Thesis for the degree Master of Pharmacy

ACTIVE LOADING OF GEMCITABINE INTO LIPOSOMES

By

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Tormund Møkleby

CONTENTS

1 ABSTRACT

Gemcitabine is a well established anticancer compound, and is in use today against several types of cancers. Gemcitabine has a short half life. Formulations of gemcitabine containing liposomes could extend it's half life, thereby maybe improving its effectiveness. Also, liposomes in the smaller size range have an advantage when it comes to treating cancer. They accumulate at the site of the tumor, and stay there for a longer time than it would have done in normal tissue(Massing and Fuxius 2000).

Previous attempts to actively load gemcitabine into liposomes have used a pH gradient with acidic pH inside compared to more neutral pH on the outside of the liposomes, accomplished by an ammonium sulphate gradient. But this approach showed some difficulties; among other things that gemcitabine had a tendency to leak out in very short time. (Gravem 2006).

In this thesis I have among other things investigated the possibility of loading gemcitabine into liposomes by precipitation. The hope was that this could give higher trapping efficiency and reduced leakage compared to the ammonium sulphate approach.

Firstly, for comparable reasons and method development, an approach to load liposomes via an ammonium sulphate gradient was tried. Thought after encountering several problems the experiment was ended without any results indicating loading, most likely due to heavily diluted liposomes.

Secondly I investigated if I could make gemcitabine precipitate. Firstly I tried a great range of different phosphate and sulphate salts, to test if any of them would cause a precipitation. Precipitation seemed to be independent of which salt used, but enhanced by factors such as high concentration of gemcitabine, alkaline conditions ($pH \geq 6$), and low temperature. The two last conditions were the total opposites of loading via an ammonium sulphate gradient, and contained several contradictions as it meant that the outer pH had to be significantly lower to avoid precipitation and that a low temperature made it harder for gemcitabine to cross the membrane of the liposomes.

An attempt to load gemcitabine into liposomes, using a pH 4 in the outer phase and pH 7 in the inner phase of the liposomes, with repeated cooling and freezing cycles, revealed poor loading. Thought optimizing conditions such as pHs, and time and temperatures in the cooling freezing cycles might enhance the loading a bit it is difficult to see this approach becoming a success.

2 ABBREVIATIONS

AO	Acredine Orange
APD	Ammonium phosphate (dibasic)
CME	Cellulose mixed ester
DAC	Dual asymmetric centrifugation
dFdC	Gemcitabine
EPC-3	Hydrogenated egg Phosphatidyl Choline
HPLC	High Performance Liquid Chromatography
MLVs	Multi Lamellar Vesicles
M_r	relative molecular weight (The ratio of the mass of a molecule, compared to 1/12 of the mass of ^{12}C)
PCS	Photon Correlation Spectrometer
PDP	Potassium dihydrogen phosphate
RPM	rounds per minute
SUVs	Small Unilamellar Vesicles
VPGs	Vesicular Phospholipids Gels

3 INTRODUCTION

3.1 Liposomes

Liposomes are small self assembling vesicles containing an inner aqueous compartment that is surrounded by a lipid bilayer of phospholipids, and often also cholesterol. Because of its properties, both having an aqueous compartment and a lipid bilayer makes it suitable for drug carriers for both hydrophilic and lipophilic drugs (see figure bellow) (Massing and Fuxius 2000).

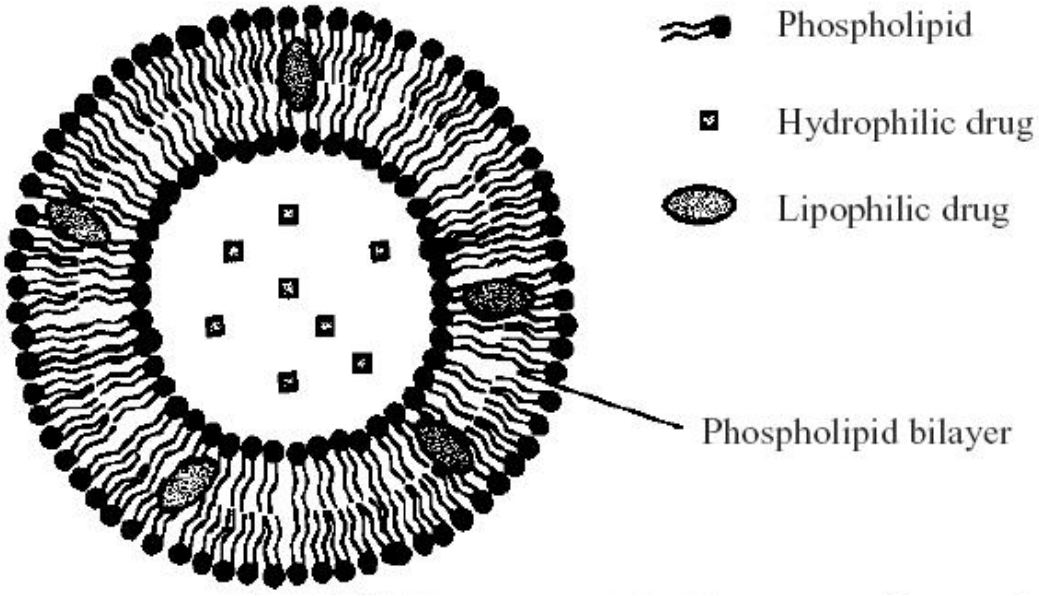


Figure 3.1. Illustration of how liposomes are suitable as drug carriers for both hydrophilic and lipophilic drugs. (taken with permission from (Massing and Fuxius 2000))

3.2 Vesicular phospholipids gels

Vesicular phospholipids gels (VPGs), are concentrated liposomal dispersions, with high lipid content, and the water content entrapped within the vesicles is about the same as the water content on the outside (Brandl, Drechsler et al. 1997). The morphology of VPGs were first described by (Brandl, Drechsler et al. 1997). When redispersing VPGs in water, they form conventional liposomes (Brandl, Drechsler et al. 1998).

3.3 Gemcitabine into liposomes

3.3.1 Gemcitabine mechanism of action

Gemcitabine (dFdC) is a nucleoside-analogue of the pyrimidine type. It's a prodrug that is converted intracellulraly to its active metabolite difluorodeoxycytidine di- and triphosphate (dFdCDP and dFdCTP). dFdCDP possess anti cancer activity by inhibiting ribonucleotide reductase, and thereby decreasing the deoxynucleotide pool available for DNA synthesis. dFdCCTP gets incorporated in DNA and results in DNA strand termination and apoptosis. (N.N. 2009a)

3.3.2 Property's of gemcitabine, and the advantage of a liposomal formulation

As mentioned above gemcitabine is a prodrug, which is activated to its active metabolite intracellurlaly. Its half-life in the body is relatively short, only between 42 and 94 minutes, depending on gender and age (N.N. 2009c). As any drug entrapped inside a liposome vesicle would be protected against metabolic breakdown and elimination, liposomes could enhance the short half-life of gemcitabine. Liposomes also serve several other beneficial properties, among other things liposomes in the smaller size range (up to a diameter of 400-600 nm) will have enhanced permeability and retention effect at the site of the tumor (see figure bellow). This is because of the special characteristics this tissue holds that differs from that in normal healthy tissue; the blood vesicles in tumor sites are leakier due to their accelerated growth to enable rapid tumor growth, and the cells are often not as densely packed as cells in healthy tissue. In addition the lymphatic system is often less expressed in tumor tissue. (Massing and Fuxius 2000; Brandl 2001).

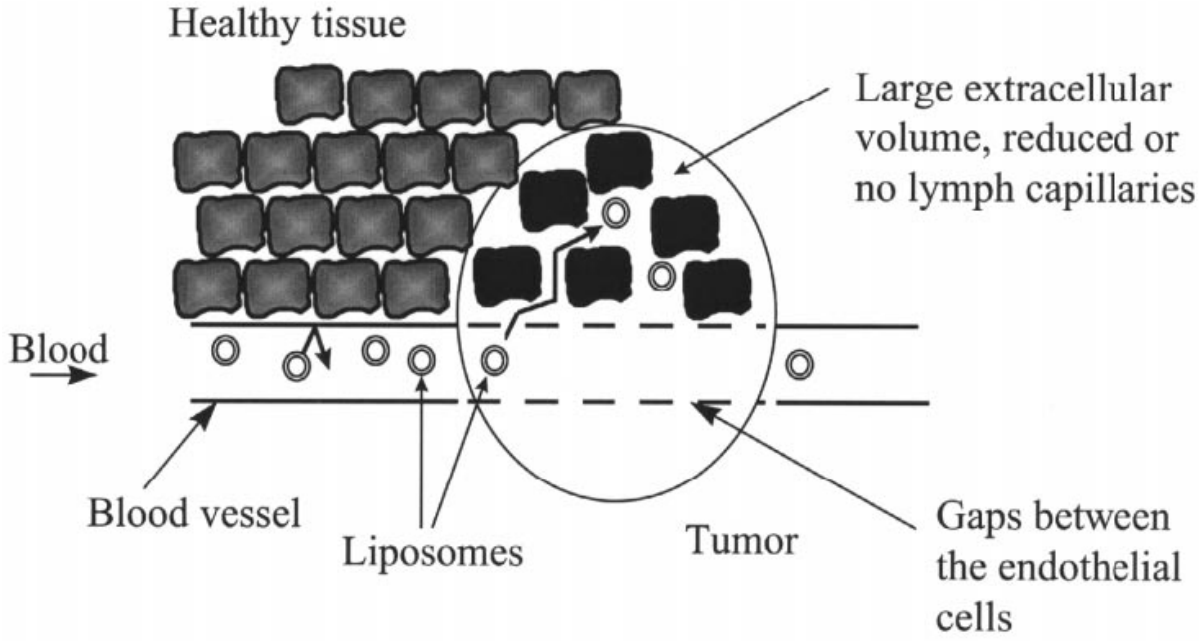


Figure 3.2. Illustration of gemcitabines enhanced permeability and retention effect in it tumor-tissue (taken with permission from (Massing and Fuxius 2000))

3.3.3 Previous attempts to load gemcitabine into liposome's

There has been made some attempts to make gemcitabine containing liposomes, though none of them has ever reached clinical trials. One problem with dFdC is that the small dFdC molecules has shown efflux very rapidly out of the vesicles (Brandl and Massing 2003). dFdC also induce hydrolytic degradation of phospatidylcholine. This degradation occurs especially at higher concentrations of gemcitabine, elevated temperatures, or extreme pHs. (Moog, Brandl et al. 2000).

In an approach to avid the above problems and increase shelf life one approach entrapping dFdC passively into VPGs were carried out by (Brandl and Massing 2003). The loading efficiency was about 35% (so solution also contained 65% non entrapped drug). Since the VPGs were not diluted until directly before use this gave a shelf life of >14 months. Testing of the formulation in mice showed promising results. It was never tested in clinical trials in humans.

In an approach loading dFdC into liposomes actively by (Gravem 2006), an ammonium sulphate gradient was used (mechanisms described further below). Loading efficiencies ranged from 3% to 28%. The liposomes showed pure stability, with 80% of dFdC-content leaking out within 24 hours.

3.4 Active loading

3.4.1 Loading drugs using a transmembrane ammonium sulphate gradient

Gemcitabine (pKa 3.58), and the model substance Acredine Orange (pKa 10.45), are weak bases(N.N. 2005; Barenholz 2007). When the pH in a solution is lower then the pKa, more then 50% of the drug will be on its protonated form. The amount of protonated base can be calculated according to Henderson-Hasselbalch equation:

Equation 1: $$pH = pKa - \log\frac{[BH^+]}{[B]}$$

If we want to solve the equation with consideration on the amount of protonated base (BH^+), the equation would look like this:

Equation 2: $$\log\frac{[BH^+]}{[B]} = pKa - pH$$

The idea with a transmembrane pH-gradient is that the neutral (not protonated) form of the drug has the ability to penetrate the liposome membrane, while the protonated form of the drug doesn't. So if the pH inside the liposome is low enough to protonate a high amount of the basic drugs inside the liposomes, and the pH outside is likewise higher, the drug will accumulate on the inside of the liposomes. The liposome formulation of Doxorubicin, Doxil®, is an example of a drug actively loaded by a pH gradient, by a so-called ammonium ion gradient. (Barenholz 2007).

The ammonium ion can dissociate to form ammonia and protonated hydrogen (see equation 3). This dissociation is pH-dependent, so when a week base such as dFdC enters the liposome and binds H^+ the pH will be stabilized because then more NH_4^+ can dissociate to ammonia. The ammonium ion is basically trapped inside the liposome, while the non-ionic ammonia has a very high permeability coefficient. The leaving NH_3 leaves a H^+ behind, and thereby the pH inside the liposomes is lowered. (Barenholz 2007)

Equation 3: NH_4^+ **(aq)** $\leftrightarrow$ NH_3 **(g)+** H^+ **(aq)**

(the equation being displaced to the left at low pH, and to the right at high pH values)

3.5 Dual asymmetric centrifugation

Dual asymmetric centrifugation (DAC), also called speed-mixing, is a technique for blending highly viscous samples. Its use for producing liposomes was described by (Massing, Cicko et al. 2008). DAC differs from normal centrifugation, in which the vial not only rotates around one rotation axis, but also around its own center. So instead of the sample material being pushed outwards, as in a normal centrifuge, the additional rotation pushes the sample to the center of the vial (see figure 5.1). This combination of two rotating forces makes DAC a very good homogenizer.

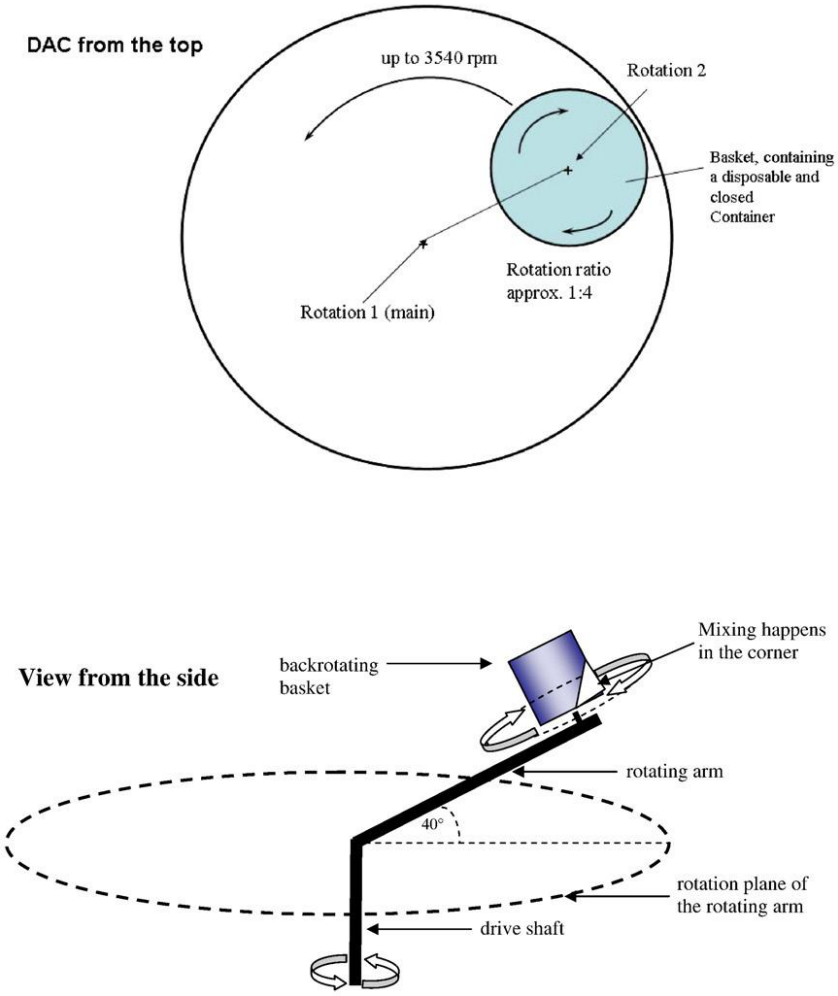
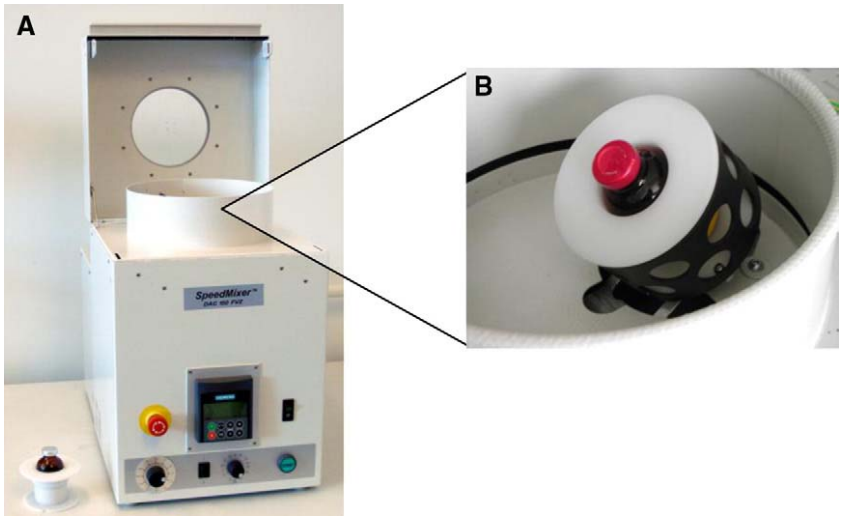


Figure 3.3. Schematic drawings of the principle of dual asymmetric centrifugation, (taken with permission from (Massing, Cicko et al. 2008))

Viscous liposome formulations are very suitable for being made on DAC. Therefore it is ideal to produce VPGs.

Liposomes produced by DAC have shown to be able to produce SUVs in a highly reproducible manner. The fact that it needs only one step, and that it is able to make very small batch sizes, makes it very suitable for experimental purposes.

(Massing, Cicko et al. 2008)



Figur 3.4. The speed mixer and a picture of the vial placed inside it. (Taken with permission from (Massing, Cicko et al. 2008))

4 AIM

The aim of this study was to investigate different methods for actively loading gemcitabine, an anti cancer agent, into liposomes. The gold was to obtain a high trapping efficiency, and long shelf life.

The primary aim for this study was to investigate whether there was possible making gemcitabine precipitate, and use this method to entrap it inside liposomes. Previous observations had shown that gemcitabine precipitated in certain salt-solutions. Using the same salt-solutions inside liposomes to precipitate the gemcitabine could be a way of increasing trapping efficiency inside liposomes. This could also increase the stability and shelf life of the formulation compared to previous attempts. First approach was to find a suitable salt-solution for this purpose and the best conditions under which to conduct the experiment. Second approach was to test how well this method worked in liposomes.

In order to develop the method and to have some trapping efficiencies to compare with I firstly made some experiments loading dFdC into liposomes via an ammonium sulphate gradient, an approach that had previously been made by (Gravem 2006).

