Thesis for the degree Master of Pharmacy

GEMCITABINE-CONTAINING LIPOSOMES

by

Hilde Gravem

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Supervisor:

Martin Brandl

Department of Pharmaceutics & Biopharmaceutics

Institute of Pharmacy

Faculty of Medicine

University of Tromsø

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Hilde Gravem

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1 ABSTRACT

Gemcitabine, an anticancer agent, is currently in clinical use for the treatment of several types of cancer. Unfortunately, gemcitabine is rapidly metabolised with a short plasma half-life and its cytostatic action is strongly exposure-time dependent. In order to achieve the required concentration over sufficient periods of time, repeated application of relatively high doses is required. This, in turn, leads to dose-limiting systemic toxicity. In order to improve both the efficiency and the toxicity profile of gemcitabine the use of liposomes appears promising. In literature, only a few attempts to entrap gemcitabine within liposomes are found, however none of these liposomal formulations has reached clinical practice. In this study, an ammonium sulphate gradient was tried for active loading of gemcitabine into liposomes.

Firstly, unsaturated egg phosphatidyl choline liposome dispersion was prepared with ammonium sulphate as hydration medium by the hand shaken method followed by filter extrusions with decreasing filter pore sizes down to 0.1 μm. Then, a transmembrane ammonium sulphate gradient was generated by removing extra-liposomal ammonium sulphate by size exclusion chromatography. Quantitative determination of the ammonium sulphate concentration, both outside and inside the liposomes, via electric conductivity measurement revealed that a gradient of external to internal ammonium sulphate of about 1:58 was achieved.

Secondly, the liposomes were loaded with gemcitabine by incubation at different conditions. Among the loading conditions tested, a total loading time of 24 hours including heating for 2 hours at 60 ˚C seemed advantageous in achieving efficient loading. A higher starting concentration of gemcitabine resulted in enhanced loading efficiency, calculated on a molar basis. Comparing these results to a vesicular phospholipid gel passive loading technique, the active loading technique resulted in a gemcitabine:lipid ratio of about 1:20 versus 1:140 for the vesicular phospholipid gel loaded liposomes. Unfortunately, the actively loaded liposomes revealed poor storage stability with 80 % leakage after 24 hours. Further studies are needed in order to optimise loading and stability of the liposomes.

2 ABBREVIATIONS

AUC	Area under the curve
CI	Contraindication
conc	Concentration
dFdC	difluorodeoxycytidine, gemcitabine
dFdCDP	difluorodeoxycytidine diphosphate
dFdCTP	difluorodeoxycytidine triphosphate
dFdU	difluorodeoxyuridine
E-80	Unsaturated egg phosphatidyl choline
EE	Encapsulation efficiency
HPLC	High performance liquid chromatography
LUVs	Large unilamellar vesicles
MLVs	Multi lamellar vesicles
MW	Molecular weight
NSCLC	Non-small cell lung cancer
PC	Phosphatidyl choline
PCS	Photon correlation spectroscopy
RP-HPLC	Reversed-phase high performance liquid chromatography
SEC	Size exclusion chromatography
SD	Standard deviation
SUVs	Small unilamellar vesicles
t½	Elimination half life
UV	Ultraviolet
VPG	Vesicular phospholipid gel
v/v	Volume ratio
w/w	Weight ratio
χ^2	Chi square

3 INTRODUCTION

According to the Cancer registry of Norway, 24 434 new cases of cancer were detected in 2004. Among males prostate cancer was most frequent with 3818 new cases, whereas breast cancer was most frequent among females with 2754 new detected cases. With more than 10 % of all new cases for males, the lungs are one of the most common cancer sites and since the seventies, lung cancer has been increasing among females as well. Bladder cancer is a common type of cancer among males. (1) The current therapy for cancer consists mainly of three approaches; radiotherapy, surgery and chemotherapy with antineoplastic drugs.

The ideal prototype of an antineoplastic drug should display anti tumour activity by targeting and damaging cancer cells without causing adverse effects or toxicity to healthy cells. Until now there are no such drugs. A drive has thus emerged for developing new antineoplastics or improving the efficacy and the toxicity profile of already existing cytotoxic agents. Formulations based on liposome technology are one strategy in approaching this requirement.

3.1 Liposomes

Liposomes are spherical vesicles composed of lipid bilayers arranged around a central aqueous core. They can be composed of natural constituents such as phospholipids and may mimic naturally occurring cell membranes. Liposomes have the ability to incorporate lipophilic and amphiphilic drugs within their phospholipid membrane or they can encapsulate hydrophilic compounds within the aqueous core as shown in Figure 1. Liposome formulations can therefore increase safety and efficiency in reaching the site of action. (2-4)

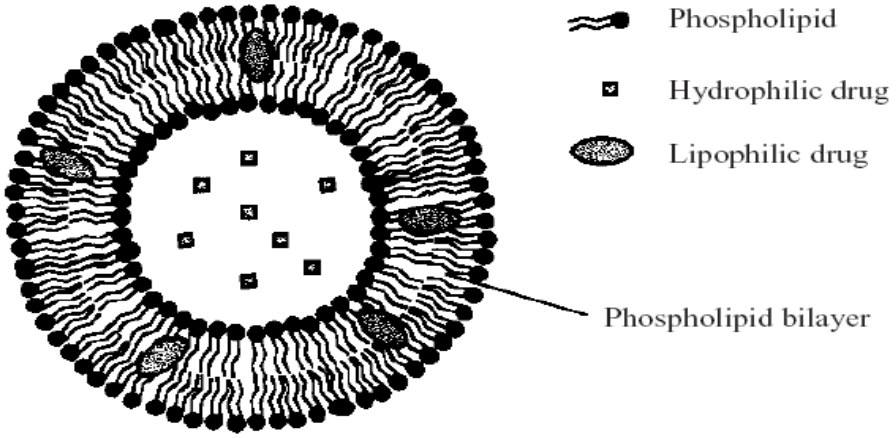


Figure 1: A schematic representation of incorporation and encapsulation of drugs into a liposome (Figure reprinted with permission from Elsevier. (3))

3.1.1 Liposome composition

A phospholipid exists of a hydrophilic head group and lipophilic tails. The polar head can be charged or uncharged and the lipophilic tails are composed of fatty acids chains (Figure 2).

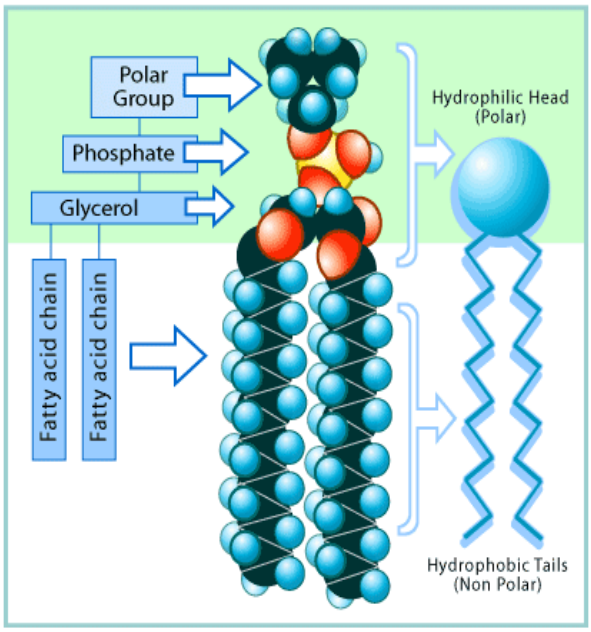


Figure 2: A schematic representation of a phospholipid (Figure taken with permission from: http://www.bioteach.ubc.ca/Bio-industry/Inex/. Artist: Jane Wang)

Phosphatidyl choline (PC), the primary lipid used in liposomes, belongs to the group of phosphodiglycerides, which are naturally occurring phospholipids. The amphiphilic PC is composed of the phosphocholine, a hydrophilic head group, linked to two lipophilic acyl hydrocarbon chains via glycerol (Figure 3).

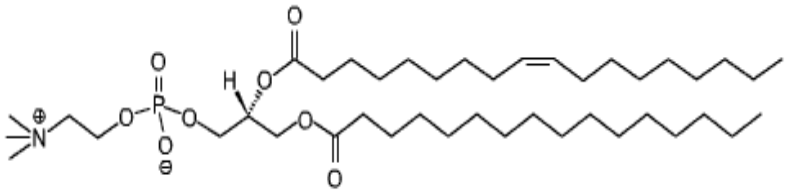


Figure 3: Chemical structure of phosphatidyl choline

Since PC's are not water soluble they self-assemble upon contact with aqueous media orienting their hydrophobic chains away from the aqueous media. They orient in a membrane like structure reducing the interaction between the hydrophobic fatty acids and the aqueous medium (Figure 4). Liposomes form spontaneously from lipid bilayers under the influence of mechanical agitation when dispersed in aqueous media (4, 5).

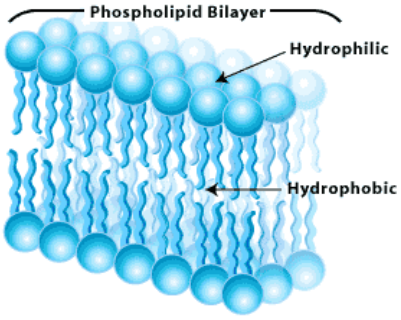


Figure 4: Phospholipid bilayer (Figure taken with permission from: http://www.bioteach.ubc.ca/Bio-industry/Inex/ Artist: Jane Wang)

PC can be produced chemically, though it is easily and inexpensively derived naturally from egg yolk and soybeans. (4) PC is zwitterionic and produces chemically inactive liposomes without a net charge. (2, 4) Hence, it is a widely used lipid. (4)

Liposomal membranes can exist in different phases depending on the temperature. The membranes physical state is changing from a tightly organised gel phase to a more moveable liquid-crystal phase with increasing temperature. This results in more flexible and permeable bilayers. The chain length and the degree of saturation of the alkyl chains determine the phase transition temperature. (2, 4) Egg PC has a phase transition temperature between -15 and -7 ˚C. (4)

A liposomal membrane composed of egg PC allows small neutral molecules to diffuse through the membrane. However, in the combination with other lipids or with cholesterol the stability is improved by making the membrane more tightly organised. This tighter packed membrane will reduce leakage. Cholesterol is a steroid and reduces the movement of the long fatty acid chains due to its rigidity. (2, 4) The chemical structure of cholesterol is shown in Figure 5.

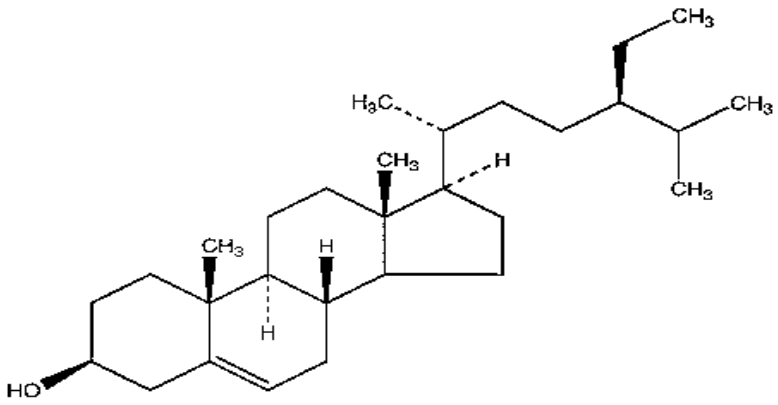


Figure 5: Chemical structure of cholesterol

3.1.2 Relevance of liposome size

The sizes of liposomes can be divided into large, intermediate and small and display different features depending on their size. (2) Even though large liposomes have the largest entrapped volume they are not ideal for intravenous administration since they are too big for escaping the macrophages during circulation in the blood pool. Intermediate liposomes can escape macrophages and stay in the blood pool long enough for reaching targets close to the circulation. Small liposomes have the smallest captured volume and a shorter circulation time compared to intermediate liposomes due to capillary extravasation. However, this gives them

the unique ability to reach targets outside the blood pool such as solid tumours. (2) One possible classification according to size is listed in Table 1. (4)

Table 1: Classification of liposomes according to size.

Liposome classification	Size (nm)	No of lamellae
Multilamellar vesicles (MLVs)	100-1000	>5
Small unilamellar vesicles (SUVs)	<100 nm, lowest possible size*	1
Large unilamellar vesicles (LUVs)	>100 nm	1

*) Varies according to ionic strength, aqueous medium, lipid composition of membrane

Liposomes can change in shape during storage of dispersions. There are two different types of alterations increasing the size of liposomes, aggregation and fusion. (2) This alters the liposomal features. Aggregation is a process where liposomes are linked together without breaking the membranes. This process is reversible. However, fusion is an irreversible process where liposome membranes are broken and melted together with other liposomes forming bigger particles. An increase in size is thus a direct result of a change in shape.

3.1.3 The rationale for using liposomes in chemotherapy

Intravenous route of administration is considered as the most promising route for liposomal formulation. The role of the liposome (containing the drug) is to circulate in the blood in order to reach the desired organ or tissue. The liposomal membrane acts as a barrier protecting the drug from premature elimination or metabolism. At the same time, the liposome membrane is controlling the release of the cytotoxic agent. The liposome carrier may also direct drugs to the tumour site. Thereby, the therapeutic window and toxicity profile of drug compounds can be improved. (2)

The liposomal accumulation at tumour site is referred to as the enhanced permeability and retention effect and is based on dissimilarities of healthy and cancerous tissues. The endothelial walls of blood vessels in tumours are leakier than those in healthy tissues because

of an increased number of bigger gaps. Thus, small liposomes are able to extravasate and penetrate into solid tumours. At the same time, the liposomes stay longer within the tumour site since the removal by the lymphatic system is greatly reduced in cancerous tissue. These special characteristics of cancerous tissue facilitate an accumulation and retention of small liposomes at tumour site, respectively. (2, 3) This can be seen from Figure 6:

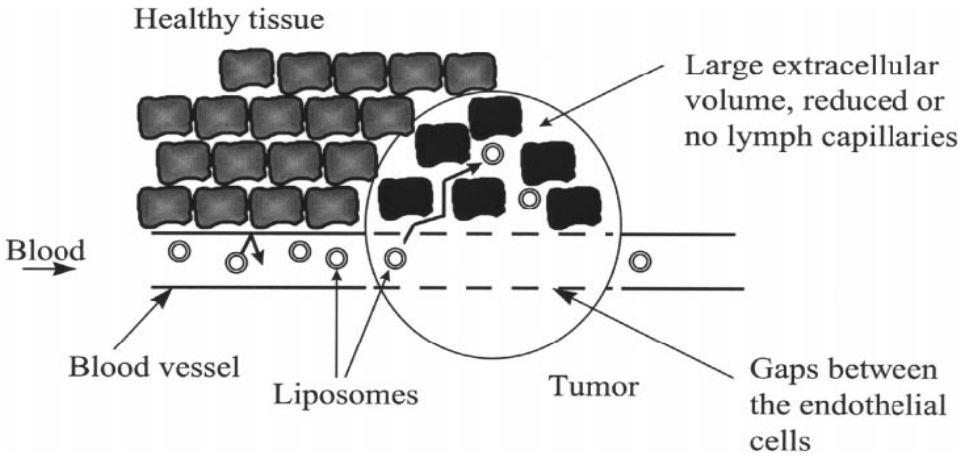


Figure 6: The enhanced permeability and retention effect (Figure reprinted with permission from Elsevier. (3))

3.1.4 Challenges with liposome formulations

Upon entering the general circulation the liposomes must be able to avoid uptake by macrophages. Once plasma proteins adsorb to the liposomal surface the liposomes are easily taken up by macrophages resulting in a decrease in liposomal elimination half life (t½). Adsorption of plasma proteins to liposomes can be reduced by producing small liposomes with rigid uncharged membranes, such as PC and cholesterol. The stability of liposomes can also be affected prior to administrating the drug. The shelf life of the product is dependant upon the chemical and physical stability. The different components in the final liposomal product can interact with each other resulting in degradation. In addition, hydrophilic drugs with a low molecular weight (MW) are prone to diffuse through the liposomal membrane, reducing the shelf life of the product. In this case a tightly packed membrane is essential. Thus, the liposomal membrane composition, i.e. lipid composition and lamellarity is important since it together with the physiochemical characteristics of the drug determines the retention of the active ingredient within the liposome. (2, 3)

3.2 Gemcitabine

Gemcitabine is a cytotoxic drug available on the market in the freeze-dried form of an aqueous solution of the HCl salt known as Gemzar. After reconstitution Gemzar is used for intravenous administration as an infusion only.

