Pham, Jennifer., Development and *In Vitro* Characterization of Gemcitabine Loaded Nanoparticles for Pancreatic Cancer Therapy. Master of Science (Medical Science Biochemistry and Cancer Biology Track), May 14th, 2021, 37 pp.; 10 tables, 18 figures, bibliography.

Introduction: Pancreatic Ductal Adenocarcinoma (PDAC) is the 4th leading cause of cancer deaths worldwide and the most common type of pancreatic malignancy (90%). With a poor five-year survival rate of only 5-8%, complete surgical resection remains the only curative treatment. However, most patients are diagnosed at a later stage where chemotherapy and radiotherapy are the only options. Gemcitabine is the FDA-approved treatment for PDAC, but the current therapy leads to more severe side effects due to the instability of gemcitabine in the blood stream and its poor membrane permeability. Nanoparticles are effective in cancer therapy because they allow modifications that make for a more effective delivery method and also reduces the toxicity to normal tissue.

Methods: In this proposed study, we aim to formulate, optimize and evaluate the *in vitro* effectiveness of gemcitabine loaded nanoparticles in a PDAC cell line in order to improve the effectiveness of current chemotherapy treatments for pancreatic ductal adenocarcinoma.

Results: We found out of the three types of nanoplatforms used for encapsulating gemcitabine (GEM-NPs): polymeric, liposomal and lipid polymer hybrid, the liposomal nanoparticles were the most effective in the encapsulation of gemcitabine according to the physicochemical properties, such as average particle size, zeta potential, drug loading and encapsulation efficiency. *In vitro* functional evaluation of liposomal formulation was done in a PDAC cell line (PANC-1).

Conclusion: This study suggests that the use of liposomal nanoparticles is the most beneficial in the encapsulation and delivery of gemcitabine.

DEVELOPMENT AND *IN VITRO* CHARACTERIZATION OF GEMCITABINE LOADED NANOPARTICLES FOR PANCREATIC CANCER THERAPY

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DEVELOPMENT AND *IN VITRO* CHARACTERIZATION OF GEMCITABINE LOADED NANOPARTICLES FOR PANCREATIC CANCER THERAPY

INTERNSHIP PRACTICUM REPORT

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By

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CHAPTER 1

BACKGROUND AND LITERATURE

Pancreatic Ductal Adenocarcinoma (PDAC) is the 4th leading cause of cancer death (*1*) which claims over 340,000 lives worldwide each year (*2*). With a five-year survival rate of only 5-8% (*1*), PDAC is classified as a recalcitrant cancer under the Recalcitrant Cancer Research Act of 2012 (*3*). It is estimated that PDAC will become the second leading cause of cancer-related deaths by 2030 (*4*).

Exocrine tumors are the most common type of pancreatic cancer with adenocarcinoma being the most common of these tumors. They usually originate in the ducts and are the reason why pancreatic ductal adenocarcinoma covers about 90% of pancreatic cancer cases (*5*). Surgical resection with chemotherapy is still the best treatment option for PDAC. However, the lack of early detection methods and symptoms showing up only in the later stages leave only 15% of patients that can undergo surgical resection while the remaining 85% are left with chemotherapy and radiation (*6*).

The main cause behind the poor prognosis of patients with PDAC is the dense, fibrous stroma which takes up roughly 80% of the tumor mass (*7*). The desmoplastic stroma causes poor vascularization, high intratumoral pressure with a reduction in drug diffusion. This is due to the production of the extracellular matrix (ECM) by cancer-associated fibroblasts (CAFs) and pancreatic stellate cells (PSCs) (*8*). The pressure on the tumor blood vessels and high metabolic demand creates a lack of nutrition and oxygen in the micro-environment, making it acidic as well (*9*). Other problems that prevent an effective treatment includes the mutations in signaling pathways and PDAC's high metastatic potential (*5*). Mutations in genes such as KRAS, which is present in almost 100% of PDAC cases, drive the growth

of cancerous tumors through changes in the signaling pathway.

Gemcitabine (GEM) has been used as the first-line treatment for pancreatic cancer since 1997 (*10*). It serves as a nucleoside analogue of deoxycytidine preventing DNA synthesis and has demonstrated to improve the overall survival rate to 21% after five years (*11*). Even so, gemcitabine has also shown to be metabolically unstable due to its rapid deamination in the bloodstream, primarily by cytidine deaminase (*12*). It has a short plasma half-life with more than 75% of gemcitabine metabolized and excreted through urine in the first 24 hours (*13*).

Because of its hydrophilic nature, gemcitabine does not cross the cell membrane easily by diffusion but has to use the membrane nucleoside transporters such as hENT1 and hENT2 instead (*14*). This poor membrane permeability requires a high dosage of gemcitabine (1000 mg/m^2) to reach the desired effect which may lead to serious side effects including kidney failure (*15*). Additionally, chemoresistance and the dense desmoplastic stroma, which serves as a physical and biological barrier, also prevents the effective delivery of gemcitabine resulting in unfavorable pharmacokinetics (PK) profile and hence decrease treatment outcomes.

Nanoparticles are effective in cancer therapy because they allow modifications for specific site targeting, deliver high drug concentrations with prolonged pharmaceutical activity leading to decreased dosing frequency while reducing the toxicity to normal tissues (*16*). There are multiple types of nanoparticles currently available, including polymeric, liposomes, gold, micelles, etc.

Polymeric nanoparticles are usually made of derivatives of naturally occurring polymers which makes them highly biologically compatible and biodegradable (*17*). The polymer poly lactic-co-glycolic acid (PLGA), for example, degrades to lactic and glycolic acids, which are nontoxic, biocompatible, and promptly eliminated from the body (*18*). However, polymeric

nanoparticles are more effective at encapsulating hydrophobic drugs (*17*).

The lipid polymer hybrid nanoparticles have gained more attention due to their ability to combine both the biodegradable favorability of polymeric nanoparticles to form the core for hydrophobic drugs and the lipid outer layer that enhances stability and systemic circulation of the nanoparticles. They can be flexible in terms of lipids, polymers, lipid-to-polymer combination selection and having the attachment of polyethylene glycol (PEG) on the surface to protect the nanoparticles in the blood stream (*19*).

Liposomal nanoparticles, such as the FDA-approved liposomal Doxil (*20*) and albumin-bound paclitaxel Abraxane, can be easily manipulated to target specific tissue by adding molecules to the outer layer of the lipid bilayer (*21*). Just like the hybrid nanoparticles, a process called PEGylation can also be used to add PEG molecules to the surface of the nanoparticles for added protection *in vivo* by preventing the opsonization with proteolytic enzymes and antibodies (*22*). Phosphatidylcholines (PC) are usually the lipids of choice in liposomal formulation and cholesterol is also often added to stabilize the lipid bilayer, just like its use in the biological cell membrane. Liposomes are good at the encapsulation of both hydrophobic and hydrophilic drugs due to their amphipathic nature, consisting of a hydrophilic core and a hydrophobic outer layer (*23*).

The favorable size for nanoparticles ranges from 10 to 100 nm. This is to avoid filtration by the kidneys for particles smaller than 10 nm and capture by the liver for particles larger than 100 nm (*24*). Other physicochemical properties used to analyze nanoparticles can include the polydispersity index (PDI), zeta potential, drug loading, and encapsulation efficiency along with other characteristics. The polydispersity index, a unitless measurement, analyzes how uniform the size distribution of a sample may be. Ranging from 0 to 1, with 0 being seen as highly uniformed and 1 indicating a broad size distribution, a PDI of 0.2

and below is deemed acceptable for polymeric nanoparticles and a PDI of 0.3 and below is acceptable for liposomal nanoparticles (*25*). The zeta potential is a way to relatively measure the surface charge of a particle. A slightly charged surface, whether it is positive or negative, can decrease self-aggregation and increase the stability of the nanoparticles (*26*). Negatively charged particles are better at delivering drugs deep into the tissue due to their high diffusion rate and preventing filtration by the kidneys (*24*).

Using a nanoparticle system will reduce the systemic toxicity by reducing the effective dose of GEM required and deliver the drug at the tumor site more efficiently, which reduces the off-target side effects (*27*). However, the hydrophilic nature of gemcitabine makes encapsulation difficult with polymeric nanoparticles because the polymers are dissolved in the organic solvent (*28*). This has led to poor drug loading and encapsulation efficiency. The use of liposomal nanoparticles, which is more preferred with hydrophilic drugs, seems to be a better fit for gemcitabine.