5 MATERIALS AND METHODS

5.1 Chemicals

Table 1: Lipids

Name of lipid	Batch numbers	Manufacturer
EPC-3/ Chol – blend 55/45 molar ratio Costume made	899362-1/020	Lipoid GMBH, Ludwigshafen, Germany

Table 2: Chemicals

Chemical	Quality	Batch number	Manufacturer
4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (Short: HEPES)	>99%	391607/1 33399	Fluka
Acetonitrile	> 99,9 %	I426830 816	Merck, Germany
Acridine Orange	N/A	02921PE	SIGMA-ALDRICH, Germany
Ammonium phosphate (monobasic)	> 98,5 %	097K0133	SIGMA-ALDRICH, Germany
Ammonium phosphate dibasic	> 98%	038K0134	SIGMA-ALDRICH, Germany
Ammonium sulphate ($H_8N_2O_4S$)	>99,5 %	2663731	ROTH, Carl Roth KG, Germany

Chemical	**Quality**	**Batch number**	**Manufacturer**
Ammonium hydrogen sulphate	>99%	1319144 42908161	Fluka analytical, SIGMA-ALDRICH, Germany
Anion exchange resin (DOWEX 1 X 8)	N/A	1220345 31906134	Fluka, SIGMA-ALDRICH, Germany
Cation exchange resin (DOWEX® 50WX8-200)	N/A	14518LE	SIGMA-ALDRICH, Germany
Cross-linked dextran gel (Sephadex G-50 Fine (Amersham Biosciences), Sephadex® G-50 (SIGMA-ALDRICH,))	N/A	290784 9048-71-9	Amersham Biosciences, Sweden SIGMA-ALDRICH, Germany
D(+)-Glucose monohydrate	N/A	325 K19714474	Merck, Germany
di-Sodium hydrogen phosphate dihydrate	$Na_2HPO_4*2H_2O$	Lot: K26627780 927	Merck, Germany
Distilled Water	N/A	0C355642 EG2317912	Merck, Germany ROTH
Ethanol denatured	>99,8	38790760 EG 2005786	ROTH, Carl Roth
Gemcitabine In the form of the drug	45,40 %	A437454	Lilly

Chemical	Quality	Batch number	Manufacturer
product Gemzar 1g, which also contains mannitol, sodium acetate, hydrochloric acid and sodium hydroxide		A450409A	
Hydrochloric acid (HCl)	1 mol/L	0C346964	Merck, Germany
Methanol	> 99,8 %	I443718 830 K36943118	Merck, Germany
Octylphenol ethylene oxide condensate (Triton X 100)	N/A	(unable to read)	ROTH, Carl Roth KG, Germany
Ortho-Phosphoric acid	85%	Lot: K28790773 107	Merck, Germany
Ortho-Phosphoric acid 85%	N/A	K28790773 107	Merck, Germany
Potassium dihydrogen phosphate	99,5-100,5	A837173 719	Merck, Germany
Sodium bisulphate anhydrous	> 95,0%	1341669 53308015	Fluka analytical, SIGMA-ALDRICH, Germany
Sodium Chloride	> 99,5 %	20786510	ROTH, Carl Roth KG, Germany
Sodium dihydrogen phosphate monohydrate	99.0 – 102,0 %	A191846 001	Merck, Germany
Sodium Dihydrogen	99,0-102,0%	7910634610-2412997	MERCK, Germany

Chemical	Quality	Batch number	Manufacturer
Phosphate monohydrate GR (NaH_2PO-H_2O)			
Sodium hydroxide solution (NaOH)	2mol/L	OC292933	Merck, Germany

5.2 Equipments

Table 3: Equipments

Equipment	Type	Manufacturer
Analytic balance	Mettler AT26119 Delta Range N85171	Mettler Toledo, Giessen Germany
Bench top centrifuge	Sigma 112 ~10.000 G Serial number: 41922	Sigma
Centrifuge, Benchtop	Sigma 112 ~10.000 g Serial number: 41922	Sigma
Cuvettes	Einmal.Küvetten aus Polystyrol, 4.5 ml	ROTH, Carl Roth, Germany
Eppendorf tubes (1,5 ml)	LOT: T118728N	Eppendorf, Germany
Eppendorf tubes (2 ml)	LOT: X132218J	Eppendorf, Germany
Fluorescence detector	Luminescence Spectrometer LS 50 B	The Perkin – Elmer Corporation
Fluorescence detector software	FL WinLab Versjon 3.00	The Perkin – Elmer Corporation
Glasperlen (Glass beads), 1 mm	BBI-8541809	B. Braun Biotech International, Germany
Heater plate	Ikamag RCT	IKA Labortechnik
Heating/drying oven	Heraeus, Serial-nr: 60393	Heraus Instruments GmbH, Germany
HPLC – Main Columns:	LiChrosphere 60 RP, Select B,	E. Merck (Darmstadt, Germany)

Equipment	Type	Manufacturer
	250*4 mm (5 µm) LiChrosphere 100 NH2, 250*4 mm (5 µm)	E. Merck (Darmstadt, Germany)
HPLC Precolumn	LiChrosphere 60 RP, Select B, 4*4 mm (5 µm)	E. Merck (Darmstadt, Germany)
HPLC Software	Empower 2, version 6.00.00.00	Waters, Eschborn, Germany
HPLC-system: Autosampler - Waters 717 Column oven Pump - Waters 625 LC System System controller – Waters 600 E UV-Detector - Waters 486 Tunable Detector	 717 003252 CHM-012670 MX5MM5736M 625EPA871 MX4MM8469M	 Waters, Eschborn, Germany Waters, Eschborn, Germany Waters, Eschborn, Germany Waters, Eschborn, Germany Waters, Eschborn, Germany
Injection vial	10 ml, glass vial	
Laboratory balance	Mettler PM 4000 (N88736)	Mettler Toledo, Giessen
Mini column separation	LiChrolut incl. PTFE frits and glass columns	Merck, Darmstadt, Germany
PCS Software	NICOMP Particle Sizing Systems CW388 Application Version 1.68	Samta Barbra, California, USA

Equipment	**Type**	**Manufacturer**
pH meter	Microprocessor PMX 3000	WTW GmbH Weilheim, Germany
Photon Correlation Spectroscopy (PCS)	PSS Nicomp 380	PSS, NICOMP, Santa Barbra, California, USA
Pipettes	Pipetman 200µL and 1000µL Eppendorf 20 µL, 100 µL, 50-250 µL, 200-1000 µL Eppendorf multipett	Gilson, USA Eppendorf, Germany Eppendorf, Germany
SpeedMixer	DAC 150 FVZ (DAZ)	Hauschild, Hamm
Sterile Filter	Disposable filter holders Rotilabo sterile 0,45µm 60027042	ROTH, Carl Roth, Germany
Vacuum pump	-	KNF Neuberger
Vortexer	Reax 2000, 89447392	Heidolph, Germany
Water bath	Certomat® WR	B. Braun

5.3 Media and solutions

25 mM Potassium dihydrogen phosphate solution

Potassium dihydrogen phosphate buffer (KH2PO4) 25 mM, pH 6.9:

i.	Potassium dihydrogen phosphate	3.40 g
ii.	Distillated water	1000 ml
iii.	Hydrochloric acid for pH adjustment	q.s.
iv.	Sodium hydroxide for pH adjustment	q.s

50 mM Potassium dihydrogen phosphate solution:

- Used as dilution media for the VPG's and as for eluting on Cross-linked dextran gel and ion exchange -columns. The reasoning behind this was to have a significant difference in pH between the outer and inner of the liposomes, the same osmotic pressure both outside and inside the liposomes, and, for AO, to have a stable pH when measuring at fluorescence.

Potassium dihydrogen phosphate buffer (KH_2PO_4) 50 mM, pH 7.4:

i.	Potassium dihydrogen phosphate	6.80 g
ii.	Distillated water	1000 ml
iii.	Hydrochloric acid for pH adjustment	q.s.
iv.	Sodium hydroxide for pH adjustment	q.s.

Buffered Triton-X solution, 10%:

- Used for cracking Acredine Orange (AO) liposomes in the preliminary experiments.

Triton-X 10% (v/v) solution in KH_2PO_4-solution, adjusted to pH 7.4:

i.	Triton-X	10 g
ii.	Potassium dihydrogen phosphate -buffer	90 g
iii.	Hydrochloric acid for pH adjustment	q.s.
iv.	Sodium hydroxide for pH adjustment	q.s.

Ethanol 20% solution

- Used for preservation of the Cross-linked dextran gel columns when they were not in use

20% Ethanol solution, 100 ml:

i.	Ethanol	15.8 g
ii.	Distilled water	80 g

Gemcitabine 1mg/ml

- Many different solutions of gemcitabine were made during the experiments. This one though, was used the most.

Gemcitabine 1 mg/ml solution in water, 10 ml:

i.	Gemcitabine hydrochloride (Gemzar®)	22.0 mg
ii.	Distilled water	ad 10 ml

The gemcitabine was weighted into a volumetric flask, and then filled with the desired volume of distilled water.

Gemcitabine 38 mg/ml

- Mainly used in the precipitation experiments.

Gemcitabine 1 mg/ml solution in water, 10 ml:

i. Gemcitabine hydrochloride (Gemzar®) 1,674.0 mg

ii. Distilled water ad 20 ml

The gemcitabine was weighted into a volumetric flask, and then filled with the desired volume of distilled water.

Glucose solution, 50 g/L

- Used for maintaining a stable osmotic environment around liposomes containing 120 mM ammonium sulphate.

50 g/L glucose solution, 500 ml:

i. Glucose 25 g

ii. Distilled water 500 g

Mobile phase for HPLC analysis, for analyzing cholesterol

- Used as the mobile phase when running dFdC and cholesterol on HPLC in the 1st set of experiments, and afterwards for just the running of cholesterol.

Acetonitrile:Methanol:H_2O, 67/30/3%, v/v/v, 1000 ml eluent:

i. Methanol, according to 30% 237.00 g

ii. H_2O pH 2.3, according to 3% 30 g

iii. Acetonitrile, according to 67 % 526.62 g

The solution was then stirred by magnetic steering, and degassed for about 10 min with Helium, 100 ml/ml before use.

Mobile phase for HPLC analysis, for analyzing gemcitabine

- Used as mobile phase for gemcitabine detection.

25 mM Potassium dihydrogen solution : Methanol, 92.5:7.5 %, v/v, 700 ml eluent:

i.	25 mM Potassium dihydrogen solution pH 6.9, according to 92,5%	647.5 g
ii.	Methanol, according to 7.5%	41.5 g

The solution was stirred by magnetic steering, and degassed for about 10 min with Helium, 100 ml/ml before use.

Sodium chloride solution, 0,9 % (w/v)

- Various uses for sustaining physiological osmolarity conditions

0,9 % (w/v) Sodium chloride solution, 100ml:

i.	Sodium Chloride	900 mg
ii.	Distillated water	100 g

Sodium chloride solution, 10% (w/v)

- Used for preparation of the anion exchange columns.

10 % (w/v) Sodium chloride solution, 500 ml:

i.	Sodium Chloride	50 g
ii.	Distillated water	500 g

Sodium chloride solution, saturated

- Used in the preparation of the cation exchange columns.

Saturated sodium chloride:

i.	Sodium chloride	200.0 g
ii.	Distilled water	500.0 g

The solution was stirred with magnetic stirrer for about 15 min, and then stayed for about 10 min for the extra NaCl to sediment. Then it was filtrated into a flask via a 0.45 µM cellulose mixed ester (CME) syringe filter.

Triton-X solution, 10 %:

- Used for cracking dFdC containing liposomes

Triton-X 10% solution:

i.	Triton-X	10 mg
ii.	Distillated water	90 mg

Various salt solutions for testing precipitation of dFdC:

- Used among other things in precipitation experiments with dFdC.

120 mM Ammonium hydrogen sulphate($(NH_4)HSO_4$) solution:

i.	Ammonium hydrogen sulphate:	276.26 mg
ii.	Distilled water:	ad 20.0 ml

120 mM Ammonium sulphate ((NH4)2SO4) solution:

- Also used in the experiments trying to lade dFdC into liposomes via an ammonium sulphate gradient

i.	Ammonium sulphate:	1.586 g
ii.	Distilled water:	ad 100.0 ml

120 mM Ammonium phosphate (dibasic) ((NH4)2HPO4) solution:

i.	Ammonium phosphate (dibasic):	316.94 mg
ii.	Distilled water:	ad 20.0 ml

133 mM Ammonium phosphate (dibasic) ((NH4)2HPO4) solution, pH 7:

- Used in the 4th sets of experiments, precipitating dFdC inside liposomes

i.	Ammonium phosphate (dibasic):	352 mg
ii.	Distilled water:	16 ml
iii.	Hydrochloric acid for pH adjustment	q.s.
iv.	Sodium hydroxide for pH adjustment	q.s.
v.	Distilled water:	ad 20.0 ml

pH was adjusted to 7, then filled up with water to get the desired concentration.

120 mM Ammonium phosphate (monobasic) ((NH4)H2PO4) solution:

i.	Ammonium phosphate (monobasic):	276.07 mg
ii.	Distilled water:	ad 20.0 ml

120 mM di-Sodium hydrogen phosphate dihydrate ($Na_2HPO_4*2H_2O$) solution:

i. Sodium hydrogen phosphate dihydrate: 427.181 mg

ii. Distilled water: ad 20.0 ml

120 mM Potassium dihydrogen phosphate (KH_2PO_4) solution:

i. Potassium dihydrogen phosphate: 326.62 mg

ii. Distilled water: ad 20.0 ml

120 mM Sodium bisulphate ($NaHSO_4$) solution:

i. Sodium bisulphate anhydrous 288.14 mg

ii. Distilled water: ad 20.0 ml

120 mM Sodium dihydrogen phosphate (NaH_2PO_4) solution:

i. Sodium dihydrogen phosphate monohydrate: 331.18 mg

ii. Distilled water: ad 20.0 ml

The salt was weight into a volumetric flask, and then filled up with the desired volume of distilled water.

5.4 Preparative methods

5.4.1 Preparation of VPGs by dual asymmetric centrifugation

Preparation of VPG:

Hydrogenated egg phosphatidyl choline and cholesterol (EPC-3/ Chol) mixture in a 55/45% molar ratio was mixed with an aqueous solution, in a 35/65% lipid/aqueous solution ratio.

Glass beads, in the size of 1 mm in diameter, were added in equal weight with respect to the total weight of the batch size prepared. Shortly after weighing the constituents, the VPGs were prepared by DAC. The speed was set to 3540 rounds per minute (rpm), the maximum speed of the mixer. The mixing time was 6*5 min (total 30 minutes, 5 min is maximum runtime, so the speed mixer was restarted after every 5 minute)

Dilution of VPGs:

The VPGs were diluted with a 50 mM Potassium dihydrogen phosphate buffer, in the ratio 1:3, then speed mixed for 1.5 minutes * 2, handshaken a little bit between each time.

5.4.2 Size exclusion chromatography

Theory:

Gel filtration, or size exclusion chromatography, is a simple and mild chromatography technique, separating molecules on the basis of difference in size. When separating components into two major groups according to their size range, it is called group separation. This technique can be used to remove high or low molecular weight contaminants.

The gel filtration is based on the fact that in a gel, sufficiently small molecules (such as ions or drug) have the tendency to enter the pores of gel particles while bigger species such as liposomes, may not be able to enter into the pores of the gel at all (see figure 5.3). When not entering the pores, the large molecules and particles instead travel through the column at the same speed as the aqueous media used for elution of the column.

(N.N. 2002)

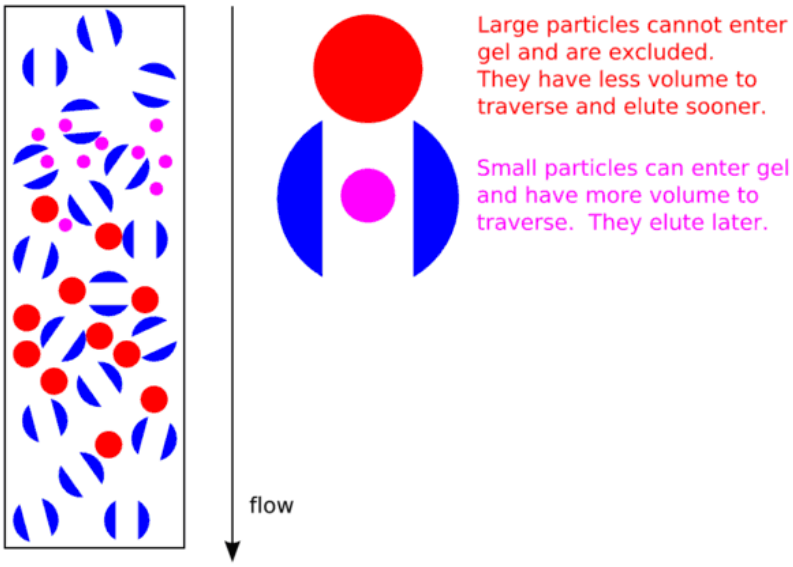


Figure 5.1. Description on how SEC works. Big molecules (such as liposomes) pass around the pores and eluate first, while the smaller molecules can enter the gel, thereby having more volume to traverse the smaller molecules using longer time through the column. (Picture taken with permission from http://en.wikipedia.org/wiki/Image:Liposome_scheme-en.svg)

Sephadex is a gel made from cross-linking dextran with epchlorohydrin. Different type of sephadex gel vary in degree of cross linking, and hence in their degree of swelling, and their selectivity for molecular sizes. The sephadex gels have names that start whit a G, and ends with a number. For example in Sephadex G-50, the "G" stands for gel, and "50" stands for the water regain of the gel, in the instance of G-50, 5.0 g water per g dry gel (N.N. 2007). Sephadex G-50 is suitable for separating large molecules, relative molecular weight (M_r) >30 000, from molecules with M_r under 1 500. That makes it suitable for separating liposomes from a salt in the outer phase. (N.N. 2002)

Procedure:

Packing of column

The columns were prepared according to an internal method given to me by Vitorio.

I. First I weight inn 250 mg Sephadex G-50 into a 10 ml glass column with frits.

II. Added 4 ml of buffer (in the preliminary experiments: Potassium dihydrogen phosphate), and let it swell for one hour at 37°C.

III. Exhausted the supernatant on maximum vacuum, constantly doubling the column volume of buffer. I did this one time with steering the content (to remove air), and the last time without steering. At the end I made sure the gel was covered by a little aqueous phase.

IV. Next I added glass wails into the vacuum chamber.

V. Added 500 µL liposomes to one of the columns, and exhausted with 500µL x the amount buffer needed to get all the liposomes out of the column. The liposomes could easily be distinguished from the surrounding water by that they color the water gray.

VI. As described by the manufacturer (N.N. 2007), the column was washed with 2 column volumes of 0.2 M NaOH, and then re equilibrated with 50mM potassium dihydrogen phosphate. Under longer brakes the column was stored in 20 % ethanol solution.

5.4.3 Active loading of dFdC into liposomes

Experiments

A fixed amount of dFdC was added to a fixed amount (volume) of liposomes solution. The liposomes were then loaded on water bath for 60 minutes at 65^0C. The water bath also had a steering option which was set to 200 rpm.

5.4.4 Removal of outer drug by ion exchange

Theory:

Ion exchange is a technique used among other things for purification purposes. The principle of the method is to apply the sample we want to purify to a column with either positive charged resin material (anion exchanger), or negatively charged resin material (cation exchanger). The charge of the molecules in solution will decide if they will stay on the column or not.

Gemcitabine is positively charged at low pH's, where it binds to the negatively charged cation resin

material. Liposomes are pH neutral, making them pass through the column, together with its content of entrapped material. Separating entrapped from untrapped material is essential for determination of trapping efficiency.

Packing columns for dFdC:

I prepared the columns according to an internal method given to me by Vittorio Ziroli:

I. 2 gram cation exchange resin was weight into a glass column with frits.

II. The column was filled with water, then rested for 5 minutes for sedimentation.

III. Water was exhausted with vacuum, but enough water was left to just cover the resin bed.

IV. Column was loaded 3 times with saturated NaCl, 3*2 ml. I let the saturated NaCl stay in the column for 1 minute each. Column was exhausted with vacuum between each time, but enough water was left to just cover the resin bed. The column never ran dry.

V. Column was then flushed with water 3* 2 ml, then rested for one minute each time, as for the NaCl solution.

VI. Then 5 minutes of full vacuum, the column now ran completely dry.

The ion exchange columns can only be used once, but the storage time is very long, since it is stored dry.

Preparing columns for AO:

The columns for AO were prepared in the same way, but with a few exceptions:

- Anion exchange resin was used, not Cation exchange.
- 10% NaCl was used instead of saturated NaCl.

Applying of samples:

Volumes up to 1000 µL were applied slowly to the columns, and sucked off with about ~4-5 Hg

vacuum. Column was then eluated with 5 times 500 µL of water. Eluate was collected in test tubes of about 10 ml.

5.5 Analytical methods

5.5.1 Photon Correlation Spectroscopy – PCS

Theory:

The PCS technique lets us see size and size distribution of particles in a liquid. Because liposomes will have different properties with different sizes as to loading and possibilities to accumulate in tumor cells this is quite useful information when making these vesicles.

The principle behind PCS is based on that when light hits small particles compared to the wavelength used the light scatters in all directions. When using laser light one observes a fluctuation in the scattering intensity due to the fact that small molecules in a solution is undergoing Brownian motion. The scattered light can then undergo interference by surrounding particles. This intensity fluctuation contains information about the time scale of the movement of the scatters. In the PCS-machine a laser beam of specified intensity polarization and wavelength are focused into the sample. A detector measures scattered light, and with the information gained from this the software is able to calculate size and distribution of the particles. (Svanberg 2005; N.N. 2009b)

Some rough intern reference values at the lab at Klinik für Tumorbiologie for "good" liposomes were as follows: Auto channel width: ~30, Mean diameter: <60 for the lowest channel widths 5 and 10, variance: 0.3, Chi squared: <30.

Procedure:

The VPGs were diluted to give an intensity between 250-350 kHz (ca a 1/2000 dilution). Measurements were done for 2*5 minutes on channel-width 5, 10 20 and AUTO. The data was then compared to internal reference values.

5.5.2 Quantification of Acredine Orange by fluorescence spectroscopy

Theory:

Fluorescence spectroscopy is a type of electromagnetic spectroscopy that measures fluorescence from a sample. To get fluorescence a beam of light, usually ultraviolet light, is used to excite electrons in

molecules on certain compounds. When the electrons go back into position it causes them to emit light of a lower energy. At low wavelengths the fluorescence intensity will generally be proportional to the concentration of the substance we analyze. The wavelengths emitted depends on the molecule we analyses, and different substances emits lights in different wavelengths. Because of this substance specificity the substance can often be analyzed without separating it from other substances in the sample (unlike for example UV).