3.2.1 Therapeutic indications

Gemcitabine has numerous applications for cancer and is indicated for breast cancer, non-small cell lung cancer (NSCLC), bladder cancer, ovarian cancer and pancreatic cancer as seen from Table 2. (6-9) Gemzar can also be used in patients displaying the following conditions: Cancer of the lymph system, the bile ducts, the gallbladder and germ cell tumours of the ovaries and testes. (10)

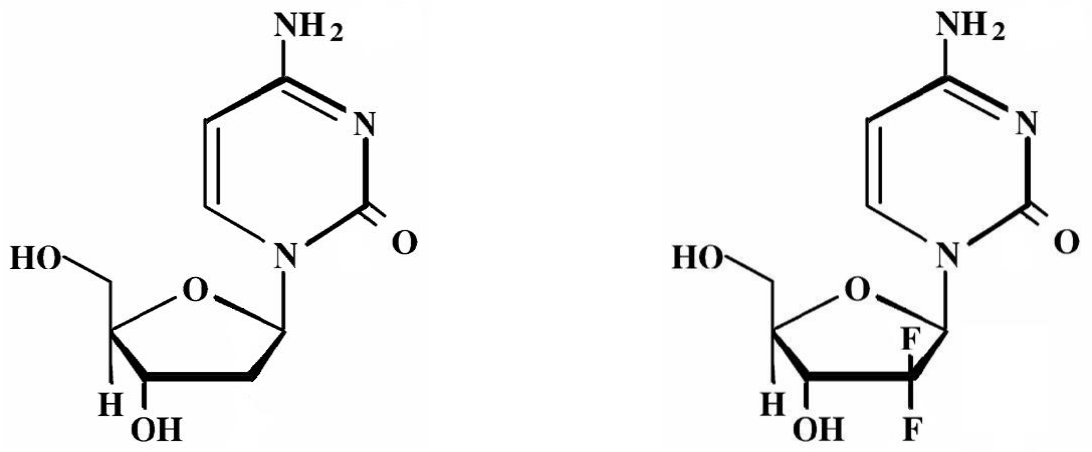
Table 2: Therapeutic indications of Gemzar.

Cancer type	**Combination**	**Stage in cancer**
Breast cancer -2nd line	paclitaxel	metastatic
NSCLC – 1st line	cisplatin	IIIA & IIIB (inoperable locally advanced), IV (metastatic)
NSCLC – palliative treatment	NIL	
Pancreatic cancer: 1st and 2nd line**	NIL	II & III (inoperable locally advanced), IV (metastatic)
Bladder cancer	cisplatin	IV (muscle invasive)
Epithelial ovarian cancer	carboplatin	III (locally advanced), IV (metastatic)

*) after failure or CI of anthracycline treatment
**) for patients previously treated with 5-FU

3.2.2 Chemical characteristics

The chemical name of gemcitabine is 2′-deoxy-2′,2′-difluorocytidine monohydrochloride. Gemcitabine, or difluorodeoxycytidine (dFdC), is an antimetabolite cytotoxic. It is a chemical analogue to the natural nucleoside deoxycytidine (Figure 7). (6, 11, 12)



Deoxycytidine Gemcitabine

Figure 7: Chemical structure of deoxycytidine and the antimetabolite gemcitabine

From Figure 7 it can be seen that two hydrogen atoms of the carbon on the second position of deoxycytidine are substituted with two fluoride atoms giving gemcitabine. The pair of fluoride atoms is contributing to the low pKa value of 3.58 for the gemcitabine HCl salt due to the increased electronegativity. (13) Gemcitabine has a MW of 263.199 g/mol whereas gemcitabine HCl has a MW of 299.66 g/mol due to the presence of hydrochloride. Gemcitabine HCl is a white to off-white powder soluble in water. (6)

3.2.3 Mechanism of action

The prodrug gemcitabine is converted intracellularly via deoxycytidine kinase to difluorodeoxycytidine monophosphate, which is further converted to two active metabolites, dFdCDP and dFdCTP, di– and triphosphate, respectively. Firstly, dFdCDP inhibits the catalysing enzyme ribonucleotide reductase resulting in a reduced amount of deoxynucleotide, deoxycytidine triphosphate (dCTP), available for DNA synthesis. Secondly, dFdCTP competes with dCTP for incorporation into DNA. Incorporating dFdCTP results in chain termination after the further addition of one more nucleotide and thus to apoptosis. Thus dFdC affects the synthesis phase of cell metabolism in two different ways and exhibits a self-potentiating effect. (6, 8, 11, 12, 14)

3.2.4 Metabolism

DFdC is rapidly metabolised in the blood, liver, kidneys and other tissues. Gemcitabine displays a short t½ ranging from 8 to 17 minutes. Less than 10 % is excreted unchanged in the urine, however large amounts of its primary metabolite, difluorodeoxyuridine dFdU were detected. (12, 15) Only a small portion of dFdC will convert into dFdCDP and dFdCTP (6) whereas 91-98 % of administered dFdC will turn into the inactive difluorodeoxyuridine (dFdU). (12, 15)

3.2.5 Toxicity

The cytotoxic activity of gemcitabine in vivo is schedule dependent. (6, 12, 15) This means the activity and the toxicity are related to the dose given and the dosage interval of the treatment. The problem with Gemzar is its short plasma t½ and its quick metabolism into dFdU followed by elimination from the body. Therefore high doses of dFdC are required in order to achieve sufficient cytotoxic concentrations of dFdCTP. (13, 15, 16) Due to the narrow therapeutic window, high administered doses increase the possibility of toxicities and concentration dependent side effects for patients. According to clinical studies, the primary dose limiting toxic effect is myelosuppression; neutropenia, leucopenia, anaemia and thrombocytopenia. In addition, together with other side effects, such as hepatic abnormalities, nausea and vomiting, 10 % of patients ceased treatment. (12)

3.2.6 Approaches for improving formulation

Some attempts, including the use of liposomes, have been tried in order to overcome the problems seen with Gemzar. DFdC is uncharged at physiological pH and is a low MW molecule, which will make it diffuse quickly through the liposomal membrane. (15) Gemcitabine has successfully been entrapped within liposomes, however it leaked out of the vesicles very quickly. In addition, dFdC appears to induce degradation of the liposomal membranes. (17) In order to avoid these problems Gemzar was encapsulated into vesicular phospholipid gel (VPG) reaching an encapsulation efficiency (EE) of 33 % and a shelf life above 14 months. A pilot study proved the formulation encapsulating dFdC into VPG had biphasic elimination (due to free dFdC and encapsulated dFdC) resulting in an increased concentration in plasma compared to Gemzar. (15)

Despite the increased potency of the VPG formulated gemcitabine, GemLip, it also revealed increased toxicity. The increased toxicity was most likely due to the prolonged and fractioned administration of gemcitabine. However, GemLip consists of 33 % encapsulated dFdC and 67 % of extra-liposomal dFdC. This amount of free dFdC will exhibit the same rapid metabolism and the same toxicities as conventional Gemzar.

4 AIM

The primary aim of this study was to investigate, whether an active loading approach can be used for encapsulating gemcitabine, an anticancer agent, into liposomes. Active loading was tried as an alternative loading approach for dFdC due to the positive literature reports. Literature indicated that drugs could efficiently be encapsulated within liposomes and that these liposomes were especially stable during storage. The active loading approach may also be suited to reduce the amount of free dFdC outside liposomes compared to the VPG method. A high amount of dFdC in the aqueous compartment of liposomes with a negligible amount of dFdC outside the liposomes could increase the potency and decrease the toxicity associated with Gemzar treatment.

The active loading method is carried out by using a transmembrane gradient approach. An ammonium sulphate pH gradient has shown to facilitate loading of drugs into the liposomes and in certain cases also induce aggregation (precipitation) of the drug inside the vesicle.

Upon establishment of an active loading method two secondary aims were exploited. The first goal was to optimise dFdC-loading into liposomes displaying an ammonium sulphate gradient and the second goal was to investigate the stability of active loaded liposomes by checking the encapsulated amount of dFdC within the liposomes during storage.

5 MATERIALS AND METHODS

5.1 Chemicals

Table 3: Lipids

Name of lipid	Batch	Producer
Cholesterol recrystallised in methanol (18)	01107101	Croda chemicals Plc, Goole, UK
Unsaturated egg phosphatidyl choline E-80	103670-3	Lipoid GmbH, Ludwigshafen, Germany

Table 4: Chemicals

Chemical	Quality/ Purity	Control no/ Batch no	Producer
Acridine orange base	N/A	235474-5G/ 08202ED	Sigma Aldrich, Chemie GmbH, Steimheim, Germany
AG 50 W-X8 Resin	N/A	210000354	Bio-Rad Laboratories, Inc, Hercules, USA
Ammonium sulphate	99.999 %	204501-250G/0681OPB	Sigma Aldrich, Chemie GmbH, Steimheim, Germany
Chloroform	99.8 %	K30427544 211	Merck, KGaA, Darmstadt, Germany
D(+)Glucose	N/A	607425208	Ferak Berlin GmbH, Berlin, Germany
D(+)Glucose	Ph Eur	05J100011	Prolab, Austin, USA

Ethanol	96 %	N/A	Arcus Kjemi AS, Vestby, Norway
Gemcitabine In the form of the drug product Gemzar, which also contains mannitol, sodium acetate, hydrochloric acid and sodium hydroxide	N/A	FF5K29G, FF4L39J	Eli Lilly and Company, Indianapolis, USA
Hydrochloric acid	N/A	60169847	Merck, KGaA, Darmstadt, Germany
Methanol	99.9 %	K34283707 508	Merck, KGaA, Darmstadt, Germany
Potassium di-hydrogen phosphate	99.5 %	A116773834	Merck, KGaA, Darmstadt, Germany
Sephadex G-50	N/A	17004301/250321	Pharmacia Biotech, AB, Uppsala, Sweden
Sephadex G-50	N/A	51-1858-00-AA/250321	Pharmacia Biotech, AB, Uppsala, Sweden
Sodium Chloride	99.5 %	K31137233236	BDH Laboratory Supplies, England, UK
Sodium Hydroxide	98-100.5 %	B252082 830	Merck, KGaA, Darmstadt, Germany
Triton X-100	N/A	053K0027	Sigma Aldrich, Chemie GmbH, Steimheim, Germany
Triton X-100	97–105 %	L667143 221	Merck, KGaA, Darmstadt, Germany

5.2 Equipments

Table 5: Equipments

Equipment	Type	Manufacturer
Conductivity meter	Conductivity Hand-held meter LF340, Standard Conductivity cell Tetra Con 325/S	WTW, Wissenschaftlich-Technische Werkstätten GmbH, Germany
Dialysis membrane	Dialysis Tubing-Visking Size 9 Inf Dia 36/32" – 28.6 nm: 3	Medicell International Ltd, London, UK
Filter extruder – Hand driven	LiposoFast	Avestin Inc, Ottawa, Canada
Filter extruder – Pressurised air driven	Maximator MSF 27 LS-02-K	Schmidt, Kranz & Co GmbH, Velbert, Germany
Gel filtration column	Gel column, with Sephadex G-50	Produced in-house
Glass beads – 5 mm	Lot no: K34518177 514	Merck, KGaA, Darmstadt, Germany
High pressure homogenisator	Gaulin APV, Micron Lab 40, 99L15450	APV, Lübeck, Germany
HPLC Software: Detector: Column:	Waters 2695 Separations module Millennium^{32} Version 4.00 Waters 2487 dual λ Absorbance Detector LiChrospher 60, RP- Select B (5 µm) 250mm long	Waters Corporation, Milford, USA Waters Corporation, Milford, USA Waters Corporation, Milford, USA Merck, KGaA, Darmstadt, Germany
Incubator	TV10B 677491	Memmert GmbH + Co KG, Schwabach, Germany

Mini column separation	LiChrolut incl. PTFE Frits and 8 ml glass columns	Merck, KGaA, Darmstadt, Germany
pH meter	744 pH Meter Metrohm Ion Analysis	Metrohm Ltd, Herisau, Switzerland
Photon Correlation Spectroscopy - PCS	Submicron Particle Sizer Model 370 (CW 370)	Nicomp Particle Sizing systems, Santa Barbara, USA
Rotavapor	Büchi Rotavapor R-124	Büchi labortechnik AG, Flawil, Switzerland
Scales	Sartorius LP 620S	Sartorius AG, Goettingen, Germany
Stirrer	RTC basic B, IKA color squid	IKA Werke GmbH & Co.KG, Staufen, Germany
Centrifuge	Biofuge Stratos	Kendro Laboratory products GmbH, Hanau, Germany
Solid phase extractor	Supelco Visiprep	Sigma Aldrich, Chemie GmbH, Steimheim, Germany
Ultrasonic cleaner	Bransonic Ultrasonic Cleaner 1510E-MT	Branson Ultrasonic Corporation, Danbury, USA
Vacuum controller	Büchi Vacuum Controller B-721	Büchi labortechnik AG, Flawil, Switzerland
Vacuum machine	Büchi Vacuum Controller B-721	Büchi labortechnik AG, Flawil, Switzerland
Vacuum pump	Millipore Vacuum Pump XF5423050	Millipore S.A., Molsheim, France
Water bath	Büchi Waterbath B-480	Büchi labortechnik AG, Flawil, Switzerland
Filters: For media filtration for PCS	Corning 430513, 500 ml bottle top filter w/45 mm neck 0.22 μm cellulose acetate,	Corning Incorporated, Corning, USA

For liposome filter extrusion	Millipore Isopore Membrane filters: 0.8µm batch no:R2NN87234 0.4µm batch no:R5SN28296 0.2µm batch no:R8MM92556 0.1µm batch no:R8NM25306 respectively	Millipore S.A., Molsheim, France
For organic HPLC eluent filtration	Sartorius sartocon polyamide 0,2µm filter batch no: 0700 25007 9904663,	Sartorius AG, Goettingen, Germany
For aqueous HPLC eluent filtration	Nitrocellulose VCWP 0.1 µm filter: lot no: H5JN 02154	Millipore S.A., Molsheim, France

5.3 Media and solutions

Ammonium sulphate solution:

- Used as hydration medium in MLV production

120 mM ammonium sulphate solution:

I	Ammonium sulphate:	1.584 g
II	Distilled water:	ad 100.0 ml

Glucose solution:

- Used as diluting agent in PCS
- Used as separation medium in gel chromatography
- Used as hydration medium in VPG production

50 g/L glucose solution:

I	Glucose:	50.0 g
II	Distilled water:	ad 1000.0 ml

20 % Ethanol:

- Used as preservative for Sephadex G-50 gel during storage

20 % v/v ethanol:

I	96 % ethanol:	20.0 ml
II	Distilled water:	80.0 ml

Triton solution:

- Used for dissolving liposomes

10 % v/v triton solution:

I	Triton X-100:	100.0 ml
II	Distilled water:	ad 1000.0 ml

Saturated sodium chloride solution:

- Used for preparing ion exchange column

Saturated sodium chloride:

I	Sodium chloride:	200.0 g
II	Distilled water:	ad 500.0 ml

The solution was heated close to its boiling point before it was filtrated into a flask for storage.

9 mg/ml sodium chloride solution:

- Used as hydration medium for gemcitabine

9 mg/ml sodium chloride:

I	Sodium chloride:	450.0 mg
II	Distilled water:	ad 500.0 ml

Phosphate buffer:

- Used as dilution media for gemcitabine in standard curve
- Used as dilution agent for HPLC measurements

25 mM phosphate buffer (pH 6.9):

I	Potassium dihydrogen phosphate:	6.805 g
II	Distilled water:	ad 2000.0 ml
III	Hydrochloric acid for pH adjustment	q.s.
III	Sodium hydroxide for pH adjustment	q.s.

Mobile phase for HPLC analysis:

Phosphate buffer with methanol v/v:

I	Phosphate buffer:	92.5 ml
II	Methanol:	7.5 ml

5.4 Preparative methods

5.4.1 Preparation of multi lamellar vesicles (MLVs)

MLVs were prepared according to the following methods:

5.4.1.1 Egg-PC raw dispersion: Hand shaken method:

10 % w/w E-80 in 120 mM ammonium sulphate solution:

I	E-80:	10.0 g
II	120 mM ammonium sulphate:	90.0 g

The raw dispersion was gently stirred using a magnetic stirrer for approximately 2 hours until E-80 was dispersed.

