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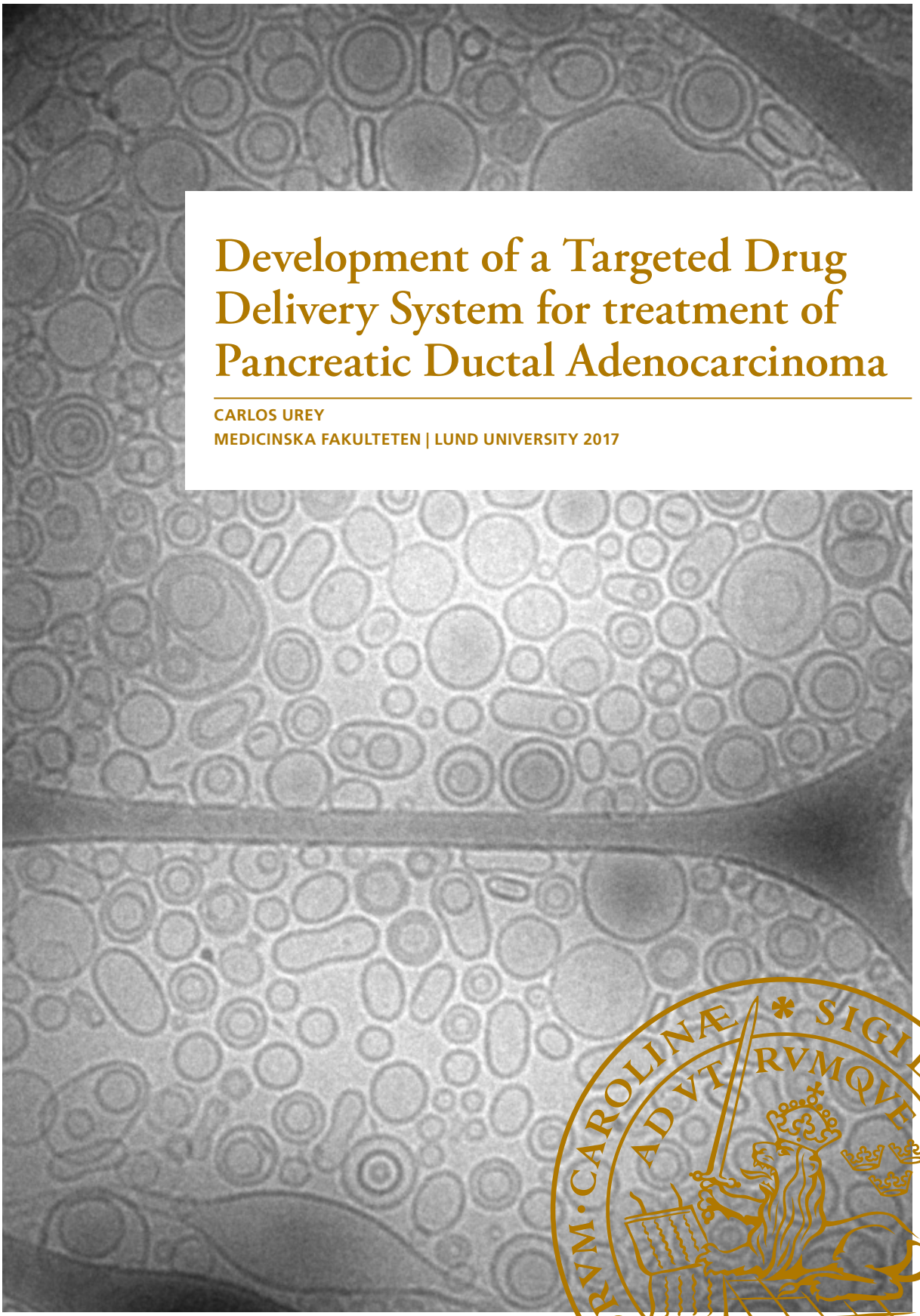

Development of a Targeted Drug Delivery System for treatment of Pancreatic Ductal Adenocarcinoma

CARLOS UREY
MEDICINSKA FAKULTETEN | LUND UNIVERSITY 2017

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Abstract

Pancreatic cancer is the fourth leading cause of cancer-related death with a 5-year survival of less than 6%. The only potential cure for pancreatic cancer is by surgical removal of the tumor. However, most patients present with an advanced and metastasized disease at the time of diagnosis, leaving only around 15% of patients eligible for surgery. For the majority of patients, palliative chemotherapeutic treatment is the only option.

Gemcitabine, a nucleoside analogue, has been the standard chemotherapeutic treatment for pancreatic cancer in both the palliative and adjuvant settings. However, the use of gemcitabine is problematic, as it presents several drawbacks such as a short half-life (~15 min), drug resistance, deficient drug delivery, poor cellular uptake and hence, a suboptimal therapeutic response.

The aim of this thesis was to develop a nanoparticle-based drug delivery system for a targeted and improved delivery of gemcitabine for treatment of pancreatic ductal adenocarcinoma.

To address this issue, we used a liposomal drug delivery system as the delivery system of choice. In a first stage, we developed and extensively characterized the liposomal system by use of several measurement techniques, such as DLS, cryo-TEM, nES GEMMA and AF4, evaluated the system stability and studied the biodistribution profile of the liposomal system by use of radiolabeled liposomes and SPECT/CT imaging. In a second stage, we proceeded to develop a targeted treatment. We first identified a potential targeting protein, MUC4, which is highly expressed in pancreatic cancer but not expressed in the healthy pancreas and studied its clinical impact on resected pancreatic cancer patients. Finally, we developed a MUC4-targeted immunoliposome (iGemLip). iGemLip showed a significantly higher binding affinity, cellular uptake and antiproliferative effect on a MUC4-positive pancreatic cancer cell line, Capan-1, compared to both free and liposomal gemcitabine.

Key words Pancreatic Cancer, Liposomes, Drug Delivery Systems, nanomedicine, MUC4

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Para mi familia

“I wish that I knew what I know now when I was younger”
- Rod Stewart

Contents

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List of Papers and Manuscripts

The following published articles and manuscripts are included in this thesis

I. *Nano Electrospray Gas-phase Electrophoretic Mobility Molecular Analysis (nES GEMMA) of Liposomes: applicability of the technique for nano vesicle batch control.* Weiss V, **Urey C**, Gondikas A, Golesne M, Hoffman T, von der Kammer F, Andersson R, Marko-Varga G, Marchetti-Deschmann M & Allmaier G. *Analyst 2016; 141:6042-6050*.

II. *Combining gas-phase electrophoretic mobility molecular analysis (GEMMA), light scattering, field flow fractionation and cryo electron microscopy in a multidimensional approach to characterize liposomal carrier vesicles.* **Urey C**, Weiss VU, Gondikas A, von der Kammer F, Hofmann T, Marchetti-Deschmann M, Allmaier G, Marko-Varga G, Andersson R. *International Journal of Pharmaceutics 2016; 513:309-318*.

III. *Size Dependent Biodistribution and SPECT/CT imaging of ^{111}-In labeled PEGylated Liposomes in a Pancreatic Cancer mouse model*. **Urey C**, Said-Hilmersson K, Andersson R. *Manuscript*

IV. *Low MUC4 Expression is Associated with Survival Benefit in Patients with Resectable Pancreatic Cancer Receiving Adjuvant Gemcitabine.* **Urey C**, Andersson B, Ansari D, Sasor A, Said-Hilmersson K, Nilsson J, Andersson R. *Scandinavian Journal of Gastroenterology. 2017; 52:595-600*

V. *Development, characterization and in vitro antitumor effect of a gemcitabine-loaded MUC4-targeted immunoliposome against pancreatic ductal adenocarcinoma*. **Urey C**, Said-Hilmersson K, Andersson B, Ansari D, Andersson R. *Accepted by Anticancer Research (2017-04)*

Abbreviations

AF4	Asymmetric Flow Field Flow Fractionation
AFM	Atomic Force Microscope
CT	Computed Tomography
DDS	Drug Delivery System
DLS	Dynamic Light Scattering
DPPC	Dipalmitoylphosphatidylcholine
DSPE-mPEG$_{2000}$	Distearoylphosphoethanolamine-PEG$_{2000}$
DSPE-PEG$_{2000}$-Mal	Distearoylphosphoethanolamine-PEG$_{2000}$-Maleimide
EM diameter	Electrophoretic Mobility diameter
EPR	Enhanced Permeability and Retention
Gem	Gemcitabine
GemLip	Liposomal gemcitabine
hCNT	human Concentrative Nucleoside Transporter
hENT	human Equilibrative Nucleoside Transporter
HSPC	Hydrogenated Soy Phosphatidylcholine
iGemLip	Immunoliposomal gemcitabine
IPMN	Intraductal papillary mucinous neoplasm
IgG	Immunoglobulin G
Mal	Maleimide
MALLS	Multi-angle laser light scattering
MCN	Mucinous cystic neoplasm
MUC4	Mucin 4
NP	Nanoparticle
NTA	Nitrilotriacetic acid
PanIN	Pancreatic intraepithelial neoplasia
PEG	Poly(ethylene glycol)
PEG$_{2000}$	Poly(ethylene glycol)$_{2000}$
PC	Pancreatic Cancer
PDAC	Pancreatic Ductal Adenocarcinoma
SPECT	Single-Positron Emission Computed Tomography

Summary in English

Pancreatic cancer is the fourth leading cause of cancer-related death despite not being among the top ten most common cancer types. The prognosis for pancreatic cancer is dismal with a 5-year survival of less than 5-6%. The only potential cure for pancreatic cancer is by surgical removal of the tumor. However, pancreatic cancer presents no symptoms in the early stages of the disease and most patients (>80%) at the time of diagnosis present with an advanced and metastasized disease, leaving only around 15% of patients eligible for surgery.

Gemcitabine, a nucleoside analogue, has been the standard chemotherapeutic treatment for pancreatic cancer in both the palliative and adjuvant settings. However, the use of gemcitabine is problematic as it presents several drawbacks such as a short half-life (~15 min), drug resistance, deficient drug delivery, poor cellular uptake and hence, a less than optimal therapeutic response.

The aim of this thesis was to develop a nanoparticle-based drug delivery system for a targeted and improved delivery of gemcitabine for treatment of pancreatic ductal adenocarcinoma.

To address this issue, we used liposomes as the drug delivery system of choice. Liposomes are versatile and biodegradable artificial vesicles that have been widely used for drug delivery during the last three decades. Liposomes have shown that by encapsulating drugs in their aqueous interior they can protect the drug from fast degradation and excretion while being passively directed and accumulating in tumors and hence, reducing unwanted side effects on healthy tissue.

In this thesis, we first developed a liposomal gemcitabine system that was thoroughly characterized with measuring techniques such as Dynamic Light Scattering (DLS), cryo-Transmission Electron Microscopy (cryo-TEM), AF4-MALLS and nES GEMMA. Finally, an optimal liposomal size for drug delivery was established by evaluating different liposome sizes using SPECT/CT imaging of radiolabeled liposomes in a pancreatic cancer mouse model.

In a second stage of the thesis work, we proceeded with developing a targeted treatment. First, we identified the membrane-bound protein MUC4 as a potential biomarker for a targeted treatment. MUC4 is overexpressed in pancreatic cancer but not expressed in the normal pancreas. We also observed that resected pancreatic cancer patients with low MUC4 expression, receiving adjuvant gemcitabine treatment had a prolonged survival as compared to patients with high MUC4 expression. MUC4 is therefore a highly interesting marker not only because of its high expression in pancreatic cancer but also for its modulation of sensitivity towards gemcitabine.

In the final part of this work, we developed a MUC4-targeted immunoliposome (iGemLip) treatment. iGemLip showed a higher binding and increased cellular uptake on MUC4-positive pancreatic cancer cells compared to non-targeted liposomes. Finally, iGemLip showed a higher antiproliferative effect compared to both gemcitabine and non-targeted liposomal gemcitabine on a MUC4-positive pancreatic cancer cell line.

In summary, in this thesis we have shown the progression and many steps required from development to application of a targeted liposomal pancreatic cancer therapy. We identified a potential biomarker for targeted treatment and have shown that in an *in vitro* environment this targeted therapy yields the desired improved effect compared to the free drug. Hopefully, the results contained in this thesis may help the progression and development of improved treatments in the field of pancreatic cancer.

Populärvetenskaplig sammanfattning

Cancer i bukspottkörteln är en av de minst förekommande cancerformerna men en av de dödligaste. Bukspottkörtelcancer går enbart att bota om sjukdomen upptäcks tidigt och tumören kan opereras bort. Dessvärre finns det inga tidiga symptom som visar på sjukdom och större delen av patienter, över 80%, diagnostiseras först när cancern hunnit sprida sig till andra delar av kroppen. För dessa patienter återstår enbart en palliativ behandling med cellgifter, dvs. en behandling som fokuserar på att förlänga patienternas livslängd, förbättra deras livskvalitet och minska smärta i största möjliga mån.

Av de cellgifter som används har gemcitabine under de senaste tjugo åren varit standardbehandling mot bukspottkörtelcancer. Detta till trots, visar gemcitabine på en rad allvarliga brister, bland annat att behandlingen bryts ner redan efter en kvart i kroppen, läkemedelsresistens från många patienter samt en undermålig transport av läkemedlet till tumörområdet. Utöver detta behöver gemcitabine aktivt tas upp av celler för att kunna utföra sin verkan i kroppen. Problemet är att enbart en tredjedel av patienter uttrycker det nödvändiga proteinet för upptag av gemcitabine i cellerna vilket gör att större delen av patienterna inte är mottagliga för behandlingen.

