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**Development and Characterization of Polymeric
Nanoparticulate Delivery System for Hydrophilic
Drug: Gemcitabine**

By

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A THESIS

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ABSTRACT

Gemcitabine is a nucleoside analogue, used in various carcinomas such as non small cell lung cancer, pancreatic cancer, ovarian cancer and breast cancer. The major setbacks to the conventional therapy with gemcitabine include its short half-life and highly hydrophilic nature. The objectives of this investigation were to develop and evaluate the physiochemical properties, drug loading and entrapment efficiency, *in vitro* release, cytotoxicity, and cellular uptake of polymeric nano-particulate formulations containing gemcitabine hydrochloride. The study also entailed development and validation of a high performance liquid chromatography (HPLC) method for the analysis of gemcitabine hydrochloride.

A reverse phase HPLC method using a C18 Luna column was developed and validated. Alginate and Poly lactide co glycolide/Poly- ϵ -caprolactone (PLGA:PCL 80:20) nanoparticles were prepared by multiple emulsion-solvent evaporation methodology. An aqueous solution of low viscosity alginate containing gemcitabine was emulsified into 10% solution of dioctyl-sulfosuccinate in dichloro methane (DCM) by sonication. The primary emulsion was then emulsified in 0.5% (w/v) aqueous solution of polyvinyl alcohol (PVA). Calcium chloride solution (60% w/v) was used to cause cross linking of the polymer. For PLGA:PCL system, the polymer mix was dissolved in dichloromethane (DCM) and an aqueous gemcitabine (with and without sodium chloride) was emulsified under ultrasonic conditions (12-watts; 1-min). This primary emulsion was further emulsified in 2% (w/v) PVA under ultrasonic conditions (24-watts; 3-min) to prepare a multiple-emulsion (w/o/w). In both cases DCM, the organic solvent was

evaporated (20- hours, magnetic-stirrer) prior to ultracentrifugation (10000-rpm for PLGA:PCL; 25000-rpm for alginate). The pellet obtained was washed thrice with de-ionized water to remove PVA and any free drug and re-centrifuged. The particles were re-suspended in de-ionized water and then lyophilized to obtain the dried powdered delivery formulation. Particle size and surface charge of the nano-particles were measured using zeta-sizer. The surface morphology and microstructure were evaluated by scanning electron microscopy. The drug loading and entrapment efficiencies were evaluated by a HPLC method (Luna C18 column (4.6 X 250 mm), 95/5 (v/v) 0.04M ammonium acetate/acetonitrile mobile phase (pH 5.5), 1.0 ml/min flow rate and 268 nm UV detection). Differential scanning calorimetry (DSC) was used to determine the physical state of gemcitabine in the nanoparticles. The cytotoxicity in pancreatic cancer cells (BxPC-3) was evaluated by MTT assay. The cellular uptake of gemcitabine solution and gemcitabine loaded alginate nano-particle suspension in BxPC-3 cells was determined for 15, 30 and 60 minutes.

The particle-size and surface-charge was 564.7 ± 56.5 nm and -25.65 ± 1.94 mV for PLGA:PCL and 210.6 ± 6.90 nm and -33.21 ± 1.63 mV for alginate. Both the nano-particles were distinctly spherical and non-porous. The drug load was 5.14% for PLGA:PCL and 6.87% for alginate-particles, and the practical entrapment efficiency was found to be 54.1% and 22.4% respectively. However, in case of PLGA:PCL particles, a two-fold increase in the entrapment efficiency was observed with the addition of sodium-chloride. The absence of endothermic melting peak of the drug in the DSC thermogram was an indication of the non-crystalline state of gemcitabine in the nanoparticles. In addition, there was no cytotoxicity associated with nanoparticle concentrations at-or-below 5

mg/mL. The uptake of nano-particles was around 4 times higher than the solution with treatment for 15 minutes and increased to almost 7 times following treatment for 60 minutes.

Gemcitabine hydrochloride could be successfully formulated into a sustained release nano-particulate formulation using calcium cross-linked alginate and dioctyl sulfo succinate system. The nano-particulate delivery system exhibited better cytotoxic activity and also significantly enhanced the accumulation of the drug in BxPC-3 cell monolayers.

PREFACE

ABSTRACTS:

1. J. Khurana, T. Beeson, A. Stanley, and A. K. Dash, The effects of various additives and set accelerators on the physical and set properties of Mineral Trioxide Aggregate (MTA)

American Association of Pharmaceutical Scientists Annual meeting 2009, Los Angeles, California.
2. N. Jain, A. Pandit, Jatin Khurana and A. K. Dash. Development and validation of HPLC method for Ciprofloxacin Hydrochloride.

American Association of Pharmaceutical Scientists Annual meeting 2009, Los Angeles, California.
3. J. Khurana, W. J. Trickler, A. K. Dash. Development and characterization of nanoparticulate delivery system for a hydrophilic drug: Gemcitabine.

American Association of Pharmaceutical Scientists Annual meeting 2008, Atlanta, Georgia.
4. J. Khurana, W. J. Trickler, A. K. Dash. Fortified Orange Juice Affects the Trans-Epithelial Transport of Ciprofloxacin and Doxycycline.

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BOOK CHAPTER:

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PUBLICATIONS:

W.J. Trickler, J. Khurana, A.A. Nagvekar, A.K. Dash, Chitosan and Glyceryl Monooleate Nanostructures containing Gemcitabine: Potential Delivery System for Pancreatic Cancer Treatment. (Submitted to AAPS PharmSciTech).

Dedicated to my family and friends

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CHAPTER 1

Introduction

A cell is the smallest living entity in the body and is often known as the structural and functional unit of all living organisms. Normal body cells grow, divide and die in an orderly fashion. All the functions of a cell including rate of cell growth, division, differentiation and death are regulated by a specified set of genes, which act as triggers. A loss of function of these genes, sometimes lead to a state of uncontrolled cell growth without any differentiation; termed as “Neoplasia or Cancer”.

Cancer is the second leading cause of death in USA after cardiovascular diseases, in both males and females. It is estimated that there were about 1,437,180 new cases and 565,650 deaths from cancer in 2008, forming 23% share of all mortality (Jemal, et al., 2008). The most common sites for new cancers in males include prostate, lung and colon whereas in females, cancers of breast, lung and colon are most prevalent. The cancers of breast, prostate lung and colon represent over 50% of cancer deaths in USA (Jemal, et al., 2008). Studies have also shown that the incidence of cancer varies within different races and ethnicities and that African Americans are affected the most (Gorey and Vena, 1994). The prevalence of cancer, as estimated according to the American Cancer Society (ACS) in 2005 is summarized in table 1.1.

Table 1.1 Prevalence of cancer

Estimated cancer prevalence in the United States, 2005			
Primary site	Estimated prevalence		
	Total	Males	Females
All sites	10,701,000	4,955,000	5,746,000
Brain & other nervous system	109,000	58,000	51,000
Breast	2,521,000	13,000	2,478,000
Cervix	195,000	0	195,000
Colon & rectum	1,168,000	570,000	598,000
Endometrial cancer & Uterine sarcoma	554,000	0	554,000
Esophagus	32,000	24,000	8,000

Hodgkin disease	144,000	74,000	70,000
Kidney & renal pelvis	280,000	166,000	114,000
Larynx	98,000	79,000	20,000
Leukemias	231,000	131,000	100,000
Liver & bile duct	24,000	16,000	8,000
Lung & bronchus	418,000	199,000	219,000
Melanoma of skin	725,000	361,000	364,000
Multiple myeloma	63,000	35,000	28,000
Non-Hodgkin lymphoma	431,000	222,000	209,000
Oral cavity & pharynx	246,000	157,000	88,000
Ovary	170,000	0	170,000
Pancreas	34,000	17,000	17,000
Prostate	2,244,000	2,244,000	0
Stomach	70,000	40,000	30,000
Testis	168,000	168,000	0
Thyroid	362,000	82,000	281,000
Urinary bladder	575,000	425,000	151,000
Childhood cancer (age 0 -19 years)	249,000	128,000	121,000

A cancer on the basis of its characteristics can either be “Benign” or “Malignant”.

A benign neoplasm is localized; can't spread to other sites or doesn't invade the neighboring tissues. On the other hand malignant neoplasms collectively known as cancers are invasive in nature and also metastasize to other locations in the body via lymph or blood. Benign tumors differ from malignant cancers both morphologically and biologically. Benign tumors are generally well differentiated and resemble closely to the corresponding parenchymal cells. Malignant cancers on the other hand, exhibit anaplasia (lack of differentiation). Anaplasia is frequently associated with some other morphological changes which collectively are considered a hallmark of malignancy. These morphological changes include; Pleomorphism (variation in size and shape), Abnormal nuclear morphology, Loss of polarity etc. Benign tumors can easily be treated by local surgical removal whereas malignant tumors are much more difficult to treat.

The major cause of cancer is the accumulation of gene defects that play crucial role in regulating cell proliferation, differentiation and apoptosis (Digweed, 1993). Various genetic studies have shown that carcinogenesis is marked by a series of changes at both phenotypic and genetic levels resulting from the accumulation of multiple mutations (Loeb, et al., 2003). Molecular and genetic analysis of some common cancers signify that at least five gene defects are frequently present in cancers of the colon (Fearon and Vogelstein, 1990: Wood, et al., 2007), breast (Wood, et al., 2007), lung (Sato, et al., 2007) and pancreas (Maitra, et al., 2006), whereas fewer gene defects may cause development of precancerous precursor lesions found in these cancers. Non-lethal genetic damage to any one of the following types of genes may lead to carcinogenesis:

The growth promoting proto-oncogenes

The growth inhibiting, tumor suppressor genes

Genes regulating programmed cell death (apoptosis)

Genes involved in DNA damage repair

These mutations can be either caused by chemical & environmental mutagens or by radiations. The carcinogenic effects of environmental and occupational carcinogens date back to 16th century. However the first ever association of such kind was documented in the 18th century. Waldron indicated that the high incidence of scrotal cancer in chimney sweeps could be related to their occupational exposure to soot and tar (Waldron, 1983). Dipple et al.; later identified the carcinogens in soot and tar to be polycyclic aromatic hydrocarbons (PAHs) (Dipple, et al., 1984). The work of Case et al.; depicted an association between tumors of the urinary bladder and azo dyes (Case, et al.,

1954). Workers employed in chemical manufacturing and textile dying, were found to be at a greater risk for bladder cancers (Case, et al., 1954). Studies have indicated that cigarette smoking increases the risk for the cancers of the lung, oral cavity, pharynx, larynx, esophagus, bladder, renal pelvis and pancreas (Vineis, et al., 2004).

Another very important physical carcinogen causing genetic mutations is radiations. The ultra violet radiations from the sun constitute the major physical carcinogen present in our environment. It has also been identified as a primary cause of skin cancer in humans. Certain electromagnetic rays such as x-rays, γ -rays are capable of both directly and indirectly damaging the genetic material (Preston, et al., 2002).

Certain human pathogens such as viruses and bacteria are capable of causing cancers. It has been established that human tumor viruses contribute to 15% of total human cancers. Burkitt's lymphoma was identified to have an infectious etiology caused by Epstein-Barr virus (EBV) (Burkitt, 1962: Epstein, et al., 1964). Another common human virus; Hepatitis B virus has been shown to be associated with hepatocellular carcinoma (HCC) (Blumberg, 1977). The work of Beasley et al.; provided significant data to establish the role of Hepatitis B virus in causing HCC (Beasley, et al., 1981: Beasley, 1988). Human papillomaviruses (HPVs) have been recognized to cause cervical cancer. This association was first studied by Harald zur Hausen and group in 1980's. Further studies provided strong evidence for HPVs causal role in the development of cervical cancer (Schwarz, et al., 1985: Yee, et al., 1985).

Hormonal imbalances have also been shown to stimulate excessive cell growth. Androgen imbalances were found to be associated with development of breast cancer

(Deshpande, 1975). The role of excessive estrogen levels has been well studied and established in promoting the development of endometrial cancer.

Studies have shown that there exists a genetic predisposition to a large number of cancer types (Knudson, 2002; Narod, 2006). Inheritance of a single autosomal dominant mutant gene greatly increases the risk of developing the tumor. Such cancers are termed as autosomal dominant inherited cancer syndrome. BRCA1, BRCA2, p53, PTEN are some of the genes associated with hereditary breast cancer predisposition (Easton, et al., 2007). In addition to the inheritance of a single autosomal dominant mutant allele, recent studies have shown that familial susceptibility to cancer may also depend on multiple low-penetrance alleles (Easton, et al., 2007).

Current treatment modalities available for cancer include surgery, radiation therapy, chemotherapy, hormone therapy and gene therapy. The use of natural and synthetic chemical agents to kill rapidly growing cancerous cells is the most common choice of treatment for different stages of benign and malignant cancers. It generally involves use of chemotherapeutic drugs either as a single agent treatment or a combination therapy. These drugs differ in their mechanism of action, site of action and also the cell growth phase they act on. Most of the chemotherapeutic agents cause cell cycle arrest during the DNA synthetic phase also known as the S phase for example 5 Fluoro Uracil, methotrexate, cytosine arabinoside etc., some other drugs act on the mitotic phase also known as the M phase, like vincristine, vinblastine, paclitaxel etc. However, some of the drugs are phase unspecific; these include cisplatin, procarbazine, dacarbazine, nitrosoureas etc.

The use of chemical agents to treat cancer goes several decades back with the development of alkylating agents in the 1950's. With constant research for over five decades, the list of these chemicals has been updated, with the addition of various conventional natural products like paclitaxel, etoposide etc. This study primarily focuses on the drug "Gemcitabine". On the basis of its mechanism of action it can be classified as an antimetabolite/nucleoside analogue. It is a synthetic molecule. Its activity was first tested by Hertel and group in 1990 against human leukemia cell line (Hertel, et al., 1990). Gemcitabine is a difluoro analogue of deoxycytidine and the chemical IUPAC name of its hydrochloride salt is 4-amino-1-[(2R, 4R, 5R)-3, 3-difluoro-4-hydroxy-5-(hydroxymethyl)-oxolan-2-yl] pyrimidin-2-one monohydrochloride. The molecular formula of gemcitabine hydrochloride is $C_9H_{11}F_2N_3O_4.HCl$ and its molecular weight is 299.66. Gemcitabine HCl is a white to off white colored solid powder and is soluble in water, slightly soluble in methanol and is practically insoluble in ethanol and other organic solvents. It was first approved by FDA in 1996 and is indicated for use in breast cancer, ovarian cancer, non-small cell lung cancer and pancreatic cancer.

Pancreatic cancer is one of the most fatal cancers. It has a high mortality and very short survival of around 8-12 months for patients with locally advanced disease and 3-6 months for metastatic cases (Oettle, et al., 2007). According to the cancer statistics, it has been identified as the fourth most common cause of deaths by cancer with an estimated number of 37,680 new cases and 34,290 deaths in USA for 2008 (Jemal, et al., 2008). The high mortality rate of this disease can be attributed to a high incidence of metastases and unavailability of potent systemic therapies.

Gemcitabine has been used as the reference regimen for the treatment of both locally advanced and metastatic stages ever since its approval in 1996. 5 FU has been tried and tested for treating pancreatic cancer before the advent of gemcitabine. In a study with patients being treated with different 5 FU, the median survival time was found to have increased to 33 weeks compared to 15 weeks among patients receiving no therapy (Palmer, et al., 1994). However studies suggested no improvement in survival time or clinical benefit with 5 FU combinations over 5 FU as a single agent (Fung, et al., 2003). Gemcitabine in a phase III randomized trial was found to increase the median survival time to 5.65 months whereas patients receiving 5 FU showed a median survival time of 4.41 months (Burris, et al., 1997).