Procedure:

Samples of acredine orange were diluted to a measurable concentration for the PCS, and then measured with an excitation wavelength of 490, and emission wavelength was set to 520. Integration time was 30secounds.

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

HPLC theory:

HPLC is much used method for separating substances. Much of its success probably lies to it's simplicity. In simple terms we can say that a HPLC consist of a mobile phase (also called eluate), a pump, injector port, a sampler, a pre-column, main-column and a detector (often UV). The mobile phase acts as a carrier for the sample solution. The sample is being injected to the mobile phase through the injector port, and floes through the column were substances in the solution get separated. To separate substances the column must have properties that delay their flow based on their structure. In reversed phase chromatography (which is what I use) the stationary phase is non polar, while the mobile phase is polar. This increases retention time for non polar substances, while polar molecules elute faster. When the sample goes through the detector we get a peak in our chromatogram which aria under the curve corresponds to its concentration (at least to a certain extent).

Procedure

Before HPLC the liposomes were cracked by adding 10 % triton-X to the liposomes, giving a triton-X concentration of > 3% in the liposome-sample. The Solution was then further diluted by a 25 mM Potassum dihydrogen solution to. This was done to give a concentration of triton-X of under 1%, and to

make sure the pH was in the right range for measuring, before applying the sample on the HPLC.

In the first set of experiments I used an HPLC-method for running and analyzing Gemcitabine and Cholesterol in the same run. This method however, did not separate triton-X (used for cracking the liposomes) from the gemcitabine. For that reason I went on to use another internal method.

Procedure for analyzing dFdC and cholesterol in the same run:

This method was obtained from Vittorio Ziroli, and was called "Simultaneous detection of Gemcitabine and Cholesterol in a vesicular phospholipids gel by HPLC to determine encapsulation efficiency". The advantage of being able to analyze both dFdC and cholesterol in one run is that it saves time, and also makes it possible to compare amounts of dFdC and cholesterol directly.

The parameters on the HPLC were set according to Ziroli, and are listed in table 4.

Table 4: HPLC parameters used in the preliminary experiments for quantification of gemcitabine

Injection volume	10 µL
Mobile phase	Acetonitrile / Methanol/ H_2O (acidified) 67:30:3
Flow rate	1.5 ml/min
UV detection wavelength	0-6.5 min: 278 nm 6.5-10 min: 215 nm
Column temperature	30^0C
Columns	LiChrosphere 60 RP, Select B, 250*4 mm (5 µm) with a guard column: LiChrosphere 100 NH2, 250*4 mm (5 µm) and LiChrosphere 60 RP, Select B, 4*4 mm (5 µm)

According to Zirolis method the sample was suppose to be diluted in ethanol/methanol 90/10. To crack liposomes with a dilution like this, and without triton-X you will need a dilution of about 1:100. However, this was impossible in my case, as the dilutions made on the Cross-linked dextran gel and ion

exchange columns made the lipid concentration to small for much further dilution.

Analyzing dFdC and cholesterol separately by the "old" method

This method was given to me by Ziroli.

HPLC determination of dFdC

Table 5: HPLC determination of dFdC

Injection volume	50µL
Mobile phase	Phosphate buffer (PBS) 25 mM, pH 6.9 / MeOH 92.5 : 7.5 (vol/vol)
Flow rate	1 ml/min
UV detection wavelength	278 nm
Column temperature	40^0C
Columns	LiChrospher 60 RP-select B, endcapped, 5µm, 250 x 4 mm, with a guard column LiChrospher 60 RP-select B endcapped, 5µm, (4 x 4mm).

HPLC determination of Cholesterol

Table 6: HPLC determination of Cholesterol

Injection volume	10µL
Mobile phase	acetonitrile/methanol/H2O 67/30/3
Flow rate	1.0 ml/min
UV detection wavelength	215 nm
Column temperature	40^0 C
Columns	LiChrosphere Select B, 5 µm, 250 x 4mm (C8-column), with gard column LiChrosphere Select B, 5 µm (4 x 4mm)
Autosampler temperature	4^0C
Ca retention time	5,7 min

6 RESULTS AND DISCUSSION

6.1 Preliminary experiments

6.1.1 Acredine Orange

Procedure

Liposomes were prepared by DAC, having an ammonium sulphate gradient. 50mM Potassium dihydrogen phosphate solution was used as dilution medium. Outer Amonium sulphate was removed by size exclusion chromatography. A solution of Acredine orange (AO) was then added to the solution of liposomes, and the samples were incubated on water bath at 65^0C, for about 1 hour. After samples were cooled to room temperature, free Acredine orange were removed by anion exchange chromatography. Samples were cracked with Triton-X 10 %, and then further diluted with 50mM Potassium dihydrogen phosphate solution down to a measurable concentration for the fluorescence spectrometry. For comparison reasons part of the sample that had not undergone anion exchange, thus containing bouth free and entrapped dFdC, I choose to call 100% samples. These were also cracked with triton-X and diluted in a similar manner, before being measured at fluorescence. Amounts of AO were determined by comparing the intensity yield in the spectrometry to a standard curve. Trapping efficiency was determined by dividing the amount of entrapped AO on the amounts measured in the 100% sample.

Making of standard curve

Because Acredine orange is a base it was assumed that pH could affect its fluorescence properties. I also proved this by trying to measure AO diluted in a highly acidic solution. This gave very low intensity in the spectrometer, showing that the pH is an important factor. Because of that, all samples were buffered to pH 7.4, using a 50mM Potassium dihydrogen phosphate solution. To test if this was a stable range I measured intensity of 1µM and 2µM dFdC at the pH 6, 7.4, and 8. The result can be seen in table 7. The intensity showed little variation, showing that 7.4 is a stable pH for fluorescence measurement.

Table 7: Variation of intensity measurements on AO at different pH'es

Concentration of AO	1 µM	2µM
pH 6	278	594
pH 7,4	226	526
pH 8	236	503

From this conclusion I made a standard curve in pH 7.4, ranging from 0.5 µM, 1µM, 2µM, 2.67 µM, and 4 µM, 2 parallels of each concentration (see figure 6.1).

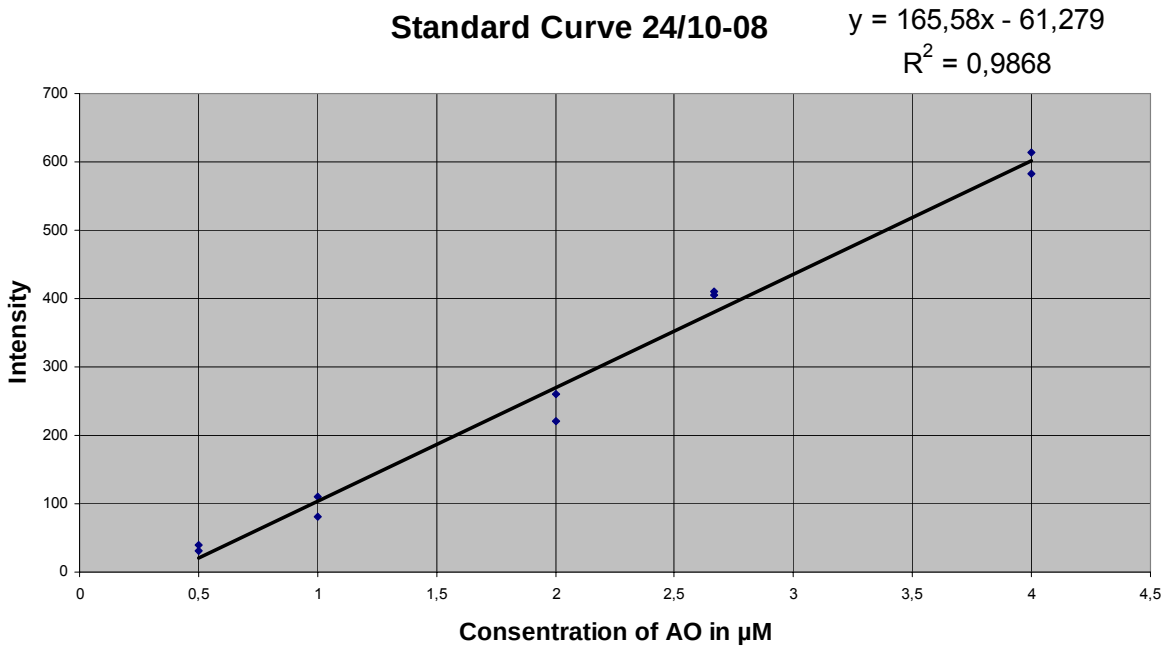


Figure 6.1. Standard curve of Acredine Orange measured at fluorescence spectrometer, ranging from 0.5 to 4 µM in concentration.

Results

The measured absorbance was much higher than the theoretical values, both in the 100 % samples (the samples that contained both free and encapsulated AO), and in the entrapped samples. For example in

one experiment the concentration should theoretically be around 0.8 µM AO, but the intensity indicated 1.80 µM. By comparing the entrapped samples with the 100 % samples though, I was able to calculate trapping efficiencies, varying from 60 to over 80%.

After 3 experiments that both showed the trend with intensity higher than it theoretically should be, and after lots of recalculations revealing no errors, I got the idea that something was influencing my samples. I tested about everything I could think of, measuring many of my solutions on the fluorescence to see if they gave any peak on the fluorescence (they were all blank, including the triton-X). Then I got the idea to mix AO solution with 5 % triton-X (the next step would have been to mix them with cholesterol to see if that had any effect). The results from this showed that samples containing triton-X gave a higher intensity in the fluorospectrometer than we could expect from just acredine orange. (see table 8):

Table 8: Intensity measurements of similar solutions of AO, with and without 5 % triton-X.

Concentration of AO	**Intensity with 5% triton-X**	**Intensity without triton-X**
2 µM	999 (to high to measure)	600
1 µM	523	-
0.5	-	139
0.25 µM	101	68

To prove this correlation between triton-X and intensity, and to show that it also gave effects down to concentrations as low as 1% triton-X a new experiment was made with more parallels. This showed the same thing, and also that a triton-X concentration of only 1% also affected the measurement of Acredine Orange (see table 9):

Table 9: Intensity measurements of similar solutions of AO, with and without 1% Triton-X.

Concentrations	Intensity with 1% triton-X	Intensity without triton-X
4µM AO, 1%	999.999	975.043
3µM AO, 1%	999.999	732.653
2µM AO, 1% Triton-X	768.760	461.728
1µM AO, 1% Triton-X	306.021	162.190

As the concentrations with triton-X was higher in the entrapped samples than in the 100 % samples, this also lead to some serious problems on how to proceed.

One way this could have been solved was to have the exact same concentration of triton-X in all cuvettes. Another is to run these substances on HPLC, separating AO and triton-X before measurements. But at least the HPLC approach would have needed lots of adjustments finding a perfect column and mobile phase. Since this was only a preliminary experiment it was limited how much time and effort it should be given, so instead it was decided to move on to experiments on gemcitabine.

But at least the experiment showed that there was an encapsulation of AO into liposomes, as triton-X did not give any absorbance alone. Encapsulation could also be observed as the liposomes filled with AO sediment in the test tubes when left over night (Free AO in water doesn't sediment).

6.2 Experiments loading gemcitabine into liposomes via an ammonium sulphate gradient

6.2.1 1st set of experiments; loading of dFdC via a Ammonium Sulphate gradient, followed by detection dFdC and cholesterol analyzes in one run

The dFdC liposomes were prepared in a similar manner to the AO, biggest exception being the use of a cation exchange column instead of an anion exchange column. Before HPLC the solution of cracked liposomes and triton-X were diluted with 25mM potassium dihydrogen solution.

As method for analyzing amount of dFdC HPLC was used. The parameters that were used can be found under section 5.5.3, Procedure for analyzing dFdC and cholesterol in the same run.

Results

As the internal method for the HPLC, Procedure for analyzing dFdC and cholesterol in the same run, had been used several times in the lab at "Klinik für Tumorbiologie", we did not expect any big problems to occur. Especially after producing a perfect calibration line including both dFdC and Cholesterol (not included here, as it was never used). The problems, though, occurred as soon as we started applying the samples of loaded liposomes. All the samples contained a big top right where gemcitabine was supposed to be (see figure 6.2 and 6.3). Problem was that it was much bigger than the concentration of gemcitabine should be after all the dilution steps it had gone through.

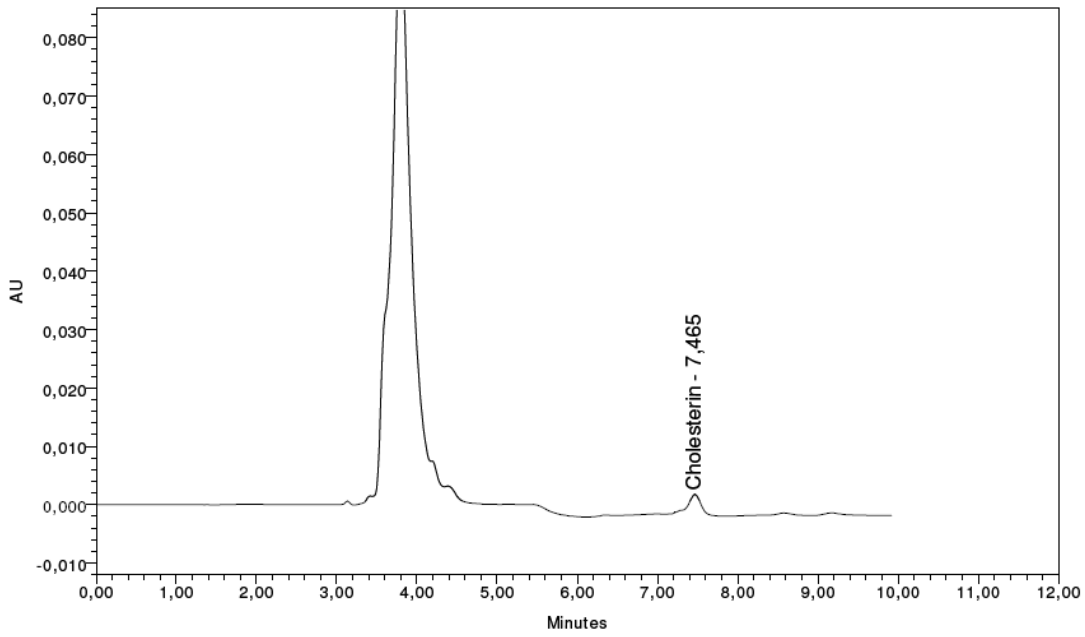


Figure 6.2. HPLC run of a 100% sample, containing dFdC and cholesterol, liposomes cracked with triton-X.

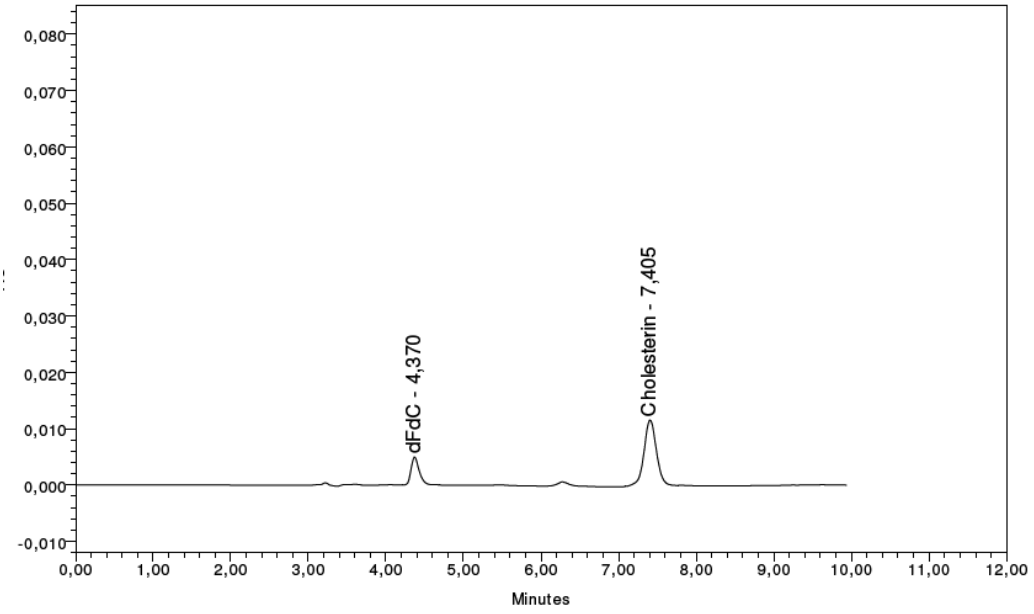


Figure 6.3. One of the standards. Sample contains dFdC and Cholesterol, but no Triton-X.

First thought was that I had made a mistake, either in the calculations, or somehow in the preparations. It was decided I should conduct a new experiment, and make the calculations one more time. But the result was the same. The thought that some of the solutions I used in the experiment might have caused this peak, one way or the other entered my mind. So I started applying everything from the water (in

case it was polluted) to my buffer solution on the HPLC (but coincidently the Triton-X was left out). Also Ziroli vent over my calculations of the dilution steps, finding no errors.

The answer first appeared when looking for cholesterol content in not loaded liposomes. According to procedure the liposomes were cracked with Titon-X, and then further diluted, before being applied to the HPLC. As the result looked just like figure 6.2, one big top and one small cholesterol top, this gave a clear indication as to what was wrong. Since this sample contained no dFdC, but only liposome content and Triton-X, it had to be Triton-X. This was proved by applying different concentrations of Triton-X to the column, which gave the same top at the same location.

The reason why this had not been discovered earlier in the method was that when it had been used at the lab they did not crack the liposomes with triton-X, but by heavily diluting them in ethanol/methanol. This was possible since in their experiments they only passively loaded liposomes, and thereby did not have to use size exclusion chromatography. Thereby their liposomes were not so diluted at the end. This was not a possibility for me, so I had to find another way.

My first thought was to adjust some of the preferences on the HPLC, such as the flow speed, adjusting the content of the mobile phase, and even removal of one of the columns. After trying several modifications, we gave up, and moved to an older method, used previously without problems at the Klinik für Tumorbiologie.

6.2.2 2nd set of experiments; loading of dFdC via a ammonium sulphate gradient, followed by separate detection of dFdC and cholesterol

Procedure

Preparation

The liposomes and the dFdC were prepared in a similar manor to the 1st set of experiments, and descriptions of the methods used can be found in section 5.4, Preparative methods. I prepared liposome batches of 1000mg each time, with 350mg lipids and 650µL 120mM ammonium sulphate. VPG's were diluted 1/3 adding 2000µL 50mM potassium dihydrogen phosphate solution (PDP-solution). The diluted liposomes were then separated into 4 parallels, and diluted once more, ½, before applying them to columns containing cross-linked dextran gel according to section 5.4.2, "Size exclusion chromatography". 2000µL of each eluate from the cross-linked dextran gel was extracted and 250 µL

1.0 mg/ml dFdC was added to this, resulting in a dFdC-concentration of 0.11111 mg/ml. The 1.0 mg/ml dFdC solution was gemzar mixed with water.

Because of variations in amounts of eluate used on the cross-linked dextran columns the concentration of cholesterol that came out of the column varied a bit. Normally the dilution was about 1/7.

The liposomes + dFdC 0.111 mg/ml, total volume being 2250µL, were transferred to sealed test tubes of 10ml. These were placed on water bath at 65^0C for 1 hour, at 150 RPM (to avoid sedimentation and enhance loading).

Removal of outer drug before analysis was preformed by ion exchange as described in section 5.4.4, Removal of outer drug by ion exchange. In addition to running the samples through these columns, a control-sample containing the equal or higher concentration of dFdC as the liposome-samples was also rand through a column, with the same conditions as the other columns. The reason behind this was to check that the columns were working and able to remove all free dFdC that was applied.

The samples ran through the ion exchange columns (thus containing only entrapped dFdC) I choose to call "loaded samples", while the samples containing both free and entrapped dFdC I choose to call 100% samples.

Analyzing

Parts of the liposome-batch produced was extracted and diluted with 50mM Potassium dihydrogen phosphate solution to measurable concentrations for the PCS, according to section 4.5.1, "Photon Correlation Spectroscopy – PCS" (except in experiment 6, where I diluted two of the samples with a 50g/L glucose solution prior to the PCS).

Prior to the analyses on the HPLC the dFdC-samples were diluted to a theoretical concentration of about 6µg/mL. For the loaded-samples this means the theoretical concentration assuming there is 100% loading-efficiency. The parameters of the HPLC can be found in section 5.3, "Analyzing dFdC and cholesterol separately by the "old" method".

Things not specified in this paragraph (procedure) or in the paragraphs which that are referred to, together with prospective changes from these methods, are outlined further under the description of each experiment.