CHAPTER 2

DEVELOPMENT AND *IN VITRO* CHARACTERIZATION OF GEMCITABINE LOADED NANOPARTICLES FOR PANCREATIC CANCER THERAPY

2.1 SIGNIFICANCE

The advance staged diagnosis of 80-85% pancreatic ductal adenocarcinoma cases where surgical resection is no longer possible (*6*) implies that chemotherapy and radiotherapy become even more necessary in the treatment of PDAC. However, the poor membrane permeability of gemcitabine, via the human equilibrative type transporters (hENT1, hENT2) (*14*) requires a higher dosage of the drug to elicit its effect which can then lead to serious side effects, including breathlessness, neutropenia, and kidney failure (*15*). Additionally, the delivery of gemcitabine to the tumor site is further hindered by the dense desmoplasia of the stroma surrounding the site (*8*). Therefore, it is extremely important to develop a gemcitabine loaded nanotherapy which delivers gemcitabine successfully to the tumor site with fewer side effects.

2.2 SPECIFIC AIMS

In this study, we aim to:

- **Aim 1:** Formulate, characterize and optimize the synthesis of gemcitabine loaded nanoparticles by the utilization of three types of nanoplatforms, two different creation techniques and various methods and tools to analyze the physicochemical properties.

This aim comprises of three tasks:

Task 1: Formulation of gemcitabine encapsulated nanoparticles in three types of nanoplatforms: polymeric, liposomal, and the lipid polymer hybrid form.

Task 2: Characterization of nanoparticles based on physicochemical properties.

Task 3: Optimization of nanoparticles based on particle size and drug loading.

- **Aim 2:** Evaluate the *in vitro* effectiveness of the most suitable nanoparticle platform in a PDAC cell line (PANC-1). This aim comprises of two tasks:

  Task 1: *In vitro* cellular uptake study in PANC-1.

  Task 2: *In vitro* cell viability study in PANC-1.

2.3 MATERIALS AND METHODS

- **2.3.1 Formulation, characterization and optimization:**

  Formulation: Within this task, gemcitabine was encapsulated in three types of nanoplatforms (GEM-NPs), namely polymeric, liposomal and a hybrid of the polymeric and the liposomal forms. GEM-loaded nanoparticles were prepared using two different methods. The first one used the microfluidic-assisted nanoprecipitation method to create the polymeric PLGA nanoparticles (GEM-PNPs) (*29*). By using microfluidics, the parameters that are used during small scale production of batches can be easily optimized and later used for the scale up process (*29*). PLGA was dissolved in acetonitrile (ACN) for the organic phase and gemcitabine was dissolved in 2% poly-vinyl alcohol (PVA) for the aqueous phase. The settings for the NanoAssemblr (Precision

Nanosystems) were: Total Flow Rate: 8 mL/min, Flow Ratio: 5:1, Total Volume: 1 mL. Dialysis was performed to remove the organic solvent.

The GEM-loaded liposomes (GEM-Lip) were prepared using a microfluidics-assisted technique to form unilamellar liposomes from solvent (*30*). One method of passive loading and three methods of active loading were used: remote loading, hypertonic loading, and a combination of remote and hypertonic loading. A ratio of 3:1:1 of 1,2-dipalmitoyl-snglycero-3-phosphocholine (DPPC), cholesterol, and DSPE-PEG(2000) at 15 mg/mL total was used for the lipids. Gemcitabine was added at 25% of the lipid weight. The samples were then purified with milli-Q water three times at 135,700 rcf at 4 ℃.

, 500 µL acetone and 25 mg lecithin. After probe sonication, the solution was then added to 3 mL of 2% PVA with 15 mg of DSPE-PEG(2000). The solution underwent more sonication, and the solvent was removed by vacuum evaporation. The sample was then centrifuged at 30,000 g at 25 ℃ for 45 min and resuspended in a 5 mg trehalose solution.

Characterization: These developed GEM-NPs were characterized for physicochemical properties including average particle size, zeta potential, drug loading, encapsulation efficiency, and stability analysis as protocols described elsewhere (*31*).

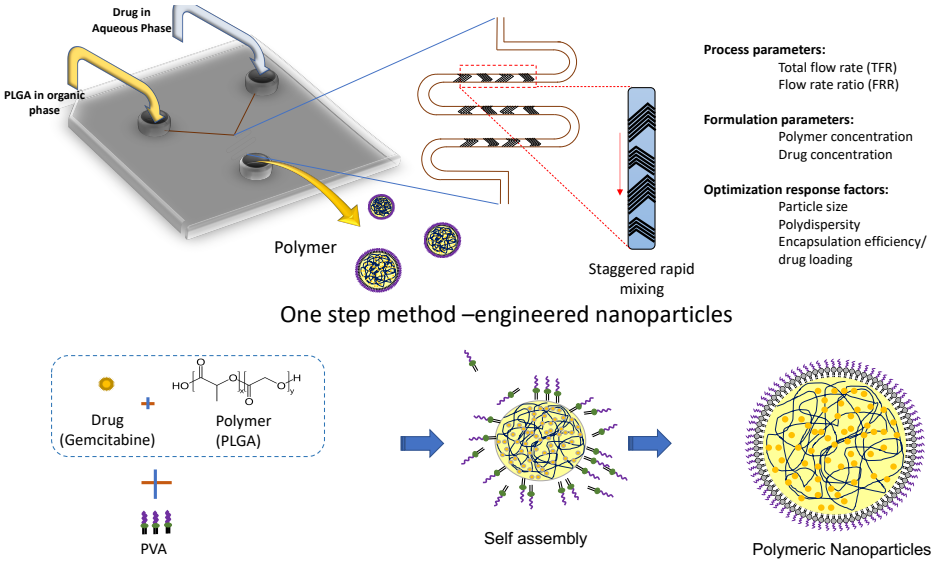


Figure 1: Microfluidics-assisted nanoprecipitation technique for polymeric nanoparticles.

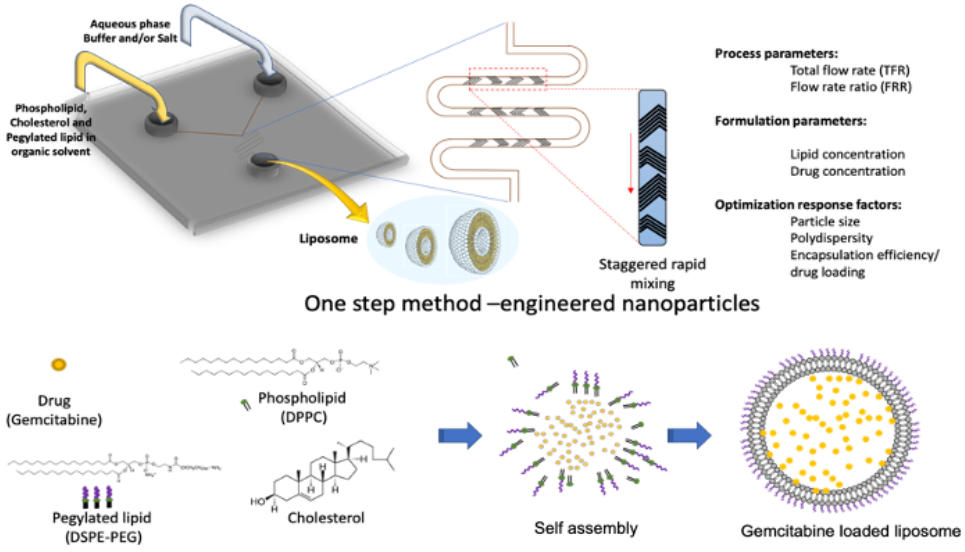


Figure 2: Microfluidics-assisted technique for liposomal nanoparticles.

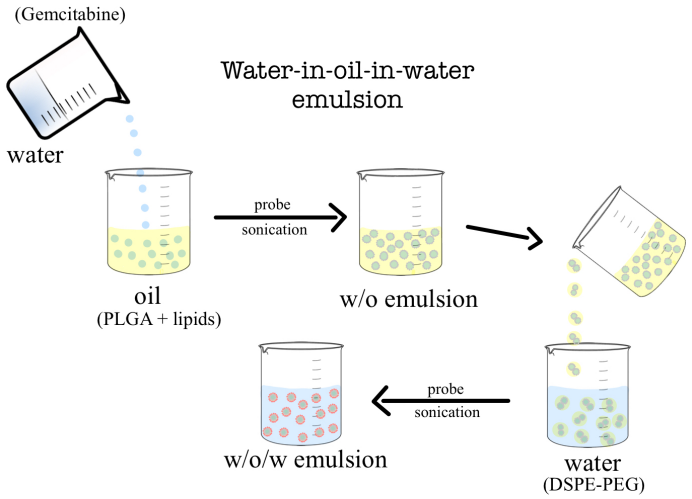


Figure 3: Water-in-oil-in-water emulsion technique for lipid polymer hybrid nanoparticles.