Different groups of researchers have tested the efficacy of various available chemotherapeutic agents in combination with gemcitabine. The combination therapy did not seem to provide any significant benefits over the use of gemcitabine as a single agent in most cases (Pliarchopoulou and Pectasides, 2009). Administration of a combination therapy of gemcitabine, tegafur and uracil in metastatic pancreatic cancer patients only showed moderate clinical benefits in a phase II trial (Lee, et al., 2004). Reni and coworkers investigated the usefulness of cisplatin, epirubicin and 5FU in combination with gemcitabine, the combination therapy was called PEFG. A higher percentage of one year overall survival was observed in the patient group receiving PEFG (38.5 % compared to 21.3 % in patients on gemcitabine alone). While, a significant increase in incidence of grade 3-4 neutropenia and thrombocytopenia was observed with PEFG (Reni, et al., 2005). The use of capecitabine along with gemcitabine has produced conflicting results in two different studies. In a phase III randomized trial, the patients

receiving the combination therapy showed evidence of an increased median survival time with similar grade 3 & 4 toxicity profile (Herrmann, et al., 2007). Whereas, in another phase III trial by Cunningham et al., the use of gemcitabine alone was found to have a higher median survival time (Cunningham, et al., 2009). Different amounts of capecitabine were administered in these studies. The higher survival time with the combination therapy in the study by Hermann et al. could be attributed to a high amount of capecitabine administered to the patients.

A combination of oxiplatin, cisplatin and gemcitabine was tested by Louvet et al. The combination therapy resulted in an enhanced survival time but at the same time increased the incidence of grade 3 & 4 toxicities (Heinemann, et al., 1988; Louvet, et al., 2005).

Gemcitabine is the most potent chemotherapeutic agent among its class of cytidine analogues. It has a dual mechanism of action; it causes cell cycle arrest at S phase. When transported into cells by nucleoside transporters (Mackey, et al., 1998; Pastor-Anglada, et al., 2004), it is phosphorylated to difluoro deoxycytidine diphosphate (dfdCDP) and difluoro deoxycytidine triphosphate (dfdCTP). DfdCDP acts by inhibiting ribonucleotide reductase thus reducing the availability of deoxyribonucleotides essential for DNA synthesis. On the other hand dfdCTP competes with cytidine triphosphate (CTP) to get incorporated into DNA and leads to DNA strand termination (Heinemann, et al., 1988). A combination therapy comprising of oxiplatin, irinotecan, leucovorin followed by 5 FU (FOLFIRINOX) has also been tested against gemcitabine. The combination therapy showed slight improvement in survival time (Ychou, et al., 2008). In a recent study, Fine and group have explored the use of capecitabine, gemcitabine and

docetaxel in combination for treating metastatic pancreatic cancer. The combination therapy produced significant results by extending the survival time to 11.2 months along with a 2 year survival rate of 20% (Fine, et al., 2008).

To date gemcitabine still remains the principal drug for the treatment of both locally advanced and metastatic pancreatic cancer. The standard dosing schedule for gemcitabine is 1-1.2 mg/m² as a 30 minute intravenous infusion on day 1, 8, 15 and 28 of every dosing cycle.

Even after exhibiting extremely promising antitumor activities against various kinds of tumors, the use of gemcitabine is limited to breast cancer, ovarian cancer, non-small cell lung cancer and pancreatic cancer. The most important drawback associated with the current clinical treatment with gemcitabine is its very short half life. Gemcitabine is rapidly metabolized in plasma into dFdU by the enzyme cytidine deaminase (Plunkett, et al., 1995). Therefore it requires administration of high doses leading to dose-limiting adverse effects. Also, a very hydrophilic molecule gemcitabine requires assistance of nucleoside transporters to be transported into the cell (Mackey, et al., 1998; Pastor-Anglada, et al., 2004). A deficiency of nucleoside transporters is the most common mechanism of development of resistance to gemcitabine (Gourdeau, et al., 2001; Mackey, et al., 1998). All these drawbacks necessitate an improved delivery system that is target specific and also possesses the ability to release the drug in a sustained fashion thus eliminating the systemic toxicity.

The goal of any drug delivery system is to achieve a therapeutic amount of the drug at the appropriate site in the body and also it should deliver the drug at a rate required to meet the needs of the body over time. Hence the two most important

objectives of any delivery system are spatial placement and temporal delivery. An appropriately designed controlled delivery system is an ideal answer to achieving the aforementioned objectives. The primary goal of this investigation is to develop a sustained release bio-adhesive nano-particulate delivery vehicle for targeted local delivery of gemcitabine for the treatment of pancreatic cancer.

A wide array of synthetic and naturally occurring polymers has been used by different groups to attain a sustained release delivery system. Natural polymers like gelatin, albumin, starch, alginate, chitosan etc are some of the biocompatible and biodegradable compounds approved by FDA for use in pharmaceutical and food industry.

Alginic acid is a water soluble linear polysaccharide extracted from brown algae. The most common species are *Laminaria hyperborean*, *Ascophyllum nodosum* and *Macrocystis pyrifera* (George and Abraham, 2006). Alginate is also found in some bacteria. It has been isolated from *Azotobacter vinelandii* and some species of *Pseudomonas* (Skjak-Braek, et al., 1986). Alginate is formed by alternating blocks of 1-4 α -L-guluronic and β -D-mannuronic acid residues as shown in figure 1.1.

Alginate is commonly used in food industry as a thickener, emulsifier and stabilizer. It has been approved by the FDA as safe upon oral administration. It has been shown to be nontoxic and biodegradable when given orally. However ambiguous results were obtained upon intravenous administration of alginate. Certain studies have shown commercially available alginate to induce foreign body reaction and fibrosis (Cole, et al., 1992; De Vos, et al., 1996), but other studies have proven it to be devoid of any kind of immunoresponse (Zimmermann, et al., 1992). The immunogenic response observed upon

intravenous administration in some cases can be attributed to toxic adulterants in commercial alginates.

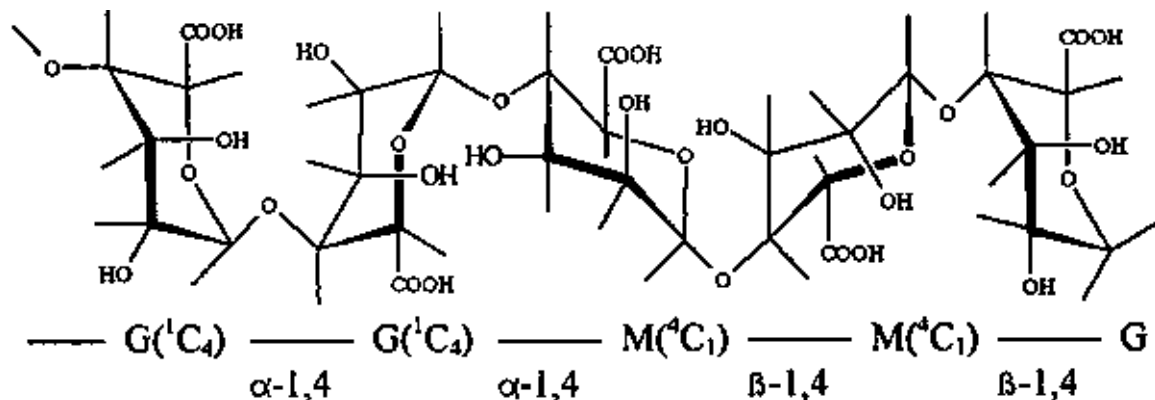


Figure 1.1 Chemical Structure of Alginic acid.

Another important quality of alginate that makes it a suitable candidate for use in drug delivery is its bioadhesiveness. It has been proven that polymers with high charge densities exhibit strong mucoadhesive properties (Ch'ng, et al., 1985), the negative charges on the surface of alginate interact with positively charged mucosal surface leading to strong electrostatic attractive forces. Further studies have provided evidence that alginate has higher mucoadhesive strength compared to some other bioadhesive polymers like chitosan, carboxymethyl cellulose, poly lactic acid and poly styrene (Ch'ng, et al., 1985).

Alginate has been used for a number of pharmaceutical applications, specifically for controlling the rate of release of therapeutic agents. The delivery systems comprised of alginate include beads, hydro-gels, microspheres, hydrocolloids and nano-particles.

Alginate hydro-gels have been reported to be formed under mild conditions. The most widely used strategy is the crosslinking of alginate using divalent ions like Ca^{2+} , Sr^{2+} or Ba^{2+} . However, no gels were formed using Mg^{2+} ions (Morris, et al., 1977).

Alginate beads have been used for sustained delivery of a wide variety of drugs including, pindolol (Ferreira Almeida and Almeida, 2004), Diclofenac sodium (Zhang, et al., 2009), ketoprofen (Del Gaudio, et al., 2009), bovine serum albumin (BSA) (Gao, et al., 2009), thiram (Singh, et al., 2009) etc. As microspheres for the controlled delivery of loratidine (Mishra, et al., 2008), diltiazem hydrochloride (Sultana, et al., 2009), terbutaline sulfate (Moebus, et al., 2009). As hydrogels, for heat shock protein (HSP27) (Lee, et al., 2009), theophylline (Colinet, et al., 2009). Alginate nano-particles prepared by crosslinking with Ca^{2+} ions have been reported to efficiently entrap and control the release of water soluble drugs like doxorubicin, clonidine, fluorescein etc. (Chavanpatil, et al., 2007b), methylene blue (Khdair, et al., 2008).

Alginate nano-particles formed by multiple emulsion solvent evaporation technique were investigated for entrapment efficiency, in vitro release and cellular uptake of gemcitabine hydrochloride. The underlying hypotheses of this investigation were:

Hypothesis I: “Gemcitabine hydrochloride can be successfully formulated into a sustained release nano-particulate delivery system using alginate cross linked with calcium ions”.

Hypothesis II: “The sustained release gemcitabine hydrochloride nano-particulate formulation can significantly enhance the cellular uptake of the drug in BxPC-3 cell lines”.

In order to test the aforementioned hypotheses, the present study had the following three specific aims:

Specific aim 1: To develop and validate a HPLC method for the assay of gemcitabine hydrochloride.

Specific aim 2: Formulation of a sustained release delivery system containing gemciatbine hydrochloride using alginate cross linked with calcium ions.

Specific aim 3: To evaluate the cellular uptake of gemcitabine hydrochloride from the delivery system using BxPC-3 cell lines as an *in vitro* model.

CHAPTER 2

Gemcitabine (Physicochemical properties and Pharmacology)

2.1. Description

2.1.1. Drug type

Gemcitabine is a small molecule (with a molecular weight of 300) and a highly hydrophilic drug, first approved by FDA on May 19th, 1996 for the treatment of breast cancer in combination with paclitaxel. On July 14th, 2006 it was further approved for ovarian cancer in combination with paclitaxel and has been recently approved as the first line of treatment for pancreatic cancer that is advanced or has metastasized.

2.1.2. Nomenclature

2.1.2.1. Chemical IUPAC name

The IUPAC name of gemcitabine hydrochloride is 4-amino-1-[(2R, 4R, 5R)-3, 3-difluoro-4-hydroxy-5-(hydroxymethyl)oxolan-2-yl]pyrimidin-2-one monohydrochloride.

2.1.2.2. Proprietary names

DDFC

DFDC

GEO

Gemcin

Gemcitabina [INN-Spanish]

Gemcitabine HCl

Gemcitabine hydrochloride

Gemcitabinum [INN-Latin]

Gemtro

Gemzar

2.1.3. Formulae

2.1.3.1. Empirical

The molecular empirical formula for gemcitabine hydrochloride is $C_9H_{11}F_2N_3O_4 \cdot HCl$.

2.1.3.2. Structural

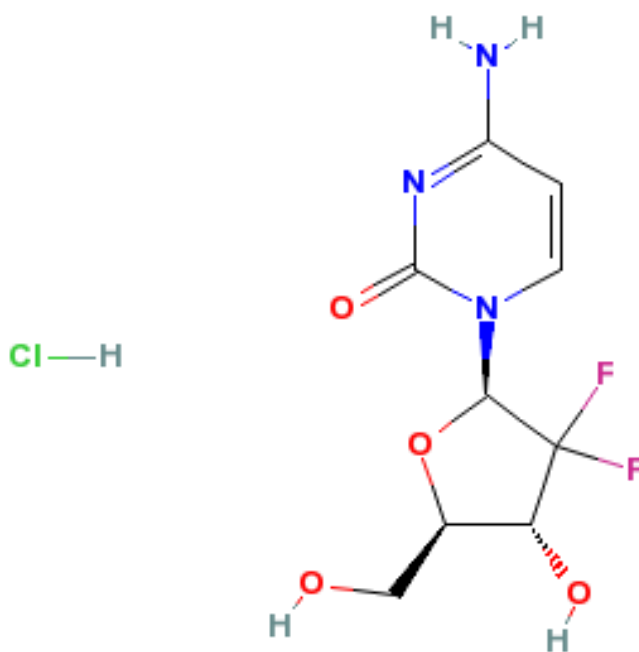


Figure 2.1: Structural Formula of Gemcitabine Hydrochloride

2.1.4. Molecular weight

299.66

2.1.5. CAS number

95058-81-4

2.2. Physicochemical properties

2.2.1 Physical state & appearance

Gemcitabine HCl is a white to off white solid powder.

2.2.2. Melting point

168.64°C

2.2.3. Partition coefficient

Gemcitabine has a log(P) value of -1.4.

2.2.4. Solubility

Gemcitabine hydrochloride is soluble in water, slightly soluble in methanol and practically insoluble in ethanol and polar organic solvents.

2.2.5. Pka

3.6

2.3. Pharmacology

2.3.1. Drug Category

Gemcitabine on the basis of its mechanism of action has been categorized as anti-metabolites, enzyme inhibitors, on the basis of its uses as anti-neoplastic agents, antiviral agents, immunosuppressive agents and radiation-sensitizing agents.

2.3.2. Mechanism of Action

Gemcitabine is a difluoro analog of deoxycytidine. It is transported into the cells through active nucleoside transporters (Mackey, et al., 1998). Once in the cell, gemcitabine is first phosphorylated into difluoro deoxycytidine by the enzyme deoxycytidine kinase and eventually into difluoro deoxycytidine di-phosphate and tri-phosphate. The cytotoxic action of gemcitabine is a result of the synergistic effects of dfdCDP and dfdCTP on various steps of DNA replication. The cytotoxic activity of dfdCDP can be attributed to its ribonucleotide reductase inhibiting potential, which results in the diminution of deoxyribonucleotides essential for DNA synthesis. Whereas,

dfdCTP competes with CTP to get incorporated in to DNA and leads to DNA strand termination (Heinemann, et al., 1988). Hence, gemcitabine causes cell cycle arrest not just at S phase but also during the logarithmic growth phase, which makes it a more potent drug than cytarabine with a broader spectrum of clinical activity (Iwasaki, et al., 1997).

2.3.3. Indications

Gemcitabine hydrochloride is manufactured and marketed by Eli-Lilly and company. The marketed formulation is available as Gemzar, a white colored powder. The therapeutic indications of Gemzar include:

Breast cancer. Gemzar used in combination with paclitaxel has been indicated as the first line treatment therapy for patients with metastatic breast cancer after failure of prior anthracycline-containing adjuvant chemotherapy, unless anthracyclines were clinically contraindicated (FDA label).

Ovarian cancer. Gemzar used in combination with carboplatin has been indicated for the treatment of patients with advanced ovarian cancer when relapsed at least 6 months after completion of platinum-based therapy (FDA label).

Non-Small Cell Lung cancer. Gemzar used in combination with cisplatin has been indicated as first line treatment therapy for patients with inoperable, locally advanced (Stage IIIA or IIIB), or metastatic (Stage IV) non-small cell lung cancer (FDA label).

Pancreatic cancer. Gemzar has been indicated as the first line treatment therapy for patients with locally advanced (nonresectable Stage II or Stage III) or metastatic (Stage IV) adenocarcinoma of the pancreas. Gemzar is also indicated for patients preVIOUSLY treated with 5-Fluoro Uracil (FDA label).