Det finns idag ett stort behov av att förbättra befintliga behandlingar mot bukspottkörtelcancer, alternativt att utveckla nya effektivare behandlingar. Med detta i åtanke var det huvudsakliga målet med denna avhandling att utveckla en riktad behandlingsform mot bukspottkörtelcancer som på ett effektivare sätt kan leverera gemcitabine till tumörområdet. Genom att specifikt rikta behandlingen mot tumören kan cellgifternas verkan på frisk vävnad minskas och därmed även minska oönskade biverkningar hos patienterna.

I en första del av avhandlingen fokuserade vi på utvecklingen av ett så kallat ”drug delivery system” som kan transportera och skydda läkemedel mot nedbrytning i kroppen. Vi gjorde detta genom användning av en sorts nanopartiklar kallade ”liposomer”. Liposomer består av lipider, dvs. fetter, och har en vätskefylld insida som kan fyllas med bl.a. läkemedel. Deras uppbyggnad av lipider gör att de har en struktur som liknar cellernas membran och de kan därför tas upp av celler utan att vara skadliga mot dessa. När liposomer tas upp av cellerna frisätts det inneslutna läkemedlet i cellens insida och kan därmed verka. För cellgifter som gemcitabine innebär detta att läkemedlet först skyddas mot snabb nedbrytning i kroppen och att det sedan kan kringgå behovet av ett aktivt cellulärt upptag. Det liposomala system vi utvecklade, GemLip, visade en lång stabilitet och ett minimalt utsläpp av det inneslutna läkemedlet. Liposomerna storleks- och ytbestämdes med flera tekniker och vi kunde därmed bekräfta en metod där vi kontinuerligt kunde framställa

GemLip med en diameter runt 100 nanometer samt med den önskade mängden inneslutet läkemedel.

Vidare studerade vi möjligheterna för en riktad behandling. För att åstadkomma detta var vi först tvungna att hitta en lämplig ”biomarkör”, t.ex. ett protein, som behandlingen kan riktas mot. En biomarkör för riktad behandling bör uttryckas högt i den sjuka vävnaden men helst inte uttryckas i den friska vävnaden. Vi identifierade ett sådant protein, MUC4, som inte uttrycks i den friska bukspottkörteln men som uttrycks i bukspottkörtelcancer och vars uttryck alltmer ökar under sjukdomsförloppet.

MUC4 har dock visat ha en inverkan på uppkomsten och spridning av bukspottkörtelcancer. Studier på celler har även visat att ett högt uttryckt av MUC4 i cancerceller skapar en resistens mot behandling med gemcitabine. För att undersöka den kliniska påverkan av MUC4 i bukspottkörtelcancer studerade vi hur uttrycket av MUC4 påverkar överlevnad hos patienter med bukspottkörtelcancer som behandlades med gemcitabine. Vi fann att de patienter som hade ett lågt MUC4-uttryck i sina tumörer hade en längre överlevnad än de som hade ett högt MUC4-uttryck. Studien bekräftar därmed experimentell data för första gången i en klinisk miljö och visar även att MUC4 kan användas som en markör för patienters mottaglighet för en behandling med gemcitabine.

I en sista fas av projektet fortsatte vi med att utveckla en liposomal gemcitabine-behandling riktad mot MUC4, s.k. immunoliposomer. I försök med cancerceller som uttrycker MUC4 visade immunoliposomerna en större förmåga att binda in till cellerna än vanliga liposomer. Utöver detta visade behandling av cellerna att immunoliposomerna hade en större förmåga att hämma tillväxten av cancercellerna än vad både det fria eller liposomala gemcitabinet kunde åstadkomma.

Sammanfattningsvis har avhandlingen visat den process som kan ligga bakom utvecklingen av ett riktat läkemedel för behandling av cancer. Vi identifierade ett lämpligt målprotein, MUC4, som visade sig ha en större klinisk relevans än vad vi till en början trott, och vi utvecklade därefter en behandling riktad mot detta protein. Den riktade behandlingen visade en större förmåga att hämma tillväxten av cancerceller än det fria läkemedlet. Resultaten i denna avhandling visar på vikten av att utveckla effektivare behandlingar men även att anpassa dessa efter patienternas individuella behov då en universell behandling inte lämpas för alla. Arbetet i denna avhandling kan förhoppningsvis bidra till utvecklingen av bättre och effektivare behandlingar mot bukspottkörtelcancer, ett fält där det fortfarande finns många framsteg att göra.

Introduction

Cancer is the collective name for a group of malignant diseases characterized by the transformation and uncontrolled proliferation of normal cells into malignant cells [1]. The cancer cells can invade and spread via e.g. the lymphatic or hematogenous systems to distant parts of the body. On their way to becoming a malignant disease, the transformation the cells undergo may be summarized by the following hallmarks: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing growth of new vasculature (angiogenesis), activating invasion and metastasis, reprogramming of energy metabolism and evading immune destruction [2, 3]. In Sweden and Europe, cancer is the second leading cause of death [4, 5]. During 2014, 65 000 new cases of cancer were reported from over 60 000 patients in Sweden alone. These numbers have been steadily increasing since the 70s [6].

Surgery and radiotherapy have been the most effective ways of treating local and non-metastatic tumors. However, for treatment of advanced and metastasized tumors, the use of chemotherapy is preferred as a systemic approach is necessary [7, 8].

Pancreatic cancer

Pancreatic Cancer (PC) is a highly lethal form of cancer. In Sweden, it represents the fourth leading cause of cancer-related death, despite not being among the top ten most common types of cancer. In 2014, nearly 1200 new cases of pancreatic cancer were reported in Sweden along with 1800 cases of pancreatic cancer-related deaths [6]. The high mortality is mainly due to an aggressive nature, the often advanced stage of disease at diagnosis due to vague symptoms and the lack of effective treatments.

Epidemiology and risk factors

Pancreatic cancer has a dismal prognosis, the overall incidence and mortality rates are almost parallel [9, 10] and the relative 5-year survival, although slightly

improving, is still in the range of 5-6% [11]. Currently, the only potential cure for pancreatic cancer is by surgical removal, i.e. resection of the primary tumor. Surgical resection is, however, only feasible in up to 15% of the patients [12] and even so, most completely resected patients will recur within 5 years with survival, during the same period, being only up to 25% [9, 10]. For non-resectable patients, the standard treatment is chemotherapy with use of gemcitabine, alone or in combination with other treatments, which offers a marginal prolongation of life [13]. Other treatments for pancreatic cancer that more recently have been introduced include FLv – the Nordic fortnightly bolus 5-Fluorouracil/Folinic acid regimen and FOLFIRINOX, a regimen consisting of oxaliplatin, folinic acid, irinotecan and fluorouracil. FOLFIRINOX has showed an improved survival compared to gemcitabine but with severe side effects and high levels of toxicity [14].

There is currently no way of screening for early diagnosis, although several risk factors have been identified. Among these are family history of pancreatic cancer, smoking, chronic and hereditary pancreatitis, diabetes, obesity, age and alcohol consumption [15, 16]. Most pancreatic cancer patients are asymptomatic until the disease has reached an advanced stage. At this stage, the most common symptoms include weight loss, abdominal and back pain, nausea and vomiting, new-onset diabetes mellitus type 2 and jaundice [10, 17]. In combination with late and vague symptom expression, limited diagnostic tools make diagnosis of pancreatic cancer at an “early” stage difficult. The standard diagnostic tools include the use of the CA19-9 serum tumor marker and computed tomography (CT), among others. However, while CA19-9 is a widely used marker, when screening for pancreatic cancer, it is limited by poor sensitivity and specificity [18]. CT on the other hand is an expensive screening method which is widely used but limited by the resolution power of the technique [19].

Tumor development and genetic alterations

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer, accounting for more than 85% of all solid pancreatic tumor cases [16, 20]. PDAC commonly occurs in the head of the pancreas from where it later infiltrates surrounding tissue and organs such as lymph nodes, major vessels and the peritoneal cavity, and metastasizes to the liver and lungs [16].

Pancreatic cancer traditionally arises from precursor lesions before developing into local invasive cancers and finally an advanced metastatic disease. Most of PDAC are thought to develop from the precursor lesion known as pancreatic intraepithelial neoplasia (PanIN) [21]. PanINs are subcategorized into three different stages, Figure 1. In PanIN-1A and PanIN-1B (low-grade lesions) the normal cuboidal flat epithelium is replaced by flat or papillary mucinous epithelium with minimal atypia.

PanIN-2 lesions (intermediate-grade PanIN) show loss of cell polarity and nuclear variation and crowding. Finally, PanIN-3 lesions (high-grade PanIN) are also referred to as carcinoma *in situ* and show severe cellular atypia without signs of invasion or trespass of the basement membrane or surrounding tissue [22, 23]. To a lesser extent than PanINs, pancreatic cancer (different from PDAC) may also develop from intraductal papillary mucinous neoplasms (IPMNs) and from mucinous cystic neoplasms (MCNs) [23]. Since PDAC will be the focus of this thesis, it will hereafter be referred to as pancreatic cancer.

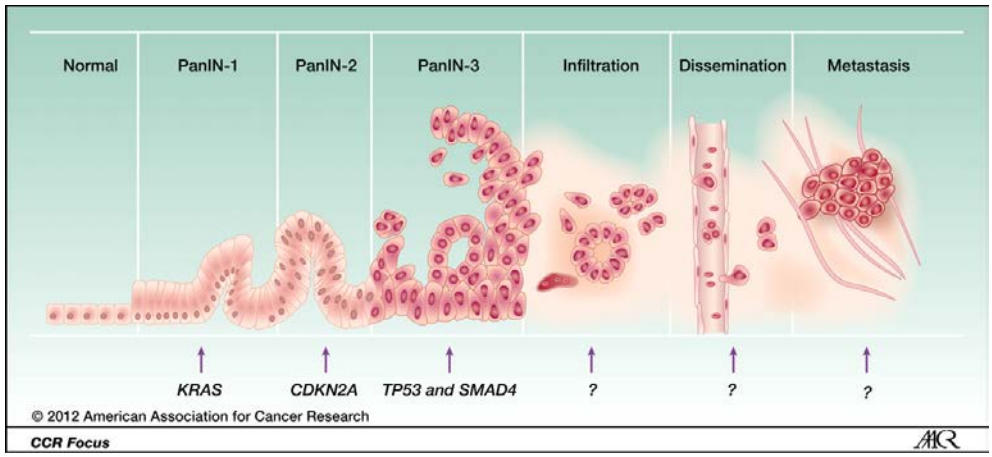

Figure 1. Pancreatic Cancer development from the PanIN precursor lesions
The image depicts the progression of pancreatic cancer from normal pancreatic cells through the stepwise PanIN lesions to invasive carcinoma. The illustration also depicts the characteristic mutations for each PanIn stage. Image printed with permission [24].

During pancreatic cancer progression, the most common genes that are targeted by mutation, amplification or deletion include the oncogenes and tumor suppressors *KRAS*, *p16/CDKN2A*, *TP53*, and *SMAD4* [20, 21, 23, 25]. The accumulation of these, and other smaller, genetic changes are a hallmark of pancreatic cancer that lead to cell cycle deregulation, cell survival, invasion and metastasis [25]. The most common mutation in pancreatic tumors is activation of the *KRAS* oncogene, present in over 90 % of cases, which typically occurs in low-grade PanIN-1 lesions [26]. Inactivation of the *p16/CDKN2A* cell-cycle regulator occurs in the intermediate PanIN-2 lesions and finally, inactivation of *TP53* DNA damage control and *SMAD4* transforming growth factor signaling occurs in late PanIN-3 lesions [25]. Characteristic mutations can be seen in Figure 1.

Clinical Staging

Pancreatic cancer progression is divided into different stages which give an indication of the tumor progression and which treatment strategy to use. In stage 1, the tumor resides in the pancreas and has not spread beyond the pancreas. In stages 1A and 1B, the tumor is less than or larger than 2 cm in any direction, respectively, but is still contained within the pancreas. In stage IIA, the cancer has started to invade nearby tissue and organs and in stage IIB the cancer has invaded nearby lymph nodes. When reaching stage III, the tumor has spread into the major blood vessels and at stage IV, the cancer has become an advanced metastatic disease and has spread to distant sites of the body.

Recent studies, both using molecular biology [27] and larger patient cohorts [28], however, demonstrated that pancreatic cancer not infrequently possess an aggressive behavior and is capable of metastatic spread already at a very small size of the tumor. These findings point at the complex nature of pancreatic cancer and that tumor biology might be more important than the size per se as comes the outcome.

Gemcitabine

Gemcitabine (2',2'-difluorodeoxycytidine), Figure 2, is a cytidine analogue with several modes of action. It is a prodrug which requires active cellular uptake in order to exert its antiproliferative effect. Inside the cell, the main mechanism of action of gemcitabine is by incorporating itself onto the DNA chain during replication and thereby disrupting the replication process, ultimately leading to cellular apoptosis.

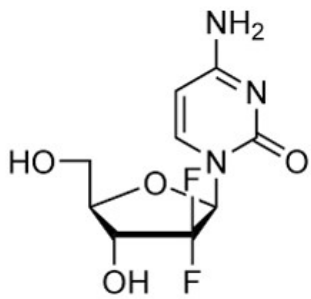

Figure 2. Gemcitabine
Molecular structure of Gemcitabine (2',2'-difluorodeoxycytidine)

In 1996, a study comparing the survival of patients with advanced pancreatic cancer receiving either gemcitabine or fluorouracil, showed an overall improved survival of 1 month among patients receiving gemcitabine [13]. Gemcitabine has since been the most widely used treatment against pancreatic cancer in the palliative setting and since 2007 [29, 30], it is also recommended as adjuvant treatment following surgical resection with curative attempt.