Optimization: We formulated different batches of GEM-NPs using different ratios of drug and nanoparticle weight and then they were optimized based on the method used for formulation. Optimal characterization of GEM-NPs based on particle size and drug loading was achieved. Our goal was to minimize the size and maximize the drug loading to obtain stable GEM-NPs nanoformulation.

- **2.3.2 *In vitro* functional evaluation of GEM-NPs in PDAC cell line:**

***In vitro* cellular uptake:** The human pancreatic adenocarcinoma cells (PANC-1) were seeded (5000 per well) in six well plates with cover slip. The cell line was derived from the primary tumors with the patient showing evidence of metastasis. The KRAS gene mutation which is commonly seen in PDAC is expressed in PANC-1 (*32*)(*33*) and PANC-1 has shown resistance to gemcitabine (*34*). The cells cultured in the plates were allowed to grow for two doubling times. Cells in plate were treated with near-infrared (NIR) dye (DiD) encapsulated NIR-NPs for 3 h at cell culture conditions (*35*).

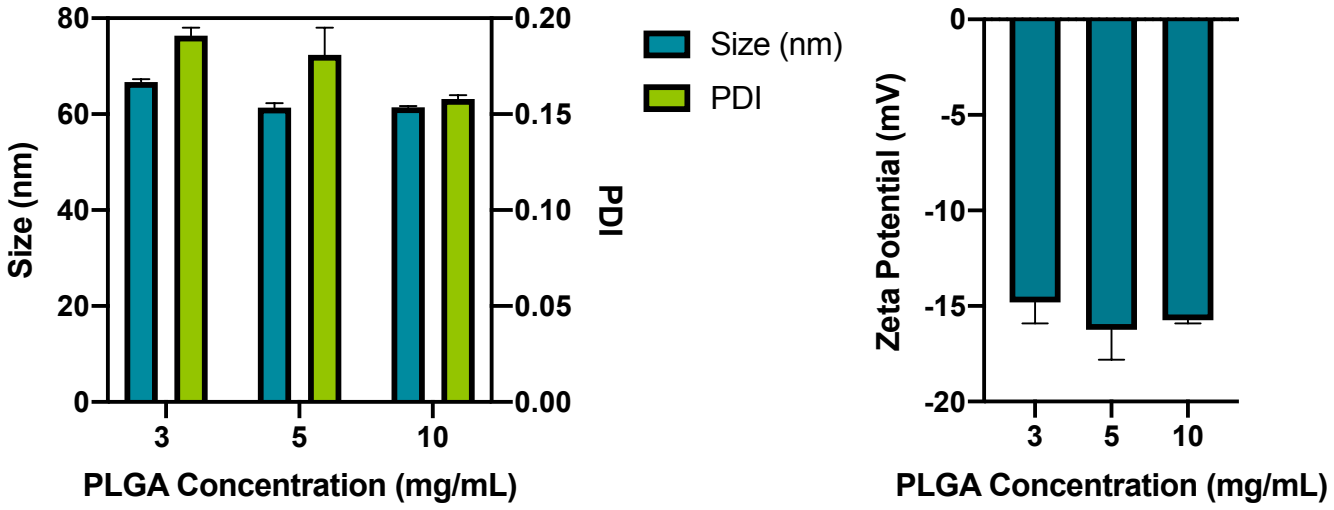
The uptake of NIR-NPs and the released dye were observed by fluorescence confocal microscopy.

Cell viability assay: The relative cytotoxicity of GEM and optimized GEM-NPs against PDAC cells (PANC-1) was assessed by the ATP based CellTiter-Glo 2.0 Cell Viability Assay. Cells were seeded in 96-well plates at a density of 7,500 cells/well and incubated for 24 h at 37 ℃ in a humidified atmosphere with 5% CO_2. These plates were treated with GEM or GEM-NPs with different concentrations for 48 h. An ATP based assay was performed for cell viability as a function of the GEM concentration of GEM-NPs and time of exposure.

2.4 RESULTS

2.4.1 GEM-loaded polymeric PLGA nanoparticles (GEM-PNPs) formulation.

The development of the polymeric nanoparticle formulation started with analyzing the physicochemical properties of PLGA at different concentrations in the desired organic solvent: acetonitrile (ACN). The physicochemical characteristics are presented in Figure 4 and Table 1. The mean particle size of the nanoparticles was 64.002 ± 1.505 nm with a mean polydispersity index (PDI) of 0.165 ± 0.014. The mean zeta potential was -15.6 ± 0.425 mV. Since there was not a significant difference in the values between 5 mg/mL and 10 mg/mL, 5 mg/mL was chosen as the concentration of PLGA going forward.

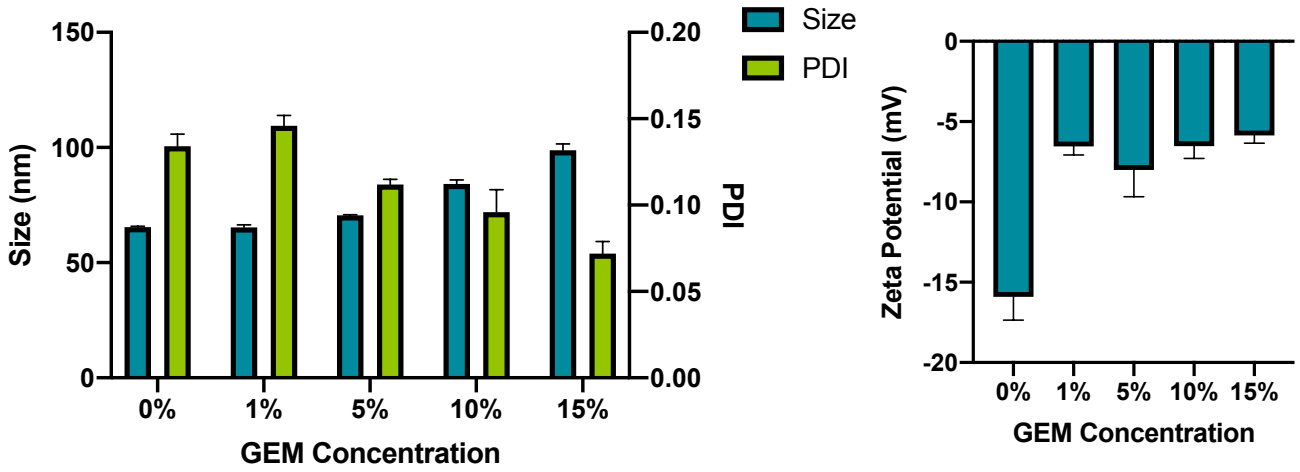


(a) Size and PDI

(b) Zeta Potential

Figure 4: Physicochemical properties (size, polydispersity index (PDI) and zeta potential) of PLGA nanoparticles at different PLGA concentrations in acetonitrile: 3 mg/mL, 5 mg/mL and 10 mg/mL.

The next step included testing the different drug concentration of gemcitabine and analyzing the physiochemical properties, including drug loading and encapsulation efficiency. The properties are presented in Figure 5 and Table 2. Different concentrations of gemcitabine were initially added according to a drug-to-polymer ratio of 0%, 1%, 5%, 10% and 15% of 5 mg/mL PLGA. The mean size of the nanoparticles was found to be 76.866 ± 6.454 nm and the mean polydispersity index (PDI) was 0.112 ± 0.013. The mean zeta potential of GEM-loaded nanoparticles was observed at -6.734 ± 0.914 mV. The encapsulation efficiency of the PLGA nanoparticles ranged from 4.64% at 15% gemcitabine concentration to 6.72% at 5% gemcitabine concentration. When compared to the other formulations, 5% GEM showed the most negative zeta potential and the highest encapsulation efficiency while 15% GEM showed the highest drug loading capacity. The drug loading capacity and encapsulation efficiency of each concentration are presented in Figure 6.



(a) Size and PDI

(b) Zeta Potential

Figure 5: Physicochemical properties (size, polydispersity index (PDI) and zeta potential) of gemcitabine loaded PLGA nanoparticles (GEM-PNPs) at different drug-to-polymer ratios: 0%, 1%, 5%, 10% and 15% of 5 mg/mL PLGA.