5.4.1.2 Egg-PC/Cholesterol dispersion: Film method:

10 % w/w E-80/Cholesterol in 120 mM ammonium sulphate solution (55 mol % E-80 and 45 mol % cholesterol):

I	E-80/cholesterol:	24.3 g
II	120 mM ammonium sulphate:	ad 243 g

The film method was carried out according to New, Lasch, Weissig and Brandl. (5, 19)

- Lipids were dissolved in chloroform: methanol in the ratio 2:1 by hand shaking
- The round bottom flask containing the resulting solution was attached to rotavapor and placed in a water bath at approximately 55 to 60 ˚C with a decreasing pressure for approximately 15 minutes.
- The resultant film was further dried at a pressure of 200 hPa for 1.5 hours followed by a decrease in pressure to 50 hPa for another 3 hours.
- The hydration medium (120 mM ammonium sulphate solution) was heated up to 55 to 60 ˚C and added to the film. The round bottom flask was shaken vigorously before placing it back in the water bath and attaching it to the rotavapor (without vacuum) until the film was completely dispersed.

5.4.2 Reduction of liposome size

Preparation of liposomes results in MLVs with a wide particle size distribution. In order to achieve more homogenous sizes of the liposomes, filter extrusions were executed.

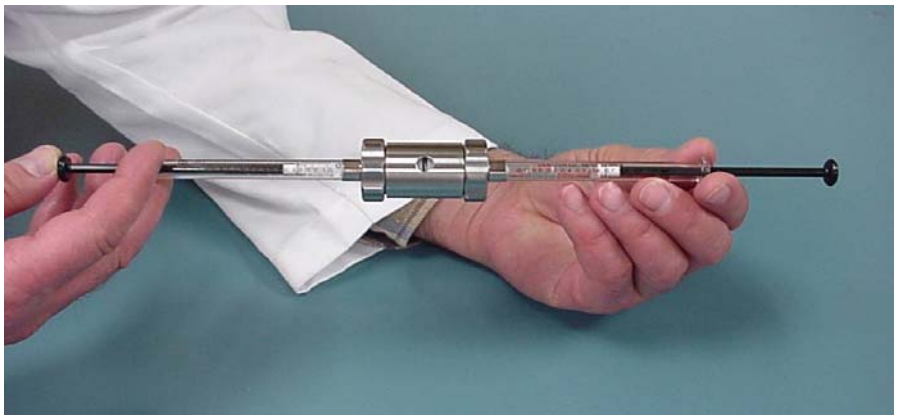
5.4.2.1 Filter extrusions

Filter extrusion involves the process of squeezing the liposome dispersion through pores of polycarbonate filters of decreasing pore sizes by applying pressure. As a result, the liposomes will break and form smaller sized liposomes. (2) Liposome membranes deform when passing through the filter pores. The deformability is reduced in liposomes containing cholesterol, which results in decreased rate of extrusion. However, increasing the temperature facilitates the extrusions. (20)

Hand driven filter extrusions are suitable for small-scale filter extrusion due to limited syringe volume whereas machine driven filter extrusions allows larger volumes and are thereby appropriate for large-scale filter extrusions.

Filter extrusion by hand

Hand driven filter extrusion was performed using LiposoFast (Picture 1). This method involves the transfer of the liposome dispersion from a 1 ml syringe through a filter holder containing Millipore Isopore filters into a second syringe. This is achieved by applying manual pressure. The transfer of liposomes is repeated five times in order to achieve a more uniform liposome size. In addition, the uneven number of extrusions will prohibit the liposome dispersion to end up in the initial syringe and thus reduce the contamination with liposomes of larger sizes. Filter extrusion was performed consecutively using filters with decreasing pore sizes of 0.8 μm, 0.4 μm, 0.2 μm and 0.1 μm.



Picture 1: Filter extrusion by hand using LiposoFast (Picture taken with permission from: http://www.avestin.com/products.html)

Continuous High Pressure Filter Extrusion

A Maximator, originally described by Schneider, Sachse, Rößling and Brandl, (21) was used for the machine driven filter extrusion. Filter extrusions took place upon initiation of air pressure (ideally at 300 kPa, but less than 500 kPa). As with the hand extruder, 5 cycles of filter extrusion per filter was performed to obtain a more homogenous size distribution of the liposome dispersion. Isopore filters with decreasing pore sizes in the same magnitude as for the hand driven extrusions were used for the continuous high pressure filter extrusions.

The applied pressure driving the liposomes through the filter varied according to the filter size and the speed of the pump. This applied pressure generated a higher pressure over the filter to execute the extrusions. Table 6 shows the pressures and the following translating factors.

Table 6: Machine driven continuous filter extrusion pressures and translation factor

Filter size (µm)	Applied air pressure (bar)	Resulting pressure over filter (bar)	Translation factor
0.8	0.75	20	26.7
0.4	0.75	20	26.7
0.2	0.75	20	26.7
0.1	1.50	60	40.0

5.4.3 VPG production:

40 % w/w E-80 in 50 g/L glucose:

I	E-80:	12.0 g
II	50 g/L glucose:	18.0 g

The VPG was gently stirred for approximately two hours using a magnetic stirrer until E-80 was dispersed.

VPG was produced for passive loading of gemcitabine into liposomes as described by Moog et al. (15) This was done in order to compare the efficiency of active loading to a standardised method.

VPG is a gel, hence, not suitable for filter extrusion. Therefore, reduction in liposome size was achieved through high pressure homogenisation.

5.4.3.1 High pressure homogenisation:

The high pressure homogeniser forces the VPG through a narrow valve applying high pressure. Liposomes are exposed to local stress due to the pressure drop occurring after VPG exits the valve. The decrease in particle size is described by two theories, the cavitation phenomenon and the turbulence theory. In brief, in the cavitation theory the liquid cavitates because of the large pressure drop when the liquid goes through the valve. When an appropriate pressure drop occurs, bubbles burst after leaving the valve, generating shock waves resulting in shear forces tearing the dispersion apart. The turbulence theory describes the generation of eddies caused by dissipating energy in the liquid. Together with the pressure differences, these eddies are responsible for shearing of the droplets. (22)

The VPG was homogenised in a Gaulin APV homogeniser. The gel was placed into the homogeniser and a pressure of 70 MPa was applied. The VPG was then collected, and the procedure was repeated nine times. (23)

5.4.4 Reconstitution of Gemzar

According to the summaries of product characteristics (SPC) for Gemzar 5.0 ml 9 mg/ml NaCl was transferred into the Gemzar vial giving a maximum concentration of 38 mg/ml of gemcitabine. (8)

5.4.5 Active loading of gemcitabine into liposomes via a transmembrane ammonium sulphate gradient

Active loading through a pH gradient is a technique based on the membrane permeability of the free base of a hydrophilic drug, whereas its charged, protonated form is membrane impermeable. The drop in pH is caused by an ammonium sulphate transmembrane gradient having liposomes with internal ammonium sulphate surrounded by an ammonium sulphate free medium. Encapsulated ammonium ions are in equilibrium with uncharged ammonia and protons. The capability to permeate the liposomal membrane is dependent on size and charge of the species according to the following relation: $NH_3 >>> H^+ >> NH_4^+ > SO_4^{2-} > (NH_4)_2SO_4$. (24) A shift in equilibrium to the right (Equation 1), thus a reduction in the pH within the liposomes, occurs when uncharged ammonia diffuses out of the vesicles, leaving the protons behind.

Equation 1: $$NH_4^+ \rightleftharpoons H^+ + NH_3$$

Simultaneously, the neutral form of the drug, in this case the free base of gemcitabine, is expected to diffuse into the vesicle where it becomes protonated, due to the low pH, and thus trapped. This decreases the proton concentration within the liposomes, however, more ammonia will subsequently be produced and diffuses out of the vesicle increasing the proton supply facilitating the drug uptake. (24, 25)

In order to create the gradient, liposome formation is carried out in ammonium sulphate solution followed by removal of external ammonium sulphate. The better the removal of ammonium sulphate in the outer aqueous phase, the greater the gradient becomes. This was executed by size exclusion chromatography (SEC) using Sephadex G50 gel. 50 g/L glucose

was used as hydration medium in the column since this concentration is expected to be isoosmotic with 120 mM of ammonium sulphate when added to the liposome dispersion. (24)

Based on the amount of substance of encapsulated ammonium sulphate in the liposome fraction compared to the amount of substance in the initial liposome dispersion, the encapsulation efficiency (EE) can be determined as given by equation 2:

Equation 2: $$\text{Encapsulation efficiency} = \frac{\text{Amount compound}_{\text{encapsulated}} * 100\%}{\text{Amount compound}_{\text{total}}}$$

The gradient is determined by comparing the concentration of external ammonium sulphate ($[(NH_4)_2SO_4]_{ext}$) in the liposome fraction after separation after SEC (see section 5.4.5.1) with the concentration of ammonium sulphate inside the vesicles ($[(NH_4)_2SO_4]_{int.}$). This gives the following equation:

Equation 3: $$\text{Gradient} = \frac{[(NH_4)_2SO_4]_{int}}{[(NH_4)_2SO_4]_{ext}}$$

5.4.5.1 Size exclusion chromatography (SEC) for generation of the ammonium sulphate gradient

Theory:

Gel filtration is a SEC method separating different particles or substances according to their size. A column is filled with a pre-swollen porous gel, completely packing the column. Particles diffuse in and out the pores of the gel and therefore pass through the column at different speed according to their size or MW. Larger particles pass through the column in a faster manner compared to smaller particles due to less diffusion into the matrix. As a result different fractions containing different particles can be collected subsequent to the separation. (26)

Packing of column:

The gel filtration column was prepared in-house using Sephadex G-50 as gel matrix according to the gel filtration handbook from GE healthcare. (26) Sephadex G-50-powder was mixed with excess distilled water and placed in a Büchi Waterbath B-480 at 90 °C for one hour in order to swell. The gel filtration column was set up with glass wool in the bottom of the column to prevent leakage of Sephadex gel matrix. Degassed 50 g/L glucose was used as a hydration medium to pack the column with Sephadex gel.

Procedure:

Three times the column volume of 50 g/L glucose was run through the column before the sample was added. (26) During separation continuous amounts of medium were added in order for the column to stay wetted. The fractions, containing the different particles, were collected and their volume was measured.

5 ml of liposome dispersion was transferred to the column and the fractions were collected. Liposomes pass through the column in a faster manner compared to ammonium sulphate giving ideally one fraction consisting of liposomes with an internal ammonium sulphate solution and 50 g/L glucose as the outer phase. The liposome fraction could easily be collected from the column since the fraction appeared turbid. After separation the column was washed with 500 ml 50 g/L glucose in order to remove residual ammonium sulphate from the gel matrix. (26) During longer breaks, the gel matrix was stored in 20 % ethanol acting as a preservative to prevent growth of micro-organisms.

5.4.5.2 Loading compounds into liposomes by ammonium sulphate gradient

The use of a pH gradient for encapsulation of drugs may reduce problems associated with other loading techniques. By applying a transmembrane gradient to the liposomes the drug is expected to cross the membrane with an increased trapping efficiency and drug retention as compared to other loading techniques. (27)

5.4.5.3 Acridine orange loading

Acridine orange was used in preliminary experiments for qualitative evaluation of the ammonium sulphate gradient. Acridine orange has advantages compared to gemcitabine,

which makes it suitable to use as a model. Firstly, acridine orange is not toxic and is therefore safe and suitable for preliminary experiments and secondly its orange colour is easy to identify visually.

Acridine orange was added to the dispersion after the gradient was established. When loading had completed the dispersion was run through the gel column again for separating the fraction of free acridine orange from the liposome fraction.

The liposomes containing acridine orange should appear orange and turbid and thus easy to collect. Before the fraction of free acridine orange there should theoretically be a small colourless fraction.

5.4.5.4 Loading of dFdC

According to Fenske, Maurer and Cullis, a transmembrane ammonium sulphate gradient is ideal for drugs supplied as an HCl salt. (25) Like gemcitabine, these drugs form an equilibrium existing either in their water soluble salt form or uncharged as a free base. This equilibrium is a function of their pKa value, which is expressed by equation 4:

Equation 4: $BH^+ + Cl^- \rightleftharpoons B + HCl$

with B as the free base and BH^+ as the cation of the salt form.

Gemcitabine HCl has a pKa of 3.58. (28) According to the Henderson-Hasselbalch equation, the amount of salt form equals the amount of basic form when pH equals pKa (equation 5).

Equation 5: $$pH = pKa - \log\frac{[BH^+]}{[B]}$$

A solution of 38 mg/ml gemcitabine has a theoretical pH of 2.2 and is protonated to 95.7 %. However, Gemzar contains NaOH and HCl for pH adjustment, which leads to a higher pH

ranging from 2.7 to 3.2 after preparation. (29) The pH of reconstituted Gemzar was measured to be 2.9. Accounting for the dilution with incubation, the pH of the outer phase was calculated to 4.85 for the sample loaded with 60 µl of dFdC HCl. At this pH, 94.9 % of gemcitabine is unprotonated and therefore able to penetrate the liposome membranes. Once inside the liposomes, the acidic environment with pH ~2.7 (25) leads to a 85.7 % protonation of the gemcitabine base, resulting in the entrapment.

Reconstituted Gemzar was added to the liposome dispersion after the gradient was considered satisfactory. After varying incubation conditions, free dFdC were separated from the encapsulated dFdC on an ion exchange column (see section 5.5.5.2) before the EE of dFdC was determined by high performance liquid chromatography (HPLC).

5.4.6 Passive loading of dFdC into VPG

Passive loading of gemcitabine into VPG was carried out according to Moog et al as follows:

- 500.0 µl of dFdC solution was transferred into a second vial already containing 3.71 g of VPG and 6.0 g of glass beads. The closed vial was centrifuged for 10 minutes at 1500 rpm before incubation for one hour at room temperature.
- The vial was centrifuged for another 5 minutes at 1500 rpm before incubation at 60 ˚C in a heater for two hours.
- The VPG was diluted with 6.4 ml 9 mg/ml NaCl solution immediately before the cation exchange chromatography was executed.

5.5 Analytical methods

5.5.1 Photon Correlation Spectroscopy – PCS

Theory:

PCS is an analytical tool to determine the size distribution of particles. The method is based on dynamic light scattering, where a laser light beam is sent through the sample of interest. The particles in the sample scatter the laser light depending on their MW, size and shape. (30)

Individual scattered waves from individual particles will interfere. The particles move in random Brownian motion and therefore, the scattered wave, or intensity at the detector site,

will fluctuate with time. (30) The scattered wave arriving at the detector site has a phase relationship dependent on the incident laser wave and the particles precise position. (30) Small particles will diffuse more rapidly compared to large particles and consequently give a more rapidly fluctuating intensity signal. (30) In contrast large particles are characterised by a signal with slowly fluctuating intensity. (30)

The temperature must be constant in order to reduce the influence of thermal convection on the intensity fluctuations. The fluctuating wave scattering signal detected at the beam determines the diffusion coefficient of the particles and the particle radius can be calculated utilising the Stokes-Einstein equation. (30)

Fitting and interpretation of the results:

The software is able to evaluate data according to two different distribution-models fitted to the particle size distribution of the sample. The Gaussian distribution is the simplest approach in determining particle size and is restricted to normal distributions. For other distributions the more advanced NICOMP model is well suited since it assumes polydisperse samples. (18)

The Gaussian model states how well a fit is approaching a normal distribution. The quality of this fit is given by Chi squared (χ^2). A good fit requires a value between one and two whereas a value equal to or below one indicates an exceptionally good fit. On the contrary, a value above three represents poor fit and it is recommended to change to NICOMP distribution. A second parameter called Baseline Adjust reveals the presence of aggregates and other contaminants such as dust particles, which is considered negligible when measured close to zero. (18)

For the NICOMP model the fit error addresses the stability of the results, with recommended values below 1.5 and preferably close to 1.0 in order to achieve reproducible and accurate results. Residual is a parameter indicating the presence of measured aggregates or other contaminants and for negligible amounts, the value should be 0.0. Significant amounts of contaminants are present at a value of 10 or larger. (18)

Experiment:

Measurements were carried out as described by Ingebrigtsen and Brandl. (31) This quality control method was employed to determine the average size of liposomes. In brief, 50 g/L glucose, filtrated through a bottle top 0.22 μm filter, was used as a medium for diluting the liposome samples. The test tubes were placed in an ultrasonic bath for 15 minutes and then rinsed with the medium solution in a LAF bench in order to reduce particle contamination. The samples were diluted with freshly filtrated medium within the LAF bench until reaching an intensity level of 250–350 kHz as stated in the Nicomp user manual. (30)

Before measurements were performed the instrument parameters were set according to correct values as listed in Table 7.