Results

Standard curve of gemcitabine

I made a standard curve for gemcitabine ranging from 100 to 7200 ng/ml dFdC. R^2=0.993410 (see figure 6.4).

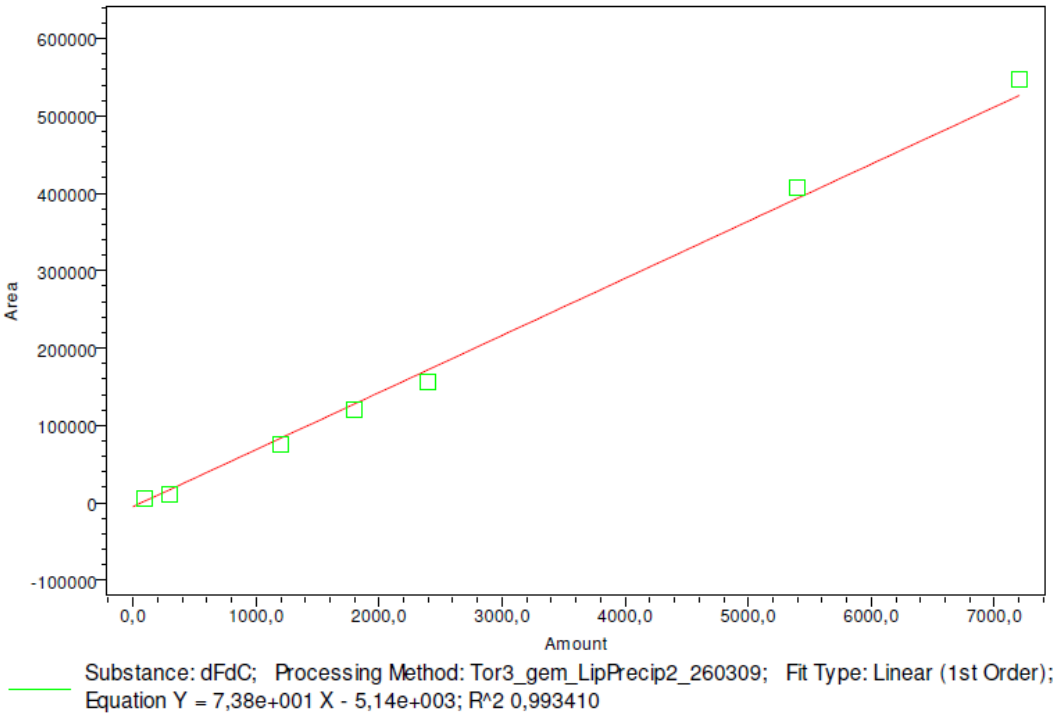


Figure 6.4. Standard curve of gemcitabine, ranging from 100 to 7200 ng/ml dFdC. X-axis: Concentration of dFdC (ng/ml). Y-axis: Aria under the curve.

Standard curve cholesterol

In a similar way I also made a standard curve for cholesterol, ranging from 400 to 3200 µg/ml, R^2=0.999055 (see figure 6.5).

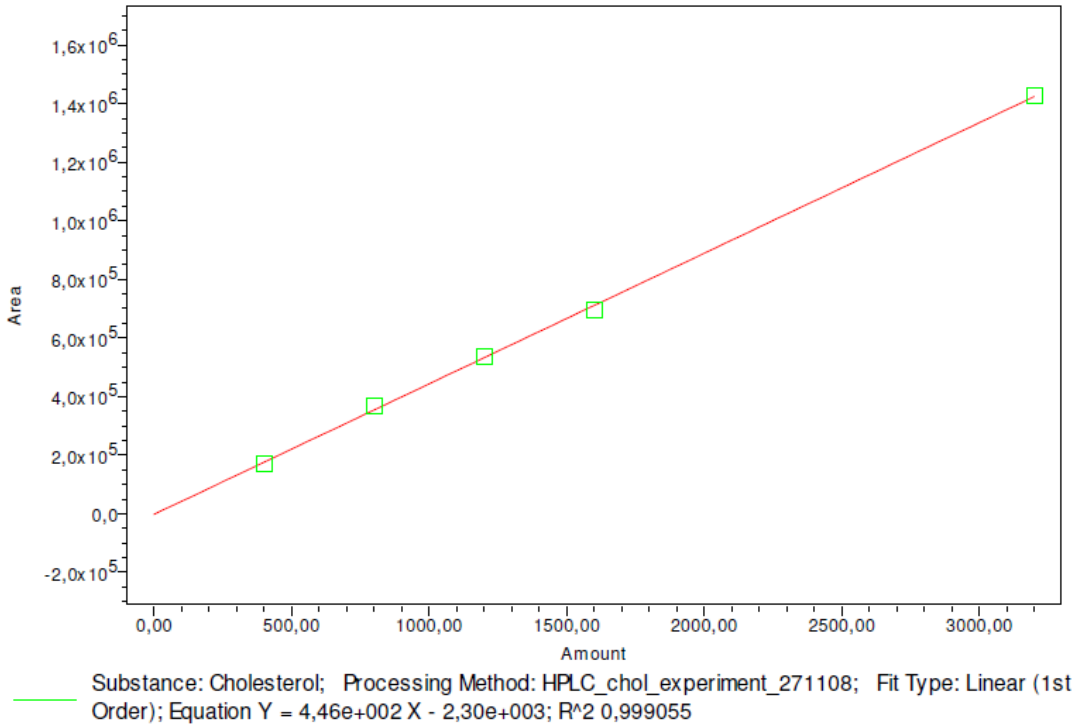


Figure 6.5. Standard curve for cholesterol, ranging from 400 to 3200 µg/ml.
X-axis: Concentration of cholesterol (µg/ml). Y-axis: Aria under the curve (AUC).

Experiment 1

To eluate the sample on the ion-exchange column the 50mM Potassium dihydrogen phosphate (PDP)-solution was used. Reason for this was that I thought it would be best to keep the liposomes in an osmotic environment until they had passed the ion exchange column completely.
All samples were diluted to a theoretical concentration of around 4µg/ml before applying them to the HPLC.

The loaded samples gave no visible peaks on the chromatogram, indicating no loading. Also, the control sample was positive indicating that the ion exchange column had not worked properly.

Analyzing the cholesterol revealed that the cholesterol-values in the expected range, with a few strange exceptions on parallel 4 of the loaded samples (78% over the expected value), and parallel 2 of the 100% samples (750% over the expected value).

The results from the PCS were in the expected range, indicating that there was nothing wrong with the liposomes size or size distribution.

The full results from experiment 1 can be found in appendix 1.

Experiment 2

In an internal method for the ion exchange column, used at Klinik für Tumorbiologie, water is used as the eluting liquid. Also (Gravem 2006) used water as eluting agent. At this point there were no clear indications that using the PDP-solution was the reason for the positive control-solution, though I saw no reason to experiment with it if the method could work without the PDP-solution as eluate.
All samples were diluted to a theoretical concentration of 6.225µg/ml prior to HPLC.

The control sample was negative (did not contain dFdC), but so were all the loaded samples. The 100% samples ranged around 6µg/ml as expected, indicating that there was nothing wrong with the dilution-steps.

The cholesterol samples showed values over their expected theoretical values, the reason for this being somewhat unclear.

The full results from experiment 2 can be found in the appendix 1.

Experiment 3

Experiment 2 showed no trapping-efficiency at all. The only big change I had made since the preliminary experiment with the Acredine Orange (were the loading had worked) were to change the eluate at the ion exchange column to water. So I thought that maybe applying the 50mM PDP- solution on the ion exchange column was necessary for the osmotic pressure. This impression was also the one of my lab supervisor, which also meant that using the solution as an eluate at the column would not affect its ability to bind free dFdC.

For that reason I used the 50mM PDP-solution when eluting the column. All samples were diluted to a theoretical concentration of 4.15ng/ml.

The loaded samples indicated a trapping-efficiency of over 50%. But the control-sample was positive, so the data was not valid and had to be discarded. The cholesterol values would probably not have revealed anything more, so they were not measured.

The full results from the 3rd experiment can be found in the appendix.

Experiment 4

My results from experiment 1, 2 and 3 indicated that the use of the 50mM PDP-solution to eluate the ion exchange columns made lots of free dFdC pass through the column. To investigate this further I made 4 columns, and applied 1000µL of 0.111mg/mL dFdC solution to each of them. Two of the columns were eluated with water, the other two with the PDP-solution. All was done according to 5.4.4, "Removal of outer drug by ion exchange". The solutions were not diluted further, but measured right out of the ion exchange-columns. This gives a theoretical concentration of 31.7µg/mL dFdC if all went through. The result can be seen in the table below.

Table 10: Testing of different eluate and they're effect on the columns ability to retain free dFdC.

Sample	**Control 1, Water**	**Control 2, Water**	**Control 3, 50mM PDP-solution**	**Control 4, 50mM PDP-solution**
AUC	0	2755	53362	200396
Calculated dFdC-concentrations (from standard curve)	0	126.3ng/mL	875ng/mL	3052ng/mL

The numbers were not totally as expected. One of the samples eluated with water contained a little bit dFdC, and the ones eluated with the PDP-solution did not reveal as high concentration as one would expect from the earlier experiments. But combined with the results from experiment 1-3, the indication that the PDP-solution did not work well with the column was quite clear.

Experiment 5

Being very optimistic I thought that the PDP-solution was the only problem, and ignored experiment 2's failure as a coincidence. I eluated the ion exchange column with water, and diluted all samples to a theoretical concentration of 6µg/mL.

The result was empty "loaded"-samples, but also a blank control-sample. The 100% samples were in the right range (around 6µg/ml).

The cholesterol samples were also in the expected range. Results can be found in appendix 1.

Experiment 6

The latter experiments gave rice to some questions whether why we did not get any loading. In (Gravem 2006) experiments with loading dFdC into liposomes via an ammonium sulphate gradient she used a solution of 50g/L glucose as hydration media for the VPG's. At the time I figured it would be a good idea to test if this could have any influence on the loading.

The point of the experiment was to see ones more how the PDP-solution affected the ion exchange column, in addition to see if the PDP-solution as a dilution medium for the liposomes had any negative effect on the loading, compared to using a glucose-solution. Also I wanted to se if any of the non existence results from the previous experiments could have any connection to the use of water to eluate the ion exchange column (because of osmotic pressure), and if e.g. the glucose solution could be used for this purpose instead.

To test this I made two batches of liposomes. One batch contained 50mM PDP-solution as outer aqua's phase, and one contained 50g/L glucose as outer aqua's phase. Each batch was then divided in two, where one part was for eluting the sample on the ion exchange column with the same liquid as used as dilution media for the liposomes (PDP-solution or glucose-solution), where the other parts were eluated with water. For each parallel I had a control-sample containing free dFdC to test the ion exchange column for the specific eluate-media. All samples were diluted to a theoretical concentration of 6.0µg/mL before they were analyzed on HPLC.

The results were much as we could have been expected out of the previous experiments. The samples eluated at the ion exchange column with the PDP-solution contained dFdC, but so did the control sample. All other samples were negative for dFdC. The results in detail can be found in appendix 1.

The PCS-results for the liposomes produced with PDP-solution as outer phase showed a very high auto channel width, at 99 (reference value is about 30), which mean diameters of over 300, and Chi squared of over 500. The other channel widths were normal. This might indicate that there were some very large liposomes in the batch. Though this could have affected the liposomes ability to load, all the other experiments give us so much info on this subject that it will not affect the conclusion.

Discussion

The results showed no loading at all. The minimum detectable amount of dFdC on the HPLC is lower than 100ng/ml. When the loaded samples are diluted to a theoretical concentration of 6µg/ml dFdC we should be able to detect loading efficiencies even lower trapping then 1.7%. So this means that loading must have been even lower than this.

Maximum theoretical loading

If we assume that the volume ratio between outside and inside of the VPG's we produce is 40%, the dilution steps before loading would result in a concentration of 0.847% of the total water content being inside the liposomes at loading (see appendix for calculations). Hence if there were only passive loading we would have gotten a loading of maximum 0.847%.

The total molar concentration of dFdC in the solution is 0.4217mM. In a solution of 2250µL, which was the amount that was used in these experiments during loading, that means that we have 0.9488 µmol dFdC in the solution. At 0.847% loading, the number we could expect from passive loading, 8.036*10^-3 µmol is loaded. (Calculations: see appendix 1)

The fact that the concentrations of the liposomes may also play an important role was unfortunately not taken into consideration, mainly because the loading with acredine orange (AO) worked fine. But there is a major difference between AO and dFdC. While AO has a pKa of 10.45 the dFdC has a pKa of 3.58 (N.N. 2005; Barenholz 2007). If the pH inside the liposomes were let's say 3, because of the ammonium sulphate gradient, then according to the Henderson-Hasselbalch equation the amount of charged AO would be near 100% (calculations can be found in appendix 1).

For the dFdC on the other hand, a pH of 3 would lead to only 3.8 times as much charged dFdC as neutral, meaning that 79% is charged. (pH 3 is not necessary the real pH inside the liposomes. If the pH inside the liposomes were 2.5, about 92% of the dFdC would be charged.) pH outside the liposomes were 7.4, meaning that under 0.02% of the dFdC (1.898*10^-4 µmol) would be charged outside the liposomes. For simplicities sake in the calculations we can therefore assume that no dFdC outside the liposomes are charged.

To the VPGs 120mM ammonium sulphate were added, giving an AS solution of 78mM in the "VPG-soup". Assuming 40% loading the volume the amount of AS would be 1.486 µmol ammonium sulphate.

(For calculations, see appendix 1)

According to theory (see section 3.4, active loading) the charged dFdC will get "stuck" inside the liposomes, while the rest will diffuse in and out randomly. Every time some dFdC get "stuck" inside the liposomes more dFdC will diffuse to the inside, so that the percentage of uncharged dFdC compared to the outside is the same all the time.

Using the numbers from above, where passive loading gives 0.847% loading and charged dFdC inside the liposomes will be 79%, we are able to calculate the theoretical maximum loading under this conditions (see appendix 1). This maximum loading would then only be 4.03%, or 0.0398 µmol dFdC loaded. This loading efficiency should have been detectable, though the loading almost never get as high as the theoretical maximum, due to liposome shape, etc. This is was also thought-experiment, and we can not be sure of all values given (such as pH inside the liposomes and the amount of water inside the liposomes). These theoretical values probably give a better loading than we would get experimentally under these conditions. So when even these values gives a maximal loading efficiency of only 4.03% it is pretty clear that the concentrations of the liposomes were to low to give high loading-efficiencies of dFdC.

Other factors

There is hard to find other factors that would lead to loading of AO but not of dFdC since there were no special differences between the preparative techniques used. Though since we have no useful loading efficiency data from the AO either we don't know how good this loading were (only that it was visible). So there is nothing wrong discussing factors that were similar between AO and dFdC.

Loading conditions

The loading of the liposomes were conducted in small sealed test tubes of about 10ml, where the liquid took 2.25ml of the space. As mentioned in the introduction chapter 3.3, "Active loading", the loading via an ammonium sulphate gradient depends on the ammonia molecule (NH_3) leaving the system by evaporating as gas. Unable to find any info from what has previously been done about this I don't know if the remaining volume in the test tube (about 6.75ml) is enough for this to take place in significant amounts.

Osmotic pressure

When looking back on these experiments I also see that factors for adjusting the osmotic pressure were not optimal. While 120mM ammonium sulphate has about the same isotonicity as 0.9% NaCl (118.2mM is the exact concentration of isotonicity of ammonium sulphate), the isotonic value for potassium dihydrogen phosphate is at 160.18mM (in my experiment I used 50mM). Also the dFdC-solution that I used was not adjusted for isotonicity (the right way to do this would have been dissolve the Gemcitabine in 0.9% NaCl instead of water, according to its SPC (N.N. 2006)).

Theoretically this would create a hypotonic environment for the liposomes, causing them to swell, or even crack. On the other hand the PCS-results look fine also when compared to the 50g/L glucose solution used in experiment 6.

6.3 Precipitation experiments

6.3.1 Background

It is known (N.N. 2006) that concentrated solutions of Gemcitabine (38 mg/ml) have a tendency to form precipitates, when stored in the fridge. Scientists at Klinik für Tumorbiologie had earlier observed that gemcitabine, especially when dissolved in a phosphate buffer then stored in the fridge shows precipitate and forms crystals. Trying to dissolve these crystals proved very difficult, even after stirring them in room temperature. Since this was undesirable it had not been documented further at this time.

Although precipitation most often is an undesirable process, it might theoretically be used when actively loading liposomes. A hypothesis was that the mechanism were that 2 dFdC(+) binds to HPO_4(2-), and leads to a insoluble salt, having liposomes with an inner phosphate solution would be a way of actively loading dFdC into liposomes. Though there were also a possibility that the precipitation were a direct consequence of low solubility at the higher pH-values combined with the storing in the fridge. Therefore all of these things were needed to be investigated further.

6.3.2 3rd set of experiments; finding the right salt and conditions for precipitation of dFdC

Experiment 1

As mentioned above the results from the precipitation of the dFdC previously observed was not documented in any way. Also we wanted to see if more than just the phosphate-salt would make this precipitation happening. To start somewhere the salts sodium bisulphate anhydrous, ammonium sulphate, ammonium phosphate (monobasic), ammonium phosphate (dibasic), sodium dihydrogen phosphate (monobasic) and ammonium hydrogen sulphate were tested first. An important thing was that I tested both phosphate and sulphate-salts.

As we had no sponsored gemcitabine, but had to pay for it ourselves it became an issue throughout the whole theses to try to minimize the use of this substance. So though the first thought when trying to make a substance precipitate is to use as high concentration as possible, it was decided to first try with only 0.5mg/ml dFdC. The concentrations of the salts were set to a concentration of 60mM. This was achieved by mixing 1mg/ml dFdC with 120mM of each salt in a 1.5ml reaction tube. I also made one sample which only contained the dFdC-solution and water, to compare to the rest of the samples.

I made 6 parallels out of every salt. Two samples of each salt-type were placed in an oven at 50^0C, two in room-temperature, and two in the fridge (~6^0C), all for about six hours. The reaction-tubes were then centrifuged on a bench-top centrifuge for 1.5 minutes, prior to extraction of 100µL of the supernatant. The extracted supernatant was immediately diluted 1/10 in water, to prevent further precipitation. Then later it was diluted to the theoretical concentration of 6µg/ml, prior to measuring at the HPLC. Since the samples contained no liposomes triton-X was of course not used, but the last 2 steps with dilution was always done using 25 mM potassium dihydrogen phosphate solution.

No precipitation could be observed by the naked eye. An HPLC analysis of the supernatant gave the results seen in the table below. The concentrations were calculated by comparing the AUC with the standard curve presented in section 6.2.3, 2nd set of experiments.

Table 11: **Precipitation experiment 1, (average and standard deviation of 6 parallels). Concentrations determined by HPLC (comparing AUC with the standard curve presented in 6.2.3, 2nd set of experiments)**

Salt	Sodium bisulphate anhydrous	Ammonium sulphate	Ammonium phosphate (monobasic)	Ammonium phosphate (dibasic)
Freezer 1	5781 ng/ml	5741 ng/ml	5919 ng/ml	5657 ng/ml
Freezer 2	6204 ng/ml	6236 ng/ml	6512 ng/ml	5985 ng/ml
Room 1	5761 ng/ml	5740 ng/ml	5648 ng/ml	5730 ng/ml
Room 2	5737 ng/ml	6497 ng/ml	6513 ng/ml	6120 ng/ml
Oven 1	5751 ng/ml	5755 ng/ml	5738 ng/ml	5524 ng/ml
Oven 2	5753 ng/ml	5382 ng/ml	4844 ng/ml	5985 ng/ml
Average	5831 ng/ml	5892 ng/ml	5862 ng/ml	5834 ng/ml
Standard deviation	167	367	570	210

Salt	Sodium dihydrogen phosphate monohydrate	Ammonium hydrogen sulphate	Control (dFdC in water only)
Freezer 1	5212 ng/ml	5057 ng/ml	4498 ng/ml
Freezer 2	6302 ng/ml	5712 ng/ml	4629 ng/ml
Room 1	5485 ng/ml	5611 ng/ml	5536 ng/ml
Room 2	6026 ng/ml	5975 ng/ml	4828 ng/ml
Oven 1	5864 ng/ml	5821 ng/ml	5719 ng/ml
Oven 2	6133 ng/ml	5131 ng/ml	5514 ng/ml
Average	5837 ng/ml	5551 ng/ml	5121 ng/ml
Standard deviation	377	342	483

Since these concentration-numbers here are the amount of dFdC in the supernatant, the less dFdC that is detected, the more should have precipitated. It might look like ammonium dihydrogen sulphate shows a little bit lower concentrations than the rest. But the control sample (containing only water and dFdC) gives an even lower concentration of dFdC. All in all it seems like the small differences seen here are random, and may for example be a result from small differences under the pipetting. So, at the extremely low concentration of dFdC chosen for this experiment (0.5 mg/ml) with none of the salts precipitation of dFdC could be detected.