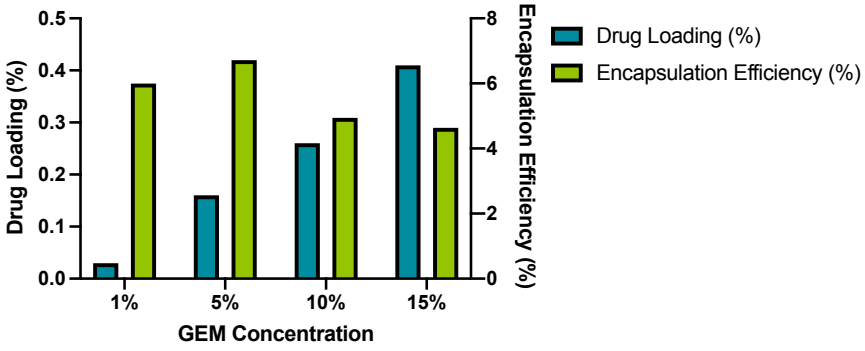
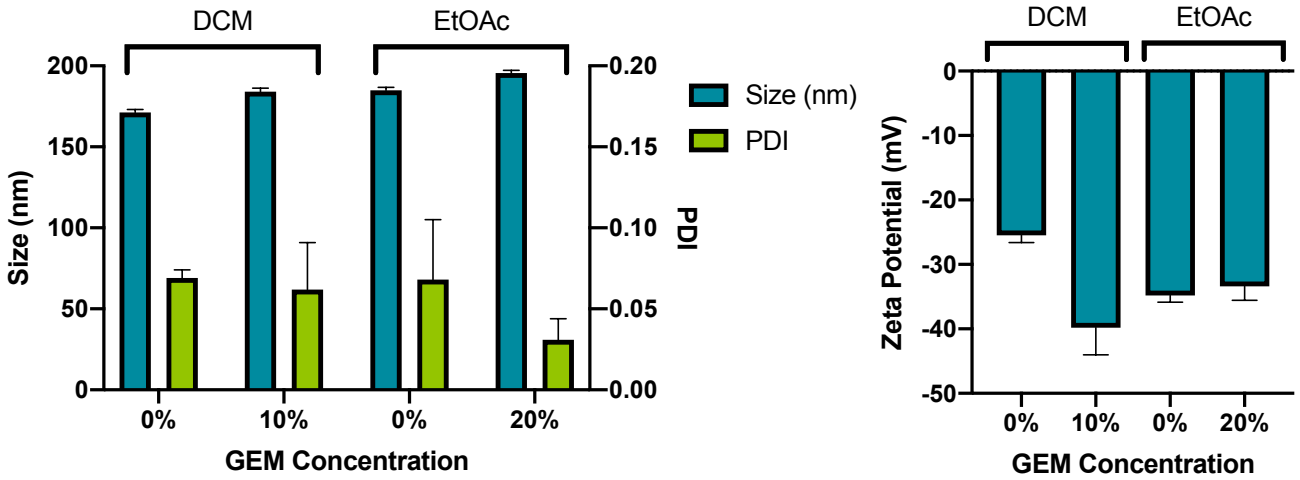


Figure 6: Drug loading and encapsulation efficiency of gemcitabine loaded polymeric nanoparticles (GEM-PNPs) at different drug-to-polymer ratios: 1%, 5%, 10% and 15% of 15 mg/mL PLGA.

2.4.2 GEM-loaded lipid polymer hybrid nanoparticles (GEM-LPHs) formulation.

The water-in-oil-in-water emulsion technique was used to develop lipid polymer hybrid nanoparticles. The physicochemical properties of these nanoparticles are presented in Figure 7, Figure 8 and Table 3. The mean particle size was found to be 183.9 ± 9.948 nm and the mean polydispersity index (PDI) was 0.058 ± 0.018. The mean zeta potential was observed at -33.375 ± 5.925 mV and the drug loading for nanoparticles made in dichloromethane (DCM) and ethyl acetate (EtOAc) was 0.31 % and 0.34%, respectively. The encapsulation efficiency was found to be 0.06% for dichloromethane and 0.03% for ethyl acetate.



(a) Size and PDI

(b) Zeta Potential

Figure 7: Physicochemical properties (size, polydispersity index (PDI) and zeta potential) of gemcitabine loaded lipid polymer hybrid nanoparticles (GEM-LPHs) in two solvents: dichloromethane (DCM) and ethyl acetate (EtOAc).

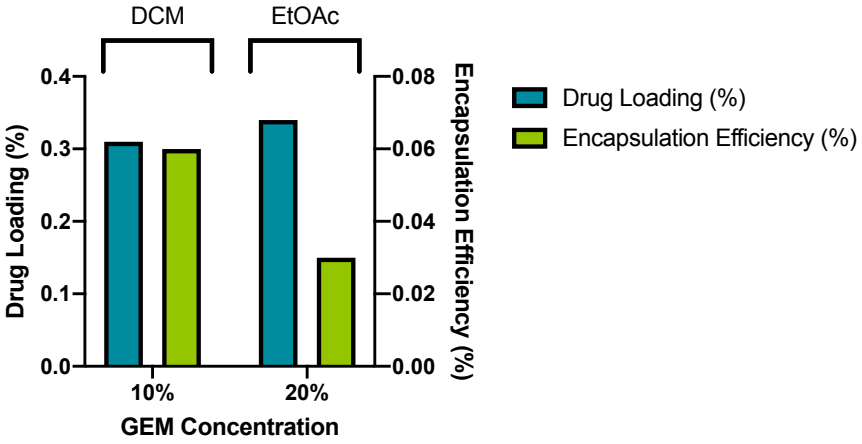
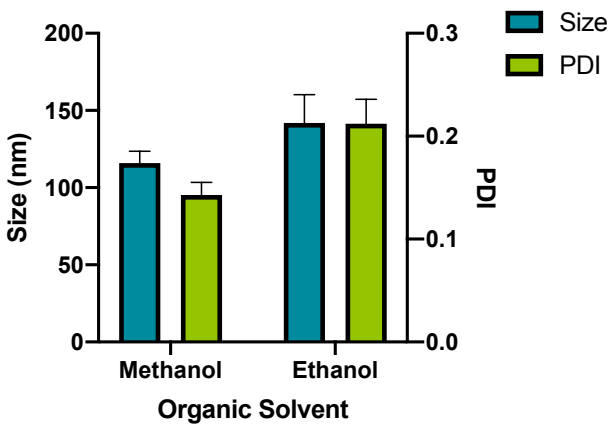


Figure 8: Drug loading and encapsulation efficiency of gemcitabine loaded lipid polymer hybrid nanoparticles (GEM-LPHs) at different concentrations in two solvents: dichloromethane (DCM) and ethyl acetate (EtOAc).

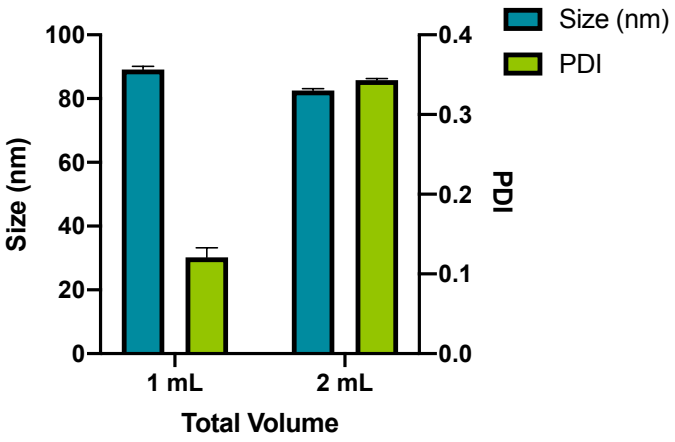
2.4.3 GEM-loaded liposomal nanoparticles (GEM-Lip) formulation.

The development of liposomal nanoparticles started with the selection of solvent: methanol or ethanol. Methanol yielded a lower particle size and polydispersity index (PDI) at 115.814 ± 7.589 nm and 0.143 ± 0.012 compared to ethanol at 141.673 ± 18.329 nm and 0.212 ± 0.024. Therefore, methanol was chosen as the solvent of choice in the formulation of the liposomes. The physiochemical characteristics of these nanoparticles are presented in Figure 9 and Table 4.

The next step in the formulation was selecting the total volume of each batch of sample created using the NanoAssemblr (Precision Nanosystems). The sample with a total volume of 1 mL had a lower polydispersity index (PDI) compared to the sample with a total volume of 2 mL and there was not a significant difference in size between the two samples. Therefore, 1 mL was chosen as the total volume of each batch of liposomal nanoparticles. The physicochemical characteristics of these nanoparticles are presented in Figure 9 and Table 5.



(a) Solvent selection



(b) Total Volume Selection

Figure 9: Physicochemical properties (size and polydispersity index (PDI)) of liposomal nanoparticles in methanol and ethanol, and in different total volumes of sample after selecting methanol as the organic solvent.

After selecting the total volume of each sample, gemcitabine was encapsulated using the standard method of passive loading by dissolving gemcitabine in the aqueous phase (10 mM PBS) and using the microfluidics-assisted nanoprecipitation technique at a flow ratio of 5:1. The passive loading method yielded low drug loading capacity and encapsulation efficiency with 0.549% for drug loading and 4.83% for encapsulation efficiency, shown in Figure 10.