Table 7: PCS parameters

Parameters	**Value**
Temperature	23˚C
Viscosity	0.9325
Liquid index of refraction – 50 g/L glucose	1.3402 (32)
Intensity set point	300
Channel width	Auto set

Three cycles of 5 to 15 minutes were run for each sample in order to achieve readings in triplicates of the mean size with a value of collected data above 1000K (1 million) in order to ensure high statistical accuracy. (33)

5.5.2 Quantification of ammonium sulphate by conductivity measurements

A conductivity meter was used for determining unknown concentrations of ionic solutions based on a standard curve. The conductivity of free ammonium sulphate can be measured whereas the encapsulated ammonium sulphate is expected to have a conductivity of zero since the liposome membranes act as insulators. For conductivity measurements and determination

of the ammonium sulphate EE, liposomes were destroyed in order to free the encapsulated ammonium sulphate.

Standard curves were prepared in order to determine the concentration of ammonium sulphate within and outside liposomes.

5.5.2.1 Ammonium sulphate in 50 g/L glucose solution

A standard curve was prepared for ammonium sulphate in 50 g/L glucose solution. The conductivity of known concentrations of ammonium sulphate was measured. This resulted in a standard curve used for determining the unknown concentrations of ammonium sulphate. Approximately the same concentrations were used as in the experiments of Haran et al (24) ranging from 0.016 mM to 120 mM of ammonium sulphate and measurements were executed at 4.0 ˚C. Preliminary tests showed that measurements at 4.0 ˚C were challenging to comply with, therefore all solutions and equipments were cooled down in an ice bath until reaching temperature of about 0.5 ˚C. Measurements were performed at temperatures ranging from 0.3 to 0.6 ˚C. The conductivity measurements are listed in Table 8, and Figure 8 shows the standard curve.

Table 8: Conductivity measurements for the standard curve – ammonium sulphate in 50g/L glucose

$(NH_4)_2SO_4$ (mM) in 50 g/L glucose	**Parallel 1**		**Parallel 2**		**Parallel 3**		**SD of triplicate**
	Conductivity (µS/cm)	**ºC**	**Conductivity (µS/cm)**	**ºC**	**Conductivity (µS/cm)**	**ºC**	
120	11300.0	0.5	11400.0	0.4	11400.0	0.4	57.7
80	8020.0	0.4	8030.0	0.6	7960.0	0.5	37.9
60	6140.0	0.3	6120.0	0.4	6150.0	0.5	15.3
24	2730.0	0.5	2740.0	0.5	2730.0	0.5	5.8
12	1454.0	0.6	1453.0	0.5	1453.0	0.6	0.6
1.2	164.8	0.5	164.9	0.5	164.7	0.4	0.1
0.12	18.0	0.5	18.0	0.5	18.0	0.5	0.0
0.012	2.7	0.5	2.8	0.5	2.8	0.5	0.1

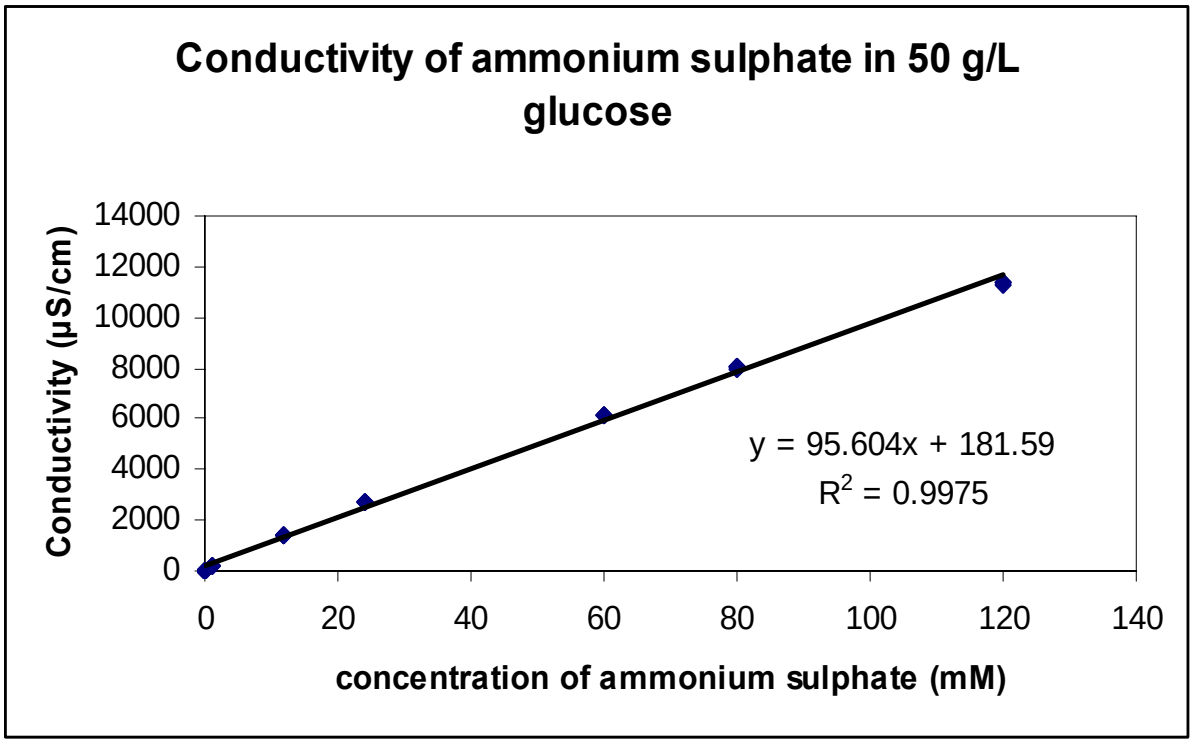


Figure 8: Standard curve of conductivity versus concentration of ammonium sulphate in 50 g/L glucose solution

5.5.3 Determination of ammonium sulphate entrapment

The liposomes entrapping ammonium sulphate were separated from non-entrapped ammonium sulphate. The liposome fraction was collected and its conductivity measured in order to calculate the remaining external amount of ammonium sulphate. Various attempts were made to destroy the liposomes (see section 6.4). The final approach was as follows: A sample of the liposome fraction was diluted with dialysed 10 % triton solution (in a ratio of 1:1) to annihilate the liposomes and measure the conductivity for calculating the amount of ammonium sulphate within the whole solution. Using these two values the amount of ammonium sulphate inside the liposomes was determined.

5.5.4 Quantification of gemcitabine through Reversed-Phase High Performance Liquid Chromatography – RP-HPLC

Gemcitabine was quantified using RP-HPLC, according to a method developed by Håkonsen. (34) Briefly, a calibration curve of gemcitabine was prepared through HPLC in order to

determine the concentration of unknown amounts of active loaded gemcitabine within the liposomes.

Theory:

The sample solution is injected into a mobile phase, which acts as a carrier for the sample. The mobile phase then flows through the stationary phase within the column. Components of the sample begin migrating through the column at the same time. However, they will travel at a speed, and separate, depending on interactions with the stationary phase. A reverse stationary column (C8) is used in these experiments to separate gemcitabine from lipids, triton solution and glucose. The stationary phase is non-polar whereas the mobile phase is polar. Samples with strong interactions with the stationary phase will stay on the column for a longer time compared to weaker interactions and consequently result in longer retention time and vice versa. An isocratic method is used in these experiments. The detector signals the resultant peak(s) seen in the chromatogram. (35)

A series of known concentrations of gemcitabine was injected into the HPLC resulting in peaks correlating to the concentrations. A calibration curve was developed, by plotting the area under the curve (AUC) from the peaks versus the concentration of the samples. Using this calibration curve, quantification of unknown gemcitabine could be executed. (36)

Experiment:

All HPLC eluants were filtrated using either a Millipore Nitrocellulose filter with 0.1 μm pore size or Sartorius polyamide filter with 0.22 μm before usage, reducing the possibility of precipitation blocking the column. The column was stored in acetonitril and had to be washed thoroughly with distilled water before changing to the mobile phase, phosphate-methanol buffer, in order to prevent salt precipitation. The parameters were set according to Moog et al and are listed in Table 9.

Table 9: HPLC parameters used for quantification of gemcitabine

Injection volume	50 µl
Mobile phase	25 mM phosphate buffer (pH 6.9)/methanol 92.5:7.5
Flow rate	1 ml/min
UV detection wavelength	278 nm
Column temperature	40˚C
column	LiChrospher 60, RP- Select B (5 µm) 250mm x 4 mm
Packing material of column	C-8

The standard curve was obtained from triplicates of each sample with concentration ranging from 50 ng/ml to 5400 ng/ml. (34) As seen from Figure 9, the calibration curve reveals a good linear fit with a R^2 value of 0.9997.

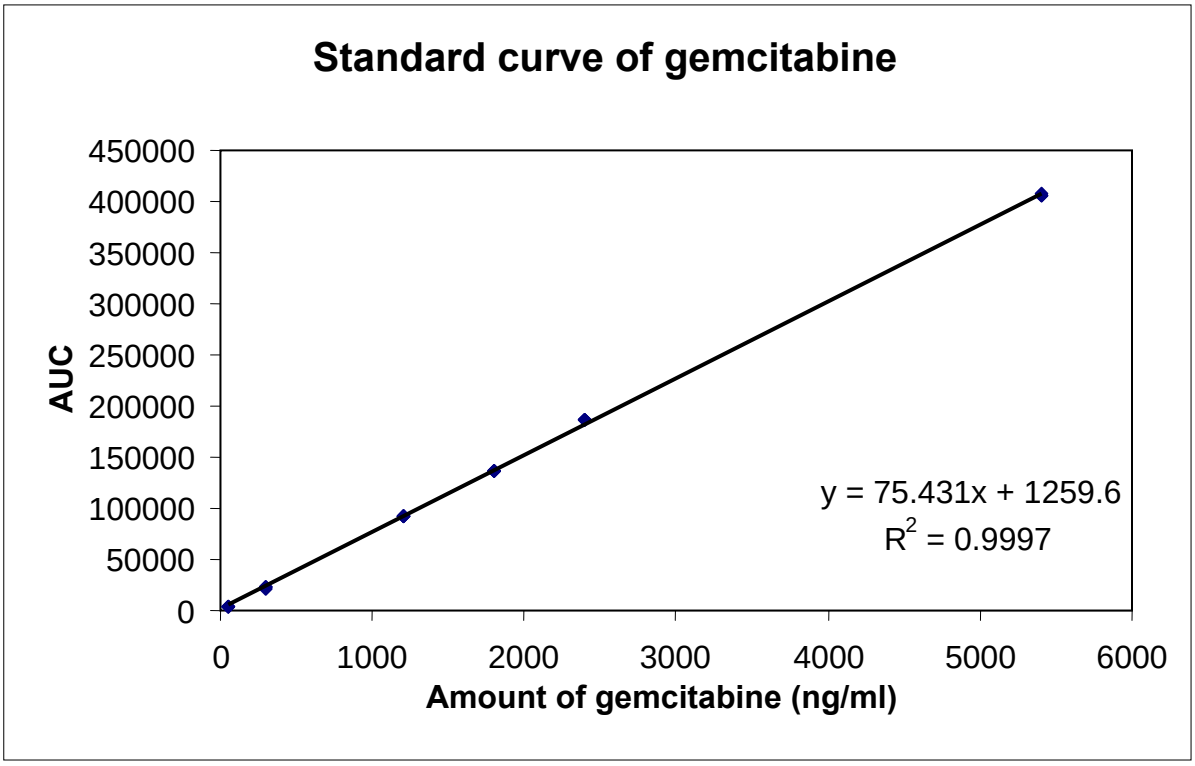


Figure 9: Standard curve used for quantification of dFdC

A typical chromatogram of a sample of gemcitabine is shown in Figure 10. This chromatogram shows a peak with an area resultant in 2935.893 ng/ml of gemcitabine.

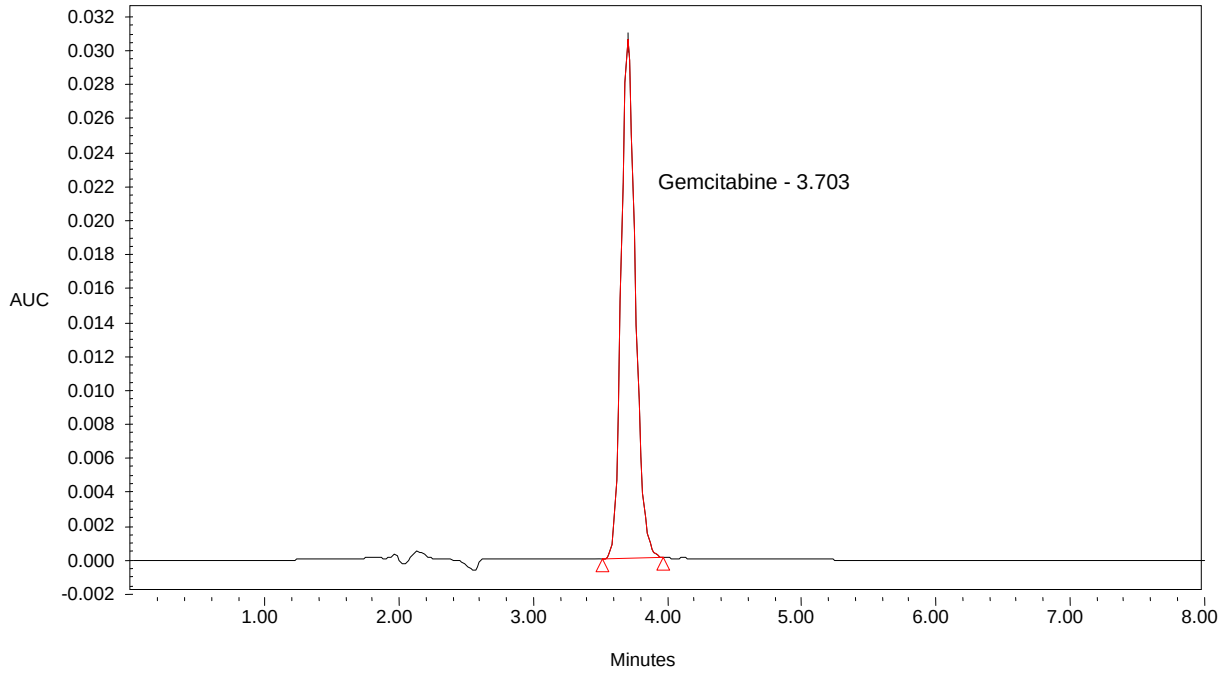


Figure 10: A typical chromatogram of gemcitabine

5.5.5 Determination of encapsulation efficiency of gemcitabine

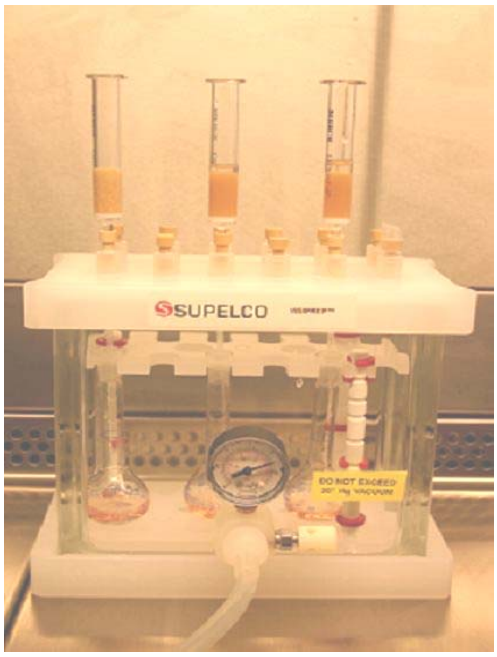
External gemcitabine must be separated from encapsulated gemcitabine before dissolving the liposomes in order to determine the EE. This separation was performed using a cation exchange column. (13) In order to quantify the amount of dFdC, and determine the EE, two samples from the same loaded sample set were compared. One sample was separated through the cation exchange column removing the external dFdC whereas the second sample, used as a reference, was not. Samples containing encapsulated and free dFdC and samples containing only encapsulated dFdC were quantified according to section 5.5.4. The column was prepared and set out according to Moog as follows: (15)

5.5.5.1 Preparation of the Lichrolut cation exchange column:

- PTFE-frits were placed within the column.
- 2.0 g of AG 50 W cationic exchanger was added to the column.

- The column was filled with excess distilled water followed by stirring for removal of air bubbles.
- After 5 minutes, when sedimentation has completed, excess water was removed by vacuum until the cationic exchanger was just covered with water.

