copolymer EPPT1 (GFLG)-Gem conjugates for the treatment of pancreatic cancer cells. The targeted conjugates proved that it could bind and internalize in pancreatic cancer cells and deliver a drug payload that can enhance the therapeutic effect to that free drug at decreasing cell viability. However, the limitations of this project call into question some of the results. Building the final polymer peptide drug conjugate is a very time consuming process. Conjugation of the EPPT1 peptide could potentially be improved if a monomer of the peptide was made and subsequently polymerized into the polymer instead of conjugation after the polymerization process. Further pre-clinical

evaluations could be done primarily to use another colocalization study to see if any of the polymer conjugate is getting confined to the endosome when internalized into the cell. The flow cytometry studies are also limited in the fact that they have no true control. To better understand and confirm the polymers pathway of internalization, non-MUC1 cell types could be used to confirm whether internalization is truly through the MUC1 receptor. It would additionally be useful to enhance the endocytosis inhibition to do a more in depth determination specifically on how the polymers enter the cell. Although the pilot studies confirmed clathrin mediated endocytosis was the route of entry, further analysis using more endocytosis inhibitors would be useful to further prove this theory. Finally, further analyses moving forward would also be required to better understand the polymer peptide drug conjugates behavior *in vivo*. Specifically, biodistribution studies may be useful to assess the polymer conjugates tumor and non-targeted organ accumulation. Although molecular weight studies have been preformed on these polymer peptide drug candidates, the overall charge of these polymers have not been assessed. Therefore, it may be important to make that determination since biodistribution is affected by molecular weight and charge. The hope would be to see significant tumor accumulation and minimum normal tissue uptake. Maximum tolerated dose (MTD) would be preformed in order to determine the best dosing regimen for tumor growth intervention. This study along with biodistribution will coincide well by focusing on doing targeted tumor accumulation and tumor growth intervention. The obvious goals here would be to see if the polymer peptide drug conjugates can actually localize near the tumor and have minimum normal tissue uptake. If that were the case, inhibited tumor cell

growth i.e. increase in Gem efficacy could be the start of a basis to begin phase I clinical trials.

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