Over the last decades, many clinical studies compared single-agent gemcitabine treatment to combination therapy, but were unable to show any consistent improved patient survival. It was not until recent years that two clinical studies showed overall prolonged survival and shifted single-agent gemcitabine treatment to combination chemotherapies; introducing the FOLFIRINOX regimen [14] and a gemcitabine and albumin-bound paclitaxel (nab-paclitaxel) combination [31]. Just recently, in 2017, a new multicenter study (ESPAC-4) showed that the adjuvant combination of gemcitabine and capecitabine had better survival and tumor response on PDAC patients following resection than gemcitabine alone, prompting the use of adjuvant gemcitabine and capecitabine as the new standard of care following resection for PDAC [32].

Even though gemcitabine has been the mainstay treatment against pancreatic cancer over the last two decades, showing improved survival with limited side-effects, it is not uncomplicated and lacking limitations. In the following sections, the molecular mechanism of gemcitabine will be explained, as well as its limitations and resistance in pancreatic cancer.

Uptake and molecular metabolism

Due to the hydrophilic nature of gemcitabine, it is unable to diffuse across cellular membranes, and hence requires active cellular uptake [33, 34]. Cellular uptake of gemcitabine is mediated by two types of human nucleoside transporters: the human equilibrative (sodium independent) and concentrative (sodium dependent) transporters, hENT and hCNT, respectively [35]. Among these transporters, gemcitabine uptake is mainly mediated by hENT1. For example, it has been shown that hENT1-deficient cells are resistant to gemcitabine [34]. Gemcitabine is also taken up by hENT2, hCNT1 and hCNT3, although to a lesser extent than by hENT1 [34].

Once inside the cell, gemcitabine undergoes a series of phosphorylation steps in the cytoplasm, as seen in Figure 3. It is first phosphorylated into gemcitabine monophosphate (dFdCMP) by deoxycytidine kinase (dCK) and then phosphorylated by nucleotide kinases (CMPK1) into its diphosphate (dFdCDP) and triphosphate (dFdCTP) active metabolites, respectively [36, 37]. The first

phosphorylation step by dCK is considered the rate-limiting step for the further phosphorylation into dFdCDP and dFdCTP [37, 38].

In the cell, gemcitabine is mainly deactivated through deamination by Cytidine Deaminase (CDA) and, when in its monophosphate form, by deoxycytidylate deaminase (dCTD) to its inactive forms difluorodeoxyuridine (dFdU) and difluorodeoxyuridine monophosphate (dFdUMP), respectively [39]. *In vivo*, deamination is also the major form of metabolic clearance due to the high activity of CDA in large body organs [33, 40]. Also, gemcitabine may be inactivated by dephosphorylation of its monophosphate form by 5'-nucelotidases (5'-NTs), catalyzing nucleotides back to nucleosides [33, 38].

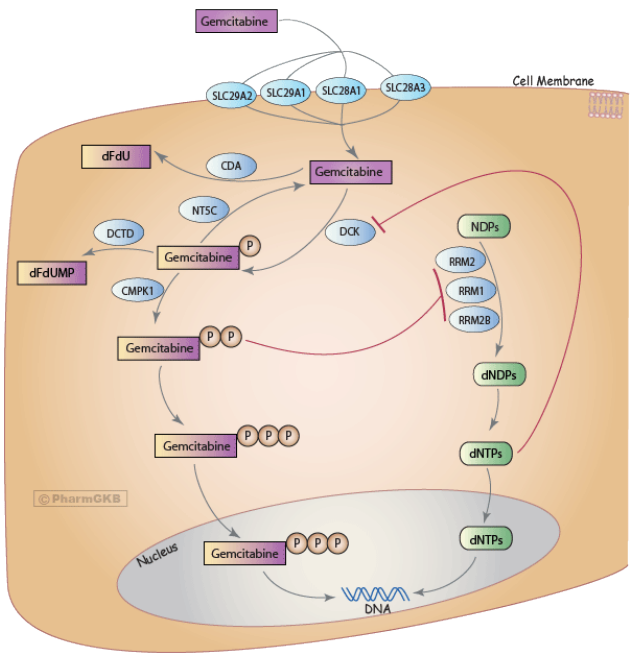

Figure 3. Cellular metabolism and mechanism of action of gemcitabine
The figure illustrates uptake and metabolic pathways of gemcitabine. To the left is the depiction of activation of gemcitabine to its active metabolites and to the right is a depiction of gemcitabine self-potentiation by inhibition of ribonucleotide reductase (RR). Image reprinted with permission by PharmGKB and the Stanford University [41].

Mechanism of action

The main mechanism of action of gemcitabine is by inhibition of DNA synthesis. When dFdCTP is incorporated into the DNA strand, only one additional nucleotide is allowed to incorporate, masking gemcitabine from DNA repair enzymes. Thereafter, DNA polymerase is unable to proceed chain elongation in a process that

is known as “masked chain termination” [42, 43]. This process is illustrated in Figure 4.

Gemcitabine also works by self-potentiation as several of its metabolites inhibit various enzymes related to deoxynucleotide metabolism (dNTPs, normally dCTP), as depicted on the right hand side of Figure 3. Longer retention of active gemcitabine metabolites in tumor cells is essential for the gemcitabine to exert its antiproliferative effect.

In the most important self-potentiation mechanism, DNA synthesis is indirectly inhibited by dFdCDP inhibition of ribonucleotide reductase (RR). Inhibition of RR leads to a depletion of cellular dCTP, which is a potent feedback inhibitor mechanism of dCK, leading to an increased gemcitabine phosphorylation [44]. In the opposite case, when increased RR activity increases the dCTP pool, a decrease in gemcitabine phosphorylation and reduction of its activity is seen [45]. Furthermore, since dFdCTP competes with dCTP and since dCK activity is down-regulated by high cellular levels of dCTP, a reduction in the cellular pool of dCTP will result in an increased incorporation of dFdCTP into DNA [33, 38, 43, 44, 46].

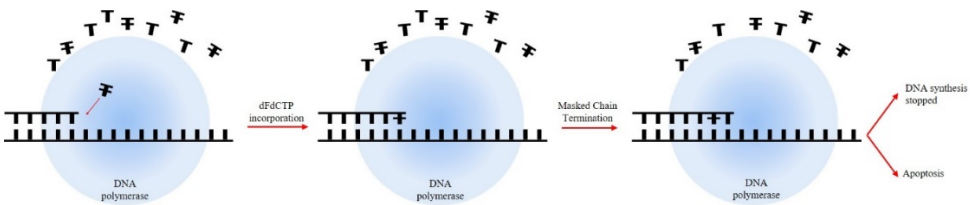

Figure 4. Gemcitabine main mechanism of action
Illustrated representation of the masked chain termination process where DNA polymerase incorporates gemcitabine triphosphate (dFdCTP) to the DNA chain during replication. Afterwards, a single nucleotide is incorporated, making the DNA polymerase unable to proceed elongation and DNA chain elongation is stopped.

Limitations and chemoresistance

As previously mentioned, gemcitabine has been the mainstay chemotherapeutic drug for treatment of pancreatic cancer. However, like many other cancer drugs, gemcitabine has its own limitations, as well as inherent and acquired resistance mechanisms from cancer cells towards the drug.

Following the course of gemcitabine in the body, from administration to metabolic activity, one of the major limitations of gemcitabine is met directly after intravenous injection as it has a very short half-life of around 15 min [47, 48]. The short half-life of gemcitabine is mainly due to deamination by CDA in the blood and in the larger organs into dFdU, the inactive metabolite of gemcitabine. dFdU is also the only gemcitabine metabolite found in human urine of patients treated with gemcitabine [40].

Nucleoside transporters play a critical role in the cellular uptake of gemcitabine to cells. Limited intracellular uptake of gemcitabine due to low expression of hENT1 is an established resistance mechanism *in vitro* [34]. This has also been confirmed in several clinical studies [49] as it has been shown that high levels of hENT1 are commonly associated with longer survival compared to patients with lower expression of hENT1 [38]. Hence, it has been shown that hENT1 can be used as a prognostic factor for gemcitabine-based treatments.

In the cell, gemcitabine needs to be phosphorylated by dCK, which is the rate-limiting step for further phosphorylation of gemcitabine into its active metabolites. Gemcitabine efficacy is usually related to dCk activity and the most commonly described form of *in vitro* acquired resistance to gemcitabine is by dCK deficiency [50]. Low dCK activity has been associated with an intrinsic gemcitabine resistance [51] whereas upregulation of dCK activity has shown proof of enhanced cell death [52, 53].

CDA, as previously mentioned, is not only responsible for deamination of gemcitabine to its inactive metabolite dFdU in the bloodstream but also inside the cells. *In vitro* studies have shown that treating human pancreatic cancer cell lines with tetrahydrouridine, a CDA inhibitor, gemcitabine cytotoxicity was significantly increased [54, 55].

Although several other proteins are involved in the inherent and acquired resistance mechanisms against gemcitabine, the most important and frequent of these (hENT1, dCK and CDA) have been covered in this section. In summary, the proteins related to metabolism of gemcitabine are the same that may be used as important prognostic factors for patient susceptibility towards gemcitabine-based treatment. Many attempts have been made throughout the years in order to overcome these obstacles, some of these have involved the direct modification of gemcitabine for improved pharmacokinetic profile and others have involved the use of nanoparticle drug delivery systems in order to prolong circulation in blood, decrease clearance and also to achieve a targeted treatment. The latter of these will be covered in the next chapter.

Mucins

In our body, epithelial surfaces are covered by a viscous layer which gives the cellular surfaces protection against the harsh environment they are exposed to. A major component of these protective layers are the proteins called mucins [56].

Mucins belong to a large family of heavy molecular weight proteins. They are characterized by long, heavily O-glycosylated, tandem repeat regions rich in serine,

threonine and proline [57]. The human mucin family consists of 21 members, MUC1-MUC21, and based on their physiological and structural characteristics they are either divided into secreted or membrane-bound mucins [58]. Membrane-bound mucins are bound to cells by a transmembrane domain and a short cytoplasmic tail. They include MUC1, MUC3A/B, MUC4, MUC11-13, MUC15-17, MUC20 and MUC21 and contribute to the protective mucous gel through their single extracellular domain of O-glycosylated tandem repeats [57]. Secreted mucins are further subdivided into gel-forming (MUC2, MUC5AC/B, MUC6 and MUC19) and non-gel-forming (MUC7) and form a physical barrier composed of viscoelastic mucous gel that provides protection for epithelial cells exposed to the external environment including the lining of the respiratory and GI tracts [58].

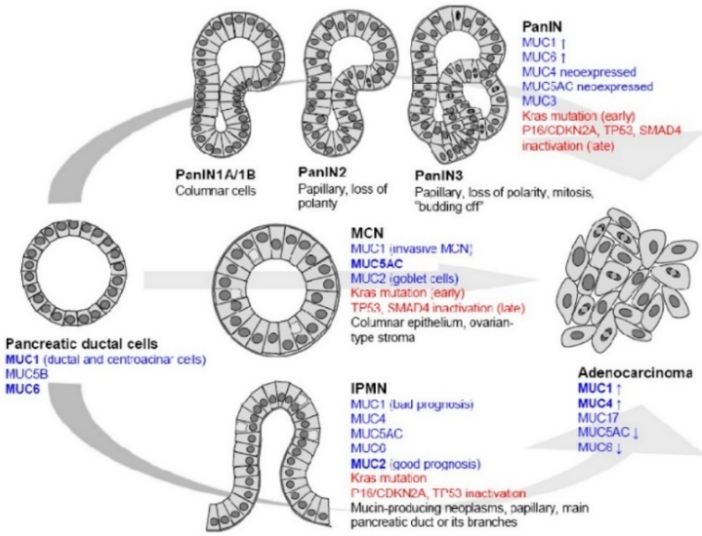

Figure 5. Mucin expression in pancreatic cancer
Altered mucin expression is seen already in early pancreatic lesions and expression of MUC4, for example, may be used as a tumor progression marker. Image reprinted with permission from [59].

Mucins in pancreatic cancer

The healthy pancreas has little to no mucin expression where MUC1 is the main, membrane-bound, mucin found [59]. In contrast to the healthy pancreas, pancreatic cancer is characterized by the altered and increasing expression of numerous mucins. During progression from the normal pancreas to malignancy, expression and glycosylation patterns for both types of mucins are altered [58], Figure 5. Also, the altered expression and glycosylation of mucins is reported to contribute to tumor growth, proliferation, invasion and differentiation [57, 58, 60].

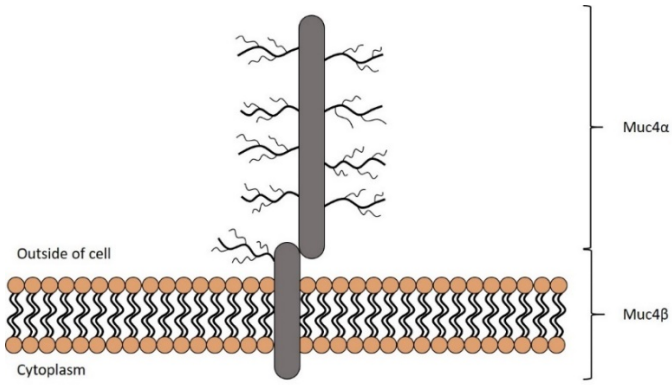

Figure 6. Structure of MUC4
MUC4 has a membrane-bound β-subunit and an α-subunit that is expressed outside the cell.