The column was loaded with saturated NaCl solution before it was flushed with distilled water followed by drying under vacuum conditions. The setup of Lichrolut cation exchange column is shown in Picture 2.



Picture 2: Setup of cation exchange columns

5.5.5.2 Cation exchange separation:

0.5 ml of loaded liposome samples was added drop wise to the column followed by 10 seconds of suction. The column was then eluted with 0.5 ml distilled water every 10 seconds, repeated four times, while the liquid was collected under continuous suction.

The eluate for the gemcitabine determination was collected in a 10.0 ml volumetric flask and diluted with 10 % triton solution up to 10.0 ml. 2.5 ml of the diluted sample was then further

diluted with 10 % triton solution in a 25 ml volumetric flask before its final dilution of 1:2 with 25 mM phosphate buffer. Gemcitabine was then quantified via HPLC.

As a reference, containing external and encapsulated dFdC, 125 µl unseparated sample, from the same sample set as used above, was diluted with 10 % triton solution in a 25 ml volumetric flask. Finally, it was diluted with phosphate buffer in the same manner as for the sample separated through the ion exchange column. The reference was then ready for injection into the HPLC.

6 RESULTS AND DISCUSSION

6.1 Preliminary experiments

6.1.1 Preparation of ammonium sulphate-containing liposomes of defined size

Lipsomes were prepared by the hand shaken method according to section 5.4.1.1 and by the film method according to section 5.4.1.2. Extrusions were carried out following the methods described under hand driven filter extrusion and continuous high pressure filter extrusion.

The liposomes mean diameter was measured using PCS. For calculating sizes, the software requires the refractive index of the dispersion medium. The refractive index of 5% glucose solution was initially not known, so calculations were done using the refractive indices for 10 % glucose and for water, respectively. The results showed a small variation in the mean liposome size (data not shown). Later on, the refractive index for 5 % glucose was found in literature (32) and used for further PCS analysis. Table 10 shows a typical result for the mean liposome sizes of liposomes prepared by the hand shaken method and filter extruded using the continuous high pressure extruder.

Table 10: Example of results from PCS analysis

Measurement 1: mean vesicle size ± SD	115.4 ± 45.4
Measurement 2: mean vesicle size ± SD	107.7 ± 44.6
Measurement 3: mean vesicle size ± SD	114.0 ± 45.1
Mean of parallels: mean vesicle size ± SD	112.4 ± 45.0
Measurement time (min)	15
Collected data in channel no 1	>1000 K

The liposomes show a slightly larger mean diameter than the filter pore size used for extrusions. This can be explained by the flexibility of the liposomal membranes leading to alterations in shape while squeezing through the filter pores.

According to Frantzen dispersions displaying Gaussian distributions, should preferably display standard deviations (SD) below 15 percent. (33) As seen from Table 10, the average SD was approximately 45 %. This high percentage indicates a wide Gaussian size distribution, (33) i.e. the presence of liposomes of different sizes.

6.1.2 Reduced conductivity of liposome dispersions

When the ammonium sulphate solution, with a conductivity of approximately 11.3 mS/cm, was mixed with E-80 the conductivity of the resultant liposome dispersion was substantially decreased. The measured conductivity of the liposome dispersions varied between 7.69 and 8.5 mS/cm depending on the batch measured. A possible explanation for this phenomenon is that entrapped ammonium sulphate is not contributing to the overall conductivity since the liposomes act as insulators. In this way only the ammonium sulphate in the outer aqueous phase will display conductivity. This is supported by Lidgate, Hegde and Maskiewicz, who discovered a reduced conductivity upon formation of liposome dispersions as compared to the conductivity of the hydrating buffer alone. (38) The reduction in conductivity was shown to be dependent on lipid concentration, the buffer ionic strength and the liposome size. They found the net loss of conductivity to be a convenient measure of the captured volume in liposomes. (38) By using the loss in conductivity as a measure for captured volume, approximately 70 % of the ammonium sulphate in this experiment was in the outer aqueous phase, whereas approximately 30 % were most likely encapsulated within the liposomes.

6.1.3 Removal of non-entrapped ammonium sulphate from the liposomes

Size exclusion chromatography was chosen as a method to separate the liposomes from non-entrapped ammonium sulphate. A series of separation attempts was carried out in order to determine the most appropriate application volume to the column for achieving the greatest separation. Tested volumes varied from 1.0 ml to 10.0 ml and their resultant conductivities are listed in Table 11.

Table 11: Results determining the appropriate application volume for size exclusion chromatography

Liposome composition	**Applied volume (ml)**	**Volume of liposome fraction (ml)**	**Conductivity of liposome fraction (µS/cm)**
E-80	10.0	32.0	1060.0
E-80	5.0	25.0	572.0
E-80/CH	5.0	35.0	37.1
E-80/CH	1.0	16.5	6.8

The conductivity of the surrounding medium of the liposome fraction was measured in order to check if the ammonium sulphate outside the liposomes was removed successfully. A conductivity of approximately 7 to 1000 µS/cm was measured at 0.5 ˚C as compared to about 8 mS/cm in the liposome dispersion before fractionation. The difference in conductivity indicates that the external ammonium sulphate was widely removed during gel chromatography. But, significant differences were observed between the different sample volumes. In general, the removal of ammonium sulphate was more complete with lower application volumes.

A distinct optimum seemed to be reached with an application volume of 5.0 ml. Although the conductivity decreased even more with smaller volumes the column diluted the samples to such a great extent, making the results less reliable due to low signal to noise ratios. 5.0 ml seemed to be the most satisfactory application volume and was therefore used in subsequent experiments.

The volumes and duration taken for collecting the different fractions, of a 5 ml application volume, are listed below in Table 12.

Table 12: Fractions from separating a 5.0 ml sample through the gel column

Fraction	**Volume (ml)**	**Duration (s)**
void fraction	20.0	155

Liposome fraction	26.4	159

6.1.3.1 Fluctuating conductivity

The conductivity of free ammonium sulphate was measured numerous times within the same batch and between different batches. However, the conductivity was varying to a greater extent than the differences in volume of the liposome fractions could account for. In addition, the size of the conductivity indicates the presence of free ammonium sulphate to a larger extent than considered optimal. This can be due to two reasons, firstly, the possible outcome of unsatisfactory separation through the column. Attempts were made in finding a suitable volume of liposome dispersion added to the gel column in order to give a satisfactory amount of free ammonium sulphate in the liposome fraction. Secondly, the measured conductivity of non-encapsulated ammonium sulphate may have been increased by ammonium sulphate leaking through the lipid membrane. It is generally accepted that liposomes prepared from natural, fluid egg PC, like E-80, do not form as tightly packed bilayers as liposomes containing cholesterol.

Attempts in reducing contaminating effects on the results:

- Sephadex matrix was replaced every 1-2 months.
- The conductivity meter was washed thoroughly with 96 % ethanol and 5 % glucose before measurements in order to eliminate a possible liposome film on the conductivity meter.
- Liposomes were freshly prepared every three weeks (liposomes composed of only E-80 showed to degrade due to discolouration).
- The gel column was rinsed thoroughly with 50 g/L glucose in order to flush through any residual ammonium sulphate ions from the previous separation. This was done experimentally by measuring the conductivity of fractions of the washing solution and a volume of 500 ml was considered appropriate in order to eliminate residual ammonium sulphate ions. This can be seen from Table 13.

Table 13: Amount of substance of ammonium sulphate in different fractions after size exclusion chromatography

Fraction	**Conductivity (µS/cm)**	**Volume (ml)**	**Conc of $(NH_4)_2SO_4$ (mM)**
Void volume	9.0	22.0	N/A
Liposome fraction	572.0	25.0	2.07
Combined fraction just before and just after liposome fraction	394.0	20.0 (6.5)*	4.30
3	2562.0	10.0	9.50
4	1793.0	20.0	16.86
5	175.9	20.0	0.60
6	9.0	300.0	N/A
7	7.0	20.0	N/A

*) Value in brackets is the original volume of the fraction. The conductivity is based on the total volume, which is adjusted for in the concentration.

6.1.4 Qualitative proof for active loading

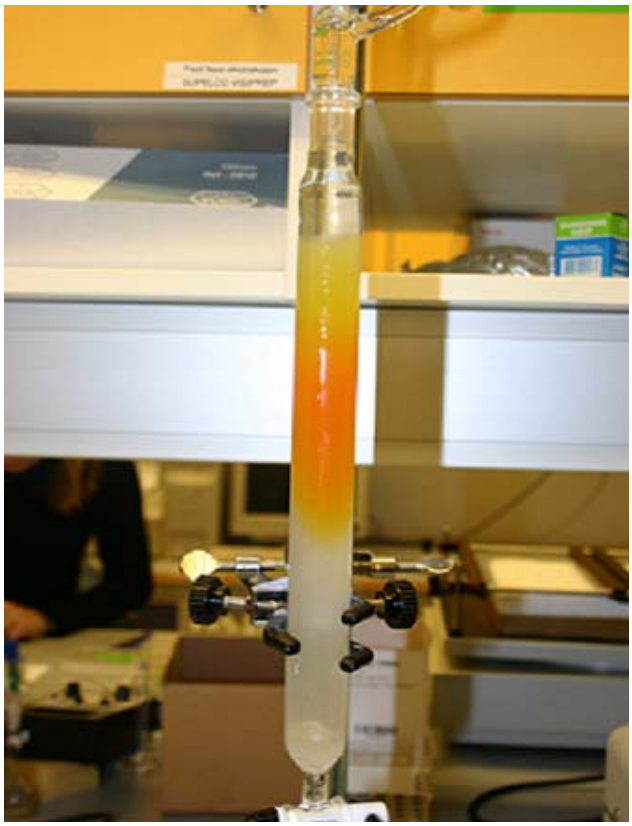
In order to check whether an ammonium sulphate gradient was established after the removal of external ammonium sulphate removal in the liposome fraction, liposomes were incubated with saturated acridine orange solution for 10 and 30 minutes, respectively. The loaded liposomes were separated from free acridine orange by SEC in the same manner as for the separation of external ammonium sulphate.

The collected fractions from the two different incubation conditions with acridine orange during separation are listed in Table 14.

Table 14: Separation results after acridine orange loading

Fraction	**10 minutes incubation**		**30 minutes incubation**	
	Volume (ml)	**Time (s)**	**Volume (ml)**	**Time (s)**
50 g/L glucose (void)	16.5	155	17.6	160
Liposome fraction	22.0	110	23.0	140
50 g/L glucose (fraction in between)	14.2	65	13.0	60
Free acridine orange fraction (excess)	22.4	120	28.0	130

A yellowish liposome fraction would indicate successful loading of acridine orange into liposomes. The visual results indicated successful loading of acridine orange both after 10 minutes and 30 minutes incubation as it can be seen from Picture 3. The orange band in the middle contains the fraction of liposomes loaded with acridine orange, whereas the free acridine orange is still within the top part of the column.



Picture 3: SEC of liposomes loaded with acridine orange

6.2 Stability of ammonium sulphate solution during heating

Since dissolving liposomes may require heating of the dispersion0, it was first examined, whether ammonium sulphate was stable at elevated temperatures. Since ammonia is known to evaporate easily and the concentration of ammonium sulphate is directly related to conductivity, the conductivity upon incubation at different temperatures was measured.

The standard curve, ranging from 0.12 mM to 120 mM (see section 5.5.2.1), was used as a reference in determining the concentration of ammonium sulphate. The conductivity of freshly prepared 120 mM ammonium sulphate solution was measured at 0.5 ˚C yielding a conductivity value of approximately 12 mS/cm. To discover the effect of different temperatures on ammonium sulphate, the conductivity of ammonium sulphate solutions was measured at different temperatures over time. This was performed in glass bottles without lids at 0.5 ˚C as well as at 60 ˚C. The solution incubated in an ice bath at 0.5 ˚C for 40 minutes had a conductivity of 12 mS/cm. The solution incubated at to 60 ˚C for 40 minutes (and

cooled down to 0.5 ˚C) showed a conductivity of 11.7 mS/cm, hence a small reduction in concentration upon air and temperature exposure.

6.3 Standard curve for quantification of remaining non-entrapped ammonium sulphate in the liposome fraction

The standard curve of ammonium sulphate in 50g/L glucose, described in section 5.5.2.1, was found not to be fully adequate for quantification of remaining non-entrapped ammonium sulphate due to low conductivity of some of the diluted samples. These samples had conductivities below 181.59 μS/cm.

The dilution factor typically occurring in the liposome fraction was derived from the experiments and a new standard curve with a more appropriate concentration range was made. The dilution steps gave rise to a dilution factor of 70 giving a theoretical maximum concentration of ammonium sulphate of 1.714 mM. A maximum concentration of 5 mM was included as the highest concentration in the standard curve by including some tolerance. Furthermore, this standard curve was obtained at a temperature of 30 ˚C. This was done because of problems in dissolving liposomes at 0.5 ˚C and since ammonium sulphate showed to be stable under the applied heat treatment. It required less time to heat samples up to 30 ˚C compared to cooling them down to 0.5 ˚C and the temperature could be easier adjusted to exactly 30 ˚C compared to 0.5 ˚C.

The conductivity measurements are listed in Table 15 and Figure 11 shows the standard curve.

Table 15: Conductivity measurements for standard curve of ammonium sulphate in 50 g/L glucose

Conc (mM) of $(NH_4)_2SO_4$ in 50 g/L glucose	Conductivity parallel 1 (µS/cm)	Conductivity parallel 2 (µS/cm)	Conductivity parallel 3 (µS/cm)	SD of triplicate
5.0	1337.0	1340.0	1341.0	2.1
2.5	696.0	698.0	6970.0	1.0
1.25	360.0	361.0	361.0	0.6
0.625	186.9	187.4	187.5	0.3
0.3125	97.7	97.3	97.1	0.3

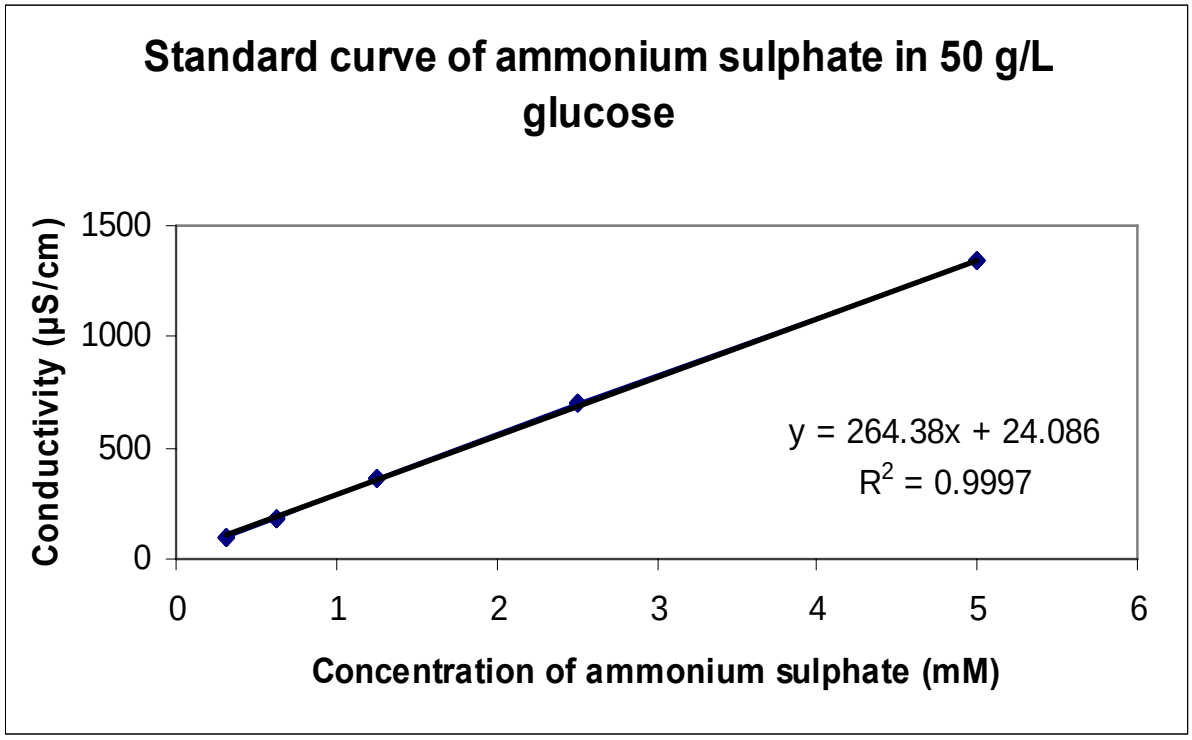


Figure 11: Standard curve for ammonium sulphate in 50 g/L glucose solution

This standard curve of ammonium sulphate in 50 g/L glucose solution revealed a good linear fit with a R^2 value of 0.9997.