2.3.4. Contraindications

Gemcitabine has been contraindicated in all the patients with a known hypersensitivity to the drug.

2.3.5. Adverse effects and Toxicity

When used as a single agent, myelosuppression is the principal dose-limiting toxicity with Gemzar. However, sometimes dose adjustments may be required for Hematologic toxicity. Studies performed on 979 patients receiving Gemzar as a single agent therapy have shown different adverse reactions and toxicities. Gemzar was administered weekly as a 30 minute infusion with starting doses ranging from 800 – 1250 mg/m² (Abou-Alfa, et al., 2006: Berlin, et al., 2002: El-Rayes, et al., 2003: Maki, et al., 2007: Moore, et al., 2007: Sederholm, et al., 2005).

Hematologic toxicity - the treatment was discontinued in less than 1% patients owing to cases of acute anemia, leucopenia or thrombocytopenia. However, 19% of the patients required Red blood cell transfusions. Not more than 1% of patients were diagnosed with sepsis, but 16 % of the patients reported hemorrhage resulting in blood loss with less than 1% patients requiring platelet infusion (Abou-Alfa, et al., 2006: El-Rayes, et al., 2003: Moore, et al., 2007). The other commonly reported adverse effects included; nausea and vomiting with mild to moderate severity, increased levels of transaminases, fever, rash and alopecia. Some of the less frequent toxicities reported include proteinuria, hematuria, paresthesias, dyspnea, extravasation, arrhythmia, myocardial infarction and bronchospasms (Abou-Alfa, et al., 2006: Berlin, et al., 2002: El-Rayes, et al., 2003: Maki, et al., 2007: Moore, et al., 2007: Sederholm, et al., 2005).

2.3.6. Metabolism

Most of the administered drug undergoes rapid metabolism. It is converted to difluoro deoxyuridine by cytidine deaminase (Plunkett, et al., 1995). The plasma half life of gemcitabine following intravenous infusion is very short; 8-17 minutes in human plasma (Abbruzzese, et al., 1991; Reid, et al., 2004) and 9 minutes in murine plasma (Moog, et al., 2002).

2.3.7. Clinical Studies

Gemcitabine exhibits cross resistance with arabinofuranosyl cytosine resistant variant P388 leukemia. However, it did not show any cross resistance to P388 leukemia resistant to doxorubicin, etoposide, vincristine, cyclophosphamide, melphalan, cisplatin and methotrexate (Waud, et al., 1996).

Gemcitabine is indicated as the first line treatment for pancreatic cancer as a single agent therapy. It is also indicated to be used in combination with paclitaxel, carboplatin and cisplatin for the treatment of breast cancer, ovarian cancer and non-small cell lung cancer respectively. The combination is generally recommended for either synergistic effects or a reduction in adverse effects or both.

The effectiveness of gemcitabine in combination with 5 fluoro uracil was tested by Hidalgo et al., it was found that the combination therapy was well tolerated by the patients and a promising antitumor effect against pancreatic cancer was observed (Hidalgo, et al., 1999). However, later on studies by Jordan and coworkers indicated no improvement in median survival of patients with advanced pancreatic cancer upon administration of 5 fluoro uracil in combination with gemcitabine (Berlin, et al., 2002). During the Phase III trials of gemcitabine in combination of cisplatin against metastatic

non small cell lung cancer, the combination regimen was found to exhibit better effects than cisplatin alone in terms of response rate, time to disease progression and overall survival (Sandler, et al., 2000). A similar result was observed during the phase II trial of gemcitabine in combination with doxorubicin for the treatment of breast cancer. Gemcitabine (800 mg/m^2) when administered in combination with doxorubicin (25 mg/m^2) in a weekly schedule exhibited promising activity against breast cancer. Phase I and pharmacokinetic studies on gemcitabine in combination with vesnarinone by Patnaik and group indicated no pharmacokinetic interaction between the two drugs (Patnaik, et al., 2000).

The effectiveness of gemcitabine in combination with docetaxel was studied by Martee L et al., for the treatment of leiomyosarcoma. It was documented that the combination regimen did not exacerbate any severe adverse effects and at the same time yielded better activity in both treated and untreated patients (Hensley, et al., 2002). In a recent study by Sederholm and group on using gemcitabine in combination with carboplatin for the treatment of advanced or metastatic non small cell lung cancer, it was found that the combination therapy did not increase any toxicity but showed significant survival benefit compared to gemcitabine use as a single agent (Sederholm, et al., 2005). Pfisterer J et al., in a similar study with gemcitabine in combination with carboplatin for the treatment of ovarian cancer suggested that the combination therapy improved the response rate with no undue increase in toxicity (Pfisterer, et al., 2006). The combination of exatecan and gemcitabine for the treatment of advanced pancreatic cancer was studied by Ghassan k et al. However, no benefits of the combination therapy over the single agent gemcitabine treatment were observed (Abou-Alfa, et al., 2006). Gemcitabine when

used in combination with erlotininb in patients with advanced pancreatic cancer was found to significantly improve the survival in patients (Moore, et al., 2007).

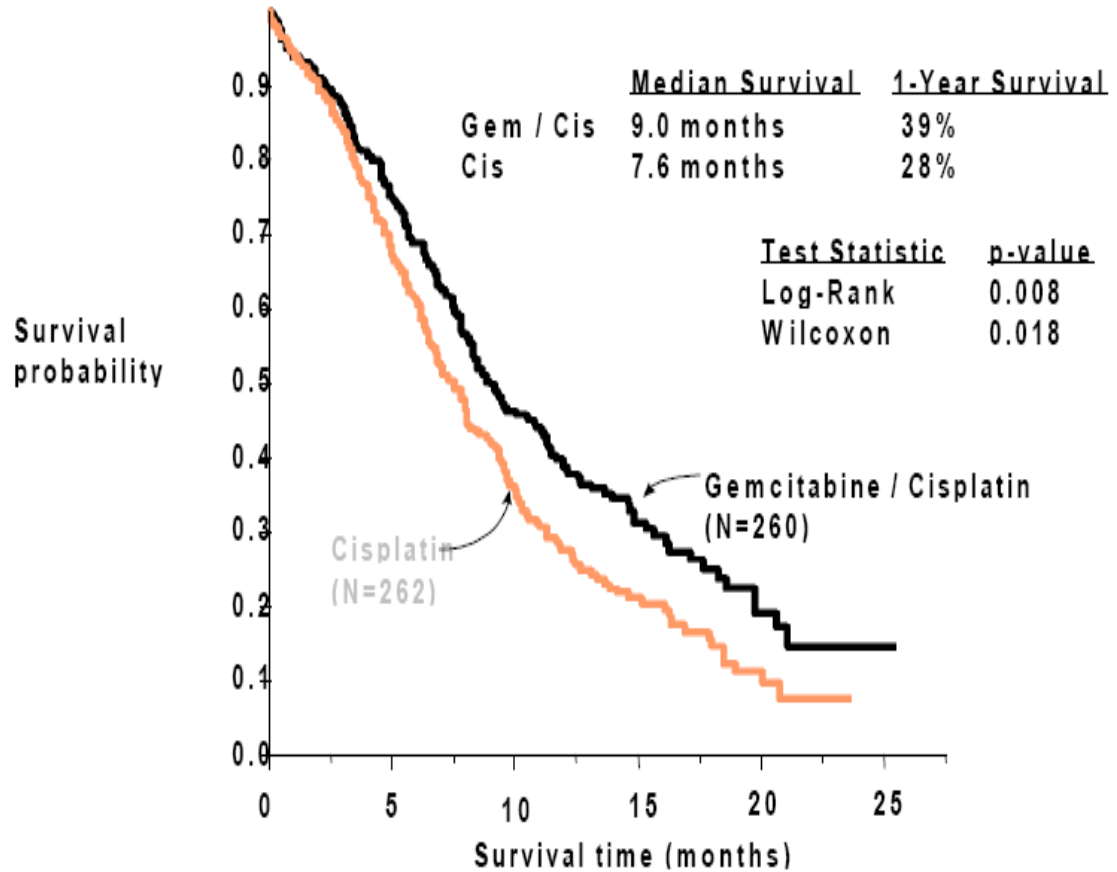


Figure 2.2: Kaplan-Meier survival curve in Gemzar plus Cisplatin versus Cisplatin NSCLC study (n=522) (Sandler, et al., 2000).

CHAPTER 3

Analytical Method Development and Validation

3.1. Introduction

Gemcitabine is a small molecule hydrophilic drug. It is classified as an antimetabolite on the basis of its pharmacological activity and as antineoplastic agent owing to its therapeutic indication. It is chemically a deoxycytidine pyrimidine analog (Abbruzzese, et al., 1991; Erlichman, 1991). The primary mechanism of metabolism of gemcitabine includes its degradation by cytidine deaminase to dFdU in liver, kidney or plasma. It is also excreted in urine unchanged (Kirstein, et al., 2006; Marangon, et al., 2008). Different analytical methods have been investigated for the separation and quantification of gemcitabine. Edzes and coworkers in 1993 described a nuclear magnetic resonance (NMR) method for the determination of gemcitabine and its metabolite dFdU. Both the compounds produced distinct spectra at the physiological pH range and a linear relationship could be developed between the concentration of the drug and its NMR intensity. However it was very difficult to separate the drug from its metabolite (dFdU) using NMR (Edzes, et al., 1993). Reverse phase HPLC and liquid chromatography in tandem mass spectroscopy are the most commonly used techniques for the identification and quantitative determination of the drug in plasma, urine and other *in vitro* media.

Several HPLC methods have been developed and validated for the analyses of gemcitabine. These methods primarily differ in the type of column used, mobile phase, flow rate and their applications. Freeman and group were the first to develop a normal phase HPLC method for the determination of gemcitabine and dFdU in both plasma and urine (Freeman, et al., 1995). This method has been successfully used to analyze samples from Phase II/III studies by various contract laboratories. Venook et al. in 2000,

developed and used a reverse phase HPLC method for the Phase I pharmacokinetic trial of gemcitabine in leukemia patients with hepatic or renal dysfunction (Venook, et al., 2000). Another reverse phase HPLC method was developed and used by Patnaik & group for the determination of gemcitabine in a phase I trial of gemcitabine in combination with vesnarinone (Patnaik, et al., 2000). However both these methods were laborious and utilized a gradient chromatography with a cycle time of approximately 77 minutes. Keith et al. in an attempt to improve the efficiency of the analysis developed another reverse phase HPLC method that offered shorter cycling time of 39 minutes (Keith, et al., 2003).

Yilmaz and group came up with an isocratic normal phase HPLC method with a very short runtime of 10 minutes for the determination of gemcitabine. The method utilized a Nucleosil 5 NH₂ column (Yilmaz, et al., 2003). Kirstein et al.; tested a reverse phase HPLC method for the separation and determination of gemcitabine and dFdU. It was observed that use of a C18 column highly improved the separation of the two compounds in comparison to the use of a C8 column. This study also suggested that best resolution is obtained when the column temperature is maintained at 40°C (Kirstein, et al., 2006). The use of C18 columns was further investigated by Losa and group. In the same study, they also analyzed the effect of ionic strength of the mobile phase on the retention time of the analytes. It was observed that an increase in phosphate salt concentration in the mobile phase resulted in a reduced retention time for all the nucleotides (Losa, et al., 2006).

Various research groups have also evaluated the use of mass spectroscopy in tandem with liquid chromatography (LC-MS). Xu et al., in 2004 successfully developed a LC-MS method for the separation and quantification of gemcitabine and its metabolite.

An electrospray ionization mode (ESI-MS) was used to fragment the drugs. The total runtime was determined to be 15 minutes per run with a retention time of 11.6 minutes for gemcitabine and 12.63 minutes for dFdU (Xu, et al., 2004). A very recent LC-MS method developed by Bowen et al.; uses a TurboIonspray interface (TIS). The method is highly efficient and very quick. It is capable of analyzing very small amounts of sample with a total run time of 1.5 minutes per sample. Chemical derivatization of gemcitabine and dFdU with dansyl chloride was found to be extremely helpful in achieving separation within 2 minutes. Chemical derivatization makes gemcitabine more hydrophobic and aids in its analysis using reverse phase HPLC (Bowen, et al., 2009). Some of the HPLC methods reported in literature, for the analysis of gemcitabine and its metabolites are listed in table 3.1.

The objective of this study was to develop and validate an efficient method for the quantitative analysis of gemcitabine. A method that is rapid and sensitive at the same time. The applications of this method encompasses determination of drug load and encapsulation efficiency, evaluation of the *in vitro* release of gemcitabine from the developed nano-particulate formulation and the cellular uptake of gemcitabine by BXPC3 cell lines.

Table 3.1 Reported HPLC methods for the quantitative analysis of gemcitabine.

Column	Mobile Phase (V/V)	Flow rate (ml/min)	Wave length (nm)	Retention time (min)	Reference
Adsorbosphere or Econosphere NH ₂ (250 X 4.6 mm) 5 μm	Cyclohexane 630 ml, 1,2-dichloroethane 150 ml, methanol 220 ml, water 1 ml, glacial acetic acid 0.5 ml, triethyl amine 1 ml.	1.5	272	20	(Freeman, et al., 1995).
C18 Cartridge (4.6 X 215 mm)	15 mM ammonium acetate pH(5.0), acetonitrile (97.5:2.5)	1.0	280	10	(Venook, et al., 2000).
Cromosil C18 (4.6 X 150 mm) 5 μm in tandem Beckman ultrasphere ODS (4.6 X 150 mm) 5 μm	Solution A (1% acetonitrile & 3 % methanol in 50 mM phosphate buffer pH 2.9), Solution B (acetonitrile and 50mM phosphate buffer pH 2.9 1/1)	Gradient non isocratic elution	272	40 minutes total cycle time	(Patnaik, et al., 2000).
Simmetry Shield C18 (300 x 4.6 mm)	30 mM Phosphate buffer pH 6.8, acetonitrile,	1	270		(Fogli, et al., 2002).

5 µm	methanol (96:2:2)				
Columbus C18 (150 x 4.6 mm) 5 µm	Solvent A (50 mM ammonium acetate pH 5.0, 2% methanol), solvent B (50 mM ammonium acetate pH 5.0 with 10% methanol)	1.5	269	18.1	(Keith, et al., 2003).
Nucleosil 5 NH ₂ (250 x 4.0 mm) 5 µm	Methanol, cyclohexane, 1,2-dichloro ethane	1.0	272	7.5	(Yilmaz, et al., 2003).
Waters YMC ODS-AQ (150 x 2.0 mm) 5 µm	Solvent A (5 mM ammonium acetate pH 6.8). solvent B (methanol)	0.2	In tandem ESI-MS detector		(Xu, et al., 2004).
Tracer excel ODSA C18 (100 x 4.6 mm) 3 µm	Solvent A (KH ₂ PO ₄ 10mM/TBACI 10 mM pH 7.0, 0.25% methanol), solvent B (KH ₂ PO ₄ 250mM/TBACI 10 mM pH 7.0:methanol 85:15) (64:36)	1.2	271	9.6	(Losa, et al., 2006).
Waters Spherisorb ODS2 (250 X	Acetonitrile, 50 mM sodium phosphate/ 3.0	1.0	267	~ 9.0	(Kirstein, et al.,

4.6 mm) 5 μ m	mM octyl sulfonic acid pH 2.9 (10:90)				2006).
Symmetry C18	Methanol, water (90:10)	0.8	266		(Stella, et al., 2007).
BEH C18 (50 X 2.1 mm) 1.7 μ m	Mobile phase A (0.1% formic acid), mobile phase B (acetonitrile)	0.7	In tandem TIS-MS detector		(Bowen, et al., 2009).