MUC4 in pancreatic cancer

The human mucin 4 (MUC4) is a large and heterodimeric membrane-bound mucin. MUC4 is composed of two subunits, MUC4β, which composes the transmembrane part of the protein and MUC4α, which composes the extracellular part of the protein containing the hallmark, glycosylated, tandem repeat regions [61], see Figure 6. In healthy tissue, MUC4 is expressed in the trachea, lungs, stomach, colon and cervix [61], but not in the pancreas.

In pancreatic cancer, the membrane-bound MUC4 is *de novo* expressed and is observed in early PanIN and IPMN pancreatic lesions. During oncogenesis, gradual expression of MUC4 increases with disease progression and the following metastasis [58, 62]. MUC4 is reported to be among the most differentially expressed genes in pancreatic cancer, with expression increasing from 17% in early lesions to 89 % in invasive pancreatic cancer [59]. Expression of MUC4 has also been described as a potential predictor for gemcitabine treatment response in pancreatic cancer patients. In *in vitro* settings, MUC4 expression has been directly related to resistance towards gemcitabine [63-65], whereas other studies reported that downregulation of MUC4 expression in pancreatic cancer cells induced sensitivity towards gemcitabine treatment [66, 67]. These *in vitro* findings were corroborated in a clinical study performed by our group where we observed that resected pancreatic cancer patients with low MUC4 expression had an increased survival benefit from receiving adjuvant gemcitabine treatment compared to patients with high MUC4 expression [68].

In summary, overexpression of MUC4 plays several roles in pancreatic cancer. It may act as an indicator of disease progression, as a potential diagnostic marker for response to gemcitabine treatment, but equally interesting, it presents potential as a

target for targeted therapy, which may prove to be of clinical benefit for pancreatic cancer patients.

Targeted Tumor Therapy

In recent years, the implementation of nanotechnology in medicine has led to a continuously increasing use of nanoparticles (NP) for drug delivery. The use of nanoparticle-based drug delivery systems has been the preferred approach for improving a pharmaceutical compounds therapeutic efficacy.

NP drug delivery systems (DDS) are widely investigated in a preclinical setting and many of these systems have already been implemented in the clinic [69, 70]. NP-based DDS for systemic administration offer several advantages over administration of their free or non-formulated drug counterparts. For example, DDS protect the drug from fast degradation and clearance, enhances drug accumulation in target tissue and decreases side effects [71].

One of the major drawbacks of current cancer chemotherapies, gemcitabine included, is the inability to deliver the drug to their specific target, i.e. poor selectivity, causing cytotoxic effects on both healthy and cancerous tissue. By delivering drugs to the treatment site, its influence on healthy tissue and undesirable effects can be minimized. An increased accumulation and retention of the drug at the tumor site warrants an overall lower systemic dose for achieving the same therapeutic effect.

During the last two decades, several attempts have been made to improve the therapeutic effect of gemcitabine. While some attempts have revolved around direct modification of the gemcitabine structure in order to improve cellular uptake or phosphorylation inside the cell, many more have focused on the improved delivery and retention of gemcitabine by use of NP-based DDS [48]. Among the tested systems were polymeric nanoparticles, micelles and liposomes in particular. Since liposomes were the systems of choice for this project, the following section will cover the basics of liposomal drug delivery systems and their antibody-targeted counterparts, immunoliposomes.

Liposomes in Drug Delivery

Liposomes are biocompatible and biodegradable artificial vesicles composed of a phospholipid bilayer enclosing an aqueous compartment, Figure 7. Liposomes were first described in the 60s by Bangham et al. [72] and proposed as drug delivery systems by Gregory Gregoriadis et al. in the mid-70s [73]. Liposome sizes range

from tens of nanometers up to several micrometers and may be composed of either one or several concentric layers, uni- or multilameller, with an estimated bilayer thickness of 5 nm [74, 75].

Like most cell membranes, liposomes are mainly composed of phospholipids which consist of a hydrophilic head group and two hydrophobic tails, Figure 7. Owing to the amphiphilic nature (both hydrophilic and hydrophobic) of the phospholipids, liposomes possess a unique set of properties which makes them suitable for entrapment and delivery of drugs. They are able to trap both hydrophobic and hydrophilic drugs in their bilayer and aqueous interior, respectively, while protecting the drugs from enzymatic degradation and fast clearance from the blood stream.

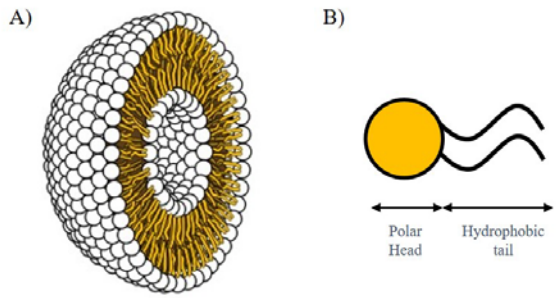

Fig 7. Liposome and their main bilayer constituents, phospholipids
A) Cross section of a liposome visualizing the structure of the phospholipid bilayer and the aqueous interior of the liposome. B) Structural image of a phospholipid depicting its hydrophilic head and hydrophobic tail

Initially, liposomes had problems with fast clearance from the blood stream by the reticuloendothelial system (RES). However, this was overcome by sterically stabilizing liposomes with the inclusion of poly(ethylene glycol) (PEG) chains to the liposomal surface. The presence of PEG-chains on the liposomal surface, i.e. stealth liposomes, dramatically increased liposome circulation time while reducing uptake by the RES system [76].

Liposomes as drug delivery systems have become increasingly popular and widely used. They have proven clinically efficient by improving on their non-formulated counterparts by increasing blood half-lives, but mainly by reducing severe side effects. This was the case for the first FDA approved liposomal drug, Myocet, which is a liposomal formulation of doxorubicin [77]. Other liposomal formulations approved by the FDA are liposomal cisplatin [78], daunorubicin, vincristine [79], and just recently, a new liposomal irinotecan formulation [80] was approved for treatment of pancreatic cancer.

In terms of drug delivery, liposomes between 70-200 nm in diameter are considered optimal as they have the advantage of passively accumulating in tumor tissue [81, 82]. In order to ensure sufficient nutrient and oxygen supply for rapid tumor growth, tumors have a more permeable and leaky vasculature compared to normal tissue. The leaky tumor vasculature allows an enhanced permeability of nanoparticles, below a certain diameter, and an impaired lymphatic drainage allows for a longer retention of nanoparticles in the tumor site. This effect is known as the Enhanced Permeability and Retention (EPR) effect and is a principle extensively used in drug delivery for targeting of nanoparticles to a tumor microenvironment [83-85], Figure 8. In a normal epithelium, the gaps between endothelial cells ranges between 5-10 nm, whereas in a tumor vasculature, depending on the type of tumor, the gaps may range between 100-780 nm [86, 87].

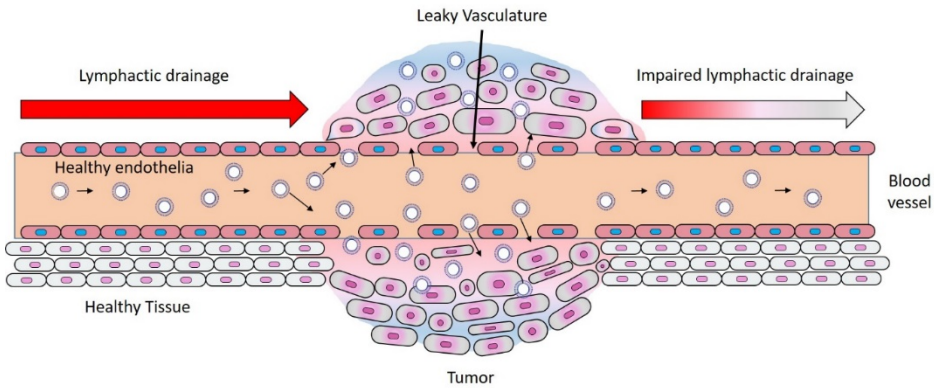

Figure 8. Enhanced Permeability and Retention effect
Passive targeting of liposomes is achieved through the Enhanced Permeability and Retention effect. Vasculature near tumor sites are leaky with large fenestrations and suffer from impaired lymphatic drainage. This allows the accumulation and increased retention of liposomes < 200 nm in the tumor tissue.

Liposome accumulation in a tumor site due to the EPR effect is commonly denominated as “passive targeting” of liposomes or nanoparticles. However, liposomes may also be actively targeted to tumors by grafting a targeting ligand to the surface of the liposomes. The targeted liposomes will accumulate in the tumor interstitial space to the same extent as regular stealth liposomes. However, liposomal retention and cellular internalization will be enhanced by an interaction between the targeting ligand and the target receptor/protein [88]. For the creation of a targeted liposome, several targeting ligands, such as small molecule ligands or peptides, have been conjugated to the liposomal surface. However, the most commonly used targeting ligands are whole or fragmented monoclonal antibodies [86], these targeted liposomes are also known as immunoliposomes. Immunoliposomes may further be divided into Type I or Type II depending on whether antibodies (or fragments thereof) are conjugated directly to the lipid bilayer

or to the distal end of the PEG-chains, Figure 9. Yet, antibodies or fragmented antibodies, are usually conjugated to the distal end of the PEG-chains as a conjugation directly to the liposomal lipid bilayer may result in reduced antigen bonding due to steric shielding of the PEG-chains.

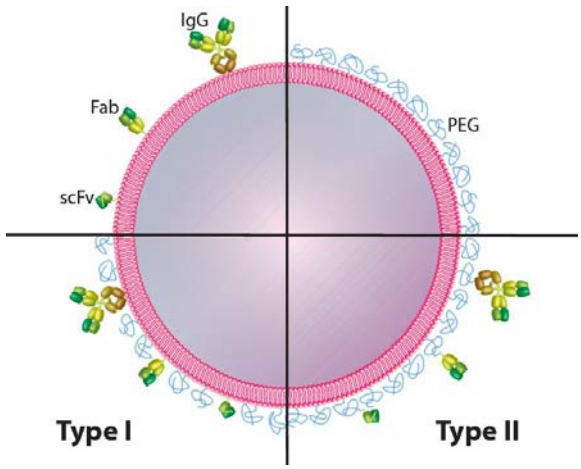

Figure 9. Immunoliposomes
Different types of immunoliposomes. In Type I, antibodies are directly conjugated to the liposomal surface whereas in Type II, antibodies are conjugated to the distal end of the PEG-chains. Copyright permission was obtained from Encapsula NanoSciences

Targeting may either be directed to cell surface proteins or receptors, the tumor microenvironment or utilizing local stimuli for enhanced and prolonged drug release [86]. Ideally, if targeting is made against a cell surface receptor or protein, the target protein should be highly expressed in the tumor tissue, but limited or not expressed at all in healthy tissue. Furthermore, the targeting efficacy of the immunoliposomes will depend on the amount of antibody conjugated to the liposome, the binding affinity of the antibody to the antigen, type of antibody (whole or fragmented) and the chemistry used for conjugation [86]. During the last decade, many studies have tried to implement the use of immunoliposomes for targeted cancer treatments, however, few of these have focused on pancreatic cancer.

Aims of the thesis

In pancreatic cancer there is an unmet need to develop new or improve existing treatments. The overall aim of the thesis was to develop a targeted drug delivery system for improved delivery, retention and uptake of gemcitabine for treatment of pancreatic ductal adenocarcinoma. The treatment would ideally do this at the same or lower doses than the free drug while reducing side effects.

The specific aims of the thesis were:

1. Develop and characterize a liposomal drug delivery system in an *in vitro* and *in vivo* setting (Study I, II and III)
2. Identify a suitable target for targeted delivery (Study IV)
3. Develop and characterize a targeted liposomal drug delivery system in an *in vitro* (Study V) and *in vivo* setting (future studies)

Materials and Methods

The studies included in this thesis are predominantly based on liposome development and characterization methods, *in vitro* evaluation of liposomes and the use of a human pancreatic cancer mouse model. Study IV is based on patient material where immunohistochemistry was performed on resected pancreatic cancer tissues. This chapter will only discuss the experimental models used in study I, II, III and V. The remaining, and detailed, methodology can be found in studies I-V in the Appendix. Table I offers an overview of methods used in each part of the project.

Table 1.
Overview of experimental modles and techniques used in study I, II, III and V

Study	Cells	Techniques
I	-	Liposome preparation, nES GEMMA, DLS
II	BxPC-3	Liposome preparation, DLS, cryo-TEM, AF4, cell proliferation
II	Capan-1	Liposome preparation, DLS, human tumor xenograft, SPECT/CT imaging and biodistribution
V	Capan-1	Immunoliposome preparation, DLS, cell proliferation assays, cell-liposome interaction assays

Liposomal methods

Throughout the project, liposomal lipid composition was changed and adapted depending on project progression, study and planned use of liposomes. Phospholipids included in this thesis are listed in Table 2 and specific compositions may be found in the Appendix (study I, II, III and V).