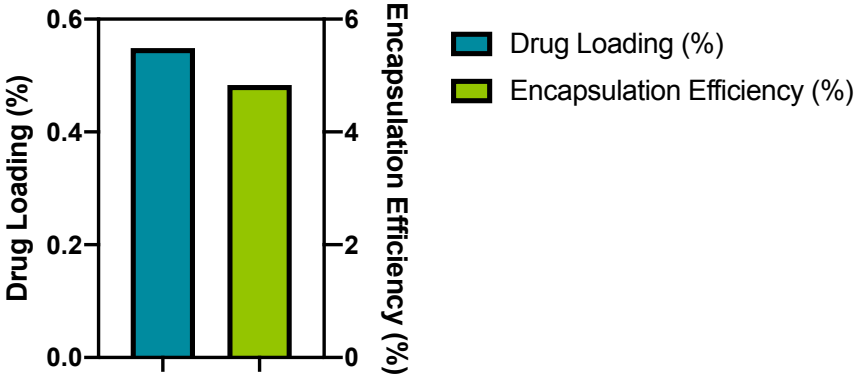


Figure 10: Drug loading and encapsulation efficiency of gemcitabine loaded liposomal nanoparticles (GEM-Lip) using the passive loading method.

Since the passive loading method yielded a low drug loading capacity and encapsulation efficiency, three methods of active loading were tested with three different flow ratios of organic-to-aqueous phase to decide on the best flow ratio for the formulation. The three methods of loading were: (1) remote loading with 250 mM ammonium sulfate (AMS) as the aqueous phase, (2) hypertonic loading with 462 mM sodium chloride (NaCl) as the aqueous phase, and (3) a combination of remote and hypertonic loading with 250 mM ammonium sulfate and 462 mM sodium chloride (AMS + NaCl) as the aqueous phase. The three flow ratios tested were: 5:1, 3:1, 1:1.

At flow ratio 5:1, the hypertonic loading method which used 462 mM sodium chloride (NaCl) had the lowest particle size at 47.36 $\pm$ 0.124 nm and the most negative zeta potential

at -6.45 ± 1.09 mV. All three drug loading methods had particle sizes under 100 nm and an acceptable PDI and zeta potential. The physicochemical properties are presented in Figure 11, Figure 13 and Table 6.

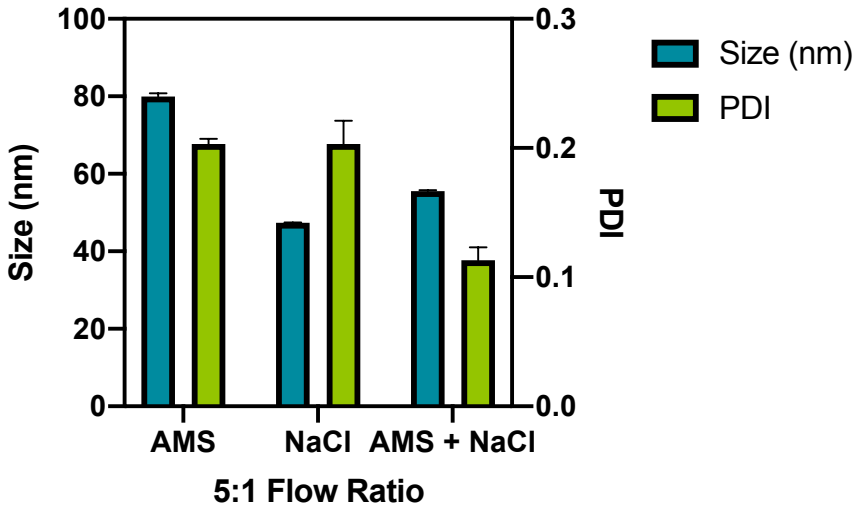


Figure 11: Physicochemical properties (size and polydispersity index (PDI)) of liposomal nanoparticles formulated using 5:1 flow ratio in three different aqueous phases: 250 mM ammonium sulfate (AMS), 462 mM sodium chloride (NaCl), 250 mM ammonium sulfate + 462 mM sodium chloride (AMS + NaCl).

At flow ratio 3:1, the hypertonic loading method which used 462 mM sodium chloride (NaCl) continued to have the lowest particle size at 51.71 ± 0.15 nm and the most negative zeta potential at -7.59 ± 0.67 mV. Only two drug loading methods had particle sizes under 100 nm but all three had an acceptable PDI and zeta potential. The physicochemical properties are presented in Figure 12, Figure 13 and Table 7.

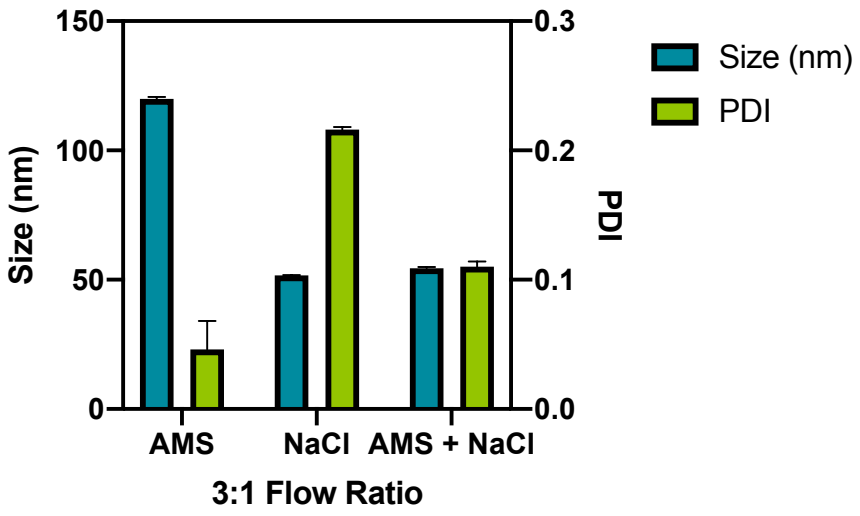
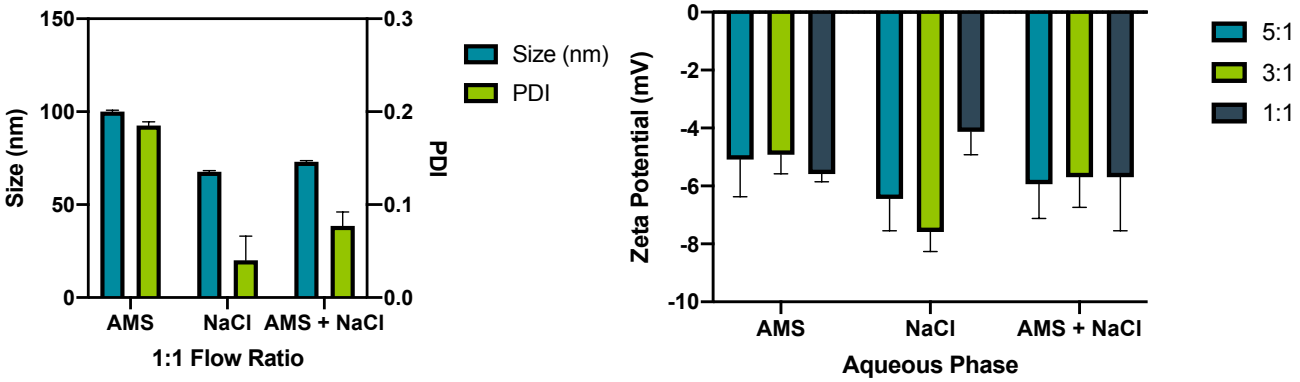


Figure 12: Physical properties (size and polydispersity index (PDI)) of liposomal nanoparticles formulated using 3:1 flow ratio in three different aqueous phases: 250 mM ammonium sulfate (AMS), 462 mM sodium chloride (NaCl), 250 ammonium sulfate + 462 mM sodium chloride (AMS + NaCl).

At flow ratio 1:1, the hypertonic loading method which used 462 mM sodium chloride (NaCl) still had the lowest particle size at 67.73 ± 0.55 nm but the combination remote and hypertonic loading method with 250 mM ammonium sulfate + 462 mM sodium chloride (AMS + NaCl) had the most negative zeta potential at -5.7 ± 1.84 mV. All three drug loading methods had particle sizes under 100 nm and an acceptable PDI and zeta potential. The physicochemical properties are presented in Figure 13 and Table 8.