3.2. Materials

Gemcitabine Hydrochloride (Gemzar FW 299.66) was purchased from Creighton University Medical Center pharmacy store. Ammonium acetate was purchased from Spectrum Chemicals (San Pedro St, CA). Sodium phosphate dibasic, sodium phosphate monobasic, dioctyl sodium sulfosuccinate, cold water soluble poly vinyl alcohol having formulae weight of 50,000 and low viscosity sodium alginate were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile and dichloro methane were from Fischer Chemicals (Fairlawn, NJ).

3.3. Methods

3.3.1. Chromatography

The HPLC system consisted of a solvent delivery module LC-10AT, an auto injector (Model SIL-9A), a UV-Vis Spectrophotometric detector (Model SPD-6AV), and a recorder (Model CR-501) all of which were obtained from Shimadzu Corporation,

Tokyo, Japan. The separation of Gemcitabine was carried out on a 250X4.60 mm 5 μ C18 Luna Column obtained from Phenomenex. The mobile phase comprised of 40 mM ammonium acetate:acetonitrile (95:5 v/v). The apparent pH of the mobile phase was adjusted to 5.5 using acetic acid glacial. The flow rate was maintained at 1.0 mL/min. The run time for each run was set for 10 minutes. The effluents were monitored at 270 nm.

3.3.2. Preparation of solutions

3.3.2.1. Ammonium acetate (40 mM)

A 40 mM solution of ammonium acetate (molecular weight 77.08) was prepared by dissolving 3.08 g of ammonium acetate in 500 mL deionized HPLC water by stirring using a magnetic stirrer. The final volume was made up to 1000 mL by adding HPLC water in a volumetric flask. The pH of the ammonium acetate solution was adjusted to 5.5 by adding acetic acid glacial drop wise.

3.3.2.2. Mobile phase

Acetonitrile (50 mL) was mixed with ammonium acetate solution, 40 mM (950 mL). The solution was agitated on a magnetic stirrer for 10-15 minutes to ensure uniform mixing. The solution was degassed for 30 minutes and then filtered through a 0.2 μ m polycarbonate filter (Osmonics, Inc.) prior to be used in HPLC. The Apparent pH of the mobile phase was 5.5.

3.3.2.3. Standard solutions

Standard solutions for gemcitabine were prepared in mobile phase. Gemzar was obtained as powdered drug. It was reconstituted with deionized water to give a stock

solution of 38 mg/mL. Various standard solutions were prepared from this stock solution after adequate dilutions with the mobile phase.

3.4. Calculations

The unknown Gemcitabine concentration was determined by interpolating from the regression equation relating to the peak area, obtained from the set of standard solutions.

3.5. Applications of the HPLC method

3.5.1. Determination of Drug Loading & Encapsulation efficiency

Drug loading in nano-particles and encapsulation efficiency were determined by measuring the left over drug in the supernatant and washing fluids. The supernatant and the wash fluid were collected in 50 ml plastic tubes. The solution was diluted accordingly and filtered through syringe filter. The amount of drug present in the solution was analyzed by HPLC. Mathematically, it can be expressed as:

Drug Loading =

$$\frac{(\textit{Total amount of Drug added} - \textit{Left over drug in supernatant and washing fluid})}{\textit{Total amount of Polymer}} \times 100$$

Encapsulation efficiency =

$$\frac{(\textit{Total amount of Drug added} - \textit{Left over drug in supernatant and washing fluid})}{\textit{Total amount of Drug added}} \times 100$$

3.5.2. *In vitro* release of Gemcitabine from the nano-particulate formulation

The *in vitro* release of the entrapped drug was determined under sink conditions. 10 mg of the formulation containing known amount of drug was added to 40 ml of phosphate buffer saline (pH 7.4) in capped Erlenmeyer flask. The flasks were kept in a shaker bath incubator maintained at 100 rpm and 37°C. Known volumes of release media were withdrawn and collected at definite intervals of time and replaced with equal volume of fresh buffer. The cumulative drug released was estimated by analyzing the drug concentration in the release media using HPLC.

3.5.3. Cellular uptake of gemcitabine by BXPC3 cell lines

The uptake of the free drug and nano-particulate was evaluated in BXPC3 cells. BXPC3 cells were seeded in six well plates according to the ATCC requirements. The cells were then incubated in a humidified incubator maintained at 37°C and supplied with 5% CO₂. Cells were observed for con-fluency under a microscope regularly. Cells when confluent; were treated with a single dose of free drug solution and an equivalent nano-particulate suspension for 15, 30 and 60 minutes. At the end of the stipulated time intervals the cells were washed 3 times with ice cold PBS to remove any free drug or particulate matter. The cells were then lysed with 1% solution of triton X. Twenty five µl of this solution was analyzed for the total protein content according to the BCA protein assay. The amount of drug present in the solution was then analyzed using HPLC.

3.6. Analysis of drug

HPLC method was developed and validated for the quantitative analyses of gemcitabine. The chromatographic separation was achieved on a LUNA C- 18 column (250x4.60 mm, 5 µ) with a flow rate of 1.0 mL/min with UV detection at 270 nm. Mobile

phase consisted of 40 mM ammonium acetate:acetonitrile (95:5 % v/v). The pH of the mobile phase was adjusted to 5.5 with glacial acetic acid. Mobile phase was filtered and degassed prior to HPLC use.

3.7. Results and discussion

3.7.1. Specificity

Analytical specificity of a method may be defined as its ability to accurately identify, measure and resolve an analyte in the presence of other closely related compounds that is, how well an assay detects only a specific substance and not the other closely related substances during an analysis. The Analytical specificity of this reverse phase HPLC method was determined by comparing the chromatograms obtained followed by the injection of just the mobile phase and the chromatogram of the mobile phase containing the drug. Twenty μL of Gemcitabine (38 $\mu\text{g}/\text{mL}$) was spiked in the mobile phase and injected onto HPLC. The representative chromatogram of the mobile phase alone (without drug) is shown in figure 3.1 and the representative chromatogram of the mobile phase containing 19 $\mu\text{g}/\text{mL}$ of Gemcitabine is shown in figure 3.2. The retention time of gemcitabine was found to be 6.8 minutes. The absence of any overlapping or extraneous peaks in both chromatograms indicates the specificity of the HPLC method. Since gemcitabine peak could be clearly distinguished from the other peaks therefore, this method was said to be specific for the analysis of gemcitabine.

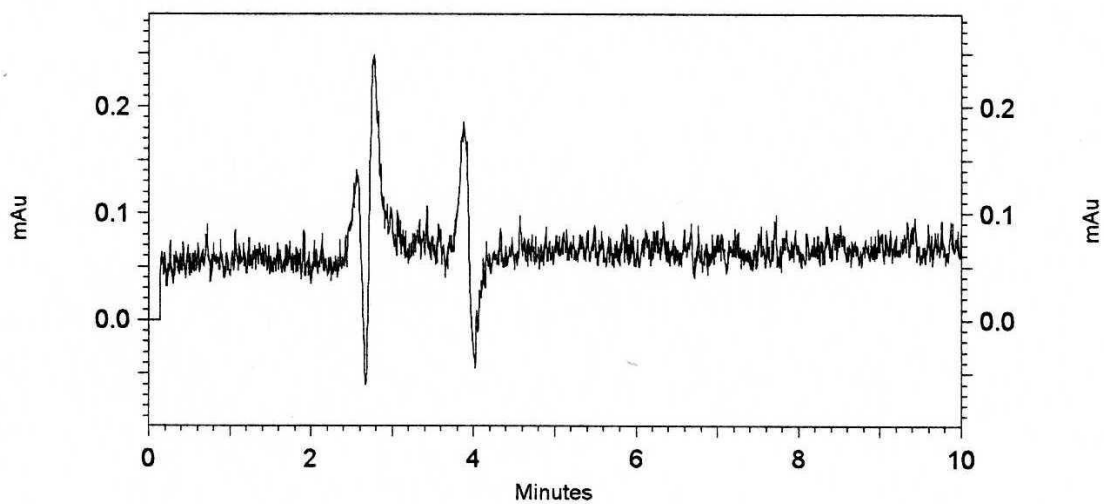


Figure 3.1 A sample chromatogram obtained following injection of mobile phase for Gemcitabine.

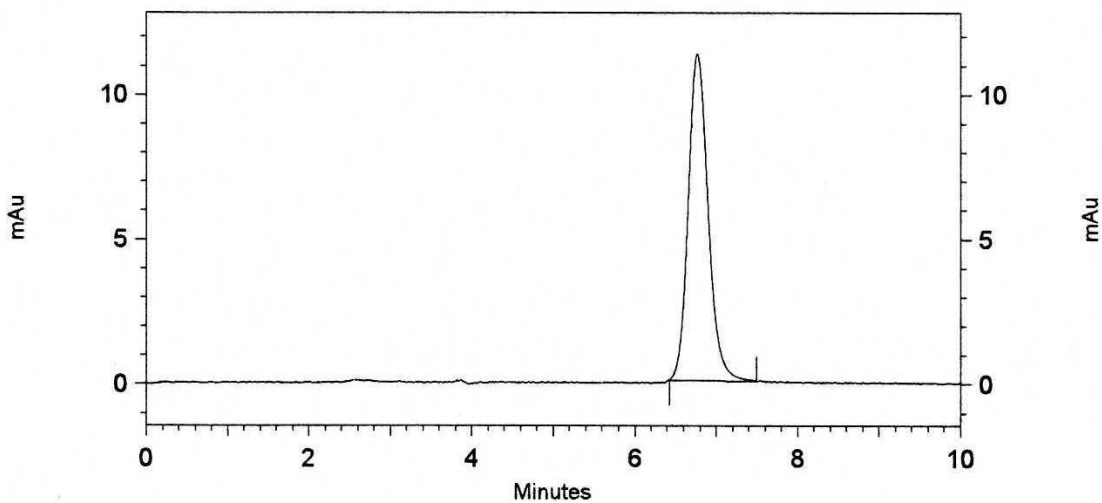


Figure 3.2 A sample chromatogram obtained following injection of a 19 µg/mL Gemcitabine sample (Retention time 6.8 min).

3.7.2. Linearity

A linear study identifies a specified concentration range where analyte's response is linearly proportional to the concentration. The standard curve was found to be linear over the concentration range of 0.95 - 76 $\mu\text{g/mL}$. The equation of the standard curve relating the peak area to the gemcitabine concentration (C in $\mu\text{g/ml}$) in this range was: Peak area = 20147 C + 1341.6, $r^2 > 0.999$. It is a statistical measure of the extent to which variations in one variable are related to variations in another. The stronger the relationship, the more the data points on the scatter plot will align them in a straight line. When $r^2 = 1$, then all data points fall on the regression line. Figure 3.3 shows a linear standard curve obtained by injecting gemcitabine standards.

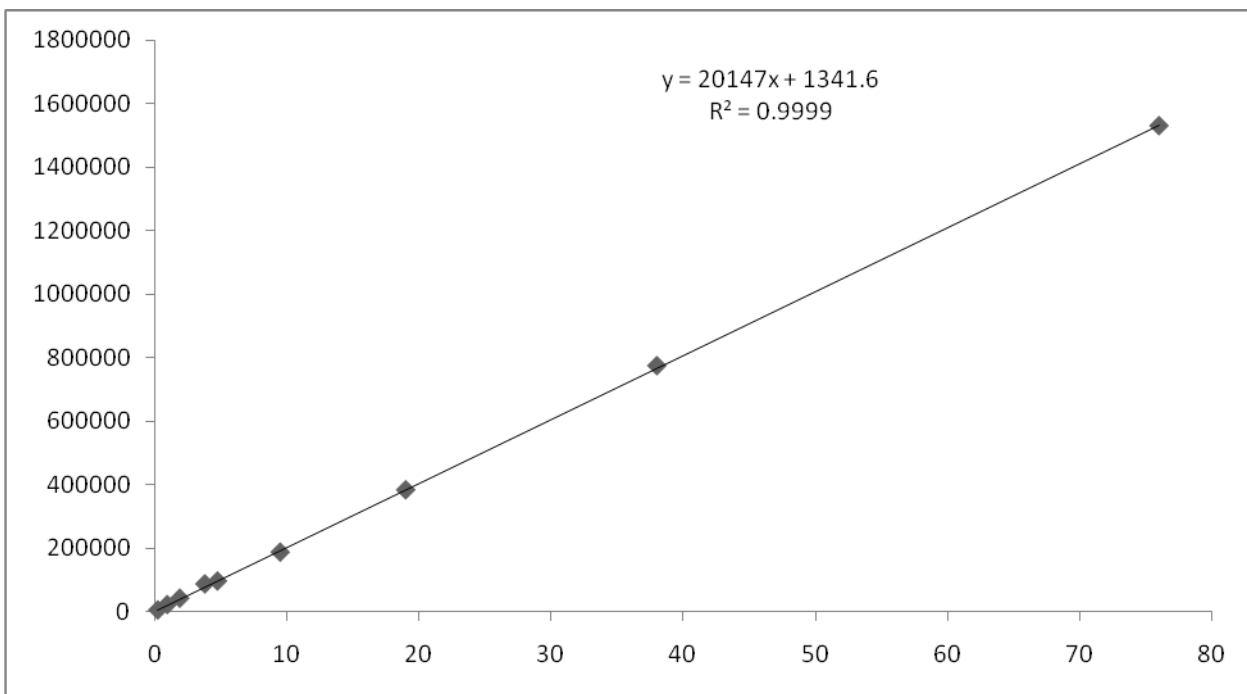


Figure 3.3 Standard Curve for Gemcitabine was found to be linear over a range of 0.95 – 76 $\mu\text{g/mL}$.

3.7.3. Precision

Precision is a measure of the consistency and reproducibility of a method. A precise method gives very close values for repeated measurements of same sample under identical experimental conditions. For current assay validation, within day precision and day to day precision were used. The within day precision was determined by repeated injection of standard solutions four times on the same day. Day to day precision was determined by injecting one set of standard solutions on four different days over a period of 30 days. The relative standard deviation (RSD) values were calculated for both within day precision and day to day precision and were found to fall within the acceptable limits. The precision results for the HPLC method are listed in table 3.2.

Table 3.2 Within day and day to day precision analysis of HPLC method for gemcitabine.

Concentration ($\mu\text{g/mL}$)	Within day		Day to day	
	Mean Peak Area	RSD (%)	Mean Peak Area	RSD (%)
0.95	19409.75	8.10	15243.6	9.48
3.8	84256.25	11.34	69046.2	2.95
4.75	92834.75	2.84	84919.8	5.84
19	384533.8	2.76	357645	4.39
76	1440369	11.37	1419695	9.86

3.7.4. Accuracy

Accuracy, sometimes also referred to as recovery is an indicator of the trueness of test measurements. To determine the accuracy of the method three quality control samples (1.9 µg/mL, 9.5 µg/mL and 38 µg/mL) were used. The samples chosen were such to represent the entire range of the standard curve i.e lower, middle and higher concentrations of the range. Samples were analyzed four times on different days. The accuracy of the assay was evaluated by comparing the estimated concentration with the known concentration of gemcitabine. The accuracy of three quality control samples was found to be 101.45%, 94.63% and 97.17% respectively. The results of this study are depicted in table 3.3.

Table 3.3 Accuracy of the HPLC method for Gemcitabine.

Actual concentrations (µg/mL)	Measured Concentrations* (µg/mL)	Accuracy** (%)
1.9	1.93 ± 0.13	101.45 ± 6.94199
9.5	9.0 ± 0.22	94.63 ± 2.27
38	36.92 ± 1.08	97.17 ± 2.84

* Mean ± SD, n=4

** Accuracy = (Measured concentration/Actual concentration) X 100, Mean ± SD, n=4

3.8. Conclusion

A rapid analytical method for the quantitative estimation of gemcitabine hydrochloride was successfully developed and validated using reverse phase high performance liquid chromatography. The chromatographic separation was carried out on a C18 Luna column (250 x 4.60 mm, 5 μ) obtained from phenomenex using a mobile comprised of ammonium acetate 40 mM and acetonitrile in a ratio of 95:5 v/v. The flow rate was maintained at 1.0 mL/min. The UV absorption by the effluents was read at 270 nm using a photo diode detector. The developed HPLC method was found to be specific for gemcitabine hydrochloride. A strong linear correlation with an $r^2 > 0.9999$ was observed between the concentration of the drug and peak area obtained upon chromatographic extraction over a concentration range of 0.24 to 76 μ g/mL. The precision of the analytical method was tested using within day and inter-day validation. Also, the method depicted an accuracy of ~ 101.45, 94.63 & 97.17 % for concentrations 1.9, 9.5 and 38 μ g/mL respectively.