6.4 Quantification of entrapped ammonium sulphate

The amount of entrapped ammonium sulphate in liposomes should be determined in order to control the encapsulation process and to evaluate the magnitude of the established transmembrane gradient. This was executed by dissolving the liposomes of the liposome fraction (upon gel fractionation) and measuring the conductivity of the resulting diluted solution.

Various attempts were tried for dissolving the liposomes in order to determine the ammonium sulphate concentration by conductivity measurement. In order to eliminate the possibility of influences on the results by dissolved E-80 and the dissolving agent, all components used in experiments were also included in this standard curve for dissolved liposomes. Thus each attempt required a new standard curve.

6.4.1 Standard curve for quantification of entrapped ammonium sulphate in the liposome fraction upon disintegration with ethanol

Attempts were made to dissolve liposomes by addition of ethanol and heating of the sample up to 30 ˚C. Initially, one part of the liposome fraction was dissolved in nine parts of 96 % ethanol giving a ratio of 1:10. The disappearance of turbidity indicated that the liposomes were dissolved. Then, a standard curve was established, where the composition of the standards was identical to those of the samples i.e. in accordance to the dilution factors. This was achieved during preliminary experiments as follows:

142.86 mg E-80 dissolved in
90.0 ml of 96 % ethanol and
10.0 ml ammonium sulphate in 50 g/L glucose solution in concentrations as lined out below in Table 16.

Table 16: Conductivity measurements of entrapped ammonium sulphate in the liposome fraction dissolved by ethanol

Conc (mM) of $(NH_4)_2SO_4$	Conductivity parallel 1 (µS/cm)	Conductivity parallel 2 (µS/cm)	Conductivity parallel 3 (µS/cm)	SD of triplicate
3	88.7	88.7	88.7	0.0
2	79.4	79.3	79.2	0.1
1	49.1	49.1	49.1	0.0
0.5	32.1	32.0	32.1	0.1
0.25	22.3	22.4	22.3	0.1
0.125	16.3	16.3	16.3	0
0.0625	13.3	13.3	13.3	0

The solution containing 3 mM ammonium sulphate turned out to be turbid at 30 ºC indicating precipitation, most probably of ammonium sulphate. Although the less concentrated standard solutions appeared clear, a small amount of particles was visible upon close inspection. The appearance of these particles led to an uncertainness whether it was dust particles or precipitating ammonium sulphate.

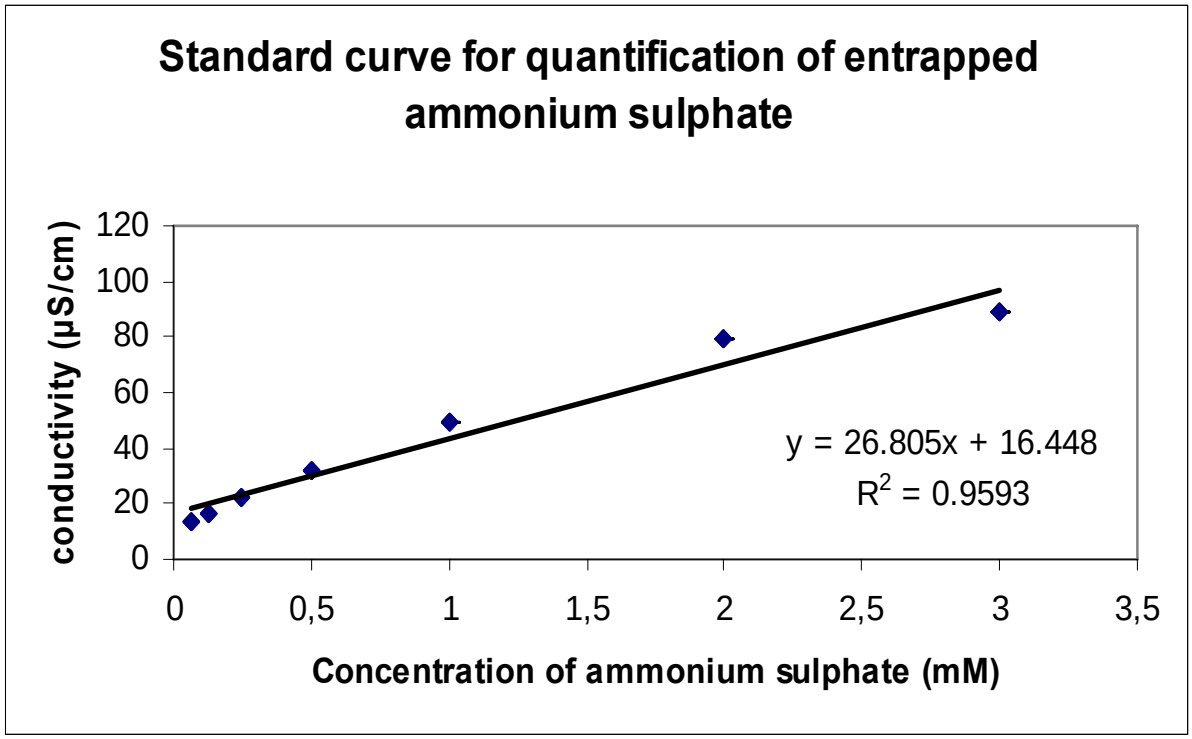


Figure 12: Standard curve for liposomes dissolved with ethanol

The graph in Figure 12 demonstrates a poor linear fit most likely due to precipitation of ammonium sulphate. The conductivity does not appear to increase linearly with increasing ammonium sulphate concentrations. This is contrary to what is expected and thus responsible for the poor linear fit. Furthermore, the fitted line of the standard curve would cross the y-axis at approximately 16 µS/cm instead of going through the zero-point. This observation also confirms a deviation from a linear fit. This means utilising this standard curve would give rise to misleading concentrations in the whole range, from very small concentrations to very large concentrations.

In the same manner, two new standard curves were executed with a decreased amount of ethanol. Instead of a liposome fraction: ethanol ratio of 1:10 the solutions had ratios of 1:7 and 1:5 (results not shown). The results were comparable to the above findings with the ratio of 1:10 with 96 % ethanol, i.e. the occurrence of ammonium sulphate precipitation. For this reason 96 % ethanol was ruled out as a suitable agent for disintegration of liposomes.

6.4.2 Quantification of entrapped ammonium sulphate in the liposome fraction upon disintegration with triton solution

In the next attempt Triton X-100 was used instead of 96 % ethanol. Triton or 2-methyl-1,3,5-trinitro-benzene is a non-ionic detergent. The liposome fraction was initially dissolved by addition of a 10 % triton solution in a 20:1 ratio. Cholesterol containing liposomes underwent a temperature treatment of 80 ˚C to facilitate disintegration whereas liposomes composed of only E-80 were dissolved at room temperature.

To check whether triton solution had an influence on the measured conductivity of the fraction containing the destroyed liposomes, the conductivity of the 10 % triton solution alone was measured. The solution of 10 % triton revealed a conductivity of 48 µS/cm at 0.5 ˚C. This means the conductivity of the triton solution was unexpectedly high, in fact in the same magnitude as the conductivity of ammonium sulphate from the liposome fraction.

In order to reduce the contribution of the triton solution to the overall conductivity, smaller amounts were tried for dissolving the liposomes. The liposome fraction, containing liposomes composed of E-80 and cholesterol, was therefore mixed with different amounts of triton solution:

1:20 1 ml liposome fraction and 19 ml 10 % triton solution
1:10 2 ml liposome fraction and 18 ml 10 % triton solution
1:5 4 ml liposome fraction and 16 ml 10 % triton solution
1:1 10 ml liposome fraction and 10 ml 10 % triton solution

As a reference, solutions of 50 g/L glucose with the same fractions of triton solution were prepared. In this way variations in the conductivity of the broken liposomes could be identified and thus a suitable amount of triton solution detected.

1:20 1 ml 5% glucose and 19 ml 10 % triton solution
1:10 2 ml 5% glucose and 18 ml 10 % triton solution
1:5 4 ml 5% glucose and 16 ml 10 % triton solution
1:1 10 ml 5% glucose and 10 ml 10 % triton solution

All solutions were preheated to 80 ˚C for 30 minutes before measurements and all solutions appeared to be clear when measured, which indicated disintegration of the liposomes. The results of conductivity measurements are listed in Table 17.

Table 17: Disintegration test. Disintegration of liposome fraction in various amounts of triton solution

	Conductivity µS/cm			
	1:20	**1:10**	**1:5**	**1:1**
Liposome fraction + triton solution	53.5	56.8	58.2	86.3
Reference (50 g/L glucose + triton solution)	47.1	45.1	41.6	28.5
Liposome fraction – reference (pr ml)	6.40	5.85	4.15	5.78

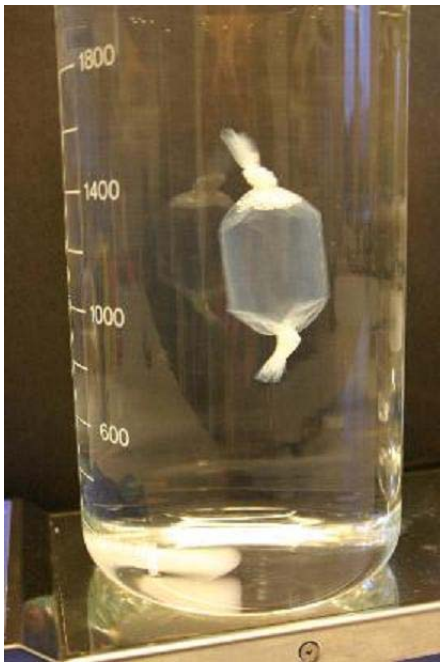
The contribution of the triton solution to the overall conductivity was regarded as unacceptable for all the investigated mixing ratios.

The conductivity of triton solution from different batches and producers may vary due to the presence of salt residues from production. In addition, the 10 % triton solution (with a conductivity of 108 µS/cm at 30 ˚C) used for the experiments was produced in house one year ago and could have been suspect for contamination over the past year. Therefore, new 10 % solutions were prepared and their conductivity measured. Triton X-100 from Sigma and Merck was used in the solutions giving conductivities of 410 µS/cm and 140 µS/cm, respectively. Triton X-100 should display the same conductivity regardless of the choice of manufacturer. However, the presence of salt residues leading to variations in conductivity of the raw material has shown to be evident. Therefore, as a result of these findings, it was decided to undertake dialysis of triton solution.

6.4.2.1 Dialysis treatment of triton solution

A dialysis treatment of the 10 % triton solution was conducted trying to reduce its conductivity by removing any possible salt residues. To maximise the dialysis effect a dialysis bag containing 20 ml of 10 % triton solution was placed in two litres of distilled water

overnight in order to generate a sink being 100 fold greater than the sample solution. This can be seen in Picture 4.



Picture 4: Dialysis treatment of 10 % triton solution

The dialysis treatment turned out to have a large impact on the measured conductivity of the 10 % triton solution. The conductivity was reduced to 4 μS/cm. However, the concentration of the solution is also influenced by dialysis, becoming slightly lower due to water molecules diffusing into the solution through the semipermeable membrane. This did not impose a problem for the experiments since 10 % triton solution was used for destruction of liposomes in excess amounts, and its exact concentration had no relevance.

6.4.2.2 Standard curve for quantification of entrapped ammonium sulphate in the liposome fraction upon disintegration with dialysed triton solution

The liposome fraction was dissolved with dialysed triton solution giving a ratio of 1:1. At this point, only liposomes containing E-80 were used for experiments and therefore a dilution factor of 1:1 seemed sufficient in order to dissolve the liposomes. The composition of the

samples for the standard curve was determined in the same manner as for the samples containing ethanol.

Accounting for the dilution step by the cation exchange column (1:7), the total dilution factor was 1:14. The conductivity measurements are given in Table 18 whereas the standard curve is illustrated in Figure 13.

Table 18: Conductivity measurements of entrapped ammonium sulphate in the liposome fraction dissolved by triton solution

Conc (mM) of $(NH_4)_2SO_4$	**Conductivity parallel 1 (µS/cm)**	**Conductivity parallel 2 (µS/cm)**	**Conductivity parallel 3 (µS/cm)**	**SD of triplicate**
10	2550.0	2550.0	2510.0	23.1
7.5	1970.0	1940.0	1960.0	15.3
5	1340.0	1330.0	1350.0	10.0
2.5	741.0	740.0	742.0	0.6
1.25	418.0	419.0	419.0	0.6
0.625	254.0	253.0	251.0	1.5
0.3125	167.0	167.0	166.0	0.6

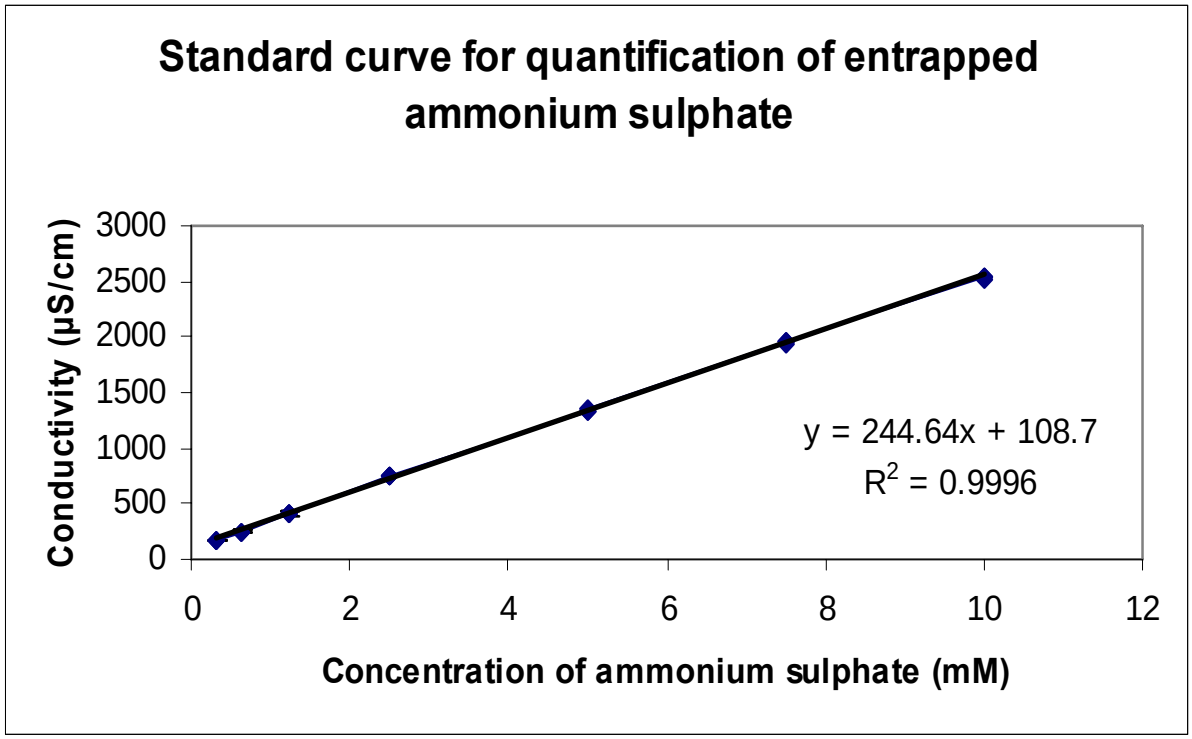


Figure 13: Standard curve for quantification of entrapped ammonium sulphate in liposomes. Dissolved with triton solution

The standard curve displays a good linear fit with a R^2 value of 0.9996. This implies the absence of ammonium sulphate precipitation by using 10 % triton solution in dissolving liposomes. The detergent has proved to be successful in dissolving the liposomes with minimal influence on the conductivity.

6.5 Method validation of quantification of ammonium sulphate inside the liposomes

Upon establishment of an appropriate disintegration method the quantification of ammonium sulphate within the liposomes could be determined. After various attempts, the development of a method was completed.

6.5.1 Determination of the recovery

In order to get reliable results, the recovery of the SEC should be close to 100 %. 5.0 ml liposome dispersion, containing 600 µmol ammonium sulphate, was added to the gel column.

By collecting all fractions from the gel column the recovery of the originally 600 µmol ammonium sulphate was determined (Table 19).