(a) 1:1 Flow Ratio

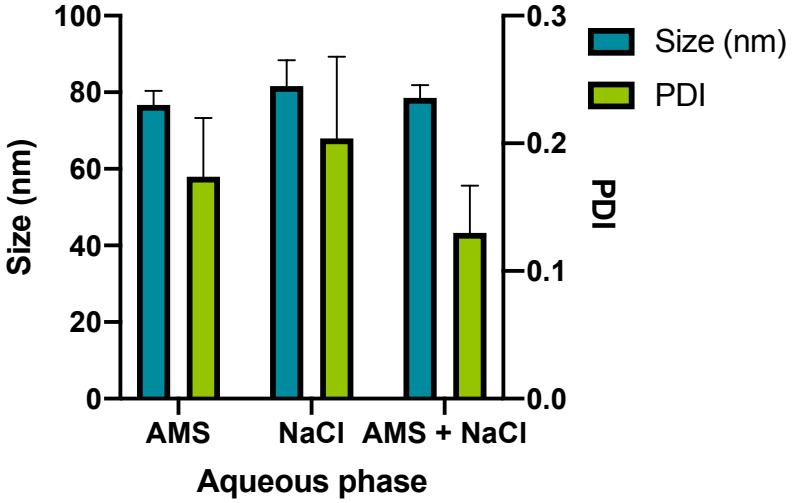
(b) Zeta Potential

Figure 13: Physicochemical properties (size and polydispersity index (PDI)) of liposomal nanoparticles formulated using 1:1 flow ratio and the zeta potential of the nanoparticles from all three flow ratios in three different aqueous phases: 250 mM ammonium sulfate (AMS), 462 mM sodium chloride (NaCl), 250 mM ammonium sulfate + 462 mM sodium chloride (AMS + NaCl).

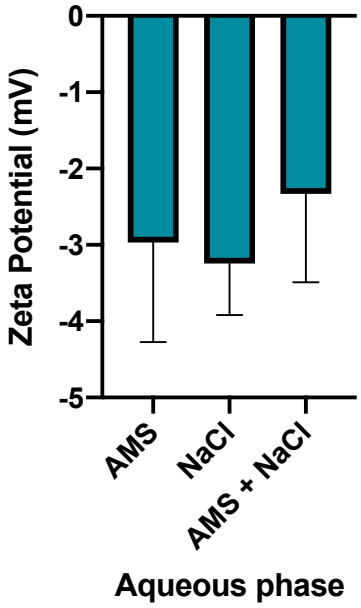
At flow ratio 1:1, the amount of lipids in each sample was maximized and the particle size, PDI value and zeta potential all fell within the acceptable values. The particle size was found to be within the range of 10 to 100 nm, the PDI value was below 0.3 and the zeta potential was observed to be negatively charged. Therefore, the flow ratio setting of 1:1 organic-to-aqueous phase was chosen for the formulation.

The three methods of loading were used once again to analyze the drug loading capacity and encapsulation efficiency of the liposomes. Gemcitabine was added to the samples

of each method at 25% of the lipids weight. The physicochemical properties of the gemcitabine loaded liposomal nanoparticles, the drug loading capacity, encapsulation efficiency and stability study are presented below in Figure 14, Figure 15, Figure 16, Table 9, and Table 10.



(a) Size and PDI



(b) Zeta Potential

Figure 14: Physicochemical properties (size, polydispersity index (PDI) and zeta potential) of liposomal nanoparticles in three different aqueous phases: 250 mM ammonium sulfate (AMS), 462 mM sodium chloride (NaCl), 250 mM ammonium sulfate + 462 mM sodium chloride (AMS + NaCl).

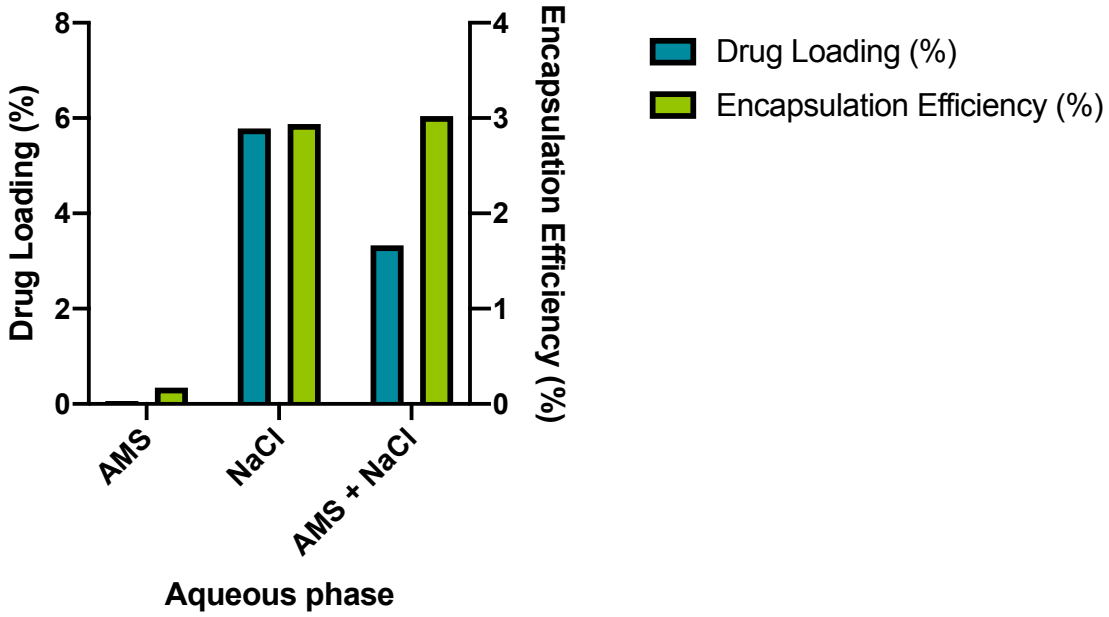


Figure 15: Drug loading and encapsulation efficiency of GEM-loaded liposomes in three different aqueous phases.

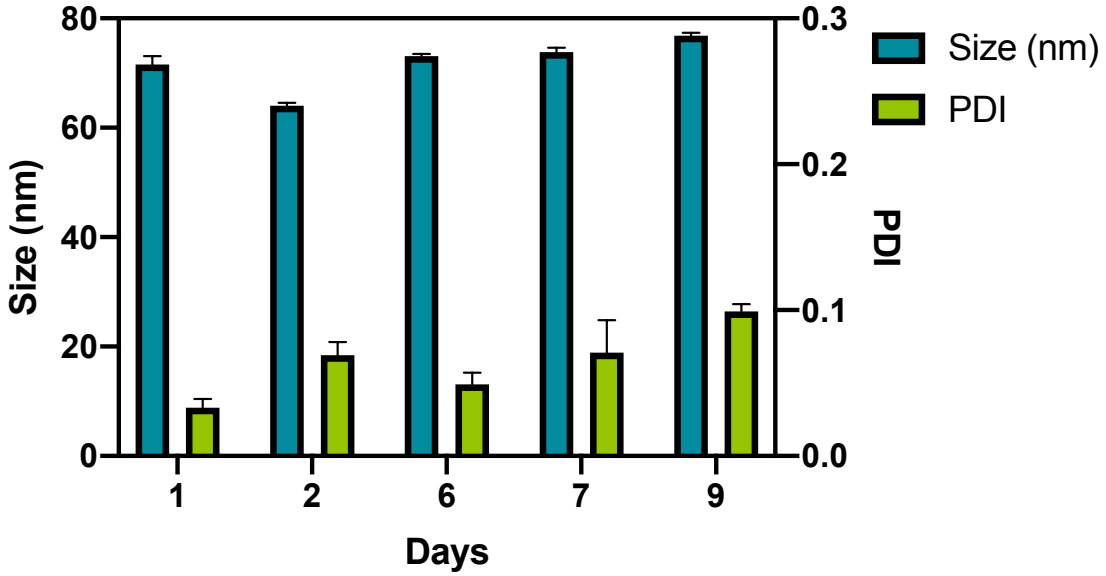


Figure 16: Stability study of gemcitabine loaded liposomal nanoparticles over 9 days with particle size and polydispersity index (PDI).

2.4.4 *In vitro* cellular uptake study

DiD (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate Salt) was loaded into the liposomes. DiD is a far-red fluorescent, lipophilic carbocyanine that can be incorporated into cell membranes and lipids for fluorescence microscopy with an excitation wavelength at 644 nm and an emission wavelength at 665 nm. After the PANC-1 cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 24 h in a 6 wells plate with cover slip, the cells were treated with the DiD-loaded liposomal nanoparticles. After 3 h, the cells were fixed with paraformaldehyde and stained with ProLong Gold Anti-fade Mountant and allowed to dry overnight. The uptake of the near-infrared (NIR) dye loaded liposomes (NIR-NPs) and the released dye were then observed by fluorescence confocal microscopy.