Table 2.
Phospholipids used in liposome preparations

Full name	Abbreviations
1,2-dipalmitoyl-sn-glycero-3-phosphocholine	DPPC
Hydrogenated Soy L-α-phosphatidylcholine	HSPC
Cholesterol	Chol
1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]	DSPE-mPEG2000
1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000]	DSPE-PEG2000-Mal

Liposome preparation

Liposomes were prepared using the lipid film hydration method [89], Figure 10. Briefly, lipids are dissolved in an organic solvent mixture of cholorform and methanol. In order to form a lipid film, organic solvents are removed under reduced pressure at 60ºC for 1h. To completely remove any traces of organic solvents, lipid films were further dried overnight using a lyophilizer. Lipid films were then hydrated with either Hepes Buffered Saline (HBS, pH 7.4) or a gemcitabine solution under rotation at 65ºC for 1 hour and further vortexed until all lipid film had dissolved. The temperature should be set above the transition temperature (T_m) of the dominant lipid. The multilamellar vesicle suspension was then serially extruded through a stacked pair of polycarbonate filters with different pore sizes (400, 200, 100 and 50 nm) to obtain a unilamellar suspension of liposomes of the desired sizes.

After extrusion, non-encapsulated gemcitabine was removed from solution by a buffer exchange through size exclusion chromatography using a Sephadex G-25 resin column. Empty liposomes were also subject to a buffer exchange in order to ensure that all liposomal samples were prepared in the same way.

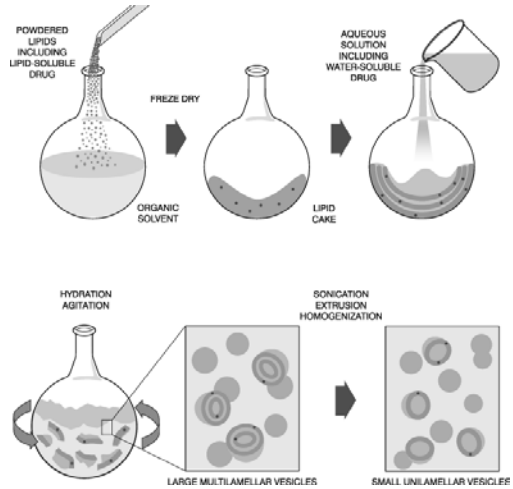

Figure 10. Liposome preparation
Depiction of the lipid film hydration technique for liposomal preparation. Briefly, lipids are dissolved in organic solvent which is subsequently evaporated to form a thin lipid film. The film is hydrated either with a saline buffer or drug solution before extruding mulilamellar vesicles to obtain unilamellar liposomes of the desired size. Printed with permission from Taylor and Francis Group LLC Books [90].

Immunoliposome preparation

For the preparation of immunoliposomes, the conventional antibody conjugation method was used [91]. In this method, antibodies are directly coupled to liposomes through conjugation to the distal end of functionalized PEG-chains. Antibodies were covalently linked to liposomes via a thioether linkage between antibody thiol groups and a maleimide-group conjugated to the distal end of the PEG-chains (DSPE-PEG_{2000}-Mal), Figure 11. Prior to antibody coupling to the liposomal surface, antibodies were thiolated using Traut's reagent in order to obtain IgG-SH.

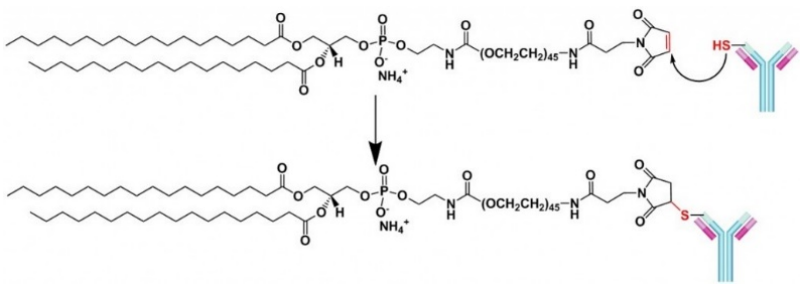

Figure 11. Conjugation of antibodies
Conjugation of thiolated antibodies to the functional group maleimide on the distal end of the PEG-chains.

Empty and drug-loaded liposomes were prepared as described in the previous section and, after removal of non-encapsulated drugs/buffer exchange, IgG-SH were coupled to liposomes for 1 h at 60ºC. After incubation, unbound antibodies were removed from the immunoliposomes by centrifugation using 300 kDa MWCO centrifugal concentrators.

Liposome radiolabeling

For biodistribution studies, liposomes were radiolabeled by a remote-loading method using ^{111}In-oxine complexes and liposomes filled with nitrilotriacetic acid (NTA).

The loading technique was adapted by Hwang et al. for liposome radiolabeling [92] from a method originally used to radiolabel cells and leukocytes [93]. Briefly, he mechanism of radiolabeling can be explained as follows: oxine is a lipophilic chelator that will form a complex of three oxines with one ^{111}In. Due to the high lipophilicity of the ^{111}In-oxine complex, it is able to diffuse across lipid bilayers. Inside the liposomes, as NTA is a stronger chelator than oxine, a transchelation of ^{111}In with NTA will occur. After transchelation, free oxine will exit the liposome, while ^{111}In will remain bound to NTA on the inside of the liposomes [93-95], Figure 12.

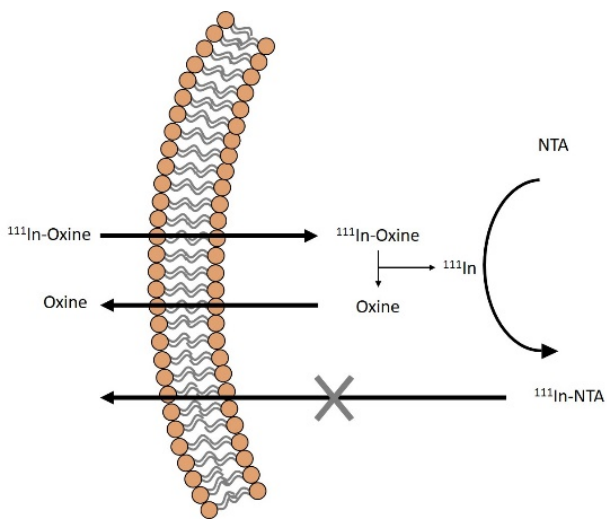

Figure 12. Remote loading of ^{111}In
^{111}In-oxine complexes cross the lipid membrane. Inside the liposomes, ^{111}In is sequestered by NTA and retained in the liposomes and free oxine is able to leave the liposomes.

Liposome Characterization

Liposome size, integrity and morphological characterization was made using several different techniques. The major characterization techniques used were Dynamic Light Scattering (DLS), cryo Transmission Electron Microscopy (cryo-TEM), Assymetric Flow Field Flow Fractionation (AF4) and nano Electrospray Gas-phase Electrophoretic Mobility Molecular Analysis (nES GEMMA).

DLS

DLS is the preferred technique for liposomal characterization due to its fast and reliable reading with little sample preparation required [96]. DLS is based on the particles light scattering intensity and yields a reading of the mean hydrodynamic radius of the sample depending on the particles Brownian motions.

For DLS readings, mean liposomal sizes were analyzed using a Zetasizer Nano SZ (Malvern Instruments, Spring Lane South, Worcestershire, UK). Samples were prepared in quartz cuvettes and diluted with either HBS or dH_2O. Samples were read immediately after preparation and mean sizes were presented as Z-averages from cumulant fit analyses.

Cryo-TEM

Cryo-TEM is widely used for size determination and for surface and morphological characterization of liposomal samples.

Briefly, liposomal samples were prepared in a controlled environment vitrification system (CEVS). Samples were prepared as thin liquid films, <300 nm thick, on a copper grid covered with a lacey carbon film and plunged in liquid ethane at -180ºC. Samples were imaged with a Philips CM120 bioTWIN Cryo electron microscope [97].

nES GEMMA

nES GEMMA is a relatively new technique for characterization of liposomes which separates liposomes in single charged droplets prior to reading, Figure 13. The droplets are obtained from an electrospray process followed by drying of the droplets and charging in a bipolar atmosphere. Separation of the individual droplets occurs in a high laminar flow of compressed air with an intersecting, tunable, electric field. By variating the field strength, only particles of a given electrophoretic

mobility diameter, i.e. size, are able to pass the differential mobility analyzer and thereafter, individually counted [97, 98].

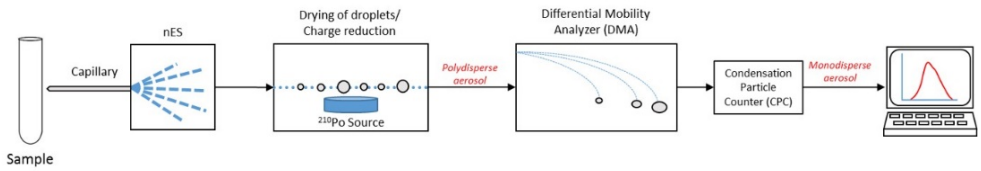

Figure 13. nES GEMMA
Illustration of the setup of a nES GEMMA

AF4

Asymmetric Flow Field Flow Fractionation, or AF4, is a fractionation technique that in combination with multi-angle laser light scattering (MALLS) may be used for characterization of nanoparticles, Figure 14. Briefly, in an AF4, samples are injected into a channel with a porous bottom membrane. The channel has a laminar flow, as well as a cross flow (or separation field). Sample separation is obtained by the difference in particle mobility in the flow field. The smaller the particles, the more extended into the channel the particles will be and the earlier they will elute [99]. When fractionation is followed by analysis with a MALS device, the AF4 allows for a more detailed characterization of heterogeneous samples compared to DLS or similar light scattering techniques.

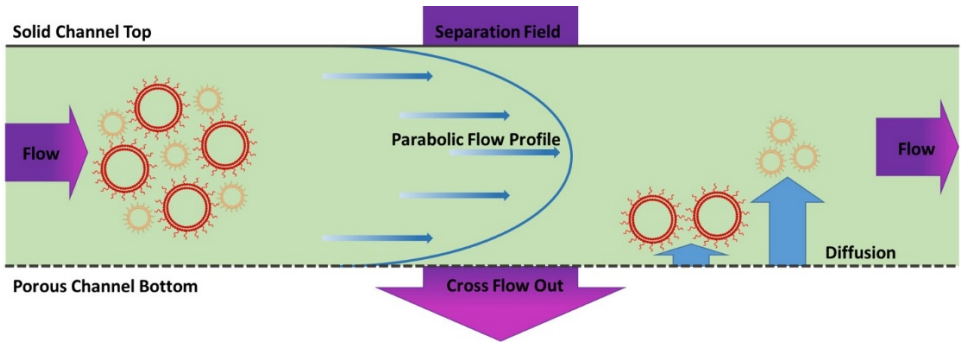

Figure 14. Schematic representation of the AF4 principle
Smaller particles will have a larger diffusion coefficient. The smaller the particle the further away from the channel they will flow and hence, they will elute first.

In vitro methods

For the studies included in this thesis, *in vitro*-based methods have been fundamental in the evaluation and optimization of liposomal treatments. The most relevant of these techniques and methods will be covered in this section.

Pancreatic Cancer cell lines

The human pancreatic cancer cell line BxPC-3 was purchased from ATCC-LGC Standards (Manassas, VA, USA) while the Capan-1 cell line was kindly provided by Professor Surinder Batra (University of Nebraska Medical Center, Omaha, Nebraska, USA). The cells were maintained in their respective cell medium supplemented with fetal bovine serum (FBS), Table 3, and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Cells were kept in a humidified 5% CO_2 atmosphere at 37°C. Culturing cell media was routinely changed and cells were passaged once a week.

Table 3.
Overview of used cell lines and their respective cell medium

Cell Line	Cell Medium
BxPC-3	DMEM + 10% FBS
Capan-1	IMDM + 20% FBS

Cell proliferation assays

For cell proliferation assays, cells were seeded in 96-well plates in standard culturing medium for 24h at 37°C to allow cell adhesion. After the initial 24h, cell culturing media was removed and replaced with medium containing the desired treatment. Cell lines, treatment and incubation times were set accordingly for each study.

Two different proliferation assays have been used in the course of this project, the MTT and WST-1 assays. Both MTT and WST-1 belong to a variety of colorimetric assays for assessing cellular metabolic activity based on reduction of a tetrazolium salt. Whereas the MTT assay produces an insoluble formazan salt that needs to be dissolved prior to spectrophotometric reading of the samples, WST-1 produces a soluble formazan salt that does not need further solubilization in order to be read [100]. The formation of the formazan is a reflection of the cell metabolic activity and hence, the number of viable cells.

Liposome-cell interaction assays

For liposome-cell interaction assays, two different types of assays were performed: cellular uptake (development stages and Study V), and cellular binding assay (Study V). For both assays, liposomes and immunoliposomes were prepared using 0.1 mol% of a fluorescently labeled phospholipid (Texas Red-DHPE).

Cellular uptake assay was performed with fluorescence microscopy (Nikon Eclipse 80i) to visualize and qualitatively compare differences in cellular uptake between different liposomal concentrations, as well as between regular liposomes and immunoliposomes. The cellular binding assay was performed as a quantitative complement to the cellular uptake assays as cellular fluorescence quantification was possible through fluorescence readings (PherasStar FS plate reader).

In vivo methods

Human Pancreatic Cancer Xenograft

Immunodeficient (Charles River, Sulzfeld, Germany) mice were housed in standardized pathogen-free conditions in individually ventilated cages. Mice were provided free and unlimited access to food, water and nesting material. All procedures were approved by the regional ethics committee and were performed in a dedicated animal operating facility in accordance with the guidelines of the Swedish Government and Lund University, Sweden.