Table 19: The table contains the amount of ammonium sulphate in fractions of the column

Fraction	**Conductivity (µS/cm)**	**Volume (ml)**	**Conc of $(NH_4)_2SO_4$ (mM)**	**Absolute amount of $(NH_4)_2SO_4$ (µmol)**
Void volume	9.0	22.0	N/A	N/A
Liposome fraction	572.0	25.0	2.07	51.8
Dissolved liposome fraction	650.0	25.0 (50.0)	2.37 (4.74)	118.5
Combined fraction just prior and after liposome fraction	394.0	20.0 (6.5)*	4.3	28.0
3	2562.0	10.0	9.5	95.0
4	1793.0	20.0	16.9	337.2
5	175.9	20.0	0.6	12.0
6	9.0	300.0	N/A	N/A
7	7.0	20.0	N/A	N/A
Σ $(NH_4)_2SO_4$ in all fractions (mg)				**591**

Values in brackets are adjusted for dilution steps.

A total amount of 591 µmol ammonium sulphate was recovered from the various fractions from the column as seen from the table. This corresponds to a recovery of 98.4 %. The high recovery, together with the reproducible and reasonable encapsulated amount of ammonium sulphate of the liposome fraction, was considered satisfactory and thus accepted as constants throughout the experiments.

6.5.2 Quantification of entrapped ammonium sulphate

The amount of substance of entrapped ammonium sulphate was established from the conductivity and thus concentration of the dissolved and the undissolved liposome fraction.

The difference of the amounts of substance between the undissolved liposome fraction and the dissolved liposome fraction gave an amount of 66.8 μmol. This amount represents the amount of substance of entrapped ammonium sulphate. The gradient was determined by dividing the concentration of external ammonium sulphate in the liposome fraction with the concentration of encapsulated ammonium sulphate. This yielded a gradient of about 1:58.

A gradient of 1:58 is quite small with respect to gradients published in the literature. Haran et al. achieved a 1000 fold gradient, however, by using other lipids. (24) Liposomes composed of E-80 are expected to result in phospholipid membranes not as densely packed compared to membranes containing cholesterol. Therefore, upon establishment of the gradient, ammonium sulphate may start to diffuse through the membrane according to its low tightness. This may be one explanation for the small gradient of ammonium sulphate between the outer and inner phase of E-80 composed liposomes. In order to reduce the magnitude of the leakage through the membrane, SEC, loading and cationic exchange was performed on the same day. Another possible explanation for the small gradient is the relatively high remaining ammonium sulphate concentration in the external medium of the liposome fraction upon size exclusion chromatography. As demonstrated in table 10 a further reduction in remaining ammonium sulphate in the external medium of the liposome fraction would probably be achieved by further reducing the sample volume applied to the column.

Comparing the absolute amount of entrapped ammonium sulphate to the absolute amount of total ammonium sulphate retained from the column gave an EE of 11.30 %. An EE of the magnitude of 11.30 % seems reasonable considering the mean size and lipid concentration of the liposome dispersion.

It is widely recognised that EE decrease with decreasing liposome size (4, 27) and decreasing lipid concentration. (37, 38) Berger et al found that liposomes with size ranging from 400 nm to 50 nm, led to an EE varying from approximately 40 % to 10 %, respectively. (37) Liposome size of 100 nm, as used in these experiments, had an EE of approximately 15 %. However, 15 % lipid concentration was used in the research of Berger et al. (37) Only 10 % lipid concentration was used in these experiments and could therefore explain the slightly deviating EE.

6.5.3 Entrapped volume of liposomes

From the EE the actually entrapped volume of the 5.0 ml fraction can be determined. This gave an encapsulated volume within the liposomes of 0.565 ml.

The encapsulated volume of 0.565 ml deviates greatly from the theoretically encapsulated volume of 1.5 ml (out of 5.0 ml) derived from the 30 % EE obtained by conductivity differences in section 6.1.2. A plausible explanation for this variation could be the effect of liposome size. Lidgate, Hegde and Maskiewicz prove MLVs are associated with larger reductions in conductivity compared to SUV. (38) This means a smaller volume gets entrapped within SUVs compared to MLVs. Lidgate, Hegde and Maskiewicz found the captured volume could be calculated according to liposome size and amount of lipid present. (38) For MLVs the captured volume was 2.5 µl per mg lipid. This would give an EE of 25 % in these experiments. The captured volume for SUVs was 0.96 µl per mg lipid yielding an EE of 9.6 %. Giving this information, it could be expected the EE of 30 %, obtained by the reduced conductivity, was reflecting a liposome dispersion consisting of MLVs whereas the calculated EE of 11.30 % was reflecting a liposome dispersion consisting of SUVs. However, this is inexplicable since the conductivity measurements of the liposome dispersion were conducted after filter extrusions were carried out.

The amount of substance of entrapped ammonium sulphate and the gradient was considered constant for all liposome fractions. Further, the amount of substance of entrapped ammonium sulphate is used for calculating active loading efficiencies.

6.6 Loading of gemcitabine

Both passive and active loading of dFdC into the interior aqueous compartment of liposomes was performed in order to compare the end results. The two methods have different variables such as lipid concentration and incubation conditions. However, the methods can be compared in the amount of encapsulated dFdC, EE and the ratio of dFdC: lipids.

6.6.1 Passive loading

The results from the HPLC determination of passively loaded dFdC are listed in Table 20.

Table 20: Detected concentrations of dFdC from passive loading

Sample	Conc run 1 (ng/ml)	Conc run 2 (ng/ml)	Mean conc of triplicate (ng/ml)
T1	2701.9	2701.9	2795.2
T2	2913.4	2916.5	
T3	2768.8	2768.8	
E1	566.1	567.2	580.3
E2	581.1	580.5	
E3	593.1	593.5	

T = Total liposome dispersion (before removal of non-entrapped dFdC)
E = Liposome dispersion after IEC-removal of non-entrapped dFdC

The samples designed E had undergone cation exchange chromatography followed by disintegration and revealed the encapsulated amount of dFdC within the liposomes. The samples designed T did not undergo cation exchange chromatography and revealed the total amount of gemcitabine in the dispersion.

6.6.1.1 Determination of amount of substance of dFdC

The addition of 500 µl Gemzar solution containing 72.2 µmol dFdC to the VPG led to an average detected amount of 67.6 µmol in the fraction (equation 6), which correlates to a percentage recovery of 93.65 % (Sample T).

Equation 6: $$\mathrm{dFdC}_{\mathrm{Vtot}}\ (\mu\mathrm{mol}) = \frac{\mathrm{dFdC}_{\mathrm{detected}}\ (\mu\mathrm{g/ml}) * 600 * \mathrm{V}_{\mathrm{tot}}\ (\mathrm{ml})}{\mathrm{MW}\ (\mu\mathrm{g}/\mu\mathrm{mol})}$$

(600 = dilution factor)

6.6.1.2 Encapsulation Efficiency:

For sample E the amount of substance of encapsulated dFdC was determined to be 14.0 µmol, according to equation 6. This gives an EE of 20.7 %. An EE of 20.7 % is smaller than previously published values. According to Moog et al, an EE of 33.2 ± 4.2 % was achieved for VPG produced of hydrogenated egg PC and cholesterol. (15)

Several effects might explain the lower EE. The difference in the liposome sizes between the VPG seems to play a major role. The homogenisation of the egg PC (E-80) lipid dispersion obviously leads to smaller particle sizes than homogenised hydrogenated egg PC/cholesterol-liposomes described by Moog et al. Moog et al described that the VPG contained vesicles of 60 to 80 nm in diameter, (15) while the largest fraction (79.6 - 99.1%) of the liposomes in our experiment had a smaller diameter of 21.5 to 37.9 nm. It is well recognised that a bigger aqueous core will result in a larger EE of hydrophilic compounds.

The smaller EE might as well be explained by the composition of the liposomes that differs in the type of egg PC used and addition of cholesterol. The VPG prepared by Moog et al contained cholesterol which leads to tighter packed membranes and therefore a reduced leakage compared to lipid membranes composed of E-80 alone. A rapid leakage of dFdC during cation exchange separation might have affected the EE negatively in our case.

6.6.1.3 Ratio of amount of encapsulated dFdC per amount of lipid

The required amount of lipid per dFdC must be determined for receiving information about the quality of encapsulation.

An amount of 14.0 µmol dFdC was encapsulated within the densely packed VPG. A concentration of 40 % E-80 with an average MW of 781g/mol (39) yields a theoretical amount of 1900 µmol PC in the sample of 3.71 g. Hence, 135.7 µmol of lipids are required in order to encapsulate one µmol of dFdC.

6.6.2 Active loading

Different incubation conditions for active loading of dFdC into E-80 liposomes were tried experimentally. Reconstituted Gemzar, containing 38 mg/ml dFdC, was used for the active loading experiments. 5.0 ml liposome fractions, from the gel column, were mixed with different amounts of dFdC solution. Upon completion of incubation, 0.5 ml of the sample was transferred to the ion exchange column in order to remove non-entrapped dFdC from outside the liposomes according to 5.5.5.2.

The amount of encapsulated ammonium sulphate in the liposome fraction was experimentally determined only once as described in section 6.5.2 and was considered constant for all subsequent samples. But the slightly varying volumes of the liposome fractions upon SEC were taken into account and the amounts of dFdC added to the samples were adjusted accordingly. The results of active loading experiments at different incubation conditions are summarised in Table 21.

Table 21: Results from different incubation conditions of active loaded samples with dFdC

Sample	Incubation condition	$dFdC_{detected}$ (µmol)	$dFdC_{entrapped}$ (µmol)	Recovery (%)	EE (%)	$dFdC_{entrapped}$ per $(NH_4)_2SO_{4\ encap}$ (%)	dFdC (mol):lipid (mol)
A	3 h in RT	7.13	0.23	81.95	3.25	1.70	1:551.7
C	2 h 45 min RT, 15 min 60 ˚C	7.14	1.14	82.07	15.97	8.50	1:112.3
F	48 h RT	7.18	1.20	87.56	16.71	9.50	1:100.0
G	5 x ↑ conc, 24 h RT	35.08	4.52	85.77	12.88	35.89	1:26.6
K	5 x ↑ conc, 24 h RT	36.86	7.62	91.24	20.67	61.63	1:15.6
O	5 x ↑ conc, 24 h RT	33.45	6.18	82.80	18.48	50.02	1:19.3
H	2 h 60 ˚C, 22 h RT	6.88	1.33	83.41	19.33	10.56	1:90.2
J	2 h 60 ˚C, 22 h RT	6.84	1.95	84.44	28.51	15.78	1:61.0
N	2 h 60 ˚C, 22 h RT	6.54	1.47	80.70	22.45	11.87	1:81.1
B	1 h RT, 2 h 60 ˚C	7.02	1.08	82.76	15.00	8.09	1:118.5
L	1 h RT, 2 h 60 ˚C	6.80	1.28	83.95	18.82	10.36	1:93.0
P	1 h RT, 2 h 60 ˚C	6.04	1.11	74.57	18.38	9.98	1:107.2
D	24 h RT	7.50	1.33	86.21	17.73	9.96	1:96.2
E	24 h RT	6.58	0.96	80.24	14.59	7.62	1:125.0
I	24 h RT	7.17	1.67	88.52	23.29	13.51	1:71.3
M	24 h RT	6.55	1.29	80.86	19.69	10.44	1:92.3

RT = room temperature
↑ = increase in

6.6.2.1 Incubation conditions and interpretation of the results:

The samples were incubated according to the conditions listed in Table 21. All incubation conditions were run in triplicates except the preliminary runs A, C and F, which were run only once.

Using equation 6, a recovery range of 74.57 % to 91.24 % of the added dFdC was obtained by HPLC for the actively loaded samples. Taking into account eventual preparation errors during this rather complicated multi-step procedure, this appears acceptable. As seen from Table 22 the recovery within parallels has a SD ranging from 1.93 to 5.11. Table 22 also lists the mean parallel values of other calculated parameters.

Table 22: Mean values of parallel results from different incubation conditions of active loaded samples with dFdC

Incubation condition	**Mean $dFdC_{entrapped}$ (µmol)**	**Recovery (%)**	**EE (%)**	**$dFdC_{entrapped}$ per $(NH_4)_2SO_{4\ encap}$ (%)**	**Lipid (mol): dFdC (mol)**
5x ↑ conc, 24 h RT	6.11 ± 1.55	86.60 ± 4.28	17.34 ± 4.02	49.18 ± 12.89	20.70 ± 5.40
2 h 60 ˚C, 22 h RT	1.58 ± 0.33	82.85 ± 1.93	23.43 ± 4.67	12.74 ± 2.72	77.40 ± 14.90
1 h RT, 2 h 60 ˚C	1.16 ± 0.11	80.43 ± 5.11	17.40 ± 2.09	9.14 ± 1.14	106.23 ± 12.80
24 h RT	1.31 ± 0.29	83.96 ± 4.05	18.83 ± 3.64	10.38 ± 2.42	96.20 ± 22.10

RT = room temperature
↑ = increased

The effect of incubation time, incubation temperature and concentration of dFdC on loading was compared for the actively loaded liposomes in three different ways. Firstly, encapsulation efficiencies were compared since they reveal to what extent added dFdC got encapsulated into liposomes. Secondly, the ratio of encapsulated dFdC to encapsulated ammonium sulphate was examined. This ratio yields information about the efficiency of loading as well as to what extent the gradient was utilised for encapsulation. Finally, the theoretical amount of encapsulated dFdC with respect to the theoretical amount of lipid in the liposome dispersion was investigated in order to evaluate the quality of the liposomes as drug carriers independent of the lipid amount, and the loading method used.

Encapsulation efficiency:

The resultant encapsulation efficiencies of the active loaded samples are listed in Table 21 and Table 22. The EE was ranging from approximately 3 % to 28 %. In general these values are low as compared to results gained with other drug compounds described in literature. For doxorubicin, an EE close to 100% is described. (24) This may have different reasons. Firstly, our conditions for loading may still be suboptimal despite some attempts for achieving improvements. Secondly, doxorubicin does upon active loading readily form precipitates inside the liposomes. Precipitation of the drug leads to a more favourable concentration gradient and in addition, it immobilises the drug and prevents leakage. The rather wide fluctuation seen between parallels and the rather small differences in mean encapsulation efficiencies between the different conditions makes it difficult to see relevant differences between the different conditions.

Relationship between the amount of encapsulated dFdC and the amount of encapsulated ammonium sulphate:

All the parallels with approximately 7 µmol dFdC appear to have similar ratios of roughly 10 %, i.e. the entrapped amount of dFdC equals about one tenth of the entrapped ammonium sulphate amount on a molar basis. A five-fold increase in the concentration of dFdC as compared to other samples yielded a higher ratio of 49.18 ± 12.89 %, i.e. the entrapped amount of dFdC reached about half of the entrapped ammonium sulphate amount on a molar basis. It remains to be tested in future studies whether a further increase in dFdC would have given even better ratios.

Interpretation of dFdC:lipid ratios:

It is evident that entrapment of dFdC is dependent upon the incubation factors tested. Concentration seems to be the variable affecting the loading to the greatest extent. By a five fold increase in the added amount of dFdC to the samples, the loaded amount escalates to an almost five fold increase in the percentage relationship between dFdC and lipid. It is, however, not investigated whether the amount would increase proportionally with a further increase in concentration.

The sample designated A (incubation for three hours at room temperature) revealed an almost

five fold lower amount of encapsulated dFdC than sample C (incubation for 2 hours and 45 minutes at room temperature and 15 minutes at 60°C) and the samples D, E, I and M (incubation for 24 hours at room temperature). The active loading process thus appeared to be not yet completed after three hours at room temperature, whereas brief warming (15 minutes 60°C) or alternatively incubation for 24 hours at room temperature led to the same result. Sample F (incubation for 48 hours) showed a similar encapsulation as samples incubated for 24 hours in room temperature. The process thus seems to be completed after 24 hours at room temperature. An incubation time of 24 hours seems reasonable in order to maximise loading. Samples C on one hand and samples B, L and P on the other hand, which were incubated for different periods at 60°C (15 minutes and two hours, respectively) contained approximately the same amount of dFdC. A longer warming period, beyond 15 minutes, seems not to improve loading. The results from sample F indicated that exceeding a loading time of 24 hours did not increase loading. A shorter incubation time is characterised by a smaller amount of entrapped dFdC, and is therefore reducing loading. Correspondingly, Haran et al found that increasing the temperature was facilitating the loading. (24) However, it has not been established whether a longer incubation time than two hours at 60 ˚C would facilitate loading.