Experiment 2

When trying to find out which salt that had been used last time that had induced dFdC to precipitate, Massing suggested that I try ammonium phosphate (monobasic) and higher concentrations of dFdC. I

made 4 samples with decreasing amounts of dFdC; 20mg/ml (76mM), 10mg/ml (38mM), 5mg/ml (19mM) and 0.5mg/ml (1.9mM). The samples were made by mixing a 38mg/ml dFdC-solution with a 120mM solution of the salt. Therefore the higher concentration of dFdC, the lower the concentration of ammonium phosphate (monobasic) was, ranging from 57mM and up. When looking at these variations in concentrations of the salt it is clear that it was not planned good enough in advance, since the sample with the highest dFdC concentration, 76mM, had a higher molar concentration than ammonium phosphate (monobasic), 57mM. Though the difference was not that big, so if there was binding between the salt and the dFdC it should give detectable amounts of precipitation.

All 4 samples were placed in the fridge over night (about 18 hours), temperature being around 6^0C. The samples were then centrifuged on a benchtop centrifuge for 1.5minutes, followed by extraction of 100µM supernatant.

No visible precipitation were observed or detected on the HPLC.

Experiment 3

It was suggested that I tried to get concentrations of dFdC as high as 38mg/ml (maximum recommended concentration according to the manufacturer). This was achieved by weighing the dFdC powder directly into the reaction tubes, followed by adding the salt-solutions. Concentrations of the salts added were 120mM. All the same salts as in experiment 2 were used. The final concentration of dFdC was 38 mg/ml (114 mM), and all the salts 120 mM (so the molar ratio was close to 1). After mixing the gemcitabine powder with the salt-solutions all dFdC dissolved, except the mix with the ammonium phosphate (dibasic), where some undissolved powder was seen. To investigate this further I made mixtures of the salt-solution and dFdC-powder, resulting in final dFdC-concentrations of 20 mg/ml (76mM), 15 mg/ml (56mM), 10 mg/ml (38mM) and 5 mg/ml (19mM). The 20 mg/ml and the 15 mg/ml did not dissolve either, while the rest seemed to dissolve. All samples were placed in the fridge (~6^0C) over night (about 20 hours). The samples were then centrifuged on a benchtop centrifuge for 1.5minutes. 100µL were taken and immediately diluted (1/10) to prevent further precipitation. Samples were later diluted down to a theoretical concentration of 5µg/ml, and analyzed on HPLC.

When investigating the samples after the night in the fridge there seemed to be no difference from the observations made the day before. dFdC-concentrations of 15 mg/ml and above in ammonium phosphate (dibasic) showed a cake indicating that they had not (completely) dissolved, while there was

no visible precipitation in the other samples. Analysis by HPLC confirmed this (see the table below).

Table 12: Calculated precipitation of gemcitabine based on the measured concentrations and in comparison to theoretical concentration of the dilution used for HPLC (5000 ng/ml).

Substance	Sodium bisulphate anhydrous	Ammonium sulphate	Ammonium phosphate (monobasic)	Sodium dihydrogen phosphate (monobasic)	Ammonium hydrogen sulphate
Final dFdC concentration	38 mg/ml	38 mg/ml	38 mg/ml	38 mg/ml	38 mg/ml
Measured concentration in supernatant	5001 ng/ml	5021 ng/ml	4987 ng/ml	4963 ng/ml	5343 ng/ml
% precipitation	0%	0%	0%	1%	-7%

Substance	Ammonium phosphate (dibasic)	Ammonium phosphate (dibasic)	Ammonium phosphate (dibasic)	Ammonium phosphate (dibasic)	Ammonium phosphate (dibasic)
Final dFdC concentration	38 mg/ml	20 mg/ml	15 mg/ml	10 mg/ml	5 mg/ml
Measured concentration in supernatant	1241 ng/ml	2585 ng/ml	2943 ng/ml	5329 ng/ml	5965 ng/ml
% precipitation	75%	48%	41%	-7%	-19%

HPLC analysis resulted in concentrations of dFdC in the supernatant close to the theoretically expected value for all salts except for Ammonium phosphate. As the HPLC-results also showed, the solubility of dFdC in a solution of ammonium phosphate (dibasic) seemed to decrease as the concentration of the dFdC decreased. If the reason for the not solving dFdC was binding to the phosphate in the salt this process would have been expected to be slow, so that we first got dissolved dFdC, and then later a precipitation. Also if this were the mechanism we would not have expected a so rapid decrease in precipitation with lower concentrations of the dFdC.

To look further into this the pH-values of the different solutions were measured. It was revealed that the salt ammonium phosphate (dibasic) had a high pH, of 8.18, while the other salts ranged from 5.5 and below. It is expected that dFdC is less soluble at higher pH. This is because at higher pH-values a smaller fraction of dFdC is charged, thereby making it less soluble. Though a binding of dFdC and phosphate was also still a possible explanation.

Experiment 4

To investigate the findings in experiment 3 further I made solutions of potassium dihydrogen phosphate, ranging from 25 to 120mM. The pH of the solution was adjusted to 7 by drop-wise adding NaOH or HCl. The dFdC concentration was 38mg/ml (114mM), and was achieved by weighing the dFdC-powder directly into reaction-tubes, as in experiment 3, then adding the pH adjusted salt (because lack of small enough pH-meter to measure pH in reaction tubes at the time the pH in the final solution was not measured). To have something to compare with I made a sample were I simply mixed water and dFdC (no precipitation is expected to happen in water). The samples were stored in the fridge over night, then centrifuged and diluted as in experiment 3. The results can be seen in the table below.

Table 13: Precipitation of dFdC when increasing concentration of potassium dihydrogen phosphate. Measurements done on HPLC.

Concentrations of potassium dihydrogen phosphate	% precipitated
25mM	14%
50 mM	19%
120 mM	55%

As can be seen the fraction precipitated dFdC increased with increasing amount of potassium phosphate concentration.

Experiment 5

In the next step, the pH of all the salts-solutions used in experiment 3 was adjusted to 7 by adding drops of NaOH or HCl –solutions of 1 and 0.5 M respectively. In addition to the salts, which had been used in experiment 3, two other salts were included; di-sodium hydrogen phosphate dihydrate and Potassium dihydrogen phosphate, both in concentrations of 120mM and pH adjusted to 7. A solution of the desired salt and a dFdC solution of 3mg/ml were mixed together, giving a final concentration of 19 mg/ml dFdC (72mM), and 60mM of the desired salt. Samples were placed in the fridge (about 6^0C) over night (about 18h). Samples were observed, then centrifuged and an aliquot of the supernatant diluted to a theoretical concentration of 5µg/ml prior to measuring by HPLC. The results can be seen in the table below.

Table 14: Observed and measured precipitation in solution with a dFdC concentration of 19mg/ml (72mM), and a 60 mM concentration of different salts. pH in the salt-solutions was prior to the mixing adjusted to 7.

Solution	% precipitated dFdC	Was the precipitation visible?
Control sample (no precipitation)	0%	no
Sodium bisulphate anhydrous	45%	yes
Ammonium sulphate	12%	no
Ammonium phosphate (monobasic)	47%	yes
Ammonium phosphate (dibasic)	53%	yes
Sodium dihydrogen phosphate (monobasic)	60%	yes
Ammonium hydrogen sulphate	8%	no
di-sodium hydrogen phosphate dihydrate	76%	yes
Potassium dihydrogen phosphate	44%	yes

The results showed precipitation with all salts, but with great variations between the different salt-solutions. There also seemed to be not so big difference between the sulphate and phosphate salts, although altogether phosphate salts seemed to generate more precipitate. Though we had no real explanation for the variations between each individual salt type.

Experiment 6

The theory was still that there were a binding between the sulphate or phosphate salt and dFdC that created the precipitation. It was discussed that maybe the binding of the dFdC(+) and the phosphate/sulphate(-) could happen faster at a lower pH then 7, because the lower the pH the more dFdC would be charged. On the other hand a high pH for the salt would make more of it charged, so I had to find a point were they both could interact and form precipitation rapidly.

It was decided to go further with 2 salts from the previous experiment, one sulphate salt and one phosphate salt, and test them at different concentrations. Ammonium sulphate was chosen as the sulphate salt and ammonium phosphate dihydrate was chosen as the phosphate salt. The salts were mixed with dFdC to form a final dFdC concentration of 19mg/ml, and a salt concentration of 240, 120, 60 and 30 mM. Tree parallels of each pH and salt was made. One parallel was stored for 2 hours at 50^0C in a in an oven, then one hour in the fridge. One parallel was stored in the fridge for 2 hours, at about 6^0C. One parallel was stored in room temperature for 2 hours. They were all compared to a control sample that had been stored similarly, but only containing water and dFdC. They were then

centrifuged and diluted as in the other experiments.

For the samples where dFdC was mixed with a salt at pH 4 there were no visible or measurable precipitation. For the pH 7-salt-mixtures there were only visible precipitation with ammonium phosphate dihydrate 240 and 120 mM, and only the samples that had been stored in the fridge. The quantitatively measurements of precipitation in the "pH 7"-salts can be seen in the table below.

Table 15: Measured precipitation on HPLC of different salt-solutions, concentrations and storing conditions.

Salt and concentration	Stored in fridge for 2h (6^0C)	Stored in room temp 2h (20^0C)	Oven for 2h (50^0C), then 1h fridge
Ammonium sulphate:			
240 mM	-7%	6%	1%
120 mM	-6%	-11%	2%
60 mM	0%	-4%	5%
30 mM	4%	-10%	0%
Ammonium phosphate (dibasic):			
240 mM	39%	6%	-2%
120 mM	49%	11%	0%
60 mM	6%	-4%	3%
30 mM	1%	-5%	0%

A few days later I was able to get my hands on a small enough pH-meter to measure pH-values in very small amounts of solutions, like the ones I had in this experiments. When using it to measure the pH in the solutions that were tested I saw that the pH was far lower than what I was expecting. Ammonium phosphate (dibasic) seemed to have the best buffer capacity, and was least affected by the adding of the acidic dFdC-solution. The pH of the solutions of salt and dFdC that was supposed to be 7 can be seen in the table below:

Table 16: pH measured on mixtures of a salt of pH 7 and dFdC.

Salt and concentration	Fridge temperature samples	Room temperature samples	Oven + fridge samples
Ammonium sulphate:			
240 mM	3.2	3.2	3.2
120 mM	3.2	3.2	3.2
60 mM	3.0	3	3.0
30 mM	3.0	3	2.9
Ammonium phosphate (dibasic):			
240 mM	6.1	6	6.0
120 mM	5.6	5.6	5.6
60 mM	3.8	3.8	4.0
30 mM	3.3	3.3	3.2
Control sample (water, no pH adjustment):	2.7	2.7	2.7

The adding of the solution of gemcitabine had clearly influenced the pH of the different solutions. This also led me to the conclusion that the pH might have been more important than the type of salt used in the solution.

Experiment 7, pH adjusted solutions

The same salts were used as in last experiment, at a final concentration of 240mM. The final concentration of dFdC was 19mg/ml. But since the pH was adjusted after the mixing the real concentration of dFdC and salt would actually be slightly lower, and also varied a bit from vial to vial. To know the exact concentration at all time the reaction tubes were weight on a balance before and after the adjustment. This was then taken into consideration in the further dilutions. The problem with this approach though was that the concentrations of dFdC vary a bit, and that may in some cases make it hard to distinguish if the precipitation was because of the pH or the dFdC concentration.

As a check to see if it was the pH alone leading to the precipitation I also prepared a sample of dFdC and water, which I tried to pH-adjust to about 7, adding sodium hydroxide or hydrochloric acid. The results can bee seen in the table below.

Table 17: % precipitation of dFdC in pH adjusted solutions, which had stayed one night in the fridge.

Target pH	Actual pH	pH after~15h	Precipitation visible?	Precipitation in %	dFdC concentration after pH adjusting
Ammonium phosphate (dibasic):					
2	1.9	2.1	no	45%	14.6 mg/ml
4	3.9	4.6	no	7%	10.7 mg/ml
5	5.1	5.3	no	8%	9.9 mg/ml
6	5.9	6	yes	72%	16.6 mg/ml
7	7	7.2	yes	62%	16.9 mg/ml
8	8.2	8.6	yes	54%	14.3 mg/ml
Ammonium sulphate:					
6	5.4	5.4	no	-52%	8.1 mg/ml
7	7	7.2	yes	68%	11.9 mg/ml
Water:					
~7, (though water has no buffer capacity)	7.2	4.4	yes	51%	12.9 mg/ml
Control, pH not adjusted…	2.7	2.7	no	0%	19.0 mg/ml

The results represent just one parallel, and the differences seen should therefore not be interpretated to much by it self. Nevertheless it is pretty clear that the pH is important for the precipitation. Also one surprising thing that clearly goes against the salt binding theory for the precipitation is that dFdC in a solution of pH-adjusted water also precipitated. This was necessary to investigate further.

Further parallels

Firstly I made 2 more parallels, parallel 2 and 3, in the similar way. Then, since some of the results was a bit unclear I made parallel 4. The results can be found in the table below.

Table 18: Precipitation at different pHs, in overnight samples, up to 4 parallels. Parallel 1, pH 5 was removed, as it differs much from the rest of the measurements.

Target pH	% Precipitation, all parallels # 1	# 2	# 3	#4	Average	Standard deviation	Precipitated - standard deviation
Ammonium phosphate (dibasic)							
2	45%	-16%	8%		12%	25%	-13%
4	7%	-20%	22%		3%	17%	-14%
5	(8%)	40%	49%	57%	49%	7%	42%
6	72%	50%	52%	62%	59%	9%	50%
7	62%	55%	49%	53%	55%	5%	50%
8	54%	50%	39%	50%	48%	6%	43%
Water							
6	-	46%	20%		33%	19%	14%
07 / 08	51%	40%	39%		43%	5%	38%
Control sample	0%	0%	0%	0%	0%	0%	0%

Putting the numbers into a diagram gives the figure below. In the table above and the figure below parallel 1, pH 5 was ignored because it differed significantly from the rest of the numbers obtained. That could maybe also have been done with parallel 1, pH 6, though the standard deviation of pH 6, 7 and 8 would still have crossed each other. One explanation for this big standard deviations may be that the parallels, except parallel 2 and 3, was carried out on different days, and that conditions such as fridge temperature, time in fridge or handling may have varied slightly from day to day. There seems to be a correlation within each parallel of which pH gives the best precipitation. pH 6 for example is the "winner" of all parallels except parallel 2.

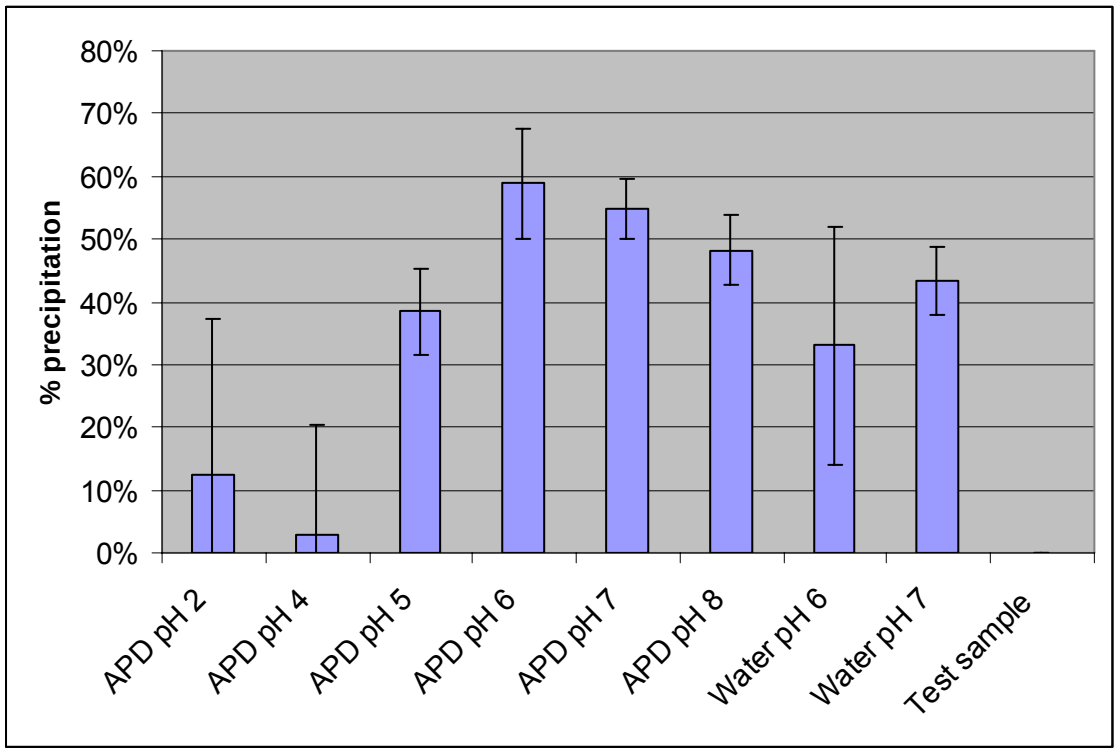


Figure 6.6. % precipitated dFdC in different pH's in solution of ammonium phosphate (dibasic) (APD) or water

Something else that might be the cause of the variations in precipitation between pH 5, 6, 7 and 8-samples might have been the concentration of the dFdC. The solutions with pH 6 and 7 had an average of 17.0 and 17.6 mg/ml respectively, while the pH 5 and pH 8 solutions had an average of 15.9 and 15.4 respectively (not counting pH 5 parallel 1). This problem could have been avoided first adjusting the pH, then fill up the sample with water so that all the samples had the same concentration of dFdC.

Crystal shape and differences

Though pH-adjusted water also gave precipitation it was not as high as the precipitation at similar pH in ammonium phosphate (dibasic). This could be caused by the low buffer-capacity of the water, for instance a sample of water and dFdC that was adjusted to a pH of about 7 was measured to pH 4.4 the next day. But it could also mean that there was a precipitation because of pH, but that it in addition was a binding of ammonium phosphate (dibasic) and dFdC. If this was the case this might have created different forms of crystals.

To investigate this I extracted some precipitated dFdC from the parallel 1 samples, and looked at them in a microscope. They can be viewed in the figures below:

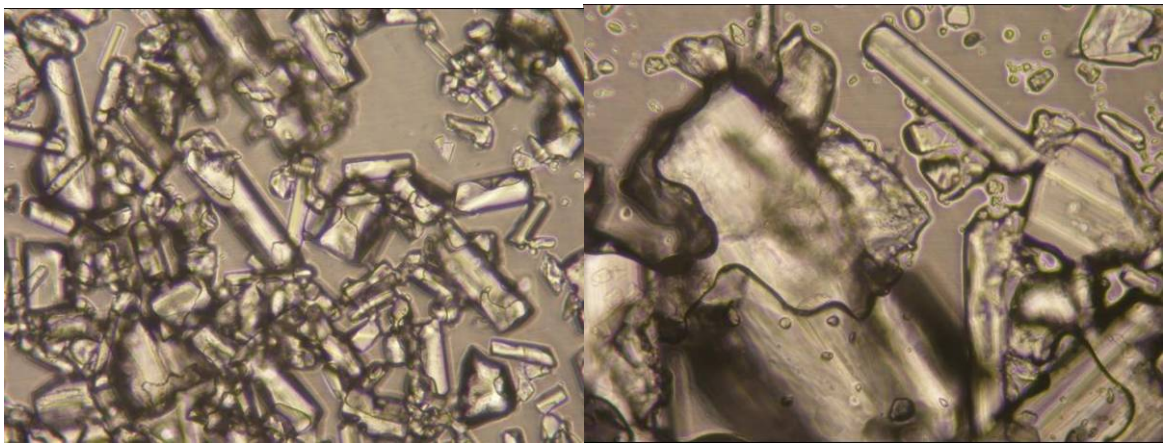


Figure 6.7. Investigation of dFdC-crystals in microscope. On the left side: Water and dFdC, pH 7. On the right side: ammonium phosphate (dibasic) and dFdC, pH 7.