CHAPTER 4

Development and Optimization of the Nano-particulate

Delivery System

4.1. Introduction

Various gemcitabine delivery systems have been formulated and investigated, which include aerosols, gemcitabine conjugates, liposome, nano-particles and in situ gel forming matrix system.

Aerosol Formulations of gemcitabine

Inhalational therapy has been used for many drugs to obtain a local effect in the lungs and to avoid the systemic toxicity. The use of aerosol delivery for the purpose of delivering antineoplastic agents to lungs for the avoidance of systemic toxicity was first studied by Hershey et al. in dogs. The dogs received paclitaxel or doxorubicin treatment by inhalational route for two weeks. The data revealed avoidance of systemic toxicity while delivering the highly potent drugs at the target site (Hershey, et al., 1999). The first aerosol formulation with gemcitabine was developed by Kawai and group. A fifty fold increase in the gemcitabine concentration in lungs was observed after pulmonary administration compared to intravenous, in rats (Kawai, et al., 1995). Gemcitabine was formulated into aerosol for the treatment of osteosarcoma in an experimental model (Koshkina and Kleinerman, 2005). Gagnadoux and coworkers studied the safety and efficacy of gemcitabine through pulmonary route (Gagnadoux, et al., 2005a: Gagnadoux, et al., 2006). The systemic toxicity was determined as a measure of weight loss and white blood cell toxicity following to administration of 2, 4, 6 and 8 mg/kg of the drug via pulmonary or oral route. It was observed that the systemic toxicity produced by the drug through inhalational route was less than compared to when an equivalent dose of

gemcitabine was administered orally (Gagnadoux, et al., 2005b). In a recent preclinical study on baboons it was observed that gemcitabine could be successfully formulated into an aerosol formulation and delivered to treat intrathoracic airway depositions via jet nebulization with no loss in its cytotoxic activity (Gagnadoux, et al., 2005b). Hence, preclinical studies have supplemented ample evidence that gemcitabine can be successfully formulated and delivered by aerosols for local therapy of lung cancers.

Gemcitabine conjugates

The chemical modification of a drug into an inactive prodrug has been widely used to improve its solubility, delivery, gastric uptake, bioavailability and half-life. In order to prevent gemcitabine from being rapidly metabolized by the process of deamination, various conjugates of the drug have been investigated. The chemical linkage of gemcitabine with lipids offer advantages in overcoming issues like its short half-life and resistance of cancer cells to gemcitabine treatment. In some cases it also improves the tissue absorption of the drug (Bergman, et al., 2004). The most widely used sites for the attachment of conjugates are 3' or 5' OH or the 4-amino group of gemcitabine (Myhren, et al.; 2000).

Gemcitabine prodrugs have been synthesized using a wide variety of compounds. Some of these include saturated or monounsaturated carbon chains, acyl fatty acids like elaidic acid, 4-(N)-stearoyl, 4-(N)- lauroyl, 4-(N)-valeroyl etc. (Immordino, et al., 2004). Target specificity can also be induced using the conjugate approach by linking gemcitabine to receptor specific ligands. Enhanced accumulation of gemcitabine conjugated with peripheral benzodiazepine receptor ligand in brain glioma model was observed (Guo, et al., 2001). This can be attributed to the overexpression of

benzodiazepine receptors in brain tumors (Black, et al., 1989; Black, et al., 1990). Gemcitabine conjugated to biphosphonate expressed high affinity to bone powder and hydroxyapatite (Zarski, et al., 2001).

Besides improving target specificity, some conjugates have also led to an enhancement in anticancer activity. Such conjugates include gamma linolenic acid (GLA) (Falconer, et al., 1994; Ravichandran, et al., 1998), eicosapentaenoic acid, docosahexaenoic acid (Guffy, et al., 1984; Rose, et al., 1995; Zijlstra, et al., 1987), folate etc.

Gemcitabine liposomes

Liposomes are phospholipids bi/multi layered vesicles with size ranging from tens to hundreds nanometers. The use of liposomes to improve the delivery of gemcitabine to tumors has been extensively studied. Studies have indicated that *in vitro* cytotoxicity of liposomes is dependent on the lipid composition and preparation conditions (Calvagno, et al., 2007). Higher cytotoxicity was observed when lipid composition of liposomes formulated from dipalmitoylphosphatidyl choline, cholesterol and distearoylphosphatidylethanolamine-methoxypolyethylene glycol was changed from 8:3:1 to 6:3:1 (Calvagno, et al., 2007). Liposomes composed of egg phosphatidyl choline and cholesterol (1:1) have also been shown to significantly increase the plasma half life of gemcitabine leading to a subsequent increase in the amount of drug levels reached in plasma, following intravenous administration (Moog, et al., 2002).

In situ gel forming matrix systems for gemcitabine

Incorporating a drug in a gel matrix to control its release has been employed for many anti-cancer agents (Jauhari and Dash, 2006; Kakinoki and Taguchi, 2007; Ruel-

Garipey, et al., 2004). Studies have revealed that release of gemcitabine can be effectively prolonged by immobilizing the drug into an *in situ* forming gel of styrenated gelatin (Okino, et al., 2003).

Gemcitabine nanoparticles

One of the recent advancements in novel drug delivery system is the advent of nano-particles. Nano sized carriers have found many applications in drug delivery (Colin de Verdiere, et al., 1994). Different polymeric systems used for incorporating gemcitabine include poly ϵ -caprolactone (PCL) (Yang, et al., 2006b), polyethylcyanoacrylate (Yang, et al., 2006a). Nano-particulate formulations have been shown to not just sustain the release of gemcitabine, but also enhance its *in vivo* antitumor activity (Gang, et al., 2007). Stella and coworkers evaluated the use of polycyanoacrylate to fabricate nano-particles for a lipophilic conjugate of gemcitabine. The gemcitabine conjugate was synthesized by covalently linking an acyl chain to the 4-amino group on the gemcitabine (Stella, et al., 2007). Nano-particles of gemcitabine were also manufactured by covalently linking gemcitabine with 1,1',2-tris-nor-squalenic acid. These nano-particles exhibited higher toxicity in murine resistant leukemia and human resistant leukemia cell lines (Reddy and Couvreur, 2008). Gemcitabine nano-particles have also been produced by entrapping the drug in polybutylcyanoacrylate (Huang, et al., 2007). Arias and group also evaluated the cytotoxicity of gemcitabine loaded polybutylcyanoacrylate nanoparticles in murine leukemia cells (Arias, et al., 2009). Gemcitabine nano-particles formulated using bovine serum albumin, were found to significantly improve its anti-proliferative activity, when tested on human pancreatic cancer lines (BxPc-3) (Li, et al., 2009).

4.2. Materials

Gemzar[®] (Gemcitabine Hydro chloride) was obtained from Creighton University Medical Center pharmacy store. It has a formulae weight of 299.66. It is available as white powder, 1 g of which when reconstituted with 25 ml of deionized water yields a 38 mg/ml solution of gemcitabine. Ammonium acetate was purchased from Spectrum Chemicals (San Pedro St, CA). Sodium phosphate dibasic, sodium phosphate monobasic, calcium chloride anhydrous, dioctyl sodium sulfosuccinate, cold water soluble poly vinyl alcohol having formulae weight of 50,000 and low viscosity sodium alginate were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile and dichloro methane were from Fischer Chemicals (Fairlawn, NJ).

4.3. Methods

4.3.1. Preparation of solutions

4.3.1.1. Alginate solution 1% (w/v)

One gram of low viscosity alginic acid sodium was weighed and dissolved in 70 ml of deionized water in a beaker. The mixture was stirred on a magnetic stirrer for 30-45 minutes. The final volume of the solution was made up to 100 ml with deionized water in a volumetric flask to yield a 1% w/v solution of sodium alginate.

4.3.1.2. PLGA:PCL (4:1) solution 4% (w/v)

One hundred and sixty mg of PLGA and 40 mg of PCL were accurately weighed and dissolve in 5 ml of dichloro methane in a 15 ml glass vial. The glass vial was capped

and kept on a shaker with gentle agitation to completely dissolve PLGA and PCL in dichloro methane.

4.3.1.3. Polyvinyl alcohol 0.5% (w/v)

500 mg of cold water soluble PVA was accurately weighed and added in small quantities from top to 80 ml deionized water in a glass beaker. The mixture was stirred on a magnetic stirrer for an hour to completely dissolve the PVA. The final volume of the PVA solution was made up to 100 ml with deionized water in a volumetric flask.

4.3.1.4. Polyvinyl alcohol 2.0% (w/v)

Two grams of cold water soluble PVA was accurately weighed and added in small quantities from top to 80 ml deionized water in a glass beaker. The mixture was stirred on a magnetic stirrer for an hour to completely dissolve the PVA. The final volume of the PVA solution was made up to 100 ml with deionized water in a volumetric flask.

4.3.1.5. Dioctyl sodium sulfosuccinate 10% (w/v)

Five grams of dioctyl sodium sulfosuccinate was weighed accurately and dissolved in 50 ml of dichloro methane in a glass beaker covered with paraffin film to prevent dichloro methane from evaporating. The beaker was kept on a shaker bath with gentle agitation to yield a 10% (w/v) solution of dioctyl sodium sulfosuccinate in dichloro methane.

4.3.1.6. Calcium chloride 60% (w/v)

30 grams of calcium chloride anhydrous was accurately weighed and dissolved in 30 ml of water in a volumetric flask immersed in an ice cold water bath. The final volume

was made up to 50 ml in a volumetric flask to yield 60% (w/v) solution of calcium chloride.

4.3.2. Formulation of the delivery system

4.3.2.1. Formulation of PLGA/PCL nano-particles

The nano-particulate formulation with PLGA/PCL was prepared using multiple emulsion solvent evaporation technique. Known amount of gemcitabine solution 38 mg/ml with and without 200 mg sodium chloride was emulsified into PLGA/PCL solution (4:1), 4 % (w/v) by sonication for 1 minute at 12 watts to prepare a primary emulsion. This primary emulsion was further emulsified into PVA solution 2% (w/v) by sonication at 18 watts for 5 minutes to obtain a (w/o/w type) multiple emulsion. This multiple emulsion was left on magnetic stirrer overnight to evaporate all the oily phase (dichloro methane). The suspended particles were separated by centrifuging at 10,000 rpm in an ultra-centrifuge. The pellet was washed twice with deionized water to remove PVA and surface bound free drug if any and re-centrifuged. The pellet was finally re-suspended in minimal amount of deionized water and the final nano-particulate formulation was procured by drying in a freeze dryer.

4.3.2.2. Formulation of Alginate nano-particles

Sodium alginate nano-particles were prepared using multiple emulsion solvent evaporation technique. Known amount of gemcitabine solution 38 mg/ml in 1% aqueous solution of low viscosity alginate was emulsified into dioctyl sodium sulfosuccinate solution in dichloro methane 10% by sonication at 12 watts for 1 minute to prepare a primary emulsion. This primary emulsion was further emulsified into PVA solution 0.5%

(w/v) by sonication at 18 watts for 5 minutes to obtain a (w/o/w type) multiple emulsion. Calcium chloride solution 60% (w/v) was then added drop wise under sonication at 18 watts. This multiple emulsion was left on magnetic stirrer overnight to evaporate all the oily phase (dichloro methane). The suspended particles were separated by centrifuging at 25,000 rpm in an ultra-centrifuge. The pellet was washed twice with deionized water to remove PVA and surface bound free drug if any and re-centrifuged. The pellet was finally re-suspended in minimal amount of deionized water and the final nano-particulate formulation was procured by drying in a freeze dryer.

4.3.3. Determination of Particle Size and Surface charge

The particle size and surface charge (zeta potential) of the formulation were determined using a zetameter. A zetameter works on the principle of photon correlation spectroscopy. 2 mg of the freeze dried nano-particulate formulation was weighed accurately and suspended in 5 ml of deionized water. The samples were sonicated prior to analysis to obtain a uniform dispersion of the particles. The samples were transferred to a cuvette and measurements were performed in triplicate using a zetameter (Zetaplus, Brookhaven instruments Corporation, NY, USA).

4.3.4. Surface morphology

Scanning electron microscopy (SEM) was used to study the morphological characteristics of blank nano-particulate formulation and nano-particulate formulation containing gemcitabine. Approximately 2 mg of sample was dispersed in deionized water. A few drops of this suspension were placed on a metal stub and allowed to air dry to form a thin uniform layer of particles on the metal stub. The stub was then coated

sputter coated with gold-palladium alloy (140 nm thickness) using a Hummer VI sputter coater (Anatech, NJ, USA). Samples were examined on a JEOL JSM 840 Scanning Electron Microscope (Jeol T-220A, Tokyo, Japan) at an acceleration voltage of 80 k V. The SEM pictures were obtained using an Orion v.6.1 digital image system.

4.3.5. Determination of thermal properties

The physical state of gemcitabine in the formulation was analyzed using a Differential Scanning Calorimeter (DSC). Three mg of both PLGA/PCL and Alginate nano-particulate formulation was accurately weighed and crimped into Aluminum crimp pans. The samples were then placed in the DSC (Shimadzu DSC-60, Japan) sample chamber and heated at a rate of 10°C/min up to a maximum of 300°C. The sample was held at this temperature for one minute before it was allowed to cool down to room temperature. The thermograms obtained were used to determine the physical state of gemcitabine in the formulation.

4.3.6. Determination of Drug Load and Encapsulation Efficiency

Drug loading in nano-particles and encapsulation efficiency were determined by measuring the left over drug in the supernatant and washing fluids. The supernatant and the wash fluid were collected in 50 ml plastic tubes. The solution was diluted accordingly and filtered through syringe filter. The amount of drug present in the solution was analyzed by HPLC. The calculations and the mathematical formulae involved in estimating the drug loading and encapsulation efficiency are previously discussed in chapter 2: analytical method development and validation.

4.3.7. *In vitro* release of Gemcitabine from the nano-particulate formulation

The *in vitro* release of the entrapped drug was determined under sink conditions. A known amount of the nano-particulate formulation was added to 40 ml of phosphate buffer saline (pH 7.4) in capped Erlenmeyer flask. The flasks were kept in a shaker bath incubator maintained at 100 rpm and 37°C. Two hundred µl of release media was withdrawn and collected at definite time intervals (2, 4, 8, 12 hours 1, 2, 4 & 7 days) and replaced with equal volume of fresh buffer. The cumulative drug released was estimated by analyzing the drug concentration in the release media using HPLC.

4.4. Results and discussion

4.4.1. Nanoparticle characterization

The average particle size and surface charges for both PLGA:PCL and Alginate particles are reported in table 4.1 along with standard deviation. Photon correlation spectroscopy and light scattering was used to estimate the average particle size and the studies were done in triplicate. Both alginate and PLGA:PCL particulate systems were found to be in nano size range, the data clearly shows that Alginate particles are much smaller in size compared to PLGA:PCL particles. The particle size for gemcitabine loaded PLGA:PCL particles is around 565 nm whereas, for alginate particles it was found to be around 210 nm. Both PLGA:PCL and Alginate particles carry a negative surface charge (~ -25.65 & -33.21 mV respectively). The negative surface charges are a resultant of anionic carboxylic acid residues on both alginic acid and glycolic acid. Particle size characterization forms an important part of nano-particulate delivery system characterization. It is very useful in understanding various properties of the nano-particles for example dispersion, aggregation and it also affects the biological uptake of the particles (Desai et al.; 1997). In studies with nano-particles it has been reported that

smaller particles are shown to exhibit higher cellular (Desai et al.; 1997) and tissue uptake (Hillyer et al.; 2001; Brooking et al.; 2001; Florence et al.; 1995).