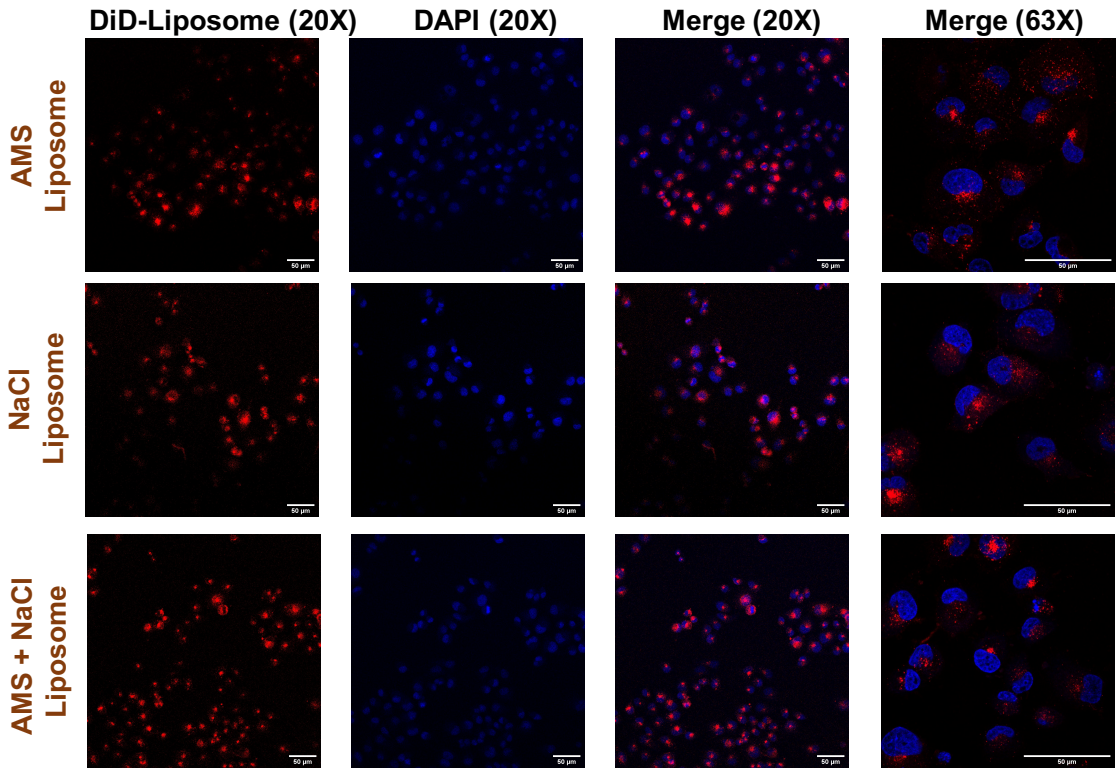


Figure 17: *In vitro* cellular uptake of gemcitabine loaded liposomes in the human pancreatic adenocarcinoma cell line PANC-1 observed under fluorescence confocal microscopy at different magnifications: 20X and 63X.

2.4.5 *In vitro* cell viability study

The viabilities of treated and untreated cells were determined by the ATP based CellTiter-Glo 2.0 Cell Viability Assay. After the PANC-1 cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 24 h in a 96 wells plate, the culture medium was replaced by a series of concentrations of drugs (0, 0.001, 0.01, 0.1, 1, 10, 100 µM) diluted with the culture medium. Five replicates were made for each measurement. In this study, gemcitabine and liposomal gemcitabine using the hypertonic loading method with 462 mM sodium chloride were co-incubated with the cells for 48 h at 37°C under the same conditions as described above. Finally, after equilibration to room temperature, the CellTiter-Glo 2.0 reagent mixed with an equal volume of cell in culture media in each well and luminescence was recorded after 10 minutes using a microplate reader. The percentage of viable cells at each drug concentration was normalized to the control and presented as cell viability. The IC_{50} value was calculated based on 50% reduction in viable cells.

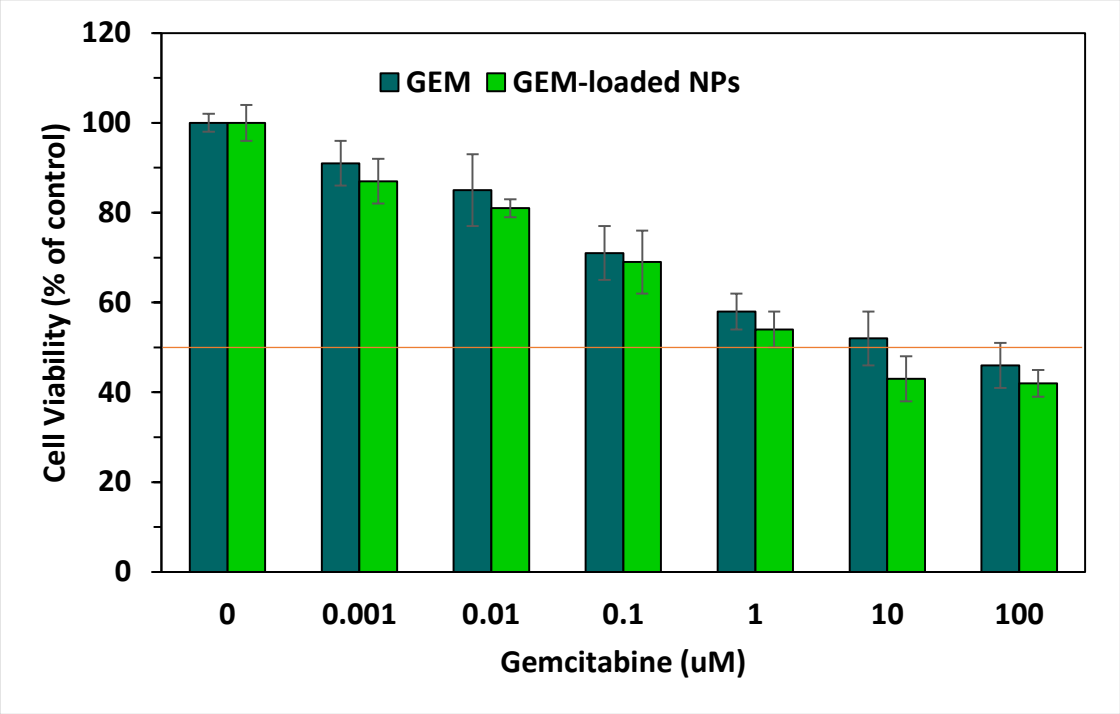


Figure 18: *In vitro* cell viability assay of free gemcitabine and gemcitabine loaded liposomes in the human pancreatic adenocarcinoma cell line PANC-1 at different gemcitabine concentrations.

2.5 DISCUSSION

All three nanoplatforms (GEM-PNPs, GEM-LPHs, GEM-Lip) and their physicochemical characteristics were analyzed. Although the polymeric PLGA nanoparticles had particle sizes under 100 nm and PDI values under 0.2, which indicates a homogeneous size distribution, the drug loading capacity and encapsulation efficiency were too low. However, it was predicted that gemcitabine's hydrophilic nature would make it difficult to be encapsulated by PLGA, which is better at encapsulating hydrophobic drugs (*17*).

The lipid polymer hybrid nanoparticles (GEM-LPHs) had a mean particle size of 183.9 ± 9.948 nm which is above the preferred range but had PDI values below 0.1 and the most negative zeta potential out of all three platforms. This nanoplatform also had a low drug loading capacity and encapsulation efficiency. Since the hybrid nanoparticles have a polymeric core of PLGA, it is possible that they have the same problem as the polymeric nanoparticles at encapsulating a hydrophilic drug.

The liposomal nanoparticles created with the passive loading method had particle sizes under 100 nm, PDI values under 0.3 and, like the two previous platforms, had a negative surface charge which is favorable for the stability of the nanoparticles. Even so, also like the two previous platforms, this method yielded a drug loading capacity below 1% and a low encapsulation efficiency. Passive loading has been proven to yield less than 10% encapsulation efficiency for hydrophilic drugs (*36*), which is the reason why active loading methods were the next to be analyzed.

The liposomal nanoparticles at the optimized settings with active loading methods had particle sizes under 100 nm, PDI values under 0.3, and a negative surface charge. Using the transmembrane chemical gradients, remote loading traps the compounds from the sur-

rounding environment into pre-formed liposomes. Hypertonic loading uses the high osmotic pressure across the membrane to induce the influx of the extravesicular water which contains gemcitabine (*30*).