The samples with a five fold increase in concentration were the only samples where the added amount of dFdC exceeded the entrapped amount of ammonium sulphate within the liposomes and could therefore in theory utilise the ammonium sulphate gradient to the full extent assuming one mol of dFdC equals one mol of ammonium sulphate. According to this assumption, these samples made use of only approximately 50 percent of the gradient. Haran et al were measuring the concentration of ammonia diffusing through the lipid membrane. (24) They found a ratio of 4:1 between released ammonia and drug (doxorubicin) uptake. In these experiments the release of ammonia was never measured. Hence, the ratio between ammonia and dFdC and thereby the required ammonium sulphate per dFdC is unknown.

6.6.2.2 Comparison of active and passive loading

The recovery of the passive loaded sample was larger compared to the active loaded parallels for an inexplicable reason. The passive loaded liposomes had an EE of 20.71 whereas the active loaded parallels had a mean EE ranging from 17.34 to 23.43. The small difference in EE does not give any information in comparing the techniques since the concentration of dFdC and lipids in the two different methods were different. The VPG used for passive

loading was prepared from a concentration of 40 % lipids whereas the liposome dispersion used for active loading had an original concentration of 10 %. This is important since an increased lipid concentration will increase the encapsulated volume and thereby automatically the encapsulated amount of a substance. There are more lipids in the passively loaded sample compared to the active loaded sample, thus reinforcing the importance of comparing the molar ratio between dFdC and the lipids of the different loading techniques.

137.5 moles of E-80 had to be present in order to passively encapsulate one mol of dFdC. For encapsulating one mol of dFdC the active method required between 15.6 and 551.7 moles (20.7–106.2 for parallels) of E-80. This means the liposomes in all the different active loaded parallels showed a greater loading capacity, compared to the passive loaded sample. The active loaded parallel incubated for 24 hours with a five-fold increase in concentration had the best ratio of 20.7 and thus the greatest loading capacity.

In order to achieve this same ratio of 20.7 between dFdC and lipids, an EE of 91.8 % would theoretically be required for passively loaded 40 % VPG dispersion composed of E-80 in order to achieve the same ratio. The literature, however, describes an EE of only 33.2 % (15) and this means the active loaded liposomes had an even greater loading capacity than the VPG described in the literature.

6.7 Stability of dFdC encapsulated liposomes

It was investigated how long the liposomes could retain their amount of encapsulated dFdC in order to evaluate the stability of the liposome membranes. High stability is important in order to achieve a long shelf life of a product.

Sample O, displaying a large amount of encapsulated dFdC, was chosen for the stability test. Samples where the external dFdC was already separated from the liposomes on the SEC column were further incubated in a fridge for 24 to 72 hours. After appropriate incubation time, the samples were run through the SEC column for a second time in order to remove dFdC leakage through the liposome membrane during incubation. Upon destruction of liposomes with triton solution, the solution was injected into HPLC for dFdC quantification. The results from the stability tests can be seen in Table 23.

Table 23: Stability tests.

Storage time	Mean amount of dFdC in triplicates (ng/ml)
0 h	542.3
24 h	109.7*
48 h	N/A
72 h	N/A

*) The dilution step from running the sample through the ion exchange column twice is accounted for.

The results reveal 20.2 % of dFdC was retained within the liposomes, that means approximately 80 % of dFdC was leaking out of the vesicles after 24 hours. After 48 to 72 hours, the remaining amount of dFdC inside the liposomes was negligible and could not be detected.

These findings can be explained by three factors. Firstly, the liposomes were made solely of the lipid E-80. E-80 composes a lipid membrane not as tightly packed as membranes composed of other phospholipids or of phospholipids in combination with cholesterol. Secondly, the sink condition imposed upon the heavily diluted liposomes may have been a facilitating factor to the leakiness. And lastly, the literature describes an enhanced PC degradation upon loading liposomes with dFdC. (15) At what point this degradation initiates, is however not described. In addition, the effect on degradation of the larger amount of protonated dFdC within the liposomes is not discussed.

7 FUTURE REFERENCES

At the present time, utilising this newly developed method for active loading of dFdC into liposomes reveals a greater loading capacity compared to passive loading. Unfortunately, the lipid membranes reveal poor stability due to dFdC leakage. However, a few alterations to the method could possibly further increase the dFdC: lipid ratio and increase the stability and thereby increase the usefulness of the encapsulation method:

- By changing the lipid composing the membrane or by adding cholesterol the phospholipid membrane would be more densely packed preventing leakage of ammonium sulphate. This could lead to the establishment of a better gradient resulting in a greater influx of dFdC.
- Appropriate adjustment of the pH of the external aqueous phase in the liposome dispersion will cause a further shift in the equilibrium of dFdC towards its basic form, increasing the encapsulation.
- The lipid concentration of 40 % used for passive loading is much higher than the final lipid concentration of approximately 2 % for the active loading. Since a higher lipid concentration would yield the potential for a higher EE an increased lipid concentration of liposomes prepared for active loading could yield a larger entrapment.
- According to Lipoid GmbH is E-80 composed of 80 - 85 % PC and does not display a tightly bound lipid bilayer. Therefore, in the combination with cholesterol, the lipid bilayer would become more tightly packed and thus have the potential to reduce leakiness and retain a high shelf life.
- dFdC has shown to increase hydrolysis of phospholipids which again is enhanced by extremes of pH, as occurs during active loading. Therefore, it might be beneficial to formulate the liposomal dFdC as a vial kit, where dFdC is added to gradient containing liposomes in a hospital pharmacy setting just prior to administration. This will increase the shelf life of the product. Myocet (doxorubicin) is currently formulated as a vial kit utilising this approach.
- To optimise loading the following conditions should be investigated:
    - The addition of larger amount of substance of dFdC
    - Increased loading time at 60 ˚C

8 CONCLUSION

In order to investigate whether gemcitabine can be actively loaded into liposomes two goals had to be reached. Firstly, a method had to be established for generating liposomes with a transmembrane ammonium sulphate gradient. Secondly, a protocol for active loading had to be developed and the liposomes analysed in order to prove the validity of the loading method.

The method for generating an appropriate ammonium sulphate transmembrane gradient comprised preparation of liposomes in ammonium sulphate solution and removing non-entrapped ammonium sulphate by size exclusion chromatography. The transmembrane gradient was quantitatively measured upon lysis of the liposomes using conductivity measurements. 10 % triton solution was favoured as a dissolving agent due to ammonium sulphate precipitation upon disintegration with 96 % ethanol. The unacceptably high contribution of the triton solution to the overall conductivity of the lysed liposomes could be reduced by dialysis of the triton solution against excess distilled water prior to the disintegration.

Active loading of liposomes due to the ammonium sulphate gradient was demonstrated qualitatively using acridine orange as a marker and followed quantitatively with gemcitabine. Variations in the loading protocol such as concentration of gemcitabine, temperature and time of incubation were shown to influence the active loading of gemcitabine. The best results were achieved with increased concentrations of gemcitabine and incubation at an elevated temperature.

Actively loaded samples revealed a more efficient loading as compared to passively loaded vesicular phospholipid gels. However, the liposomal membranes composed of E-80 were not tight enough, as almost 80 % of encapsulated gemcitabine leaked out of the aqueous core within 24 hours.

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10 APPENDICES

Appendix 1

Continuous High Pressure Filter Extrusion - pressures and translation factors

Filter size (µm)	Applied air pressure (bar)	Resulting pressure over filter (bar)
0.8	0.75	20
0.4	0.75	20
0.2	0.75	20
0.1	1.50	60

Filter size (µm)	Applied air pressure (bar)	Resulting pressure over filter (bar)
0.8	0.75	0
0.4	0.75	0
0.2	1.00	25
0.1	1.25	50

Filter size (µm)	Applied air pressure (bar)	Resulting pressure over filter (bar)
0.8	1	0
0.4	N/A	N/A
0.2	1.5	20
0.1	1.5	30

Appendix 2

PCS results

All results are from Gaussian distributions.

1 E 80 - hand extruder

2 E 80 - continuous high pressure filter extruder

3 E 80/CH - hand extruder

4 E 80/CH - continuous high pressure filter extruder

5 VPG- high pressure homogenisation

liposome preparation	Mean SUV size of triplicate(nm)	Mean SD of Triplicate (nm)	Measurement time (min)	Approximately value of channel no 1
1	149.8	46.0	5	470 K
2	106.7	33.8	5	295 K
2	83.6	25.2	5	220 K
2	69.9	22.3	5	77 K
2	112.4	45.0	15	>1000 K
2	96.7	37.4	15	>1000 K
3	153.2	49.7	5	281 K
4	153.0	74.9	5	660 K
4	170.0	73.2	5	580 K
4	173.2	75.1	5	580 K
4	155.2	76.6	5	525 K
4	160.7	73.8	5	550 K
5	*	*	45	>1000 K

*)Homogenised VPG showed a NICOMP distribution and the mean diameter are listed below:

	Peak 1		Peak 2		Peak 3	
	Mean diameter(nm)	Percent	Mean diameter(nm)	Percent	Mean diameter(nm)	Percent
Run 1	21.5	79.6	54.7	20.3	227.3	0.1
Run 2	37.9	99.1	151.5	0.9	-	-
Run 3	35.6	98.7	136.8	1.3	-	-

Appendix 3

Calculations steps for quantifying pH and dissociation

300 µl dFdC HCl solution (pH:2.9) is added to 5 ml liposome dispersion, giving a concentration of H^+ ions:

$10^{-2.9} = 1.259 \times 10^{-3}$ mol/l

giving the amount of H^+ in 300µl (3×10^{-4}):

1.259×10^{-3} mol/l * 3×10^{-4} l = 3.777×10^{-7} mol

which leads to an amount in 5 ml:

3.777×10^{-7} mol / 5×10^{-3} L = 7.554×10^{-5} mol/L

Giving a pH of:

-log 7.554×10^{-5}

pH = 4.12

The pH of 4.12 is the pH of the outer aqueous phase. In determining this value, any influence of ammonium sulphate, glucose or lipids has not been accounted for.

56 µl dFdC HCl solution (pH:2.9) is added to 5 ml liposome dispersion, giving a concentration of H^+ ions:

$10^{-2.9} = 1.259 \times 10^{-3}$ mol/l

giving the amount of H^+ in 56µl (5.6×10^{-5}):

1.259×10^{-3} mol/l * 5.6×10^{-5}l = 7.050×10^{-8} mol

which leads to an amount in 5 ml:

7.050×10^{-8} mol / 5×10^{-3} L = 1.410×10^{-5} mol/L

Giving a pH of:

-log 1.410×10^{-5}

pH = 4.85

The pH of 4.85 is the pH of the outer aqueous phase. In determining this value, any influence of ammonium sulphate, glucose or lipids has not been accounted for.

Henderson-Hasselbalch equation:

$pH = pKa - \log([BH^+]/[B])$

For:

pH = 4.12

pKa = 3.58

$4.12 = 3.58 - \log([BH^+]/[B])$

$0.54 = -\log([BH^+]/[B])$

$-0.54 = \log([BH^+]/[B])$

$10^{-0.54} = ([BH^+]/[B])$

$0.2884 = ([BH^+]/[B])$

$[BH^+] = 0.2884 * 100\ \% / 1.2884$

$[BH^+]$ = 22.4 % dissociated

A protonation of 22.4 % of gemcitabine leaves 77.6 % in the uncharged form which can permeate the liposomal membrane.

For:

pH = 4.85

pKa = 3.58

$4.85 = 3.58 - \log([BH^+]/[B])$

$1.27 = -\log([BH^+]/[B])$

$-1.27 = \log([BH^+]/[B])$

$10^{-1.27} = ([BH^+]/[B])$

$0.0537 = ([BH^+]/[B])$

$[BH^+] = 0.0537 * 100\ \% / 1.0537$

$[BH^+]$ = 5.1 % dissociated

A protonation of 5.1 % of gemcitabine leaves 94.9 % in the uncharged form which can permeate the liposomal membrane.

Appendix 4

Results from active loading

Sample	Conc run 1 (ng/ml)	Conc run 2 (ng/ml)	Mean conc of triplicate (ng/ml)	Amount of dFdC in sample (µmol)	Amount dFdC added to sample (µmol)	Amount $(NH_4)_2SO_4$ in sample (µmol)	Amount of lipid in sample (µmol)
AT1	601.9	598.1	625.4	7.13	8.7	13.4	128
AT2	641.5	638.8					
AT3	636.2	636.1					
AE1	22.2	22.1	20.4	0.23			
AE2	19.6	20.8					
AE3	19.0	18.5					
BT1	613.1	614.6	631.1	7.2	8.7	13.4	128
BT2	646.7	648.2					
BT3	632.4	631.9					
BE1	96.3	95.6	94.4	1.08			
BE2	90.1	89.6					
BE3	97.3	97.4					
CT1	620.4	620.9	626.8	7.14	8.7	13.4	128
CT2	626.3	622.3					
CT3	636.0	634.8					
CE1	101.9	102.6	99.8	1.14			
CE2	99.2	98.3					

CE3	97.8	98.9					
DT1	664.7	668.1	653.4	7.5	8.7	13.4	128
DT2	628.1	627.6					
DT3	686.3	645.2					
DE1	115.0	114.5	116.5	1.33			
DE2	120.2	117.0					
DE3	115.6	116.7					
ET1	570.6	559.8	576.9	6.58	8.2	12.6	120
ET2	578.2	576.1					
ET3	594.6	581.9					
EE1	85.9	87.8	84.3	0.96			
EE2	84.0	84.1					
EE3	82.1	81.7					
FT1	637.0	611.2	629.9	7.18	8.2	12.6	120
FT2	650.6	626.0					
FT3	634.5	619.8					
FE1	104.6	102.1	105.6	1.20			
FE2	111.0	102.7					
FE3	109.8	103.3					
GT1	3046.1	3006.4	3077.5	35.08	40.9	12.6	120
GT2	3126.7	3100.1					

GT3	3107.6	3078.1					
GE1	397.5	394.1	396.5	4.52			
GE2	398.5	392.3					
GE3	400.6	396.1					
HT1	614.0	606.4	603.8	6.88	8.2	12.6	120
HT2	613.1	606.1					
HT3	598.0	585.3					
HE1	115.8	115.0	116.7	1.33			
HE2	116.4	115.7					
HE3	119.3	117.9					
IT1	633.3	604.2	628.8	7.17	8.1	12.4	119
IT2	636.9	618.2					
IT3	615.5	664.7					
IE1	142.4	144.5	146.5	1.67			
IE2	144.1	148.8					
IE3	150.8	148.1					
JT1	617.6	605.2	600.4	6.84	8.1	12.4	119
JT2	593.9	582.4					
JT3	591.2	611.9					
JE1	176.0	168.0	170.7	1.95			
JE2	162.5	169.6					

JE3	170.9	177.4					
KT1	3246.9	3190.7	3233.6	36.86	40.4	12.4	119
KT2	3278.2	3232.6					
KT3	3180.9	3272.4					
KE1	687.8	660.7	668.5	7.62			
KE2	655.0	674.5					
KE3	662.1	670.7					
LT1	578.6	567.4	596.4	6.80	8.1	12.4	119
LT2	619.4	615.2					
LT3	607.0	597.9					
LE1	114.4	111.8	112.2	1.28			
LE2	108.7	107.6					
LE3	115.1	115.5					
MT1	582.4	583.2	574.6	6.55	8.1	12.4	119
MT2	584.0	576.7					
MT3	562.3	558.8					
ME1	148.4	117.6	113.2	1.29			
ME2	97.1	92.0					
ME3	114.5	109.8					
NT1	589.4	590.0	573.6	6.54	8.1	12.4	119
NT2	574.4	576.8					

NT3	555.1	555.9					
NE1	126.3	126.0	128.6	1.47			
NE2	128.2	127.6					
NE3	131.9	131.8					
OT1	3005.4	2930.7	2934.2	33.45	40.4	12.4	119
OT2	2926.0	2835.466					
OT3	2971.7	2935.893					
OE1	567.1	545.903	542.3	6.18			
OE2	542.1	493.359					
OE3	556.7	548.584					
PT1	587.6	587.579	529.6	6.04	8.1	12.4	119
PT2	595.9	597.365					
PT3	607.1	606.678					
PE1	93.6	94.044	97.3	1.11			
PE2	94.8	95.894					
PE3	102.5	102.918					

Loading conditions:
A = 3 h at RT
B, L, P = 1 h at RT and 2 h at 60 ˚C
C = 2h 45 min at RT and 15 min at 60 ˚C
D, E, I, M = 24 h at RT
F = 48 h RT
G, K, O = 5 x increased concentration, 24 h at RT
H, J, N =2 h at 60 °C and 22 h at RT