4.4.2. Surface morphology

Figures 4.1 & 4.2 depict scanning electron microscopic pictures of Gemcitabine loaded PLGA:PCL and Alginate particles. Both PLGA:PCL & Alginate particles were found to have spherical shape. PLGA:PCL particles however, had a smooth surface compared to alginate particles, which appeared gelatinous. Dioctyl sulfosuccinate has been shown to form reverse micelles in non polar solvents such as methylene chloride (Chavanpatil et al.; 2007). Chanvanpatil and coworkers have previously reported that in nano-particles fabricated with aerosolized dioctyl sulfosuccinate (AOT) and calcium cross linked alginate, the calcium cross linked alginate forms the central core which is surrounded by AOT head groups with AOT tails pointing away from the central core (Chavanpatil et al.; 2007). The AOT tails comprise the hydrophobic matrix and form a bilayer structure in the multiple emulsion (Israelachevilli; 1991).

4.4.3. Differential Scanning Calorimeter

The DSC thermograms for both PLGA:PCL and Alginate particles are reported in figures 4.3 & 4.4. The absence of any sharp endothermic peak in the chromatograms suggests that gemcitabine is not present in the crystalline state in the matrix.

4.4.4. Drug Load and Encapsulation efficiency

The drug load and encapsulation efficiency data for various formulations is reported in table 4.2. The drug load is expressed as percentage of the drug in the delivery system (% w/w) and the encapsulation efficiency is expressed as the percentage of the

total drug entrapped in the system (% w/w). The drug loads were found to be 5.14%, 2.8% & 6.9% (w/w) for PLGA:PCL (with NaCl), PLGA:PCL (without NaCl) and Alginate particles respectively. The encapsulation efficiencies for the formulations were found to be 54.09 % for PLGA:PCL (with NaCl), 29.3 % (without NaCl) & 22.4 % for Alginate particles. It has been previously reported that incorporation of osmotic agents such as sodium chloride, sorbitol, sodium carbonate etc. in the inner aqueous phase can improve the yield and entrapment efficiency of the emulsion system (Ohwaki et al; 1992). Sodium chloride was evaluated for improvement in the drug load for PLGA:PCL particles. An approximate two fold increase was observed in the drug loading and encapsulation efficiency upon addition of 400 mg sodium chloride in the inner aqueous phase of the multiple emulsion. The presence of sodium chloride in the innermost aqueous phase imparts osmotic pressure and hence prevents the diffusion of solvent and active ingredient across the polymeric matrix into the external aqueous phase (Hino et al.; 2001).

4.4.5. *In vitro* release studies

The cumulative percentage result of Gemcitabine from Alginate particles (6.9% w/w) is represented in figure 4.5. Nano-particles demonstrated sustained release. The release curve depicted a burst release of approximately 12% after 12 hours followed by a sustained release of per day, with around 50% maximum release at the end of 7 days. No release was observed from PLGA:PCL particles even after 7 days. *In vitro* release studies indicate that nanoparticles can sustain the release of gemcitabine. The burst release can be explained by the surface bound or free drug. It is expected that electrostatic interactions between the protonated amino residues on gemcitabine and anionic

carboxylic groups are responsible for the surface bound interactions involved in the burst release. Previous studies have proven that the release of drug from alginate system is primarily governed by the sodium – calcium proportion in the release media. Calcium alginate systems when exposed to media rich in monovalent sodium ions are changed to water soluble sodium alginate (De and Robinson, 2003). Studies have indicated that increased levels of sodium ions increase the rate and amount of drug released from the nano-particulate delivery system (Chavanpatil, et al., 2007b). It has also been show, that the release of basic drugs is more sustained compared to the release of acidic drugs (Chavanpatil, et al.; 2007b). The electrostatic interaction with the basic drug and the anionic matrix is expected to contribute to sustained drug release. Therefore, it can be said that electrostatic interaction plays an important role in the drug release from alginate systems.

Nanoparticles	Particle Size (nm) (mean \pm SEM)	Zeta Potential (mV) (mean \pm SEM)
Gemcitabine loaded PLGA:PCL	564.7 \pm 56.50	-25.65 \pm 1.94
Gemcitabine loaded Alginate	210.6 \pm 6.90	-33.21 \pm 1.63

Table 4.1 Particle size and surface charge of Gemcitabine loaded PLGA:PCL and Alginate nano-particles.

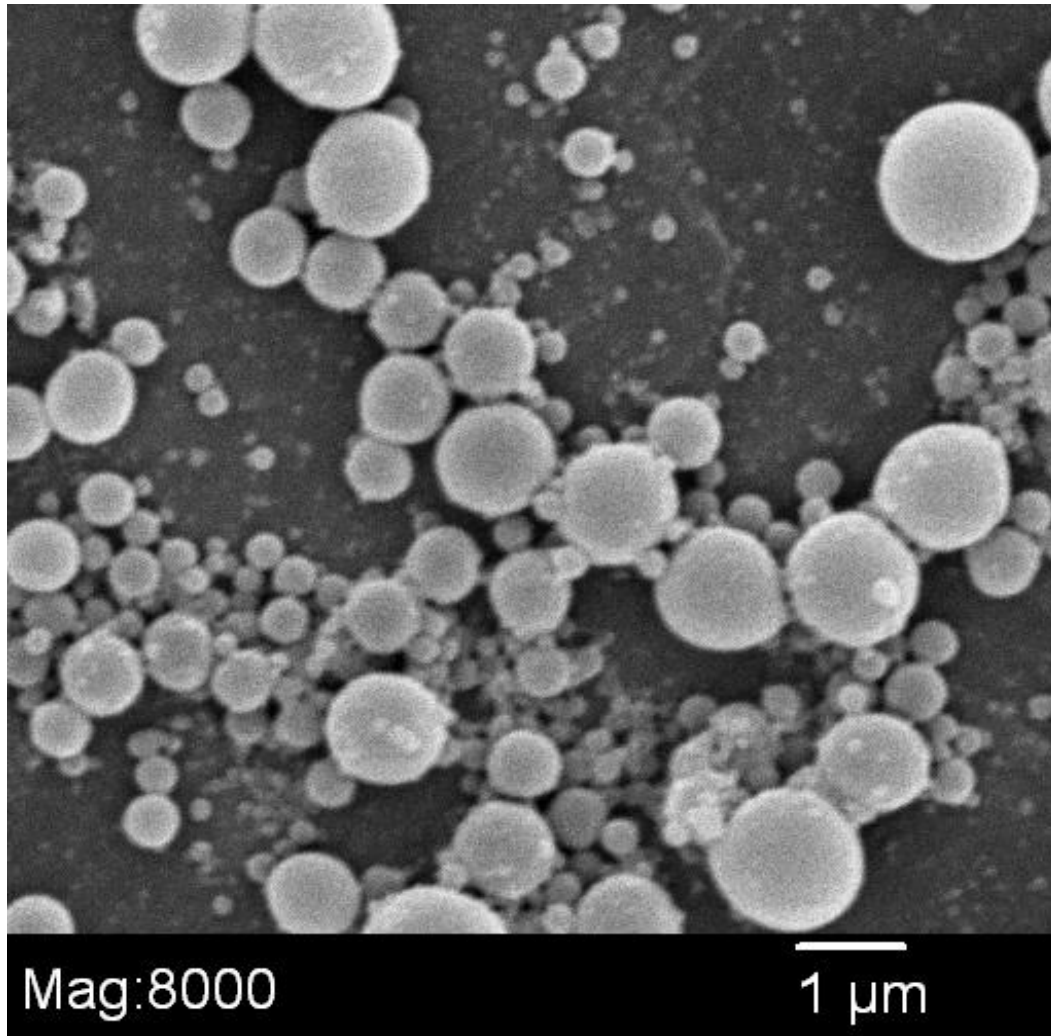


Figure 4.1 Surface morphology and microstructure of Gemcitabine loaded PLGA:PCL particles taken by Scanning electron microscopy at magnification 8000 X.

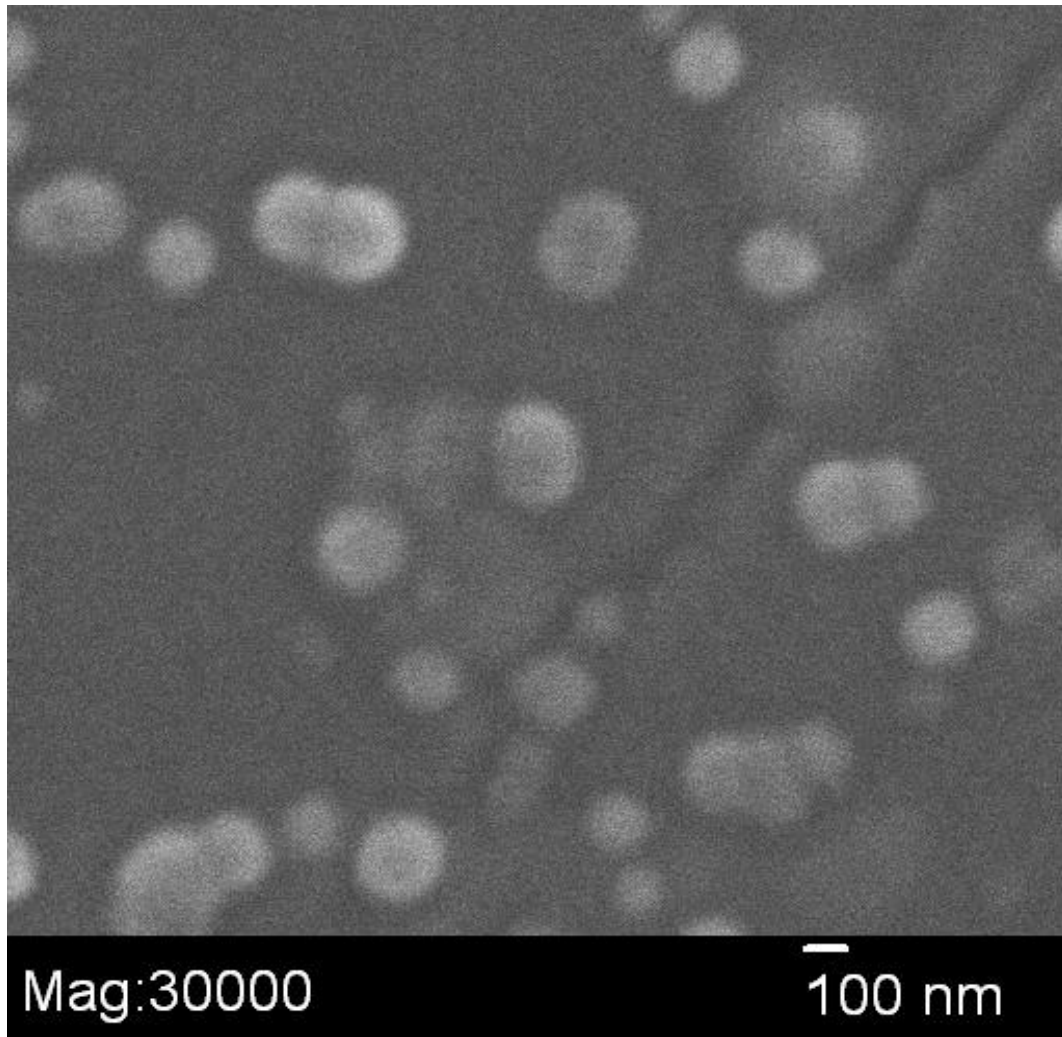


Figure 4.2 Surface morphology and microstructure of Gemcitabine loaded Alginate particles taken by Scanning electron microscopy at magnification 30000 X.

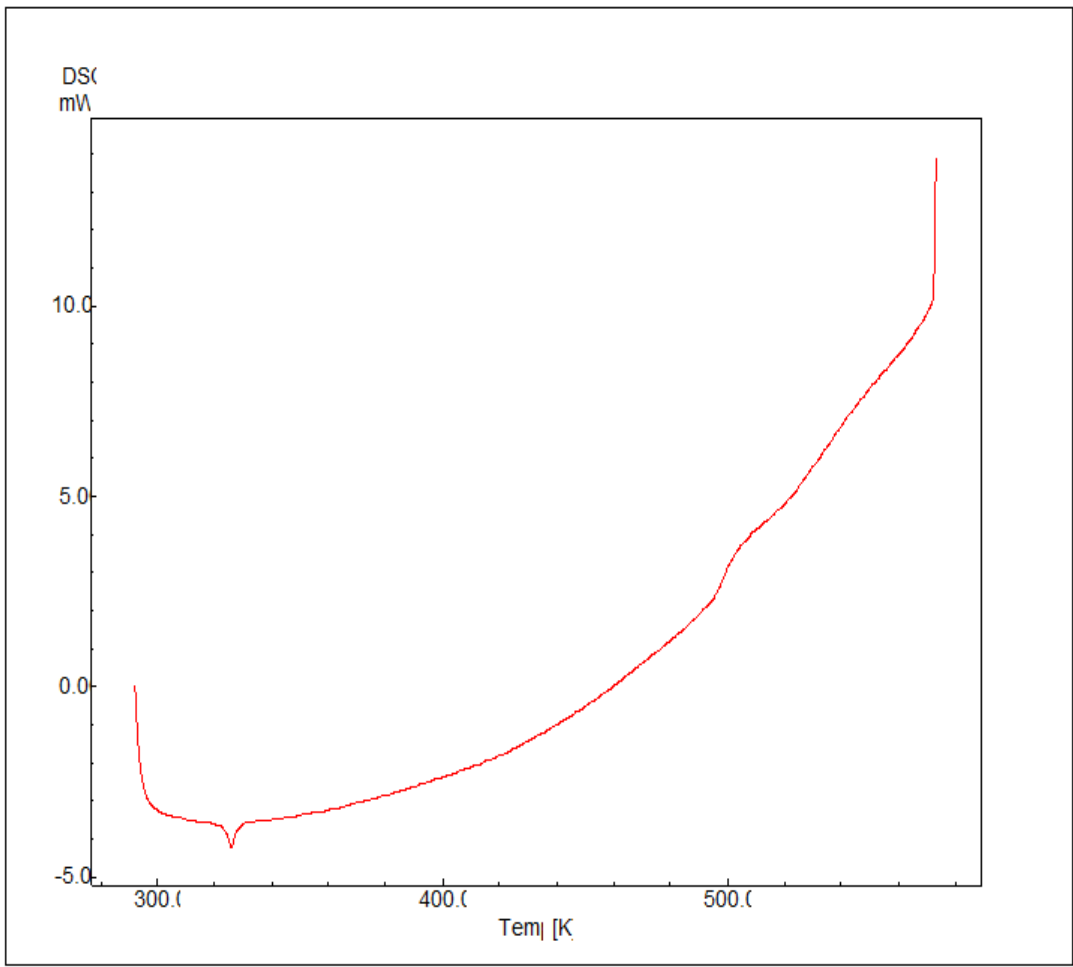


Figure 4.3 DSC thermogram of Gemcitabine loaded PLGA:PCL particles.