Since ammonium sulfate was used in the active loading method to encapsulate doxorubicin for the FDA-approved drug Doxil, it was also tested here for our formulation (*37*). However, due to the low pK_a value of gemcitabine at 3.6, it does not ionize as effectively in the acidic inner compartment of liposomes to form an ionic complex with sulfate compared to a compound with a higher pk_a, such as doxorubicin with at 8.68.

The actively loaded liposomes had the highest drug loading and encapsulation efficiency out of the three nanoplatforms which matches the hypothesis that liposomes, with their hydrophilic core, would be the best at encapsulating gemcitabine, a hydrophilic drug.

Due to the limitation of time, further optimization of the liposomal nanoparticles and replications of the cell uptake and cell viability studies could not be done. Nevertheless, fluorescence confocal microscopy showed the relatively equal amount of uptake of liposomes by the PANC-1 cells regardless of the aqueous phase. For future investigations, fluorescence signal quantification would be done to quantify the amount of liposomes taken up by the cells.

Since using the hypertonic loading method with 462 mM sodium chloride yielded the highest drug loading and encapsulation efficiency, this method was chosen as the treatment for the cytotoxicity assay for comparison to the free drug. The cell viability study showed that the gemcitabine loaded liposomal nanoparticles were comparable to the free drug in terms of cytotoxic effects at the same concentration.

2.6 SUMMARY AND CONCLUSIONS

In conclusion, out of the three nanoplatforms: polymeric, liposomal and lipid polymer hybrid, the liposomal nanoparticles had the highest drug loading capacity and encapsulation efficiency. Future studies would investigate different gemcitabine-to-lipids ratio, perform *in vitro* characterization in more PDAC cell lines, such as BxPC-3 and MIA PaCa-2. BxPC-3 has the wild type of the KRAS gene while MIA PaCa-2, like PANC-1, has the KRAS mutation but has a greater sensitivity to gemcitabine compared to PANC-1 (*34*). Future studies would also include *in vitro* efficacy evaluation of gemcitabine loaded liposomal nanoparticles on spheroid-based 3-D culture model and *in vivo* efficacy evaluation in pancreatic cancer tumor xenograft mouse model.

Table 1: Physicochemical properties of PLGA nanoparticles

PLGA Concentration	Particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
3 mg/mL in ACN	66.672 ± 0.657	0.191 ± 0.004	-14.8 ± 1.10
5 mg/mL in ACN	61.348 ± 0.967	0.181 ± 0.014	-16.25 ± 1.55
10 mg/mL in ACN	61.443 ± 0.232	0.158 ± 0.002	-15.75 ± 0.15

Physicochemical properties (particle size, polydispersity index (PDI) and zeta potential) of PLGA nanoparticles at different concentrations in acetonitrile (ACN).

Table 2: Physicochemical properties of gemcitabine loaded PLGA nanoparticles (GEM-PNPs)

GEM-to-PLGA (%)	Particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)	Drug loading (%)	Encapsulation efficiency (%)
0	65.494 ± 0.277	0.134 ± 0.007	-15.9 ± 1.450	0	0
1	65.366 ± 1.081	0.146 ± 0.006	-6.549 ± 0.529	0.03	6
5	70.569 ± 0.214	0.112 ± 0.003	-8.016 ± 1.651	0.16	6.72
10	84.145 ± 1.854	0.096 ± 0.013	-6.519 ± 0.769	0.26	4.94
15	98.754 ± 2.750	0.072 ± 0.007	-5.851 ± 0.496	0.41	4.64

Physicochemical properties of gemcitabine loaded PLGA nanoparticles (GEM-PNPs) (particle size, polydispersity index (PDI) and zeta potential) at different drug-to-polymer ratio: 1%, 5%, 10% and 15% of 5 mg/mL PLGA.

Table 3: Physicochemical properties of gemcitabine loaded hybrid nanoparticles (GEM-LPHs)

GEM concentration (mg/mL)	Particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)	Drug loading (%)	Encapsulation efficiency (%)
0 in DCM	171.2 ± 1.9	0.069 ± 0.005	-25.5 ± 1.1	0	0
10 in DCM	184.1 ± 2.1	0.062 ± 0.029	-39.8 ± 4.2	0.31	0.06
0 in EtOAc	184.9 ± 1.9	0.068 ± 0.037	-34.8 ± 1.1	0	0
20 in EtOAc	195.5 ± 1.7	0.031 ± 0.013	-33.4 ± 2.2	0.34	0.03

Physicochemical properties of gemcitabine loaded lipid polymer hybrid nanoparticles (GEM-LPHs) at different concentration in two organic solvents: dichloromethane (DCM) and ethyl acetate (EtOAc).

Table 4: Physicochemical properties of liposomal nanoparticles in methanol or ethanol

Solvent	Particle size (nm)	Polydispersity index (PDI)
Methanol	115.814 ± 7.589	0.143 ± 0.012
Ethanol	141.673 ± 18.329	0.212 ± 0.024

Table 5: Physicochemical properties of liposomal nanoparticles in 1 mL or 2 mL sample

Total volume (mL)	Particle size (nm)	Polydispersity index (PDI)
1	89.13 ± 0.954	0.121 ± 0.012
2	82.56 ± 0.571	0.343 ± 0.002

Table 6: Physicochemical properties of liposomal nanoparticles with 5:1 flow ratio

Aqueous phase	Particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
AMS	79.94 ± 0.870	0.203 ± 0.004	-5.09 ± 1.28
NaCl	47.36 ± 0.12	0.203 ± 0.018	-6.45 ± 1.09
AMS + NaCl	55.53 ± 0.22	0.113 ± 0.010	-5.94 ± 1.18

Physical properties (size, polydispersity index (PDI) and zeta potential) of liposomal nanoparticles formulated using 5:1 flow ratio in three different aqueous phases: 250 mM ammonium sulfate (AMS), 462 mM sodium chloride (NaCl), 250 ammonium sulfate + 462 mM sodium chloride (AMS + NaCl).

Table 7: Physicochemical properties of liposomal nanoparticles with 3:1 flow ratio

Aqueous phase	Particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
AMS	119.9 ± 0.81	0.046 ± 0.022	-4.92 ± 0.66
NaCl	51.71 ± 0.15	0.216 ± 0.002	-7.59 ± 0.67
AMS + NaCl	54.47 ± 0.41	0.11 ± 0.004	-5.70 ± 1.04

Physical properties (size and polydispersity index (PDI) and zeta potential) of liposomal nanoparticles formulated using 3:1 flow ratio in three different aqueous phases: 250 mM ammonium sulfate (AMS), 462 mM sodium chloride (NaCl), 250 ammonium sulfate + 462 mM sodium chloride (AMS + NaCl).

Table 8: Physicochemical properties of liposomal nanoparticles with 1:1 flow ratio

Aqueous phase	Particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
AMS	100.1 ± 0.67	0.185 ± 0.004	-5.59 ± 0.26
NaCl	67.73 ± 0.55	0.04 ± 0.026	-4.13 ± 0.80
AMS + NaCl	73.12 ± 0.62	0.077 ± 0.015	-5.7 ± 1.84

Physical properties (size and polydispersity index (PDI) and zeta potential) of liposomal nanoparticles formulated using 1:1 flow ratio in three different aqueous phases: 250 mM ammonium sulfate (AMS), 462 mM sodium chloride (NaCl), 250 ammonium sulfate + 462 mM sodium chloride (AMS + NaCl).

Table 9: Physicochemical properties of gemcitabine loaded liposomal nanoparticles (GEM-Lip)

Aqueous phase	Particle size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
AMS	76.7 ± 3.670	0.174 ± 0.046	-2.970 ± 1.303
NaCl	81.623 ± 6.764	0.204 ± 0.064	-3.243 ± 0.676
AMS + NaCl	78.537 ± 3.339	0.130 ± 0.037	-2.333 ± 1.157

The particle size, PDI and zeta potential of gemcitabine loaded liposomal nanoparticles in three loading methods: remote loading with 250 mM ammonium sulfate (AMS), hypertonic loading with 462 mM sodium chloride (NaCl) and a combination of remote and hypertonic loading with 250 mM ammonium sulfate + 462 mM sodium chloride (AMS + NaCl).

Table 10: Drug loading and encapsulation efficiency of gemcitabine loaded liposomal nanoparticles (GEM-Lip)

Aqueous phase	Drug loading (%)	Encapsulation efficiency (%)
AMS	0.05	0.17
NaCl	5.78	2.94
AMS + NaCl	3.33	3.02

Drug loading and encapsulation efficiency of gemcitabine loaded liposomal nanoparticles in three loading methods: remote loading with 250 mM ammonium sulfate (AMS), hypertonic loading with 462 mM sodium chloride (NaCl) and a combination of remote and hypertonic loading with 250 mM ammonium sulfate + 462 mM sodium chloride (AMS + NaCl)

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