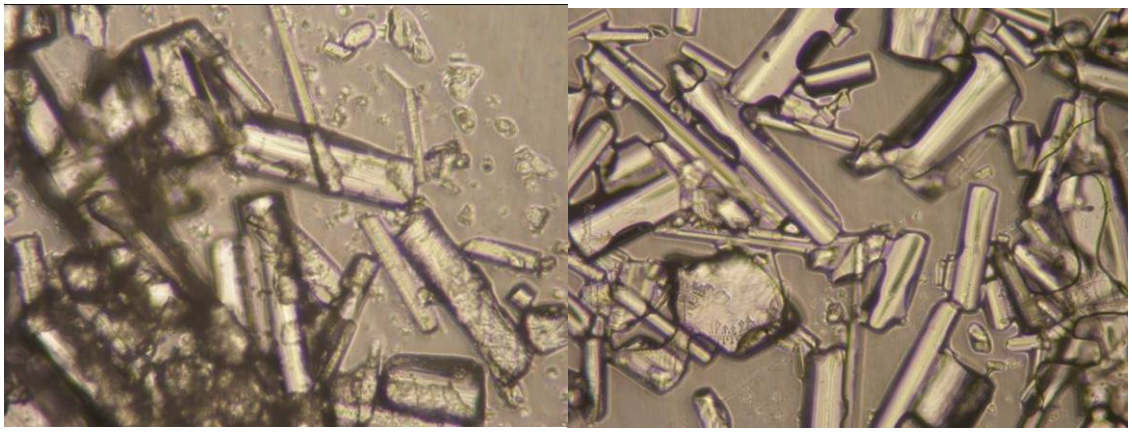


Figure 6.8. Investigation of dFdC-crystals in microscope. On the left side: ammonium sulphate and dFdC, pH 7. On the right side: ammonium phosphate (dibasic) and dFdC, pH 8.

The pictures differ slightly, and might lead you to think that it is a difference. But the crystals are mainly rod-formed, and of equal size. The difference is the spot in the sample were the picture is taken. By studying the samples carefully in the microscope I came to the conclusion that there were no visible differences that would indicate that any of the samples had an other kind of crystals than the rest.

Experiment 7, room temperature experiment

All the experiments above were carried out in the fridge. Both to get an idea of the stability of the precipitated dFdC, and as a possible approach to loading, there was decided that I should test the achieved precipitation at room temperature. The pH in 4 parallel vials containing dFdC and ammonium phosphate (APD) was adjusted to 6.5 (+/-0.2). The vials were then filled up with distilled water so that

the concentration of dFdC in all vials was 15.83 mg/ml. The vials were stored in the fridge over the weekend (about 70 hours), before samples of the supernatant were diluted to a theoretical concentration of 5.0 µg/ml and analyzed by HPLC. To determine the amount of dFdC that had precipitated the concentration of dFdC in the supernatant were compared with a control sample (a sample that had gone through the same conditions, but only containing dFdC and water, and therefore was expected not to precipitate).

The result was a average of 32% (+/- 2.7%), so a little bit lower that the results from the "fridge-samples". All vials had visible precipitation on inspection, but on the measuring on HPLC parallel number 4 showed a precipitation of only 2%. Parallel 4 was of this reason ignored. The results can bee seen in the table below.

Table 19: Measurements of precipitated dFdC in solutions of 120 mM ammonium phosphate (dibasic) (APD), pH 6.5. The value of parallel 4 differed significantly from the other numbers, and was therefore ignored.

Salt and pH	% precipitated
#1 APD pH 6.5	30%
#2 APD pH 6.5	31%
#3 APD pH 6.5	35%
#4 APD pH 6.5	~~2%~~
Control (H2O)	0%

Average (not parallel 4):	32%
Standard deviation (not parallel 4)	2.7%

Experiment 8, test of precipitation in different solutions

The results from experiment 6 gave a strong indication that the precipitation of dFdC was not a result of a binding to a salt, but rather a result from the pH in the solution. The only thing that might indicate something else is that the precipitation in pure water was generally lower than the other solutions. My theory was that this was the result of the low buffer-capacity in the water-dFdC-solution. To investigate this further I made an experiment making solutions of dFdC and 120mM of either ammonium phosphate (dibasic) or sodium bisulphate anhydrous, or 25mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 3 parallels of each solution. The pH was adjusted, and the solutions were further diluted to a concentration of 10.36 mg/ml dFdC. Samples were stored in fridge (~6^0C) over night (18 h). The samples were then investigated, centrifuged, and parts of the supernatant was extracted and investigated further. The results and can bee seen in the table and figure below.

Table 20: Precipitation of dFdC in solutions of different salts and concentrations, pH adjusted to 6.5.

Solution	Parallel 1	Parallel 2	Parallel 3	Average	Standard deviation
Ammonium phosphate (dibasic) 120mM	35%	38%	32%	35%	2%
Sodium bisulphate anhydrous 120mM	45%	28%	39%	37%	7%
HEPES 25mM	24%	31%	36%	31%	5%
Control samples	0%	0%	-	0%	0%

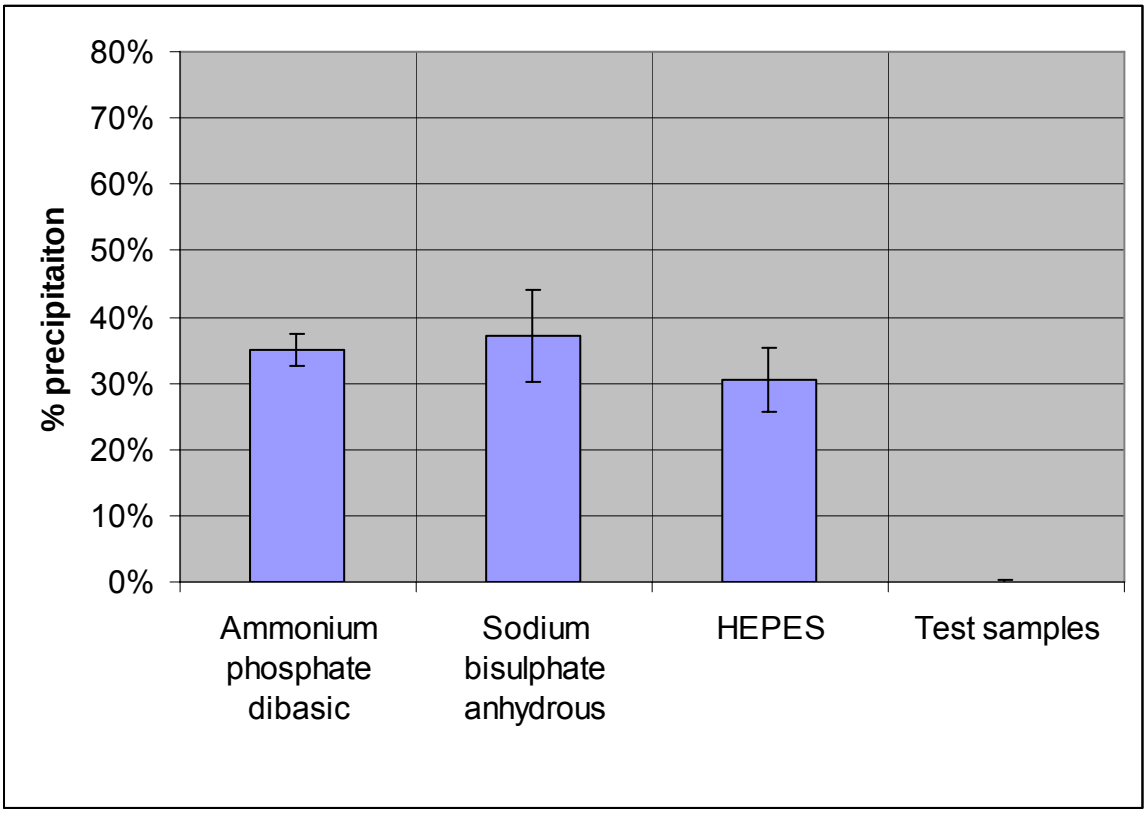


Figure 6.9. % precipitated dFdC in different pH's in solution of ammonium phosphate (dibasic) (APD) or water

As all the standard deviations overlap there were no significant difference between the types of salt used in the solutions. This is another indication for that the precipitation is a result of pH alone, and not related to the type of salt used in the solution.

Experiment 9, test of the effect of pH when concentration of dFdC is constant

Experiment 7 indicated that there might be an optimal pH-region for precipitation at around pH 6-7 (though not significantly better than pH 8). But the results might be biased by the fact that the pH 5 and pH 8 samples generally were more diluted than the pH 6 and pH 7 samples. Therefore I made one more experiment varying the pH'es. Solutions of 120 mM ammonium phosphate (dibasic) and 16mg/ml dFdC were made in the same way as experiment 8. pH'es ranged from 4 to 8, in addition to two control-solutions containing only dFdC and water. Because of the costs of dFdC, and because we already had the results from experiment 7, there were only made one parallel. Samples were stored in the fridge (about 5^0C) over night (19 h), then centrifuged and supernatant were extracted as usual.

Precipitation was observed in all samples except the two control samples. The results can bee seen in the table below:

Table 21: Precipitation of dFdC in solutions Ammonium phosphate (dibasic) 120mM and 16mg/ml dFdC, varying pHs.

Target pH	4	5	6	7	8
% precipitated dFdC	36%	29%	44%	42%	35%

The results continue to back up the previous results, that pH 6 and 7 gave higher precipitation than both pH 5 and pH 8 solutions. But the precipitation at pH 4, combined with the fact that pH 4 gave higher precipitation than pH 5 and that there were only one parallel at each pH contributes to create doubt around the result. But at least the precipitation at pH 4 can be explained by the fact that the temperature in the fridge was measured to about 1^0C colder than in the previous experiments.

Experiment 10, stability of the precipitated dFdC

In order to load the dFdC into liposomes heating/freezing cycles could be one approach. Without heating the liposomes, the crossing of the liposome membrane is believed to take significantly longer time. With the help of heating/cooling cycles it would be possible to let the liposomes passively load, then cool them down to let the dFdC inside precipitate, then heat the solution up again so that more dFdC can passively load into the liposomes. Though for this approach to become more than just passively loading it is dependent on that the dFdC inside the liposomes don't dissolve again as soon as it

is heated up. Therefore it was necessary to take a look at the stability of the precipitate from the liposomes.

Small preliminary tests without quantification

Firstly I did some small tests with some precipitated samples, which I only inspected visually. Samples were placed on a water bath, and inspected visually after a certain amount of time. RPM on the water bath was set to 50. For times under one hour it seemed like at least not all the precipitated dFdC did dissolve at temperatures under 40^0C. One new experiment at 45 and 55^0C also showed that not all of the precipitated dFdC did dissolve within an hour on the water bath. Also 60^0C for 40 minutes did not dissolve all precipitated dFdC (it did not dissolve after 40 minutes either, but the experiment were terminated).

But this experiment only shows what we already knew, that the precipitated dFdC is hard to dissolve. What we needed to know was how hard it was to dissolve, in addition to how much dFdC dissolves after a certain amount of time.

Quantifying dissolution-rate of precipitated dFdC at elevated temperatures for one hour

For this experiment I used samples from experiment 8, "test of precipitation in different solutions". The pH of the salts used were 6.5, and the salt used was 120 mM ammonium phosphate. Before this test the samples had stayed in the fridge for 2 days. The experiment was set up to take out 50µL of sample every 15 minute, to measure if more dFdC had been dissolved after a certain time period. Theoretically withdrawing aliquots from the reaction tube would not affect the concentration of dFdC being dissolved in the solution, as this is dependent on the solubility product and velocity of the dFdC to dissolve. For comparison, one reaction tube sample was placed in an oven, while one were placed on a water bath at 100rpm. Temperature in both places was set to 60^0C.

All samples were diluted to a theoretical concentration of 6µg/ml, prior to analysis on HPLC. The APD-samples were then compared to the average of the control samples. The results can be seen below:

Table 22: Estimated precipitated content left in reaction tube, based on analyses of the supernatant. Incubation temperature is 60^{0}C.

	Estimated precipitated amount left (%)			
	Water bath		Heating oven	
Time (minutes)	APD pH 6.5	Control sample	APD pH 6.5	Control sample
0	44%	4%	45%	-4%
15	46%		43%	
30	44%	0%	45%	8%
45	38%		37%	
60	39%	-5%	41%	-4%
Average		0%		0%
Standard deviation		4%		6%

As these results showed, some dFdC did dissolve though the increase were not that high after 1 hour. Also, the last measurement shows a slightly increase of not dissolved dFdC. Therefore this was needed to be investigated further.

Quantifying dissolution-rate of precipitated dFdC at elevated temperatures for two hours

For this experiment I used the samples from experiment 9, "test of the effect of pH when concentration of dFdC is constant". Both samples, and the control sample, had stayed in the fridge over the weekend. Except that the experiment was carried out during a longer period of time, the rest were as last time. Results can bee seen in table below.

Table 23: Estimated precipitated content left in reaction tube, based on analyses of the supernatant. Temperature is 60^{0}C.

	Estimated precipitated amount left (%)					
	Water bath			Heating oven		
Time (minutes)	APD pH 6	Precipitation visible?	Control sample	APD pH 7	Precipitation visible?	Control sample
0	40%	Yes	0%	43%	Yes	0%
30	37%	Yes		42%	Yes	
60	27%	Yes	1%	41%	Yes	1%
90	22%	No		35%	Yes	
120	18%	No	-3%	8%	No	4%
Average			-1%	Average		2%
Standard deviation			2%	Standard deviation		2%

Discussion

Careful considerations are necessary when analyzing these results. Especially when using them to plan how to load liposomes. Except for the fact that there are few parallels, and that samples from earlyer experiments were used, one of the biggest problems with this study is the size of the precipitated dFdC-crystals, which beforehand had been centrifuged to the bottom of the reaction tube. It was for example observed that when heating precipitated dFdC the first thing to disappear were the small crystals on the walls of the reaction tube. So one should probably be careful transferring these findings directly to what would happen in liposomes.

Experiment 10, speed of precipitation, fridge compared to ice

In this experiment I would measure how fast precipitation occurred under different conditions. What I wanted to measure was how fast the precipitation occurred in the fridge (measured to 5.7^0C that day) compared to an ice bath (which will be close to 0^0C all the time). Solutions of 120 mM ammonium phosphate (dibasic) and 19mg/ml dFdC were made. pH was then adjusted to 6.5, and dFdC concentration to 14.6µg/ml in all vials. One sample of 50µL was extracted every hour for 3 hours. It was then centrifuged and diluted as in the other experiments. The results can bee seen in the table below:

Table 24: Precipitation by dFdC hour by hour, fridge compared to ice.

	Precipitation		
Sample type	1 hour	2 hour	3 hour
Fridge, APD pH 6.5, #1	62%	59%	66%
Fridge, APD pH 6.5, #2	59%	59%	66%
Fridge, APD pH 6.5, #3*	5%	37%	52%
Fridge, H2O (Control)	-1%	-4%	5%
Avarage (excl. control)	42%	52%	61%
Standard deviation (excl control)	26%	10%	6%
Ice, APD pH 6.5, #1	69%	79%	83%
Ice, APD pH 6.5, #2	43%	63%	71%
Ice, APD pH 6.5, #3	59%	36%	80%
Ice, H2O	29%	73%	28%
Avarage (excl. control)	57%	59%	78%
Standard deviation (excl. control)	10%	17%	5%

As seen in the table above also control sample of dFdC on ice precipitated. Though it was measured

both at hour 1, 2 and 3, it was only visible at hour 2, and then it disappeared. As 29% precipitation also would have been visible the measurements of the ice control sample after 1 and 3 hours can be assumed to be false positives, reasons unknown. The precipitation after 2 hours on the other hand was visible. It therefore seems that dFdC can precipitate at very low temperatures even if the pH is low, though at low pH it also seems to dissolve easily. On the other hand this seems to hint that the temperature on the icebath was not stable, as it is expected to be, and questions the reliability of the results. Just for the record, all the precipitations were calculated against the control sample from the fridge.

6.3.3 4th set of experiments; using precipitation as an instrument to load liposomes.

The results from the 3rd sets of experiments were not the ones we had been hoping fore. The mechanism behind the precipitation seems to bee dFdC's low solubility at higher pHes, rather than complex forming with another salt such as the phosphate salt. Even on ice, with a concentration of 14.6µg/ml dFdC, the precipitation was not higher than 83% (+/-5%) after 3 hours. To make dFdC load via this method a high pH inside the liposomes is needed for precipitation to take place, while pH on the outside should be much lower so to avoid precipitation there. This is the total opposite of the active loading via an ammonium sulphate gradient described in section 3.3, active loading. As known from this section a significant amounts dFdC will get charged at low pHes, and charged dFdC does not cross liposomes membranes. This might then cause an effect, that I choose to call reverse loading, where gemcitabine get charged on the outside of the liposomes and therefore will not pass the membrane to the inside. To prevent this reverse loading the external pH must be low enough for precipitation not to take place (or at least so high that it easily redissolves on light heating), but high enough to prevent reverse loading. And also even though the heating experiments with dFdC showed good stability of precipitated dFdC the same might not be the case in liposomes. In short there were lots of uncertainties to this way of loading gemcitabine into liposomes, and the only thing I could do was to make a professional guess, then test if it worked.

Experiment 1, two weeks loading in cold-room

It was decided to first try loading in very low temperatures, since the precipitation was seen to be highest here. Hilde Gravem had shown that loading in room temperature for over 24 hours gave comparable results to room temperature for1 hour followed by heating for 2 hours (Gravem 2006).

The plan was to load the liposomes in a cold-room expected to be around ~5^{0}C. Becouse this was significantly colder than what Gravem used it was decided to load them for a significantly longer period; two weeks.

As mentioned in the introduction to this section I no longer try to load the liposomes as described in section 3.3, active loading, where ionization of the gemcitabine on the inside of the liposomes is the reason for entrapment. What I want to do here is to make gemcitabine precipitate inside the liposomes, and as found in the 3rd sets of experiments this is archived by a high pH. As pH 6 and 7 had shown to give the best precipitation (all though not significantly better than pH 8), I decided to use a pH of 6.5 as the inner pH. To buffer the solution I used ammonium phosphate (dibasic) (APD), because I by my experience with it during the precipitation experiments observed that its buffer capacity was very good. As outer pH I made one parallel with pH around 3 and another parallel with pH around 4 to see which of them gave the best loading.

The VPGs were prepared similar to the 2nd sets of experiments, with batches of 1000 mg VPGs, containing 35% lipids, and 65% of 240mM APD, pH 6.5. In advance I had experimented with how to mix the liquids to get the desired pH in the outer phase of the liposomes. The VPGs that were going to have the pH 4 in the outer phase I diluted 1/3 with more 240 ammonium phosphate (dibasic), divided them into 4 parallels, and diluted them 1/2 with a solution of 38mg/ml dFdC in water. The liposome batch which was going to have an outer pH of 3 I diluted 1/3 with 240 mM ammonium phosphate (dibasic) pH 4, then divided them into 4 parallels and diluted them 1/2 with a solution of 38mg/ml dFdC in water. The pH in each liposome batch was then measured, and the results can be seen in table below:

Table 25: pH of the outer phase of the liposome batches.

Parallel:	**#1**	**#2**	**#3**	**#4**
Target: pH 4	3.98	4.1	3.91	4.07

Parallel:	**#1**	**#2**	**#3**	**#4**
Target: pH 3	3.31	3.23	3.17	3.19

As seen the pH 4 hit quite well, while the batches targeted pH 3 got a little too high. That did not matter too much though, the most important thing was that they were significantly lower than the pH 4

samples.

The samples were then placed in the cooling room for 2 weeks. So that the liposomes would not sediment the reaction tubes were placed on a shaking machine at 50 RPM.

After the two weeks aliquots of the liposome samples were withdrawn and applied on cation exchange-columns to separate the free dFdC in the solution from the entrapped. This samples I choose to call "loaded samples", while the samples that did not undergo cation exchange, thus containing both free and entrapped dFdC, I choose to call 100% samples. Both the loaded samples and the 100% samples were cracked with triton-X, and then diluted to a theoretical concentration of 6µg/ml before being applied on HPLC.

PCS results

PCS-measurements were taken of the liposomes with dFdC before and after the 2 weeks. The values may indicate that there has been a change in the size-distribution of the liposomes within the two weeks. The mean diameter of the samples were unusually high before the two weeks in the fridge, while it seems like it had been reduced afterwards, at least in the pH 4 sample.