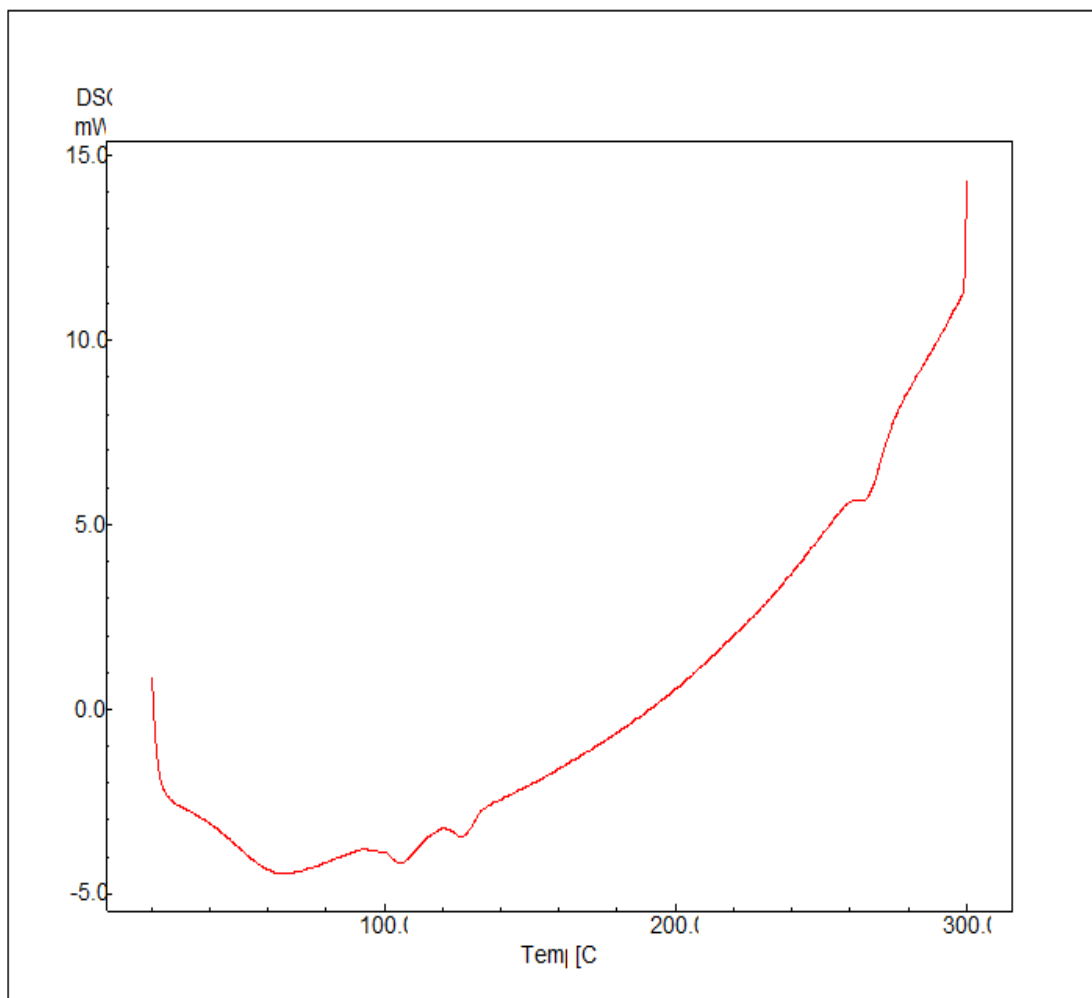


Figure 4.4 DSC thermogram of Gemcitabine loaded Alginate particles.

Nanoparticles	PLGA:PCL		Alginate
	With NaCl	Without NaCl	
Drug Load	5.14%	2.8%	6.9%
Entrapment efficiency	54.09%	29.3%	22.4%

Table 4.2 Drug load and Encapsulation efficiency of Gemcitabine loaded PLGA:PCL and Alginate particles.

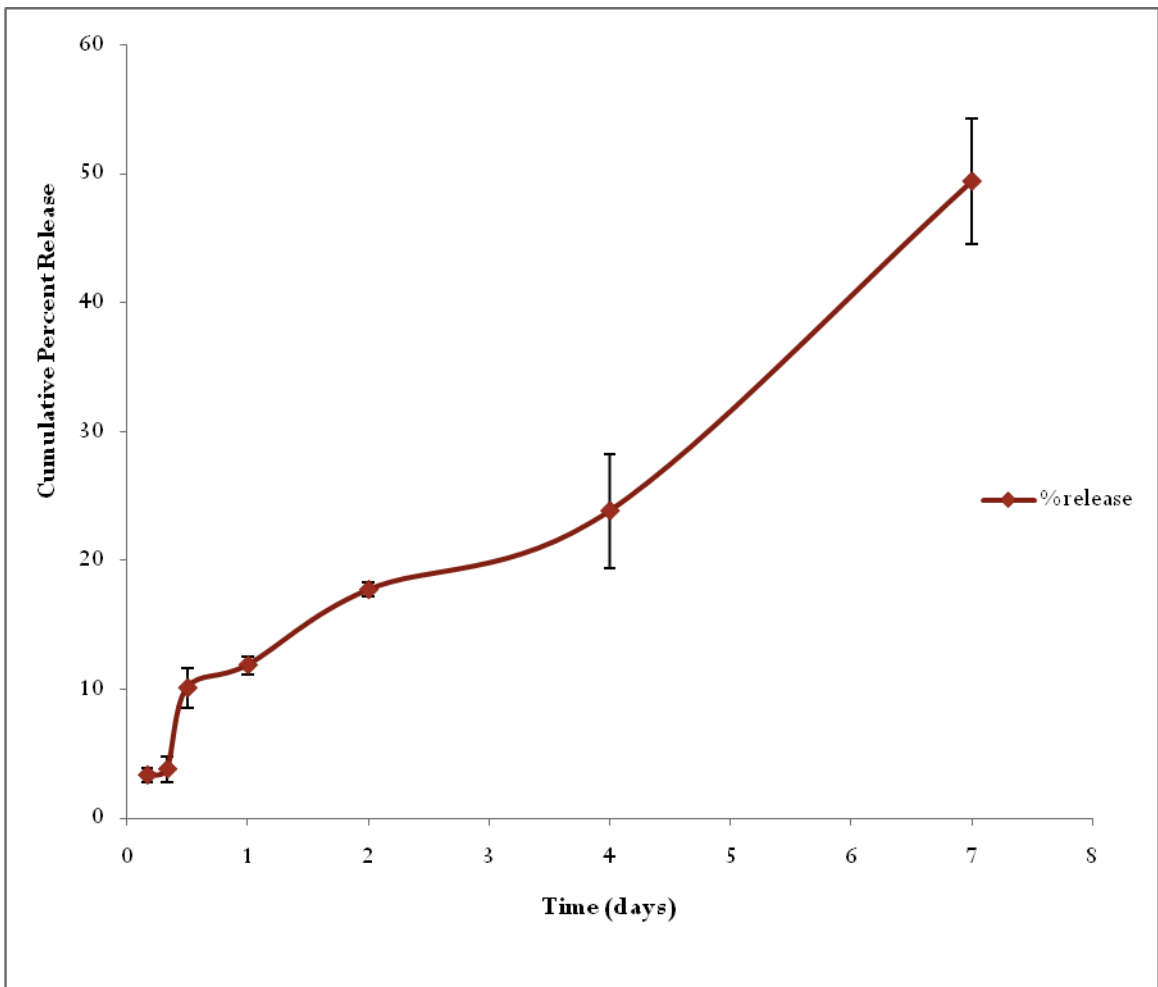


Figure 4.5 In vitro release profile of Gemcitabine loaded Alginate particles (6.9% w/w), carried out in phosphate buffer saline at pH 7.4 in a shaker bath maintained at 37°C (mean \pm SEM, n=3).

4.5. Conclusion

This study has demonstrated that gemcitabine could be successfully encapsulated and formulated into nano-particulate delivery systems composed of PLGA:PCL or calcium cross-linked alginate. The nano-particles can be formulated using multiple emulsion solvent evaporation technology. The organic phase (methylene chloride), which can form the intermediate phase can be evaporated by overnight stirring at room temperature. The suspension of the particles can then be centrifuged and lyophilized to obtain the dried free flowing powdered product. An average particle size of 210 nm and 565 nm was observed for alginate and PLGA:PCL particles, respectively. An *in vitro* release pattern from these alginate nano-particles demonstrated sustained release characteristics.

Chapter 5

Cellular uptake and Efficacy of the delivery system

5.1. Introduction

The transport of drugs across the cell membrane is a vital step for drugs having intracellular site of action (Zhao, et al., 2005). The lipophilic nature of cell membrane may present a barrier to the transport of easily ionizable weakly acidic and weakly basic drugs. The diffusion of polar compounds across the cell membrane is limited, which in turn, affects their availability at the intracellular site of action which may result in a complete loss of activity or inadequate therapeutic efficacy. The use substances that adhere to mucus layer, has been proposed to increase the cellular levels of the drugs.

Many bioadhesive polymeric systems have been successfully used to extend the mean residence time of the drug in body. Use of mucoadhesive delivery systems has also been shown to considerably enhance the bioavailability of active medicaments. Polymers that are bioadhesive in nature generally interact with mucus through weak forces such as hydrogen bonds, Van-der Waal forces, ionic interactions etc. (Roldo, et al., 2004). However, strong covalent bonds such as disulfide bonds between the polymer and mucin also exist (Bernkop-Schnurch, 2005; Leitner, et al., 2003).

Alginate has been known to possess bioadhesive properties. The polyanionic groups due to carboxyl ends make it a good mucoadhesive agent. Alginate beads, hydrogels, microspheres, nano-particles have been utilized to sustain the release of the drug.

Another important mechanism that governs the transport of molecules through the cell membrane is endocytosis. Endocytosis is an active process that requires energy. In a study, it was observed that the uptake of alginate particles was dependent on energy.

Alginate nano-particles have been previously evaluated to enhance the cellular delivery of water soluble molecules (Chavanpatil, et al., 2007a). *In vitro* studies using cell monolayers suggest that alginate nano-particles are efficient drug carriers and can significantly enhance and sustain the cellular delivery of water soluble drugs.

Cell monolayers have been widely employed for studying the cellular uptake and cytotoxicity of delivery systems. They present many advantages, including easy to culture and studies can be performed within a controlled environment. In many cases a significant correlation between the studies performed on *in vitro* cell monolayers and *in vivo* human studies has been observed. Hence, *in vitro* studies can be used as predictive tools for estimating the fate and activity of the delivery system in the actual human body (Tavelin, et al., 2002).

The aim of this study was to evaluate the cytotoxicity and cellular uptake of gemcitabine hydrochloride from the nano-particulate suspension and an equivalent dose of free drug solution using *in vitro* pancreatic cancer cell culture models (BXPC3).

5.2. Materials

Gemzar[®] (Gemcitabine Hydro chloride) was obtained from CUMC pharmacy store. BXPC3 cell lines were purchased from American Type Culture Collection (ATCC), (Manassas, VA), Ammonium acetate was purchased from Spectrum Chemicals (San Pedro St, CA). Sodium phosphate dibasic, sodium phosphate monobasic, calcium chloride anhydrous, dioctyl sodium sulfosuccinate, cold water soluble poly vinyl alcohol having formulae weight of 50,000 and low viscosity sodium alginate were purchased from Sigma-Aldrich (St. Louis, MO).

5.3. Methods

5.3.1. Preparation of Solutions

5.3.1.1. MTT Reagent

Fresh MTT reagent was prepared. MTT reagent is available as a yellow colored powder. One hundred twenty five mg of this powder was accurately weighed and dissolved in 20 ml of deionized water. The solution was transferred in a 25 ml volumetric flask and the volume was made up to 25 ml using deionized water.

5.3.2. Determination of cytotoxicity of the delivery system

The cytotoxicity/efficacy of the nano-particulate formulation was determined in BXPC3 cell lines using MTT assay. BXPC3 cells were seeded in 96 well plates at a density of 5000 cells per well in 100 μ l of DMEM supplemented with 10% FBS, 1% L-glutamine, 1% sodium pyruvate, 1% non-essential amino acids and 1% penicillin/streptomycin. The cells were incubated in a humidified chamber maintained at 37°C and 100% RH supplied with 5 % CO₂. The cells were treated with different concentrations of free gemcitabine solution and equivalent amount of gemcitabine loaded nano-particle suspension for a period of 4 hours. Following the 4 hours exposure period, the treatments were removed and the cells were supplied with fresh DMEM and allowed to incubate in humidified chamber for 24, 48 and 72 hours. After the incubation period, the cells were treated with fresh MTT reagent (250 μ l, 5mg/ml) and further incubated for 2 hours. Finally, the cells were treated with 50:50 solution of 20% (w/v) SDS (dissolved in water) and DMF (dimethyl formamide). The plates were then read on a microplate reader at 550 nm. The data is expressed as the percent cell survival of control cell monolayers receiving just the media.

5.3.3. Determination of cellular uptake

The uptake of the free drug and nano-particulate delivery system was evaluated in BXPC3 cells. BXPC3 cells were seeded at a density of 50,000 cells per well in six well plates according to the ATCC requirements. The cells were then incubated in a humidified incubator maintained at 37°C and supplied with 5% CO₂. Cells were observed for con-fluency under a microscope regularly. Cells when confluent; were treated with a single dose of free drug solution and an equivalent nano-particulate suspension for 15, 30 and 60 minutes. All the treatments were prepared in Assay Buffer II. At the end of the stipulated time intervals the cells were washed 3 times with ice cold PBS to remove any free drug or particulate matter. The cells were then lysed with 1% solution of triton X. Twenty five µl of this solution was analyzed for the total protein content according to the BCA protein assay. The amount of drug present in the solution was then analyzed using HPLC. The cellular uptake was calculated and presented as the mean ± SEM of the percent drug per mg total cellular protein (n=3).

5.3.4. Analysis of Gemcitabine

Gemcitabine was analyzed using reverse phase chromatography. The HPLC method is previously described in detail.

5.4. Results and Discussion

5.4.1. Efficacy of the formulation

The percent survival of the human pancreatic cancer BXPC3 cells following treatment with free gemcitabine solution and gemcitabine loaded nano-particles was determined using MTT cytotoxicity assay (Figures 5.1-5.3). The dose response curves

demonstrated that alginate particles loaded with gemcitabine had improved cytotoxicity compared to the drug solution alone. The results clearly indicate a higher cell death with the drug loaded nano-particulate suspension compared to an equivalent dose of free drug solution. The higher cytotoxicity can be attributed to higher concentration of gemcitabine accumulated in the cells from nano-particulate suspensions. The burst release from the nano-particles in the extracellular media makes the free drug available for transport inside the cells through nucleoside transporters expressed on the cell membrane. Also, the nano-particles are actively endocytosed. These release characteristics may be beneficial in providing both free drug and the nano-particles containing drug immediately at the site of action and therefore, one can expect a higher therapeutic concentration of gemcitabine inside the cell by both transport pathways.

5.4.2. Cellular uptake of Gemcitabine

The *in vitro* cellular uptake of gemcitabine following treatment with free gemcitabine solution and gemcitabine loaded nano-particles for 15, 30 and 60 minutes, was investigated for BXP3 cell line (Figure 5.4). The cellular uptake of nano-particulate formulation was found to be higher than the free drug solution at all-time points and increased with an increment in exposure time. However, no significant increase in the uptake of free drug solution was observed as the exposure time was increased from 15 minutes to 60 minutes. The uptake of nano-particles was around 4 times higher than the solution with treatment for 15 minutes and increased to almost 7 times following treatment for 60 minutes. Gemcitabine is an ionized nucleoside analogue and has an intracellular site of action (Achanta, et al., 2001). The internalization of most ionized drugs is limited due to barriers to diffusion across the lipophilic cell membrane

thus rendering the intracellular concentrations of the drug very negligible (Gerweck, et al., 1998). However, gemcitabine is internalized in the cell through cell membrane bound nucleoside transporters (Mackey, et al., 1998: Pastor-Anglada, et al., 2004). Higher cellular accumulation of gemcitabine with nano-particles suggests that uptake of nano-particles is governed by processes other than simple diffusion. The cellular uptake of nano-particles is dependent on two very important cellular processes; endocytosis and exocytosis (Panyam and Labhasetwar, 2003). The particle characteristics like particle size and surface charge of the nano-particles also play a major role in regulating the process of endocytosis (Desai, et al., 1996: Desai, et al., 1997: Sahoo, et al., 2002). Gemcitabine hydrochloride is a highly water soluble drug. The lipophilic nature of cell membrane can hinder the diffusion of ionized drug molecule. The process of endocytosis has also been observed with nano-particle formulations using other polymeric systems like poly(D,L-lactide-co-glycolide) (PLGA) (Panyam, et al., 2002). It has been reported that transport of the free drug inside the cells is regulated by active nucleoside transporters expressed on the cellular surfaces. Hence, the total amount of the drug accumulated in the cells is a possible combination of both, free drug entering the cells via active transport and from the nano-particles endocytosed by the cells.