For the biodistribution study performed in study III of this thesis, we employed a human pancreatic cancer xenograft previously developed in our group [101]. In order to establish the human tumor xenografts, immunodeficient mice were briefly anesthetized and injected in the right flank with $1x10^6$ Capan-1 cells. After cell inoculation, tumor development and body weight were monitored weekly and the overall well-being of the mice controlled daily.

SPECT/CT imaging and biodistribution of human tumor xenografts

Liposomal biodistribution in the human tumor xenograft models was studied in two different stages. In the first stage, mice were randomly divided into groups and intravenously injected with ^{111}In-labeled liposomes. In order to study live biodistribution, upon injection of liposomes, animals were sedated and imaged using SPECT/CT (NanoSPECT/CT, Mediso Medical Imaging, Budapest, Hungary) at different time points throughout the first 24h after intravenous injection. In the

second stage, after the last SPECT/CT imaging session at 24h after injection, animals were sacrificed by CO_2 inhalation. A blood sample was drawn and tissue of interest dissected, weighed and counted in a gamma-counter. After correction for radioactive decay, tissue and tumor uptake was presented as injected dose per gram tissue (% ID/g).

Results and Discussion

The overall aim of this thesis was to develop a targeted drug delivery system for improved drug delivery and treatment of pancreatic ductal adenocarcinoma. The work done in this thesis can be divided into two larger parts: development and characterization of a liposomal drug delivery system and development of a targeted treatment. The following section will be divided into these parts.

Development and characterization of a liposomal drug delivery system

(Study I, II, III and development work)

The first part of this thesis was dedicated to the development and characterization of a liposomal drug delivery system. Although three studies can be included in this part, a large part of the work was done previous to the conclusion of these studies.

Development work

Two main lipid compositions were used during the development of our liposomal drug delivery system. Lipid compositions were based on previous work done on liposomal gemcitabine [102-105], as well as composition from the first FDA approved liposomal drug, Doxil [77]. From liposomal Doxorubicin, we decided to use a HSPC:Chol:DSPE-mPEG$_{2000}$ lipid composition and based on previous work with liposomal gemcitabine we also decided to use a DPPC:Chol:DSPE-mPEG$_{2000}$ lipid composition. The HSPC-composition presents the advantage of being tested and approved for clinical use, whereas the DPPC-composition, although similar, presents ease of handling due to the lower transition temperature of DPPC compared to HSPC, T_m = 42°C vs T_m = 62°C [106]. DPPC and HSPC are both saturated lipids.

Furthermore, the use of saturated lipids in a lipid membrane is also preferable to use of unsaturated lipids. Membranes mainly composed of unsaturated lipids are fluid, highly permeable and susceptible to oxidation, whereas saturated lipids will offer a more stable lipid membrane [107]. Also, the mixture of a saturated and unsaturated

lipid membrane would create voids in the lipid packing making the membrane more permeable to water. The inclusion of cholesterol is also important for lipid membrane stability. Simply explained, cholesterol makes membranes stiffer but retains the necessary fluidity for membrane functions [108]. For lipid membranes above the transition temperature, cholesterol reduces fluidity to stabilize the membrane, whereas for membranes below the transition temperature it intercalates between lipid molecules to prevent them from locking up [107, 108]. This chemically complex effect of cholesterol on lipid bilayers is known as the "liquid ordered phase" which is an intermediate between the solid and liquid disordered phases of lipids [109].

For liposomal production, the thin lipid film hydration method was chosen with passive drug loading. Contrary to what has been presented in early work with liposomal gemcitabine [103, 110-112], we were unable to replicate an active gemcitabine loading process into liposomes. The proposed method was based on remote loading through a pH gradient, the same method used for loading Doxorubicin into liposomes [77]. This method is, however, difficult to apply for gemcitabine since gemcitabine has a low pKa = 3.6 and also because most of its molecules exist in a non-ionized state [113]. Hence, a passive loading method was used where the lipid film was hydrated with a gemcitabine solution. In order to achieve stable drug loading, special care was taken to osmotically balance intraliposomal pressure (300 mOsm) to the external saline buffers at which liposomes were suspended. As observed in other studies [113, 114], by maintaining isotonicity after removal of non-encapsulated drug to saline buffers, a burst release of drug would be avoided and minimal leakage ensured.

Once a proper method for production of liposomal gemcitabine through passive drug loading was established, the mean encapsulation efficiency (EE) achieved was EE = 10.98 ± 3.6 % and mean drug loading (DL) was DL = 41.11 ± 13.5 % (n = 7). A stable drug loading was also achieved, Figure 15, with a mean drug leakage of 4.1 ± 1 % of encapsulated drug over a two-week period.

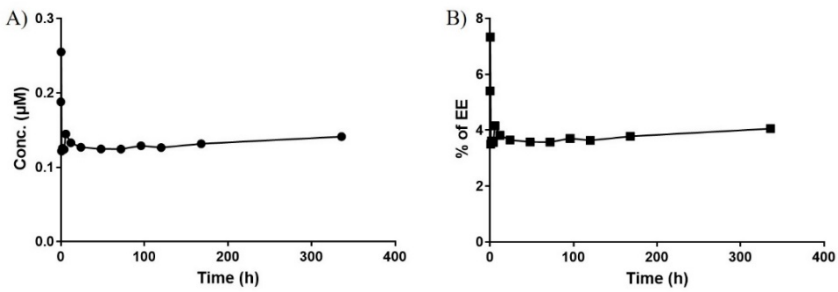

Figure 15. Liposomal drug leakage. Drug leakage of liposomes during a two-week period. Panel A) shows the total concentration of leaked drug and B) the percentual leakage compared to encapsulation efficiency of the liposome.

Liposome size and surface characterization

(Study I and Study II)

When using liposomes for drug delivery purposes, a thorough characterization of liposomes concerning liposomal size, size distribution, number of vesicles per sample volume and liposome integrity is of great importance. All of these factors have an effect on liposomal drug loading, biodistribution, passive targeting and accumulation of liposomes in the tumors through the EPR effect and in general, a liposomal drug delivery system's pharmacodynamic properties.

In Studies I and II, the overall goal was to establish whether the nES GEMMA technique would be suitable for routine liposomal characterization (Study I), in regards to liposomal size and size distribution, and if nES GEMMA would be best suited as a stand-alone or complementary technique for this purpose (Study II).

nES GEMMA is a technique that separates single-charged molecules (nanoparticles in our case) in a gas-phase according to their size in a laminar flow by means of a perpendicular, tunable electric field (see Figure 13 in the Methodology section) [98]. By variation of the field strength, only particles of a certain EM diameter are able to pass the differential mobility analyzer and subsequently individually counted [115, 116]. nES GEMMA is able to perform either a number- or mass-concentration based analysis of the measured sample.

In Study I, we evaluated the suitability of nES GEMMA for routine characterization of liposomes by studying the effect of setup parameters, liposome integrity and accumulation after passing through the instrument, as well as measurement repeatability within the same sample and between different liposomal batches. For setup parameters, we found that the use of particle free, dry compressed air was beneficial for liposomal analysis, as this reduced the amount of solvent molecules on the liposomal surface which in turn lead to more distinct peaks. Liposomal aggregates were discarded, as a dilution series of the same sample yielded highly similar size peaks upon normalization of data, Figure 16. Furthermore, membrane integrity of liposomal samples that passed the equipment was studied through AFM imaging. Contrary to what was observed in earlier studies applying electrospray for liposome characterization [117, 118], AFM images showed intact membrane integrity. This suggests that peaks observed at small sizes belong either to small liposomes or micelle formation instead of liposomal debris or lipid agglomerates.

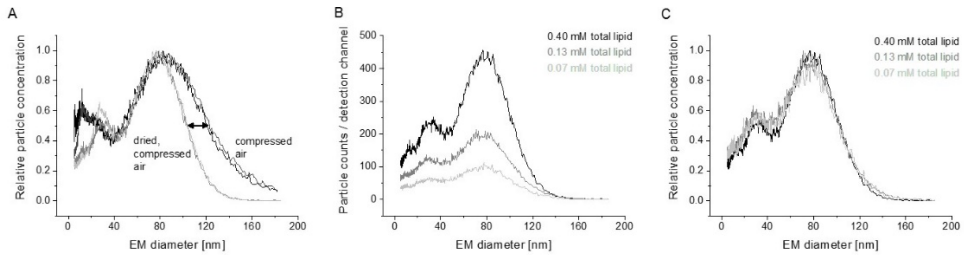

Figure 16. nES GEMMA measurement of liposomes
Measurement of liposomes using nES GEMMA showed that the use of compressed dry air yielded sharper peaks (A) and that unspecific liposome or lipid aggregates could be discarded as a dilution series of a sample yielded highly comparable results, (B) and (C) [98].

In Study II, continuing the work from Study I, we set out to evaluate the use of nES GEMMA as either a stand-alone or complementary technique for liposomal characterization. In order to do this, nES GEMMA readings from liposomal samples were compared to those of standard nanoparticle characterization techniques, such as DLS, AF4 coupled to MALLS, and cryo-TEM imaging.

Using nES GEMMA number-concentration based analysis, significant variations in the presence of low EM diameter sample components were detected between identically prepared liposomal samples. However, when using the mass-concentration based analysis this information was lost while the main vesicle peak also shifted to a higher EM diameter, Figure 17.

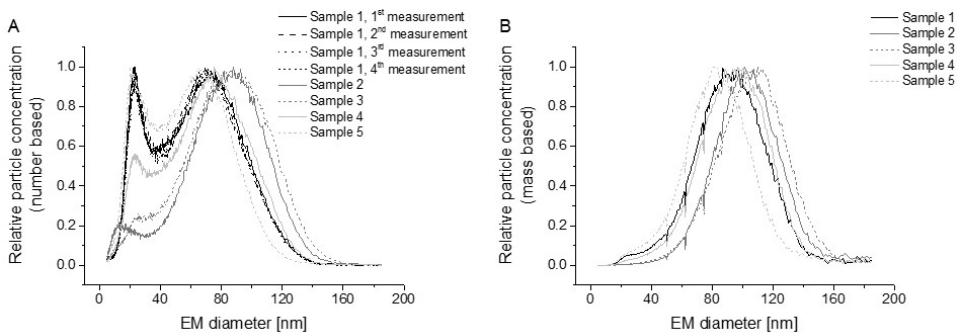

Figure 17. nES GEMMA measurement of liposomes
In (A), using a number-concentration based analysis the presence of small EM diameter sample components is clear. However, in (B) using a mass-concentration based analysis on the same samples, information on low EM diameter sample components is lost while main vesicle peak is shifted to higher EM diameters.

The differences concerning low EM diameter sample components, seen in nES GEMMA with number-concentration analysis, could not be detected by the use of DLS alone. Furthermore, even though the use of size fractionation with AF4 prior to light scattering analysis improved detection and indicated heterogeneity of

samples, the multimodal size distribution could not be detected. However, the presence of small diameter liposomes could be confirmed by cryo-TEM imaging, Figure 18A and B. By statistical analysis of cryo-TEM images, the size distributions seen using nES GEMMA could be confirmed for each sample, Figure 18C and D.

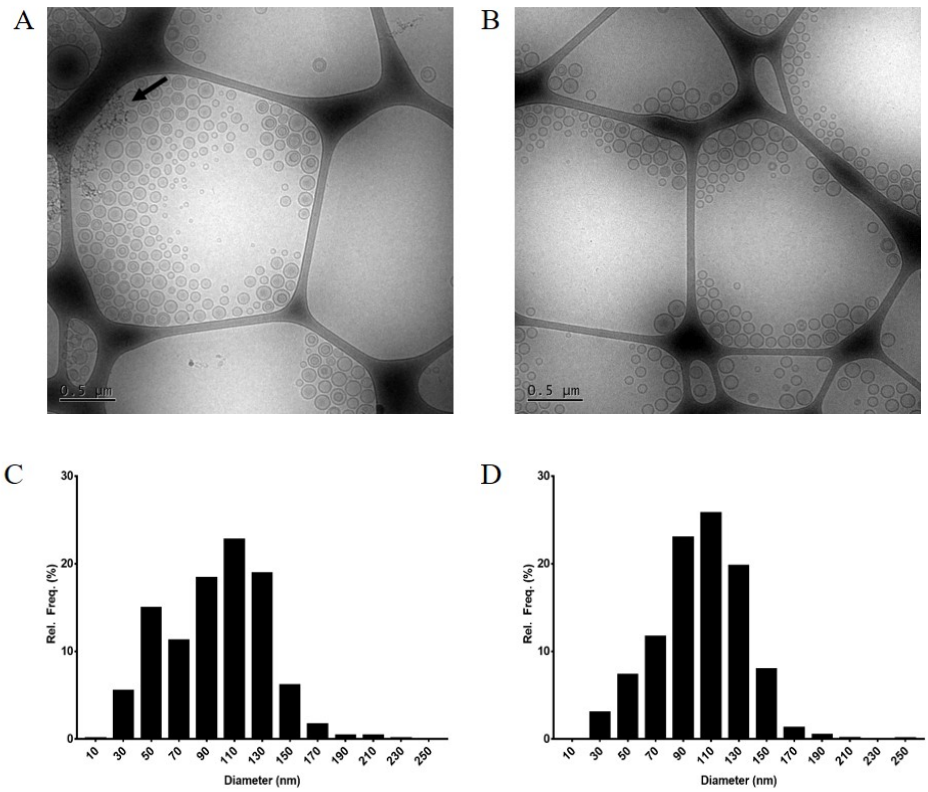

Figure 18. cryo-TEM images of liposomes
Cryo-TEM images of liposomal samples confirm a bimodal biodistribution (C) for sample imaged in (A) and a more homogeneous, Gaussian-type, size distribution (D) for sample imaged in (B).

nES GEMMA number-concentration based analysis was able to detect a multimodal size distribution in liposomal samples not detectable by nES GEMMA mass-concentration based analysis, DLS or AF4. For number-based data evaluation, each nanoparticle is counted regardless of size or mass whereas in the mass-based data evaluation, even a small number of larger nanoparticles can bias results, masking the presence of smaller particles [98]. Light scattering techniques, such as DLS, are fast and reliant for homogenous samples. However, for more heterogeneous samples, the mean particle diameter will be strongly biased toward the larger particles in the sample [119, 120] which explains why DLS was unable to detect the bimodal size distributions seen in liposome samples. The issue of particle masking

may partially be resolved by particle size fractionation prior to analysis with light scattering, as done with AF4 coupled to MALLS, but due to detection limitations for smaller particles in MALLS, only indications of a heterogeneous sample could be detected instead of the actual size distribution. Size distributions seen using the number-concentration based analysis were only confirmed by the extensive process of analyzing cryo-TEM images.