Table 26: PCS-results, outer phase pH 4, right after production

	Channel width 27 (auto)		Channel width 20		Channel width 10		Channel width 5	
Mean diameter (nm)	100.7	108.4	103.6	104.3	123.4	124.9	180.5	189.9
Variance (P.I)	0.249	0.23	0.249	0.253	0.226	0.223	0.118	0.1
Chi squared	10	18	4	4	0.394	0.895	0.893	1

Table 27: PCS-results, outer phase pH 3, right after production

	Channel width 88.0 (auto)		Channel width 20		Channel width 10		Channel width 5	
Mean diameter (nm)	336.7	334.1	90.9	92.8	81.5	81.5	80.1	69.3
Variance (P.I)	0.48	0.524	1	1	1,232	1	0.64	0.748
Chi squared	986	2,178	0.947	1	0.761	0.792	1	1

Table 28: PCS-results, outer phase pH 4, after 2 weeks

	Channel width 44.0 (auto)		Channel width 20		Channel width 10		Channel width 5	
Mean diameter (nm)	95.3	93.6	65.6	65.7	58.6	58.6	57.4	57.8
Variance (P.I)	0.548	0.573	0.803	0.808	0.799	0.797	0.656	0.646
Chi squared	82.485	188.352	5.172	10.207	0.568	0.966	1.165	1.242

Table 29: PCS-results, outer phase pH 3, after 2 weeks

	Channel width 50 (auto)		Channel width 20		Channel width 10		Channel width 5	
Mean diameter (nm)	118.0	116.7	77.5	77.6	84.7	83.5	141.5	155.5
Variance (P.I)	0.564	0.587	0.841	0.843	0.702	0.706	0.361	0.329
Chi squared	118.067	256.439	0.457	0.439	0.739	0.891	0.976	1.238

Temperatures

The temperature in the room was measured with an electronic thermometer to keep track of any big changes in temperature during these two weeks. One measurement was preformed every hour, and there were 411 measurements in total. The maximum temperature was 13.40^0C, the minimum 4.30^0C, and average was 6.55^0C. A graphical description of the temperature-variations during the two weeks can be seen in the figure on the next page.

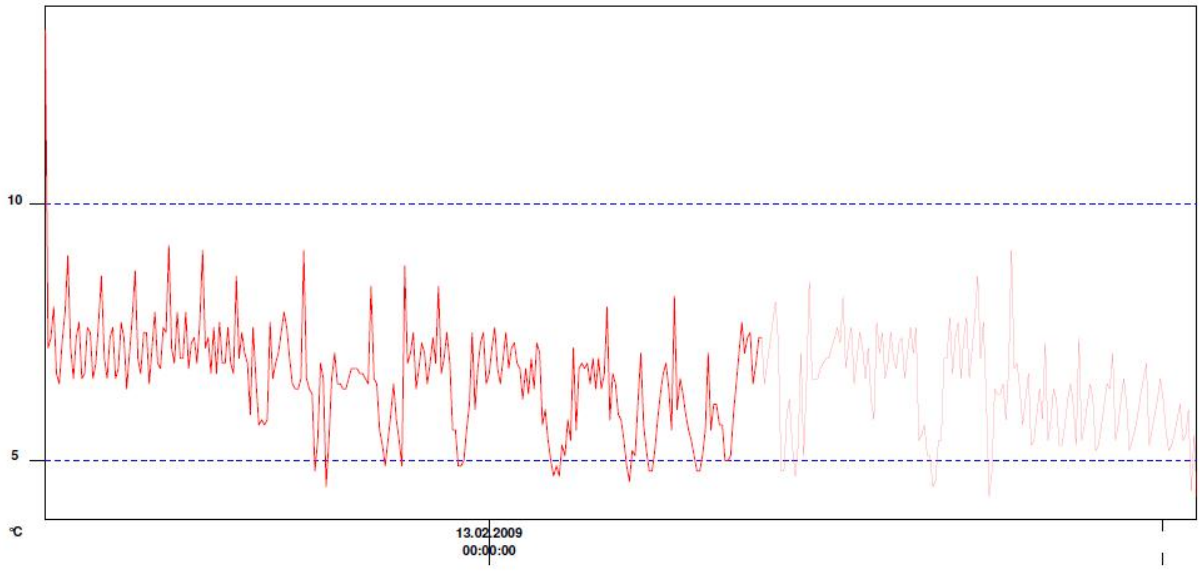


Figure 6.10. Temperature in the cold-room during the two weeks of experiment.

dFdC values

The dFdC values showed no loading, but the rest of the samples were in the expected range. Control samples were blank. The results can bee seen in the tables below:

Table 30: dFdC values, loaded samples. pH indicates the target pH in the outer phase.

Sample	Loaded pH3 #1	Loaded pH3 #2	Loaded pH3 #3	Loaded pH3 #4
Theoretical concentration	6000	6000	6000	6000
Measured concentration	0	0	0	0

Sample	Loaded pH4 #1	Loaded pH4 #2	Loaded pH4 #3	Loaded pH4 #4
Theoretical concentration	6000	6000	6000	6000
Measured concentration	0	0	0	0

Table 31: dFdC values, 100% samples. pH indicates the target pH in the outer phase.

Sample	100%sample pH3 #1	100%sample pH3 #2	100%sample pH3 #3	100%sample pH3 #4
Theoretical concentration	6000	6000	6000	6000
Measured concentration	4295	7327	4297	5133

Sample	100%sample pH4 #1	100%sample pH4 #2	100%sample pH4 #3	100%sample pH4 #4
Theoretical concentration	6000	6000	6000	6000
Measured concentration	3435	3204	4467	3189

Cholesterol value

Cholesterol values were mostly in the expected range, though there were some smaller variations. The results can bee seen in the tables below:

Table 32: Cholesterol values loaded samples. pH indicates the target pH in the outer phase.

Sample	Loaded pH3 #1	Loaded pH3 #2	Loaded pH3 #3	Loaded pH3 #4
Theoretical concentration	138.6	138.6	138.6	138.6
Measured concentration	167	146	97	105
"Loss" of cholesterol:	-17%	-18%	18%	43%

Sample	Loaded pH4 #1	Loaded pH4 #2	Loaded pH4 #3	Loaded pH4 #4
Theoretical concentration	138.6	138.6	138.6	138.6
Measured concentration	108	149	140	140
"Loss" of cholesterol:	35%	24%	19%	31%

Table 33: Cholesterol values 100% samples. pH indicates the target pH in the outer phase.

Sample	100%Samples pH3 #1	100%Samples pH3 #2	100%Samples pH3 #3	100%SamplespH3 #4
Theoretical concentration	138.6	138.6	138.6	138.6
Measured concentration	145	126	175	170

Sample	100%Samples pH4 #1	100%Samples pH4 #2	100%Samples pH4 #3	100%Samples pH4 #4
Theoretical concentration	138.6	138.6	138.6	138.6
Measured concentration	157	189	180	195

Discussion

There were no detectable loading of the dFdC. As mentioned under the discussion of the 2nd sets of experiments when diluting the samples to a theoretical concentration of 6µg/ml we should be able to detect loading efficiencies as low as 1.7% (and even lower) at the HPLC.

Maximum theoretical loading

Even though we did not use size extrusion chromatography to get rid of any ions, the liposomes was diluted to some extent before loading. We can assume the VPGs entrapped 40% of the water content in the solution. The VPG's were then diluted 1/3, and then 1/2 again when adding the dFdC-solution. That would result in 6.67% of the total water content being inside the liposomes, hence if there were only passive loading it would be expected to be in that range. Therefore this can not be the only explanation for the bad loading.

Osmolarity

One issue is that the osmolarity were not taken into consideration when mixing the dFdC and the liposomes; the dFdC powder was dissolved in water, not NaCl. Also the concentration of ammonium phosphate (dibasic) was considerably over the osmolarity of 0.9% NaCl (see calculations on isotonicity in appendix 2). Theoretically this would crate a hypotonic environment, causing liposomes to swell and crack. This might also be one of the reasons for the results of the PCS being higher then usual (see PCS results above).

Temperature

There can be discussed if any dFdC passed the membrane of the liposomes at such low temperatures. Even though the period of time was quite long this might not have been enough for the dFdC to cross the membrane.

Other factors

As discussed in the start of this experiment there is a chance that we get a "reverse loading" effect, where dFdC get charged in the outside of the liposomes, and therefore less dFdC passes the membrane. Even at pH 4 a significant amount of dFdC, 38%, will be charged. Precipitation is also a lot slower process.

Also it is known that nucleoside analogues such as gemcitabine may cause hydrolysis of liposomal phospholipids (Moog, Brandl et al. 2000). As this was a quite long experiment lasting for 2 weeks hydrolysis would have plenty of time to occur, and may also have been a contributing factor to the result.

Experiment 2, loading with heating cooling cycles

Procedure

With the experience gained from experiment 1 and the 3rd sets of experiments I tried to set up an experiment with heating cooling cycles that was likely to give a high loading. To have exact and reproducible temperatures a water bath was used for the heating, while the cooling found place on an ice bath (expected temperature of 0^0C). The cycles were set up in the following 6 steps:

1. Heating in water bath at 60^0C for 2 hours
2. Cooling on ice for 3 hours
3. Heating at 60^0C for 30 minutes.
4. Cooling on ice for 2 hours
5. Heating at 60^0C for 30 minutes
6. Cooling on ice for 2 hours

The heating at step 1 was so that dFdC could pass the liposome membrane easily. Since there is nothing entrapping dFdC inside the liposomes the loading in this process is expected to be equal or less than passive loading (less because of the "reverse loading" effect previously mentioned). This was followed by step 2, a long cooling-time so that all dFdC already inside the liposomes could precipitate. The 30 minutes at 60^0C in step 3 was a compromise between wanting more dFdC to enter the liposomes while hopefully not solving the already entrapped dFdC. The heating-experiments of dFdC precipitated dFdC showed in the 3rd set of experiments the dissolving of the precipitate happened gradually and took over an hour to dissolve completely (thought we did not know if the same would be the case inside liposomes).

This loading depended on that a large amount of dFdC inside the liposomes would precipitate during the cooling cycles. New dFdC would also have to enter the liposomes every time we heated up the solution, while little of the precipitated dFdC would dissolve. According to this theory the last step was a little bit unnecessary as no loading occurs between step 5 and step 6, just precipitation of dFdC already inside (for more dFdC to be loaded new dFdC has to enter). But in total there should be 3 loading cycles were more dFdC get into the liposomes.

The pH on the inside of the liposomes was decided to be 7. This was mainly because good precipitation were seen on this and surrounding pH, but also because that the lyso-transformation of EPC-3 liposomes showed some of the lowest lyso-transformation at this pH (Moog, Brandl et al. 2000). The outer pH was set to 4. These were a compromise between trying to avoiding precipitation on the outside of the liposomes, and at the same time reduce the extent of the "reverse loading".

To buffer the solution both on the inside and outside of the liposomes a solution with 133 mM ammonium phosphate (dibasic) was used. This concentration was calculated to be isoosmotic to 0.9% NaCl (see appendix 2). The solution of dFdC, containing 38mg/ml dFdC also consisted of 0.9% NaCl. Since most of the previous experiments on precipitation were at around 16µg/ml dFdC, used the same concentration in this experiment.

1000mg liposomes were made according to procedure, and then diluted with 1263µL 38mg/ml dFdC in 0.9% NaCl, and 737µL ammonium phosphate (dibasic) pH7. To try to find how to make the outer pH 4 I made a "simulation" of the amounts of dFdC that I was going to use. When mixing the solutions in the ratio mentioned above, simulating the inner phase by using only half of the solution, I measured a pH of 4.08, and that was the reason for the mixing regime I used. Also in this mixture of liposomes the

liposomes were not diluted so much as in previous experiments. If the inner water phase was 40% at start, the VPGs are only diluted ones, 1/3, and the inner water phase should be about 13 % (and so passive loading would be expected to be in that range). The liposomes were divided into 2 parallels of 600µL each.(Learned from the previous experiments I had seen that it were often smart to test a few parallels first to see if they work, then run a full size experiment)

I was making several withdrawals of the liposome dilutions at different times in the loading process, totally 6 times, extracting 50µL each time. This was to keep track of the course of the loading-process. The reaction tubes were vortexed before each extraction so that the lipid content extracted each time would be the same. Therefore the extraction would not interfere with the ratio of inner and outer dFdC.

After each extraction of samples they were diluted 1/10 in 0.9% NaCl, before free dFdC were removed by ion exchange. All samples were cracked with triton-x, and then diluted to a theoretical concentration of 6µg/ml prior to HPLC.

Because of lack of time in addition to a big worldwide shortage of acetonitrile, the cholesterol values were not measured. In most of the previous experiments the cholesterol values had been in the expected range, and they had not given much value to the previous experiments anyway.

First pare of parallels

Samples were taken after step 1 (heating, passive loading), step 2 (1. cooling), after step 4 (2. cooling), after step 5 (3. heating), and after step 6. Actually there was no reason for taking a sample at step 2, as only precipitation, not loading had occurred. Samples taken at step 5 and step 6 should also have about the same value.

Measurements for PCS were taken of the liposomes before the first heating and after the first cooling, the last sample to see if the cooling might have destroyed the liposomes in some way (for example by crystal forming dFdC). The PCS-results looked normal and in range, and there did not seem like any mayor differences between the first and second measurement, except maybe some strange values at the auto channel width in the first PCS-results (see below). The reason for this might be some very large liposomes in the first measurement.

Table 34: PCS-result before step 1

	Channel width 60 (auto)		Channel width 20		Channel width 10		Channel width 5	
Mean diameter (nm)	124.9	122.6	65.8	65.2	55.7	56.2	51.3	51.6
Variance (P.I)	0.679	0.697	1.020	1.006	0.974	0.939	0.753	0.745
Chi squared	480.941	948.582	9.607	16.560	0.980	1.258	0.925	0.985

Table 35: PCS-result after step 2 (cooling)

	Channel width 31 (auto)		Channel width 20		Channel width 10		Channel width 5	
Mean diameter (nm)	76.1	73.9	62.3	60.6	60.8	59.9	63.6	64.3
Variance (P.I)	0.434	0.456	0.552	0.560	0.530	0.539	0.460	0.454
Chi squared	1.551	7.286	2.698	1.976	0.466	0.463	0.777	0.851

First results of the loaded samples gave some very small tops indicating concentrations of about 300ng/ml. New dilutions were made with a theoretical concentration of 12µg/ml to get more accurate measurements. Now the values ranged around 700ng/ml. The aria under the curve of the samples were divided in half, and then divided on the aria under the curve from the 100% samples to get the loading %. Results can bee seen in table below:

Table 36: Lading efficiencies in the samples after freezing-cooling cycles

	After 1. step, heating (passive loading)		After 2. step, cooling (still passive loading)		After 4. step, cooling	
Parallel	1#	2#	1#	2#	1#	2#
Loading efficiency	5.1%	4.4%	4.4%	4.7%	4.4%	5.7%

	After 5. step, heating		After 6. step, cooling (should not be any different from last sample)	
Parallel	1#	2#	1#	2#
Loading efficiency	5.3%	6.2%	5.9%	5.6%

It is not possible to interpret if an active loading had occurred, or if this is just random variations. Dough there is a slight increase in detected dFdC in the loaded samples the increase is to small, and the parallels are too few to conclude on this numbers. The extra loading because of the precipitation (if there were any) were insignificant, and there exists methods of loading dFdC into liposomes that have

shown far better loading than this (Brandl and Massing 2003; Gravem 2006).

Second and third pare of parallels

I made two new batches of liposomes using the same procedure as last time. But one of the liposome-batches I made with an outer pH of 7. This was to compare with the ones with about pH 4 outer pH. It was made in the same way as the other samples, but the dFdC that were added were in an ammonium phosphate (dibasic) solution that had been pH-adjusted to 7. The reason why I made one sample with a pH of 7 was to see if "reverse loading" could bee the cause of the low loading-efficiency. The pH in the other batch of liposomes was measured to a pH of 4.2.

The PCS-results of the two batches did not differ significantly from the first pare of parallels or each others, and I considered them to be ok (see below).

Table 37: PCS-results, liposomes with outer pH 4

	Channel width 53 (auto)		Channel width 20		Channel width 10		Channel width 5	
Mean diameter (nm)	119.2	113.3	72.2	72.8	60	59.3	53.8	53.4
Variance (P.I)	0.607	0.676	1.024	1.086	1.107	1.126	0.852	0.834
Chi squared	224.741	422.231	3.118	9.462	0.713	0.964	1.060	1.473

Table 38: PCS-results, liposomes with outer pH of 7

	Channel width 48 (auto)		Channel width 20		Channel width 10		Channel width 5	
Mean diameter (nm)	94.3	95.7	69	68.6	59.2	60.6	52.8	56.2
Variance (P.I)	0.610	76.600	0.918	0.953	0.960	0.902	0.755	0.671
Chi squared	174.070	0.640	2.126	4.479	0.733	0.876	0.991	1.181

Both batches were divided into two parallels. For the pH 7 -samples (samples with outer phase of pH 7) one sample from each parallel were taken out after step 1 (first heating), and one sample from parallel 1 after step 5 (3rd heating). From the pH 4-samples samples were taken after step 1 (1st heating), step 3 (2nd heating), one sample of parallel 1 after step 4 (2nd cooling, should not be any differences here), and after step 5 (3rd heating). The results can be seen in the table below:

Table 39: Loading efficiencies of dFdC into liposomes.

	pH 4 samples		pH 7 samples		pH 4 samples	
Sample	Step 1, #1	Step 1, # 2	Step 1 # 3	Step 1, #4	Step 3, # 1	Step 3, # 2
Loading %	4%	5%	3%	5%	4%	7%

	pH 4 sample	pH 4 samples		pH 7 sample
Sample	Step 4, # 1	Step 5, #1	Step 5, #2	Step 5, # 3
Loading %	6%	6%	8%	4%

In accordance with big variations between the parallels we can only assume that there is a slight tendency to increased loading of dFdC from step 1 to step 5.

Discussion

All in all the loading efficiencies were too low, and did not increase significantly either. Also the loading-speed is expected to decrease after each cycle. This is because only a certain % of the dFdC inside the liposomes will precipitate, so that there is always an amount of non precipitated dFdC in the inside of the liposomes. This amount will maximally be the same amount as of passive loading, in this case 5%. Since the ratio between the 20% of the inside dFdC that is not precipitated and the 5% of dFdC that will passively load is 4, it means that only 80% / 4 = 20 % of the total amount of dFdC in the solution could precipitate inside the liposomes. Hence the maximum theoretical loading in this experiment, after infinite cycles of heating/cooling in an ideal world where none of the precipitated dFdC would dissolve again upon heating, would be 25%.

There is several ways this experiment could have been changed to improve loading. It may seem like the precipitation of dFdC is increasing with increasing concentrations of dFdC. By increasing the concentration of dFdC to in the solution at loading it is a possibility that we could have increased the loading-efficiency. E.g. 90% loading would have given a theoretical loading of 45%.

Diluting the liposomes less would also probably have increased the loading efficiency. One of the great benefits with this method of loading liposomes is that there is now need for removing any outer substances by size exclusion chromatography, thereby avoid a big dilution step. Though a certain dilution of the liposomes is probably needed because of viscosity-reduction and the adding of the gemcitabine (if dFdC were mixed directly in the dry powder of the VPGs it would be hard to control the pH of the inside of the liposomes).

7 CONCLUSION

In this project I have investigated the possibility of loading gemcitabine into liposomes by precipitation. We demonstrated that favorable conditions for making gemcitabine precipitate is a combination of high pH (< 6), a high gemcitabine concentration, and low temperatures. Our hypothesis that this precipitation was a result of complex-forming of gemcitabine and a salt can therefore probably be discarded.

Attempts trying to load gemcitabine into liposomes using a pH of 7 inside the liposomes and an external pH of 4, followed by heating and cooling cycles lead to low encapsulation efficiencies (< 8%) and inconclusive results of whether the method gave any increase in loading compared to passive loading.

Active loading via an ammonium sulphate gradient as described by (Gravem 2006) is based on the principle of an acidic pH inside the liposomes while having a more neutral external pH. Since the pH-conditions required for precipitation is the direct opposite to condition required for active loading these two methods exclude each other.

8 REFERENCES

Barenholz, Y. (2007). "Amphipathic weak base loading into preformed liposomes having a transmembrane ammonium ion gradient: from the bench to approved Doxil." Liposome Technology (3rd Edition) **2**: 1-25.

A review focuses on the use of transmembrane intraliposome high/extraliposome low ammonium ion gradients to load liposomes with amphipathic weak bases, and esp. on doxorubicin (DOX) remote loading into sterically stabilized liposomes (SSL) to form Doxil. [on SciFinder (R)]

Brandl, M. (2001). "Liposomes as drug carriers: a technological approach." Biotechnology Annual Review **7**: 59-85.

A review. Liposome researchers have created a huge variety of liposomal drug carriers in the past thirty years mainly by small-scale lab. techniques using more or less well defined raw materials. Only a few of these liposomal prepns. have made their way to approved drugs for clin. use in humans so far. The review gives a crit. literature survey over key technologies, which are used to evaluate an appropriate lipid formula and to prep., size, load and sterilize liposomes. It also deals with quality and shelf stability aspects of liposomal drug carriers. [on SciFinder (R)]

Brandl, M., M. Drechsler, et al. (1997). "Morphology of semisolid aqueous phosphatidylcholine dispersions, a freeze fracture electron microscopy study." Chemistry and Physics of Lipids **87**(1): 65-72.