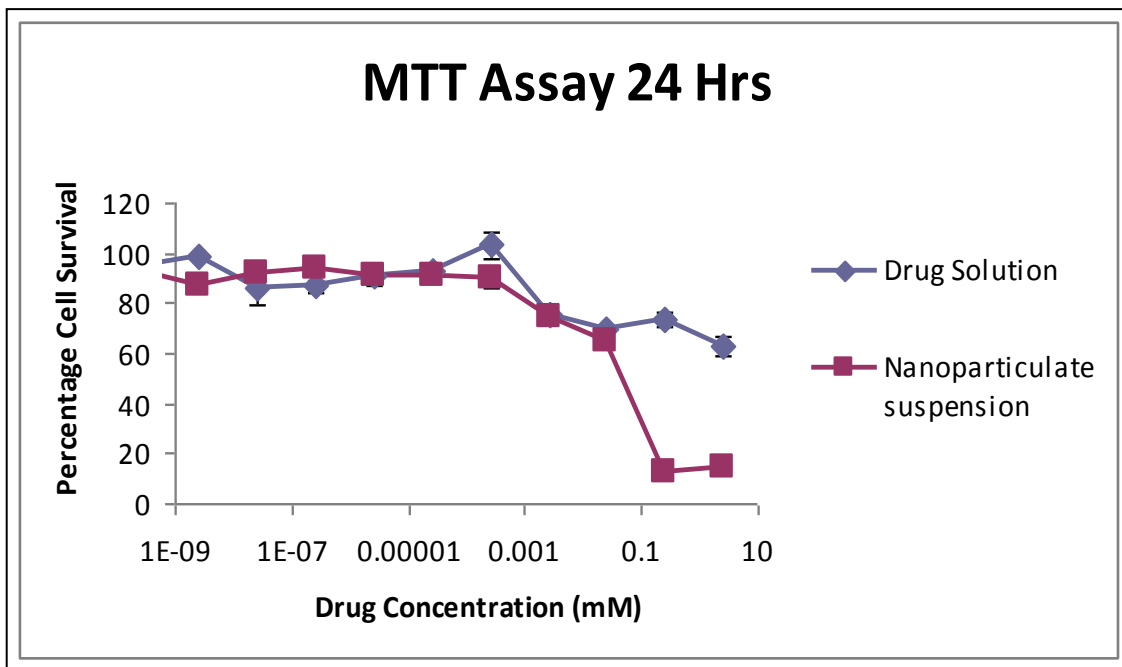


Figure 5.1 *In vitro* Cytotoxicity profile of Gemcitabine Solution and Gemcitabine loaded Alginate nano-particles at 24 Hours post treatment. BxPC3 monolayers when confluent were treated with various concentrations of Gemcitabine solution and Gemcitabine loaded Alginate nano-particles. The data is expressed as mean \pm SEM with n=3.

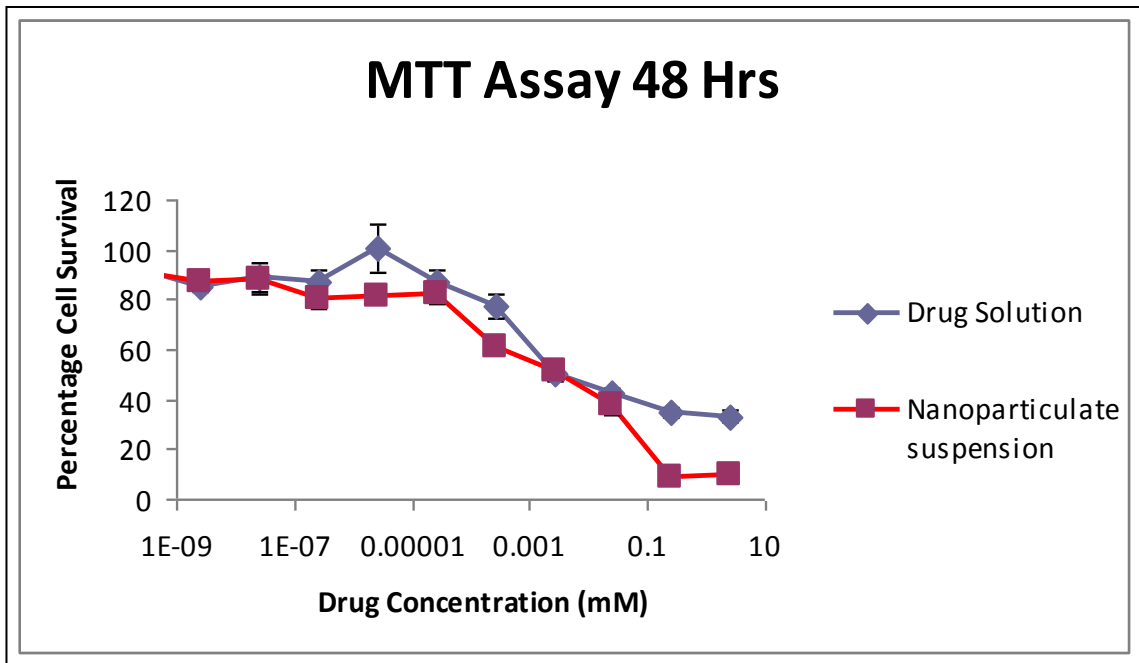


Figure 5.2 *In vitro* Cytotoxicity profile of Gemcitabine Solution and Gemcitabine loaded Alginate nano-particles at 48 Hours post treatment. BxPC3 monolayers when confluent were treated with various concentrations of Gemcitabine solution and Gemcitabine loaded Alginate nano-particles. The data is expressed as mean \pm SEM with n=3.

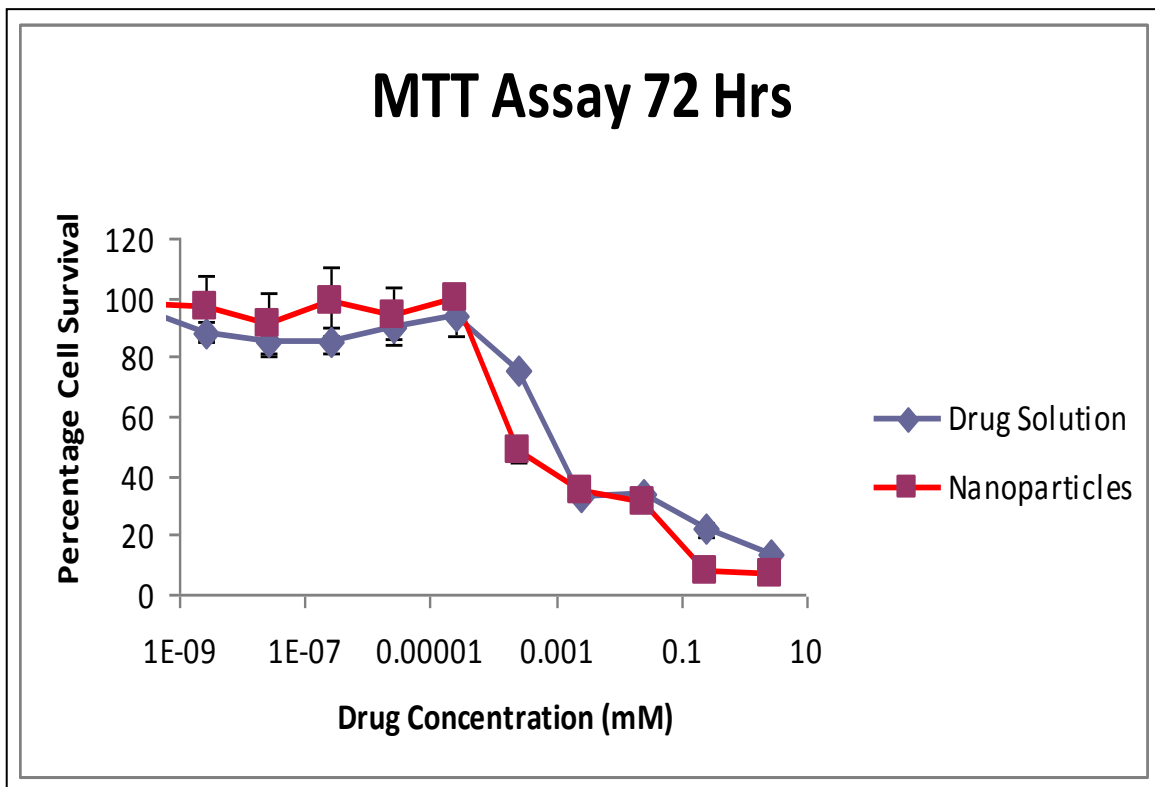


Figure 5.3 *In vitro* Cytotoxicity profile of Gemcitabine Solution and Gemcitabine loaded Alginate nano-particles at 72 Hours post treatment. BxPC3 monolayers when confluent were treated with various concentrations of Gemcitabine solution and Gemcitabine loaded Alginate nano-particles. The data is expressed as mean \pm SEM with n=3.

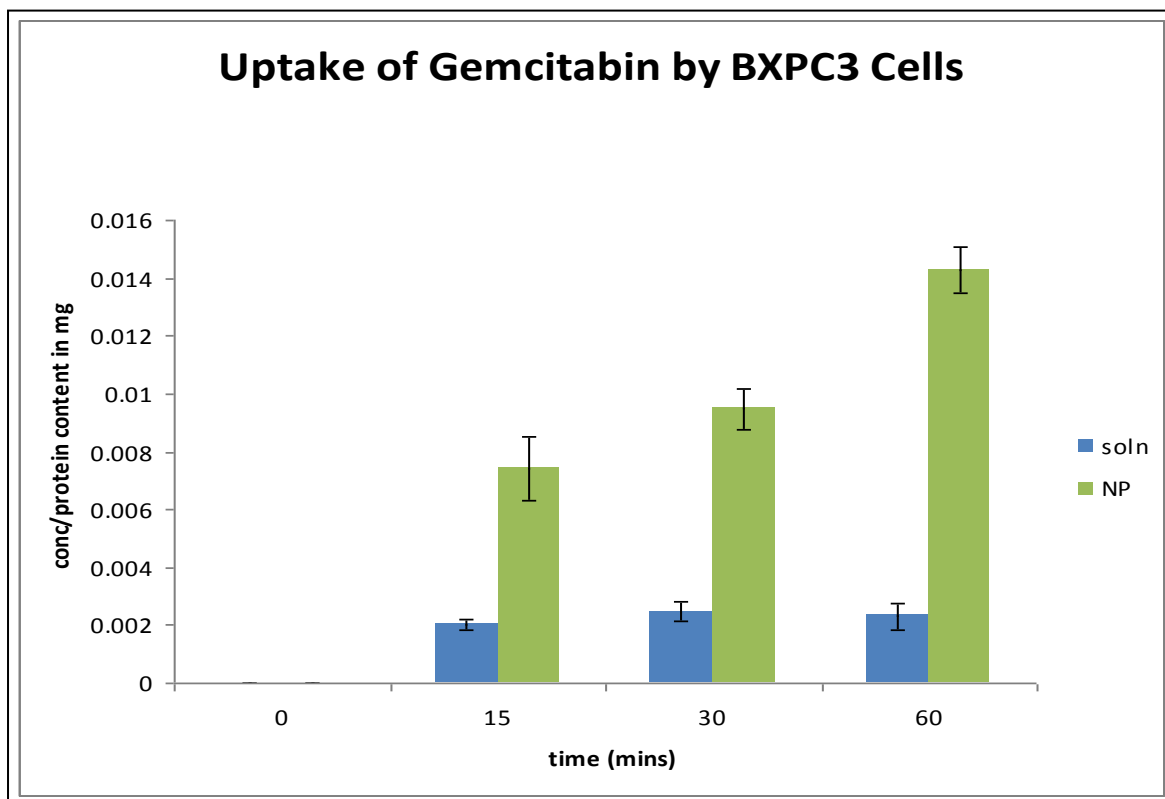


Figure 5.4: Cellular uptake of Gemcitabine solution and Gemcitabine loaded nano-particles. BXPC3 cell monolayers when confluent were exposed to Gemcitabine solution and Gemcitabine loaded nano-particles. The data is expressed as mean \pm SEM with n=3. The uptake of the nano-particulate formulation increased 4-7 times over a period of 60 minutes.

5.5. Conclusion

This study clearly reveals that formulating gemcitabine into a nano-particulate sustained release delivery system can significantly improve its cellular accumulation and cytotoxicity. These advantages offered by the nano-particulate sustained release delivery system over the conventional intravenous treatment may improve the therapeutic window allowing lower doses to be administered and thus avoiding the systemic toxicities associated with higher doses.

Chapter 6

Summary and Future Directions

6.1. Summary

A sustained release nano-particulate formulation containing gemcitabine hydrochloride was successfully developed using calcium cross-linked alginate and dioctyl sulfosuccinate system. The nano-particulate system was formulated using multiple emulsion solvent evaporation technique. The particles were found to be spherical in shape with a mean particle size of 210.6 ± 6.90 nanometers and carried an overall negative surface charge. The absence of any endothermic peak in the DSC thermograms of drug loaded particles indicates that gemcitabine is not present in crystalline state in the nano-particles.

A rapid, precise and accurate reverse phase HPLC method was developed and validated for the quantitative estimation of gemcitabine in mobile phase and phosphate buffer saline. The drug was separated on a C18 Luna column with a bore size of 5μ . The mobile phase used for the chromatographic extraction comprised of a mixture of ammonium acetate 40 mM and acetonitrile in the ratio of 95:5 (%v/v). The applications of the HPLC method included determination of drug loading, encapsulation efficiency, *in vitro* release and cellular uptake.

The *in vitro* release studies carried out in phosphate buffer saline at a pH of 7.4, showed a 50 % total release at the end of 7 days. The nano-particulate formulation also significantly enhanced the cellular accumulation of the drug in pancreatic cancer (BxPC-3) cell monolayers. The cellular uptake was found to be time dependent. A sevenfold higher uptake resulted from 60 minutes exposure of drug loaded nano-particulate suspension compared to an equal dose of free drug solution. Higher accumulation of the

drug from nano-particles suggests that the delivery system is more readily endocytosed than the free drug.

6.2. Future directions

Further continuation of this study may include evaluation of formulation's efficacy on other pancreatic cancerous cell monolayers like Mia Paca, Panc-1. The human pancreatic cancer cell lines can serve as an alternate *in vitro* cell model for the estimation of the efficacy of the nano-particulate formulation. Coherent results would ensure the effectiveness of the delivery system. Other studies may include assessment of local tissue and organ toxicity of the delivery system.

These studies can be followed up with *in vivo* safety and efficacy studies using animal (rodent) models with induced carcinomas. Human pancreatic cancer cell lines like Panc-1 can be used to set up mouse xenograft models. These models can aid in studying the therapeutic efficacy and toxicity to normal tissues/organs at the site of injection and other distant sites in immunocompetent mice. The delivery system may be compared with conventional systemic delivery to evaluate its clinical applications.

A different blend of polymeric systems for example chitosan, poly epsilon caprolactone, eudragit can be tried and tested in combination with sodium alginate to improve the drug loading and encapsulation efficiency of gemcitabine hydrochloride.

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