Furthermore, a major size difference was seen between nES GEMMA readings, using both analysis methods, compared to DLS and AF4. This difference is explained by the fact that techniques that are based on particle light scattering in a liquid phase yield readings of a particles hydrodynamic diameter [96]. nES GEMMA, on the other hand, yields a reading of surface-dry particles through their EM diameter which allows for single-particle, number- and mass-concentration based sample analysis. Differences between hydrodynamic and surface-dry particle diameters can be significant as has been shown for the characterization of silica or gelatin based nanoparticles [121-123].

In conclusion, throughout Study I and II we were able to establish the use of nES GEMMA as a suitable technique for characterization of liposomes regarding size and size distribution. nES GEMMA exhibited good measurement repeatability and did not affect liposome integrity. Using the number-concentration based analysis nES GEMMA provided valuable information on size distributions not obtainable by use of only light scattering techniques. However, considering the notable difference between dry particle- and hydrodynamic diameters detected, the need of volatile solutions for sample preparation and non-automaticity of the technique, to date, it would still have to be considered as a valuable but complementary technique for liposome characterization.

Size-dependent biodistribution of liposomes

(Study III)

In the final liposome characterization study, Study III, we proceeded to evaluate the size-dependent biodistribution of liposomes to find the optimal size for our purposes.

In order to study the effect of liposome size on biodistribution and tumor uptake, we employed liposomes of varying sizes (100, 130 and 200 nm) in a pancreatic cancer mouse model. By radiolabeling PEGylated liposomes using ^{111}Indium, biodistribution and tumor uptake could be tracked live, using SPECT/CT imaging, and postmortem, by scintillation analysis of radioactivity in mouse organs of interest.

For SPECT/CT imaging in tumor bearing mice, a continuous biodistribution of all liposomal sizes could be seen. Liposomes first accumulated in the urinary and circulatory systems and thereafter in the tumor, kidneys, spleen and lungs. For SPECT/CT imaging, biodistribution patterns in tumor bearing mice were similar for all liposome sizes and visually, accumulation in tumor was inversely related to liposome size whereas accumulation of activity in the spleen, lungs and kidneys was directly related to liposomal size.

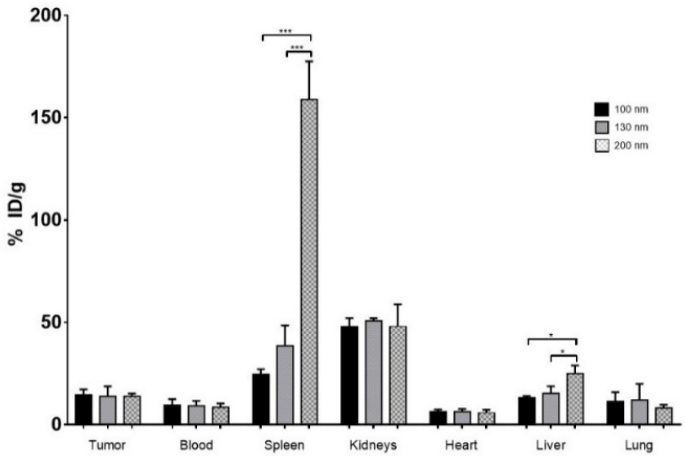

Figure 19. Biodistribution of ^{111}In-labeled liposomes 24h post IV
Biodistribution of ^{111}In-labeled liposomes of varying size in tumor bearing mice 24h post IV. Tissue uptake is presented as % ID/g (mean ± SD, n = 3). * = P < 0.05, ** = P < 0.01 and *** = P < 0.001.

Following final SPECT/CT scan, mice were sacrificed and radioactivity in vital organs was measured. For all liposomal sizes, a high uptake was observed in the spleen, kidneys and liver. However, the 200 nm liposomes had a significantly higher accumulation in spleen and liver compared to both 130 and 100 nm liposomes. No significant differences were seen in accumulation of activity in the kidneys, heart, lungs or blood clearance. Finally, a high tumor uptake of activity was registered for all liposome sizes but no significant difference could be found for 100, 130 and 200 nm liposomes (14.7 ± 2.1; 13.9 ± 3.5 and 13.95 ± 1.02 % ID/g, respectively).

Tumor accumulation and retention of liposomes is regulated by the EPR effect. The EPR arises from leaky tumor vasculature allowing extravasation of nanoparticles below certain sizes into the nearby tumor tissue [84]. Hence, the size of liposomes aimed for drug delivery plays a major role in determining several of its biological aspects, such as circulation half-life, clearance, macrophage uptake and extravasation into tumor tissue [124]. In this study we wanted to investigate the effect of liposome size on biodistribution and tumor uptake. *In vivo* studies have shown that liposomes with diameters of 70-200 nm have the highest tumor

accumulation and longest half-lives [76, 125, 126]. Furthermore, liposomes below and above these ranges have a tendency to accumulate in the liver and spleen [126], and liposomes larger than 200 nm are generally cleared faster than smaller liposomes [127]. Considering the previously mentioned as well as liposome sizes employed in previous studies with liposomal gemcitabine [111, 112, 128], we decided to employ liposomes within the narrow size range of 100-200 nm in diameter. We expected to see a higher tumor accumulation of smaller liposomes and less accumulation for the larger, 200 nm, liposomes. However, this was not the case and we were not able to detect a significant difference in tumor accumulation between the three different liposome sizes. We did, however, achieve a mean high tumor accumulation (14.2% ID/g) for all liposome sizes compared to what has been reported in previous studies [129].

In conclusion, tumor accumulation of ^{111}In-labeled liposomes did not differ significantly within the narrow range of 100-200 nm in diameter. However, the significantly increased accumulation of larger liposomes in spleen and liver prompts the use of smaller liposomes for drug delivery as a potential way of improving drug delivery and reducing unwanted side effects.

Targeted Treatments

(Study IV-V)

A biomarker for targeted treatment should ideally be highly expressed in the diseased tissue but limited or not expressed in healthy tissue. According to this definition, MUC4 seems like an ideal target as it is not expressed in the healthy pancreas but aberrantly expressed in pancreatic cancer. However, MUC4 expression has also been related to oncogenesis, as well as inducing resistance towards gemcitabine and other nucleoside analogues [57, 60, 63, 65, 130-133]. In light of these observations, we decided to first investigate the clinical impact of MUC4 expression on pancreatic cancer patients with resectable tumors (Study IV) before proceeding with development of a targeted treatment (Study V). The results of the clinical study would be helpful in the further development of the targeted treatment, considering what type of targeting and treatment options that could be considered in the future.

Clinical impact of MUC4 in pancreatic cancer

(Study IV)

In this study, 78 patients were included, the patient group comprised of 41 male and 37 female patients with ages ranging between 43 and 78 years. Immunohistochemical staining and analyses showed that no MUC4 expression was detected for the 10 normal pancreatic tissues that were stained. On the other hand, MUC4 positivity was detected in 93.6% (73/78) of PDAC specimens. Out of these, 42 (54%) specimens presented a low MUC4 expression (H-score ≤ 100) and 36 (46%) presented high MUC4 expression (H-score > 100). Representative images of no, low and high MUC4 expression are shown in Figure 20 (A), (B) and (C), respectively.

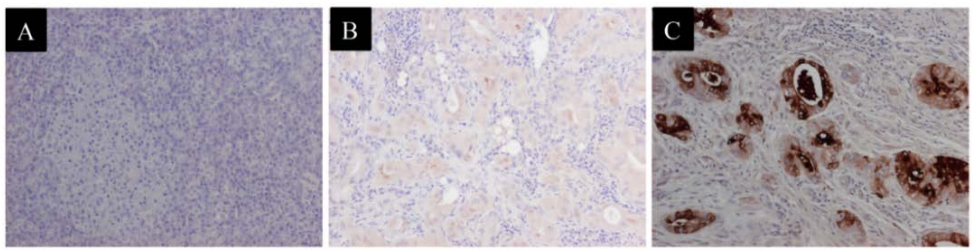

Figure 20. MUC4 expression in pancreatic tissue
(A) MUC4-negative staining from normal pancreas tissue, (B) low MUC4 expression in pancreatic ductal adenocarcinoma (H-score ≤ 100) and (C) high MUC4 expression in pancreatic ductal adenocarcinoma (H-score > 100).

Furthermore, to study the predictive impact on patient survival related to MUC4 expression, a comparison between treatment effect was made for patients receiving adjuvant gemcitabine compared to those receiving no or other adjuvant therapy not including gemcitabine. Patients were divided into low and high MUC4 expressing groups and initial Kaplan-Meier survival analysis showed a survival benefit for patients with low MUC4 expression (p=0.046). Furthermore, after adjusting for covariates, multivariable cox proportional hazard analysis showed a significant survival benefit for patients with low MUC4 expression (p=0.021) compared to those expressing high MUC4 (p=0.882) when receiving adjuvant gemcitabine, as seen by their hazard ratios, 0.37 and 1.07, respectively, Figure 21.

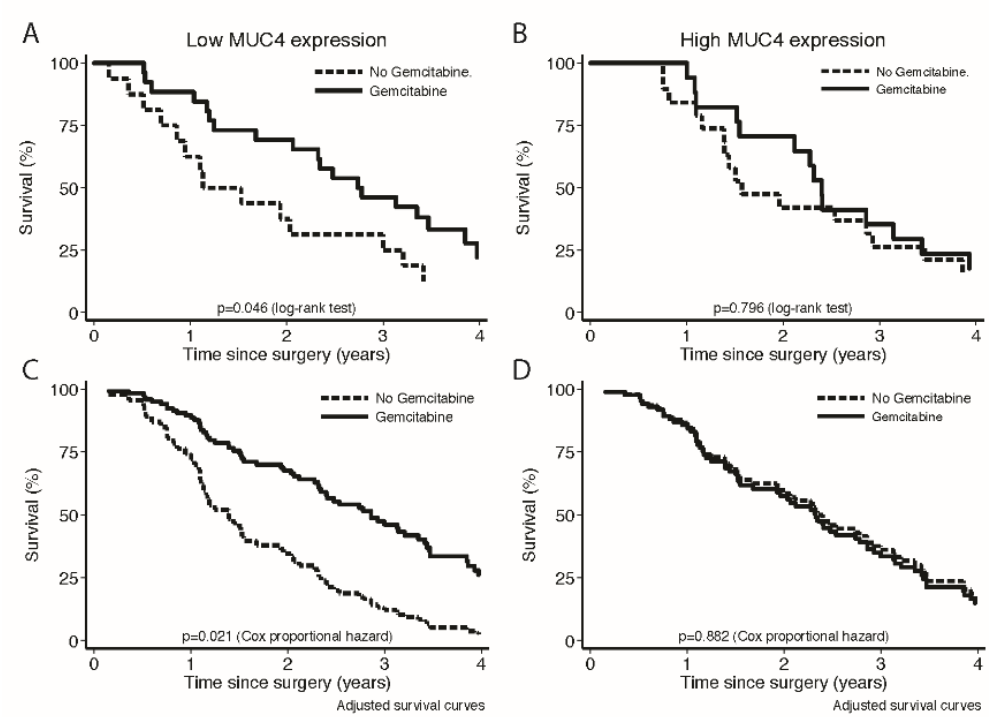

Figure 21. Kaplan-Meier Survival curves
Kaplan-Meier survival curves showing the predictive impact of MUC4 expression for patients with resectable PDAC receiving adjuvant gemcitabine (solid line) compared to patients receiving no or other adjuvant treatment (dashed line) in panels A and B, and adjusted for 11 covariates in panels C and D. In panels A and C, patients receiving adjuvant gemcitabine with low MUC4 expression have a significant survival benefit (p=0.021), compared to patients receiving no adjuvant gemcitabine. However, patients with high MUC4 expression have no added survival advantage of receiving adjuvant gemcitabine, compared to no or other adjuvant treatment, panel B and D [68].

As previously mentioned, in an *in vitro* setting, MUC4 expression was not only found to increase growth, promote survival and invasion of pancreatic cancer cells [134] but more recently, it was also found that MUC4 expression induces gemcitabine resistance in pancreatic cancer cells [63, 64, 67]. In study IV we found that low MUC4 expression is an indicator for survival benefit for patients with resectable pancreatic cancer receiving adjuvant gemcitabine compared to patients with a high MUC4 expression. Study IV serves as complement to previous *in vitro* findings on MUC4-induced gemcitabine resistance. It also strengthens the case to use MUC4, as a predictive marker for response to gemcitabine-based treatments in pancreatic cancer, but also as an interesting target for targeted treatment of pancreatic cancer.