Semisolid aq. dispersions of soy phosphatidylcholine (PC) with high PC mass fractions between 0.35 and 0.6 were prepd. using a high-pressure homogenizer. Their morphologies were investigated by freeze fracture TEM (FF-TEM) with regard to the quantity of water present. With all lipid/buffer ratios studied, vesicular structures in dense packing were seen on FF-TEM micrographs. PC ratios of up to 0.45 yielded small, unilamellar vesicles which were uniform in size. Matrixes of higher lipid/buffer-ratios (0.45-0.6) also contained these small unilamellar vesicles plus an increasing fraction of more heterogeneous large multivesicular or multilamellar vesicles. Apparently, organization of PC into small, homogeneous and unilamellar vesicles is not limited to 'classical' liposome dispersions, i.e., dil. lipid dispersions (<300 mM), but can also be achieved in more concd. lipid dispersions of PC fractions of up to 0.45 (600 mM) when lipid swelling is exerted under high mech. stress conditions. [on SciFinder (R)]

Brandl, M., M. Drechsler, et al. (1998). "Preparation and characterization of semi-solid phospholipid dispersions and dilutions thereof." International Journal of Pharmaceutics **170**(2): 187-199.

Highly concd., viscous to semi-solid phospholipid dispersions with phosphatidylcholine (PC) contents up to 600 mg/g or 780 mM were obtained by high-pressure homogenization. Diln. of these pastes with excess buffer led to 'classical' liposome dispersions. The diln. technique detd. the homogeneity of the liposome dispersions. Handshaking yielded heterogeneous dispersions, which according to cryo-electron microscopy contained large multivesicular vesicles (MVVs) as

well as small unilamellar vesicles (SUVs). By using a ball mill for diln., however, the phospholipid pastes could be completely transferred into uniform SUVs with mean diams. of about 20-40 nm. The absence of bigger particles could be demonstrated both by a membrane filtration test through 0.2 mm pore filters and photon correlation spectroscopy. Lipid paste formation and subsequent diln. into liposomes led to high encapsulation efficiencies of the hydrophilic model compd. 5,6-carboxyfluorescein. For true SUV dispersions, encapsulation efficiencies rose with increasing lipid contents up to a max. of over 45% at original lipid contents of 600 mg/g. According to geometrical considerations, the packing of SUVs reaches densest sphere packing at this lipid content. Semi-solid, vesicular PC pastes can be dild. by ball milling into homogeneous SUV dispersions with high encapsulation efficiency for hydrophilic compds. [on SciFinder (R)]

Brandl, M. and U. Massing (2003). Vesicular phospholipid gels. Liposomes. V. P. Torchilin and V. Weisssig. Oxford, Oxford Press**:** 353 - 372.

Gravem, H. (2006). Gemcitabine-containing liposomes. Tromsø, University of Tromsø**:** 87 bl.

Massing, U., S. Cicko, et al. (2008). "Dual asymmetric centrifugation as a new technique for liposome preparation." Journal of Controlled Release **125**(1): 16-24.

This is the first report on the use of a dual asym. centrifuge (DAC) for prepg. liposomes. DAC differs from conventional centrifugation by an addnl. rotation of the sample around its own vertical axis: While the conventional centrifugation constantly pushes the sample material outwards, this addnl. rotation constantly forces the sample material towards the center of the centrifuge. This unique combination of two contra rotating movements results in shear forces and thus, in efficient homogenization. We demonstrated that it is possible to prep. liposomes by DAC, by homogenizing a rather concd. blend of hydrogenated phosphatidylcholine and cholesterol (55:45 mol%) and 0.9% NaCl-soln., which results in a viscous vesicular phospholipid gel (VPG). The resulting VPG can subsequently be dild. to a conventional liposome dispersion. Since DAC is intended to make sterile prepns. of liposomes, or to entrap toxic/radioactive compds., the process was performed within a sealed vial. It could be shown that the DAC speed, the lipid concn., the homogenization time and the addn. of a mixing aid (glass beads) are all crit. for the size of the liposomes. Optimized conditions resulted in liposomes of 60+-5 nm and a trapping efficacy of 56+-3.3% for the model compd. calcein. [on SciFinder (R)]

Massing, U. and S. Fuxius (2000). "Liposomal formulations of anticancer drugs: Selectivity and effectiveness." Drug Resistance Updates **3**(3): 171-177.

A review with 52 refs. After a delay of roughly 30 yr, liposomes are becoming important as drug-targeting systems esp. in anticancer therapy. Two liposomal anthracycline formulations are now available for cancer treatment and more are in preclin. tests or in clin. trials. This review summarizes the possibilities to improve the anticancer activity of certain drugs by entrapment in liposomes. The problems and limitations of liposomal systems were discussed from a pharmacokinetic as well as from a manufg. viewpoint. Moreover, the anticancer properties of the two new liposomal anthracycline formulations will be discussed with regard to

anticancer activity and side effects and compared with the properties of the resp. free drugs. [on SciFinder (R)]

Moog, R., M. Brandl, et al. (2000). "Effect of nucleoside analogs and oligonucleotides on hydrolysis of liposomal phospholipids." International Journal of Pharmaceutics **206**(1-2): 43-53.

The hydrolysis of the bilayer forming phospholipids resulting first of all in lysophospholipids and fatty acids is one limiting factor detg. the shelf-life of liposomes. In several studies, the influence of pH, buffer, lipid compn. and other parameters on the hydrolysis of phospholipids have been demonstrated, but the influence of drugs has not yet been investigated systematically. In this study the influence of nucleoside analogs, esp. 2',2'-difluoro 2'-deoxycytidine (gemcitabine, dFdC) on the degrdn. of phospholipids was elucidated in more detail. It could be demonstrated that the interaction of dFdC with phospholipid bilayers promotes the hydrolysis of phospholipids in a concn.-dependent manner. Obviously two parts of the mol., the amino group bound to the pyrimidine moiety and the 2',2'-difluoro-2'-deoxyribose, seem to be responsible for the forced phospholipid hydrolysis. The dFdC-induced hydrolysis of phospholipids was influenced by pH, buffer, lipid compn. and different anions. Optimization of the above parameters resulted in prolonged shelf-life of dFdC liposome dispersions, which is an important prerequisite for clin. practice. [on SciFinder (R)]

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9 APPENDICES

9.1 Appendix 1

Results from 2nd set of experiments (loading of dFdC via an ammonium sulphate gradient, followed by separate detection of dFdC and cholesterol)

Here are the results from the 2nd set of experiments. Measured concentrations means concentration values obtained from comparing the aria under the curve (AUC) to a standard curve. The concentrations marked with "~" are manually compared with the standard curve, thereby making them less accurate (while the other concentrations are calculated by the computer).

Experiment 1

Table 40: dFdC-values from the HPLC.

Sample	Loaded 1	Laded 2	Loaded 3	Loaded 4	Control sample
AUC dFdC	0	0	0	0	255168
Theoretical concentration	3630 ng/ml	3630 ng/ml	3630 ng/ml	3630 ng/ml	5446 ng/ml
Measured concentration	0	0	0	0	~4000 ng/ml

Sample	100 % # 1	100 % # 2	100 % # 3	100 % # 4
AUC	1712185	1742560	1699695	1726731
Theoretical concentration	4.39	4.39	4.39	4.39
Measured concentration	~2500 ng/ml	~2500 ng/ml	~2500 ng/ml	~2500 ng/ml

Table 41: Cholesterol values from the HPLC.

	100% #1	100 % # 2	100% #3	100% #4
Theoretical concentration	236 µg/mL	239 µg/mL	267 µg/mL	247 µg/mL
AUC	383881	(3612471)	458178	515685
Measured concentration	216.59 µg/mL	(2027.34) µg/mL	258.259 µg/mL	290.512 µg/mL

	Loaded 1	Loaded 2	Loaded 3	Loaded 4
Theoretical concentration	34 µg/ml	34 µg/ml	38 µg/ml	35 µg/ml
AUC	76559	65494	70248	86508
Measured concentration	44 µg/ml	38 µg/ml	41 µg/ml	50 µg/ml

Table 42: PCS-results

	Channel width 33 (auto)		Channel width 20		Channel width 10		Channel width 5	
Mean diameter (nm)	90	80	61	61	61	65	90	100
Variance (P.I)	0.381	0.436	0.5	0.5	0.5	0.4	0.3	0.3
Chi squared	5.713	15.762	2.4	2.57	0.5	0.6	0.7	0.8

Experiment 2

Table 43: dFdC-values from the HPLC.

Sample	Loaded 1	Loaded 2	Loaded 3	Loaded 4	Control sample
AUC	0	0	0	0	0
Theoretical concentration	6225 ng/ml	6225 ng/ml	6225 ng/ml	6225 ng/ml	6225 ng/ml
Measured concentration	0	0	0	0	0

Sample	100 % # 1	100 % # 2	100 % # 3	100 % # 4	100 % control
AUC	511363	492469	479703	467553	475409
Theoretical concentration	6225 ng/ml	6225 ng/ml	6225 ng/ml	6225 ng/ml	6225 ng/ml
Measured concentration	~6000 ng/ml	~5700 ng/ml	~5600 ng/ml	~5600 ng/ml	~5600 ng/ml

Table 44: Cholesterol values from the HPLC.

Sample	Loaded 1	Loaded 2	Loaded 3	Loaded 4
AUC	286659	234288	252422	303768
Theoretical concentration	137 µg/ml	132 µg/ml	135 µg/ml	144 µg/ml
Measured concentration	~600 µg/ml	~520 µg/ml	~550 µg/ml	~600 µg/ml

Sample	100 % # 1	100 % # 2	100 % # 3	100 % # 4
AUC	245843	241763	261774	247540
Theoretical concentration	137 µg/ml	132 µg/ml	135 µg/ml	144 µg/ml
Measured concentration	~550 µg/ml	~550 µg/ml	~550 µg/ml	~550 µg/ml

Table 45: PCS-results

	Channel width 30 (auto)		Channel width 20		Channel width 10		Channel width 5	
Mean diameter (nm)	98.1	94.9	80.5	80.5	75.1	76	72.9	81.4
Variance (P.I)	0.334	0.343	0.392	0.394	0.404	0.407	0.404	0.368
Chi squared	4.59	8.291	0.539	0.862	0.497	0.638	1.089	1.126

Experiment 3

Table 46: dFdC-values from the HPLC

Sample	Loaded 1	Loaded 4	Control sample
AUC	190501	237838	209705
Theoretical concentration	4150 ng/ml	4150 ng/ml	6225 ng/ml
Measured concentration	2905 ng/ml	3606 ng/ml	3190 ng/ml

Experiment 5

Table 47: dFdC-values from the HPLC

Sample	Loaded 1	Loaded 2	Loaded 3	Loaded 4	Control sample
AUC	0	0	0	0	0
Theoretical concentration	6000 ng/ml	6000 ng/ml	6000 ng/ml	6000 ng/ml	6000 ng/ml
Measured concentration	0	0	0	0	0

Sample	100 % # 1	100 % # 2	100 % # 3	100 % # 4	100 % control
AUC	483855	486480	480244	483502	325943
Theoretical concentration	6000 ng/ml	6000 ng/ml	6000 ng/ml	6000 ng/ml	6000 ng/ml
Measured concentration	6319 ng/ml	6353 ng/ml	6273 ng/ml	6315 ng/ml	4294 ng/ml

Table 48: Cholesterol values from the HPLC

Sample	Loaded 1	Loaded 2	Loaded 3	Loaded 4
AUC	133179	101205	112854	187995
Theoretical concentration	106 µg/ml	121 µg/ml	107 µg/ml	117 µg/ml
Measured concentration	99 µg/ml	84 µg/ml	90 µg/ml	124 µg/ml

Sample	100 % # 1	100 % # 2	100 % # 3	100 % # 4
AUC	131108	140806	114854	152269
Theoretical concentration	118 µg/ml	134 µg/ml	119 µg/ml	131 µg/ml
Measured concentration	98 µg/ml	103 µg/ml	91 µg/ml	108 µg/ml

Experiment 6

Table 49: dFdC results from HPLC, Glucose used as hydration media for the VPGs

	Glucose used to eluate the liposomes on ion exchange column:			*Water used to eluate the liposomes on the ion exchange column:*		
Sample	Loaded #1 Glu/Glu	Loaded #2 Glu/glu	Control (Glu)	Loaded #1 Glu/Water	Loaded #2 Glu/Water	Control (Water)
AUC	0	0	0	0	0	0
Theoretical concentration	6,0 µg/ml	6,0 µg/ml	6,0 µg/ml	6,0 µg/ml	6,0 µg/ml	6,0 µg/ml
Measured concentration	0	0	0	0	0	0

Sample	100 % # 1	100 % # 2	100 % # 3	100 % # 4
AUC	244069	244945	233312	232276
Theoretical concentration	6,0 µg/ml	6,0 µg/ml	6,0 µg/ml	6,0 µg/ml
Measured concentration	3378.7	3387.6	3230	3215.96

Table 50: dFdC results from HPLC, PBP-solution used as hydration media for the VPG's

	PDP-solution used to eluate the liposomes on ion exchange column:			*Water used to eluate the liposomes on the ion exchange column:*	
Sample	Loaded #1 PDP/PDP	Loaded #2 PDP/H2O	Control (PDP)	Loaded #1 PDP/H2O	Control (Water)
AUC	109424	78538	48978	0	0
Theoretical concentration	6,0 µg/ml	6,0 µg/ml	6,0 µg/ml	6,0 µg/ml	6,0 µg/ml
Measured concentration	1551.8	1133.4	733	0	0

Sample	100% #1	#100% 2	100% #3	100% #4
AUC	247205	237658	261445	260750
Theoretical concentration	6,0 µg/ml	6,0 µg/ml	6,0 µg/ml	6,0 µg/ml
Measured concentration	3418.2	3288.9	3611.1	3601.7

Table 51: PCS-results, 50g/L glucose used as hydration media for the VPG's

	Channel width 45 (auto)		Channel width 20		Channel width 10		Channel width 5	
Mean diameter (nm)	139.6	137.8	104.8	101.1	100.4	104.3	151.7	152
Variance (P.I)	0.398	0.394	0.477	0.489	0.475	0.462	0.319	0.316
Chi squared	25.203	59.045	0.256	0.465	0.351	0.328	0.803	0.759

Table 52: PCS-results, 50mM PDP-solution used as hydration media for the VPG's

	Channel width 99 (auto)		Channel width 20		Channel width 10		Channel width 5	
Mean diameter (nm)	375.3	433.6	91.7	79.4	79.2	73.5	67.5	59.2
Variance (P.I)	0.45	0.391	1.219	1.188	1.173	1.192	0.887	1.038
Chi squared	520.214	890.151	0.565	1.609	0.348	0.471	0.55	0.712

Calculations of dilution of the liposomes and the inner aquas phase

These calculations are based on the assumption that the start distribution between outside and inside water content of the VPGs are 60% /40%. In the further dilution of the VPGs, a step to lower the viscosity, the liposomes were diluted 1/3. They are then diluted 1/2 prior to removal of outer ammonium sulphate by size exclusion chromatography. In the process of size exclusion chromatography the liposomes were on average diluted 1/7. At loading there was a small dilution step of 200/225. The end result is that the concentration in percent of the total amount of the total water content being inside the liposomes is as follows:

4/10 (start amount of water phase inside the liposomes) * 1/3 (dilution of VPGs) * 1/2 (dilution prior to size exclusion chromatography) * 1/7 (dilution on the size exclusion chromatography column) * 200/225 * 100% = 0.847%.

This means that at the time of loading the water-content can be expected to be around 0.847%.

Calculations of molar concentration and quantity of dFdC at loading

In the 2nd set of experiments I used a concentration of 0.111mg/mL dFdC upon loading. dFdC has a molar mass of 263.199mg/mmol. The molar concentration of dFdC is then:
0.111mg/mL / 263.199mg/mmol = $4.2173*10^{-4}$ mmol/mL = 0.4217mM.

The total volume that was used in the experiments was 2250µL, or 2.25*10^-3 L. The number of mol of dFdC in this solution is then 2.25*10^-3 L * 0.4217mM = 9.488*10^-4 mmol = 0.9488 µmol.

The amount expected to load passively, 0.847%, is 0.847% * 0.9488 µmol = 8.036*10^-3 µmol.

Calculations of amount of charged acredine orange inside the liposomes

Acredine orange has a pKa of 10.45. If we assume that the pH inside the liposomes is 3 (due to the ammonium sulphate gradient) we can calculate the amount of charged AO from the Henderson-Hasselbalch equation:

$$\log\frac{[BH^+]}{[B]} = pKa - pH = 10.45 - 3 = 7.45$$

$$\frac{[BH^+]}{[B]} = 10^{7.45} = 28183829$$

So there is about 28183829 times as much charged AO (AO^+) as there is non-charged AO, meaning close to 100% of the AO inside the liposomes will be charged.

Calculations of amount of charged dFdC inside the liposomes

Gemcitabine has a pKa of 3.58. Assuming the pH inside the liposomes is 3 (due to the ammonium sulphate gradient) we can calculate the amount of charged dFdC from the Henderson-Hasselbalch equation:

$$\log\frac{[BH^+]}{[B]} = pKa - pH = 3.58 - 3 = 0.58$$

$$\frac{[BH]}{[B]} = 10^{0.58} = 3.80$$

So there is 3.8 times as much $dFdC^+$ as there is non-charged dFdC at pH 3, meaning 79% is charged.

Theoretical amounts of ammonium sulphate within the liposomes

In the solution of VPGs and liposomes there is 650µL 120mM ammonium sulphate and 350mg VPGs. Counting all as one volume the real concentration of ammonium sulphate is 650µL * 120mM / 1000µL = 78mM. If we assume 40% of all water-content in the VPG's to be inside the liposomes at the start, we can use the calculated expected water-amount of water in the liposomes as calculated four sections above. The volume of water inside the liposomes if the liposomes contain 0.847 % of the total water-volume is:
2250µL * 0.847% = 19.06µL = 1.906*10^-5 L.
From this we can calculate the theoretical quantity of ammonium sulphate:
78 mM * 1.906*10^-5 L = 0.001486 mmol = 1.486 µmol.

Calculations of maximum theoretical loading

In the following calculation I pretend that the pH inside the liposomes is stable, and thereby also the charged amount of dFdC no mater how much dFdC that are loaded:
We can assume that passive loading with the concentrations we use gives a loading of about 0.847%. If pH inside the liposomes is 3 then 79% of the dFdC inside the liposomes will be charged, according to the calculations above. Taken the passive loading into consideration this means that 0.669 % of the total [dFdC] (outside and inside the liposomes) is now charged (ignoring the fact that 0.02% of the dFdC outside the liposomes also will be charged). 0.178 % of total [dFdC] will be inside the liposomes but not charged. Because of passive diffusion this amount will rice to 0.847% (the theoretical amount that

will passively load), meaning we get 0.669% more dFdC inside the liposomes. We now got 1.338 % of the total concentration of dFdC inside the liposomes. 79% of this, 1.057% of the total [dFdC], will be charged. This leaves 0.281% of the dFdC inside the liposome not charged. This amount will then again rice to 0.847%, adding 0.566% new dFdC inside the liposomes. Now we got 1.904% of the total amount of all dFdC inside the liposomes, and so on. The number of new dFdC coming into the liposomes after each "cycle" is decreasing. So after a while the loading will stabilize with non charged dFdC being 0.847% of the total amount of dFdC, and being 31% of the total dFdC inside the liposomes. The total percentage of dFdC inside the liposomes would then be 0.847%/21% * 100% = 4.03% loading.

In the solutions used in our experiments the total dFdC in the solution was 0.9488 µmol. The amount of dFdC in µmol that would maximum have entered the liposomes according to our model is therefore 0.0398µmol. This would have been possible, the total amount of ammonium sulphate inside the liposomes is expected to be 1.486 µmol.

Calculations of the isotonicity of solutions

Ammonium sulphate is isotonic at 1.68% (w/v), (16.8 mg/ml). It's molar weight is 142.14mg/mmol. Calculating the molar concentration of this gives us:

$$\frac{16.8mg/ml}{142.14mg/mmol} = 0.1182mmol/ml = 118.2mM$$

Potassium dihydrogen phosphate is isotonic at 2.18% (w/v), (21.8 mg/ml). It's molar weight is 136.09mg/mmol. Calculating the molar concentration of this gives us:

$$\frac{21.8mg/ml}{136.09mg/mmol} = 0.16mmol/ml = 160.18mM$$

All the isotonicity percentages are taken from the Merk-index.

9.2 Appendix 2

Calculations of the isotonicity ammonium phosphate (dibasic)

Ammonium phosphate (dibasic) is isotonic at 1.76% (w/v), (17.6 mg/ml). It's molar weight is 136.06mg/mmol. Calculating the molar concentration of this gives us:

$$\frac{17.6 mg/ml}{132.06 mg/mmol} = 0.13327 mmol/ml = 133.27 mM$$