Development and characterization of a targeted liposomal drug

(Study V)

For the final part of the thesis, after establishing the production of a stable liposomal drug delivery system and finding a suitable target in MUC4, we proceeded to develop a targeted treatment.

Immunoliposomes are the most used liposomes for targeting membrane-bound proteins. They are created by conjugating monoclonal antibodies to the liposomal surface. Immunoliposomes may be prepared by several conjugation chemistries and by two antibody-coupling methods: classic and post-insertion method. In the conventional method, antibodies are directly conjugated to the distal end of functionalized PEG-chains on the liposomal surface, whereas in the post-insertion method, antibodies are conjugated to the distal end of PEG-chains in PEG-micelles. Micelles are then added to a liposomal solution where the micelles fuse to the liposome surface [91], Figure 22.

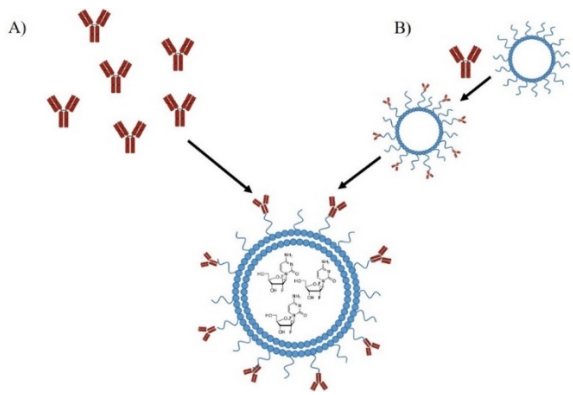

Figure 22. Antibody conjugation methods
In (A), in the conventional coupling method monoclonal antibodies are conjugated directly to the liposomal surface whereas in the post-insertion method (B), antibodies are first coupled to PEG-micelles adding antibody-micelles to liposomes.

In this study, both antibody coupling methods were used. However, as the post-insertion method yielded a significant loss of encapsulated drug upon fusion of micelles, we decided to use the conventional antibody coupling method for further immunoliposome production. The conventional coupling method had no impact on encapsulation efficiencies compared to the non-targeted liposomal drug (iGemLip vs. GemLip, respectively) and by maintaining isotonicity after removal of free drug and unconjugated antibodies, it exhibited a similar and stable drug loading to GemLip. Furthermore, when comparing sizes between GemLip and iGemLip, we saw that antibody conjugation had a negative impact on liposomal size. Upon

conjugation of antibodies, mean particle size increased by approximately 40 nm from 129 nm for GemLip to 168 nm for iGemLip. However, this increment in size should not affect *in vivo* characteristics of iGemLip, as it is still below the recommended maximum 200 nm for optimal tumor accumulation.

We then proceeded with *in vitro* characterization of immunoliposomes using the MUC4-positive pancreatic cancer cell line Capan-1. We first investigated cellular uptake and binding efficacy of iGemLip compared to GemLip. Incubating Capan-1 cells with fluorescently labeled liposomes, by fluorescence microscopy, we could visually establish that iGemlip had an increased cellular uptake compared to GemLip. Observations from fluorescence microscopy were confirmed by fluorescence readings of Capan-1 cells treated with fluorescently labeled liposomes as iGemLip had an overall, significantly, higher cellular binding to Capan-1 than GemLip, Figure 23.

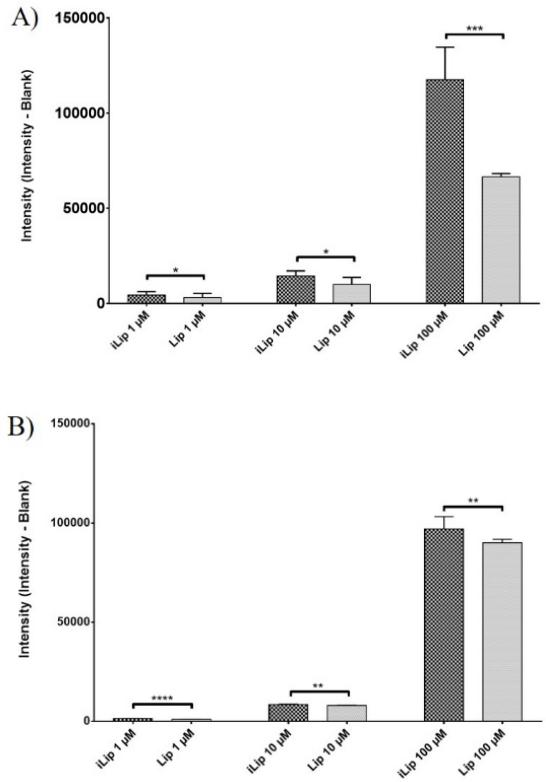

Figure 23. Binding affinity of iGemLip to Capan-1 cells than GemLip
Fluorescence intensity readings from Capan-1 cells incubated with fluorescently labeled liposomes show an enhanced binding affinity of iGemLip compared to GemLip after (A) 1h and (B) 4h incubations. Data is presented as mean ± SD. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$).

Finally, we proceeded to investigate the antiproliferative effect of iGemLip compared to that of free gemcitabine and GemLip in Capan-1 cells. Due to the significantly higher binding affinity of iGemLip compared to GemLip, we expected to see similar results reflected in the cell viability studies. However, for the lower treatment concentrations after both 24h and 48h, no difference in antiproliferative effect could be detected for the three different treatment types. It was only for the highest treatment concentration that we could see a small but significantly improved treatment effect of iGemLip compared to both free gemcitabine and GemLip, Figure 24.

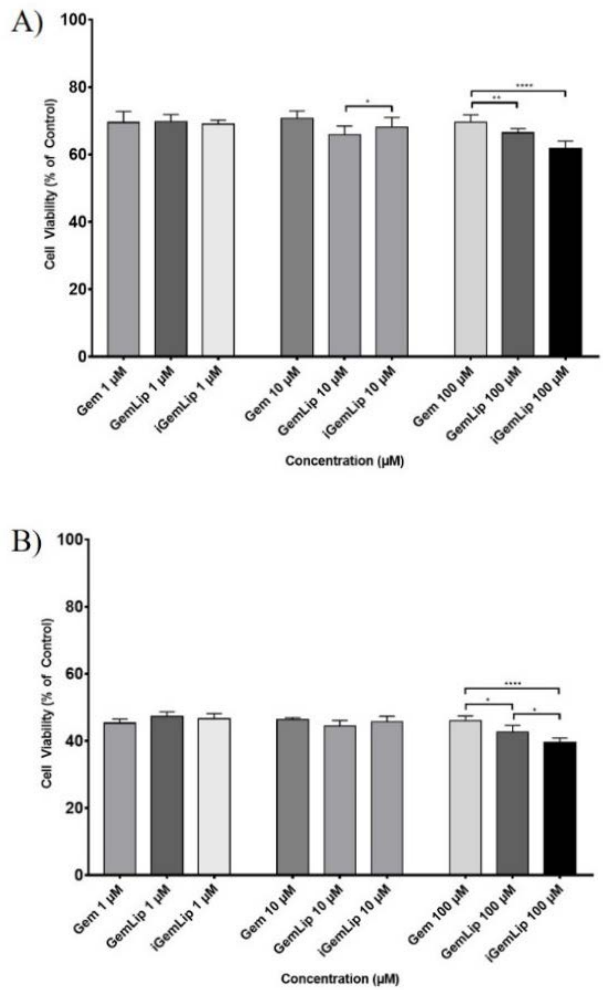

Figure 24. Cell viability assays
Evaluating cell viability in Capan-1 cells for free gemcitabine, GemLip and iGemLip for (A) 24h and (B) 48h. iGemLip has a significantly higher antiproliferative effect on Capan-1 cells after both 24h and 48h compared to Gem and GemLip. The data is presented as mean ± SD. (* $P \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$).

In Study V, we successfully produced a stable, MUC4-targeted gemcitabine-loaded immunoliposome. The immunoliposomes showed a significantly higher binding affinity to MUC4-positive pancreatic cancer cells, as well as a minor, but significantly improved treatment effect compared to both free gemcitabine and non-targeted liposomal gemcitabine.

Considering the increased antiproliferative effect of iGemLip, this could be used in an approach to target MUC4-positive gemcitabine-resistant cells. One of the underlying mechanisms of MUC4-induced gemcitabine resistance is through downregulation of necessary transporters for uptake of gemcitabine, hCNT1 [63]. A targeted liposomal approach would ensure that gemcitabine is specifically delivered to gemcitabine-resistant cells and taken up through liposomal fusion to the cell membrane, hence, circumventing gemcitabine's need for active cellular uptake.

Another approach could be to make use of the effective targeting against MUC4-positive cells presented by iGemLip. Studies have presented evidence that downregulation of MUC4 suppressed tumor cell growth and metastasis in pancreatic cancer cells [135], while further studies showed that downregulation of MUC4 sensitized gemcitabine-resistant cells to the treatment [66, 67]. Combining these two findings, a two-step treatment could be tested where MUC4 is first targeted with a MUC4-silencing agent before administering a gemcitabine-based treatment. By targeting and downregulating MUC4, oncogenesis could be suppressed and cells sensitized towards gemcitabine, thereby increasing the therapeutic effect of a gemcitabine-based treatment, while reducing the clinical impact of MUC4 in pancreatic cancer patients.

Conclusions and future perspectives

The overall aim of the thesis was to develop a targeted drug delivery system for improved drug delivery and treatment of pancreatic ductal adenocarcinoma. The work was divided into two major parts concerning the development and characterization of a liposomal drug delivery system and its further development into a targeted treatment.

After a lengthy and complicated development process we were able to develop a liposomal drug delivery system that exhibited high and stable gemcitabine loading with minimal drug leakage. Liposome size was approximately 130 nm, empty liposomes were non-toxic to cells at high lipid concentrations and the system presented no precipitation or aggregation of liposomes for up to one month at room temperature.

Continuing with the developmental work, in a small series of studies, we set out to investigate the usefulness of a relatively new technique, nES GEMMA, for its use in characterizing size and size distribution in a liposomal sample. These studies also served a parallel purpose as we were able to thoroughly characterize our drug loaded system, ensuring that the production process we had established would continuously produce liposomes of approx. 130 nm with the expected encapsulation efficiencies.

In the final characterization study, Study III, we investigated the effect that size could have on liposomal biodistribution in a human pancreatic cancer mouse model. Even though we found that there was no difference in tumor accumulation for the smaller liposomes, approx. 100 nm, the significantly higher accumulation of larger liposomes in vital organs supports the continued use of liposomes around 100 nm for drug delivery.

Once a stable liposomal drug delivery system was developed and comprehensively characterized, we proceeded with the development of a targeted treatment. The first step revolved around finding a suitable target. In MUC4 we found a potential target that is not expressed in the healthy pancreas, but *de novo* expressed in pancreatic cancer and increasingly expressed during disease progression. MUC4 expression has, however, also been associated with oncogenesis, invasion and proliferation in pancreatic cancer cells, as well as inducing gemcitabine resistance. For this reason, in Study IV, we decided to investigate the clinical impact of MUC4 in pancreatic cancer patients with resected tumors receiving adjuvant gemcitabine. We found that

for this patient group, patients with a low MUC4 expression were associated with a survival benefit compared to those with high MUC4 expression. These observations further strengthen in vitro findings of MUC4-induced gemcitabine resistance, indicating that MUC4 expression could also be used as a predictive biomarker for treatment response of pancreatic cancer patients receiving adjuvant gemcitabine.

In the final part of the thesis, we proceeded to develop a MUC4-targeted treatment using anti-MUC4 immunoliposomes, iGemLip. iGemLip exhibited successful targeting against MUC4-positive cells as they had significantly higher binding and uptake compared to their non-targeted counterpart. Moreover, iGemLip also showed an improved antiproliferative effect on MUC4-positive pancreatic cancer cells compared to both free and liposomal gemcitabine.

These initial results show great promise as the treatment could be used in different forms. In its current form, it could be targeted to MUC4-positive pancreatic cancer cells with low nucleoside transporter expression, ensuring the uptake of gemcitabine through fusion of immunoliposomes to the cell. Using the successful targeting against MUC4, MUC4-positive pancreatic cancer cells could first be targeted in an attempt to downregulate MUC4-expression before administering a gemcitabine-based treatment to the sensitized cells. Either way, MUC4 seems like an increasingly interesting target for treatment of pancreatic cancer and a MUC4-targeted treatment would possibly help to reduce the clinical impact of MUC4 in pancreatic cancer patients.

Nevertheless, before continuing the discussion on the optimal use of the treatment, further development work needs to be done, for example in optimizing antibody binding, but also to explore other targeting alternatives, such as the use of fragmented antibodies instead of whole IgG. Finally, the targeted treatment needs to be tested in an in vivo setting before any conclusions can be drawn on its translatability to a clinical setting.

Pancreatic Ductal Adenocarcinoma is a devastating disease with a dismal prognosis. There is a great, unmet need for the development of new treatments or improvement of the already existing in order to improve patient survival and quality of life throughout this affliction. In this thesis, we have combined the use of existing drugs with the development and application of a new way of administering the treatment. We have obtained promising preclinical results but much work needs still to be done in order to truly evaluate the potential of the targeted treatment developed throughout this work.

Acknowledgments

The work and completion of this thesis would not have been possible without the help and support of my colleagues, family and friends. Here, I would like to express my gratitude to all who have had an impact during these last years.

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