FACULTY OF PHARMACEUTICAL SCIENCES

EPTIFIBATIDE-LOADED LIPOSOMES

MICROFLUIDIC-BASED LOADING AND SET-UP OF AN HPLC ANALYSIS

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A Master dissertation for the study programme Master in Drug Development

Academic year: 2017 - 2018

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ABSTRACT

BACKGROUND Eptifibatide (Integrilin®) is an intravenous peptide drug that selectively inhibits ligand binding to the platelet GPIIb/IIIa receptor. It is a therapeutic strategy for management of acute ischemic coronary syndromes, such as non-Q-wave myocardial infarction or unstable angina, and acute ischemic complications of percutaneous coronary interventions. It has a short half-life and it can be accumulated in non-targeted tissues. Therefore, encapsulation in a liposome is achieved, in order to couple nanobodies, which selectively recognize dysfunctional endothelial cells or superficial thrombi.

OBJECTIVE The encapsulation efficiency for microfluidic-based manufacturing is evaluated and a purification protocol (= removal of the untrapped drug molecules) is developed. Furthermore, the aim is to establish an HPLC analysis, which quantifies the eptifibatide present in the liposome suspensions.

MATERIALS AND METHODS Preparation of DPPC liposomes is performed using the NanoAssemblr™. Different batches are made: eptifibatide solubilized in H_2O, HBS and ethanol. Additionally, a lower concentrated batch and a thin-film hydration batch are prepared. Quantification of the batches is performed applying reversed phase liquid chromatography and purification is obtained by gel filtration chromatography followed by dialysis.

RESULTS Low concentrations of eptifibatide are achieved with a microfluidic-based technique opposed to thin-film hydration. Purification of the batches is accomplished, by quantifying the filtrate (=flow-through) for eptifibatide

CONCLUSIONS Preparation via thin-film hydration leads to a significantly higher encapsulation efficiency. More research is required to enhance the drug encapsulation via this microfluidic technique. Solubilizing eptifibatide in the ethanol phase, leads to a higher eptifibatide concentration than when de drug is solubilized in the aqueous phase.

SAMENVATTING

ACHTERGROND Eptifibatide (Integrilin®) is een intraveneus gegeven peptidegeneesmiddel en een antagonist van de GPIIb/IIIa receptor. Het wordt aangewend bij een acuut coronair syndroom, zoals *non-Q-wave* myocardinfarct of instabiele angor en acute complicaties door coronaire angioplastiek. Het geneesmiddel heeft een korte halfwaardetijd en het kan opgestapeld worden in ongewenste weefsels. Daarom worden ze ingekapseld door liposomen die nanobodies dragen, om selectief beschadigd endotheel en stolsels te kunnen herkennen.

DOEL De incapsulatie-efficiëntie voor 'microfluidic-based' liposomen wordt geëvalueerd en een zuiveringsprotocol wordt opgesteld. Verder wordt er ook een HPLC methode ontwikkeld, die de gehaltes aan eptifibatide kan bepalen.

MATERIALEN EN METHODEN De DPPC liposomen worden gemaakt met behulp van de NanoAssemblr™. Er worden verschillende batches gemaakt: eptifibatide wordt opgelost in H_2O, HBS en ethanol. Daarnaast wordt ook nog een lager geconcentreerde batch gemaakt, en een batch gemaakt via 'thin-film hydration'. Concentraties worden bepaald door middel van omgekeerde fase vloeistofchromatografie en zuivering wordt bekomen door gelfiltratie chromatografie, gevolgd door dialyse.

RESULTATEN Er worden lage concentraties aan eptifibatide teruggevonden in de batches gemaakt via 'microfluidic-based manufacturing', in vergelijking met de 'thin-film hydration' batch. De zuivering is goed gelukt en wordt gecontroleerd door het filtraat van de liposomen te testen.

CONCUSIES Liposomen die gemaakt worden via 'thin-film hydration' beschikken over een significant hogere incapsulatie-efficiëntie. Er is meer onderzoek nodig om deze incapsulatie-efficiëntie te verhogen, gebruik makende van 'microfluidic-based manufacturing'. Alleszins, het geneesmiddel oplossen in de ethanolfase zorgt ook voor een hogere concentratie eptifibatide in vergelijking met wanneer het geneesmiddel in de waterige fase is opgelost.

ACKNOWLEDGEMENT

First of all, I would like to thank Prof. Dr. Raemdonck for giving me the opportunity to write my thesis abroad.

I would like to thank Prof. Dr. Schiffelers for welcoming me in the wonderful NANOCAR team, for giving me advice and reviewing my work. I am also grateful towards Dr. Urbanus for giving suggestions about my work.

This thesis would not have been possible without the support and time of my fantastic supervisor Ing. Arnold Koekman. Thank you so much for your excellent guidance.

I would also like to thank Maurice for guiding me in the laboratory at my arrival and the other colleagues of the Laboratory of Clinical Chemistry and Haematology for creating a nice atmosphere. I felt very welcome.

Finally, I would like to thank my family, especially my mother, and my lovely friends, who are always there for me.

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LIST OF ABBREVIATIONS

ACN	acetonitrile
aq. dest.	distilled water
AU	arbitrary units
AUC	area under the curve
Da	Dalton
DMSO	dimethyl sulfoxide
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DSPE-mPEG-2000	1,2-distearoyl-phosphatidylethanolamine-methyl-polyethyleneglycol-2000
EE	encapsulation efficiency
FRR	volumetric flow rate ratio (aqueous to ethanol)
GP	glycoprotein
HBS	HEPES-buffered saline
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high-performance liquid chromatography
KGD	lysine - glycine - aspartic acid
MW	molecular weight
MWCO	molecular weight cut-off
NTA	nanoparticle tracking analysis
PEG	polyethylene glycol
PPP	platelet-poor plasma
PRP	platelet-rich plasma
RGD	arginine - glycine - aspartic acid
RT	room temperature
SDS	sodium dodecyl sulphate
TFA	trifluoroacetic acid
TFH	thin-film hydration
TRAP	thrombin receptor activating peptide
UV	ultraviolet

1 INTRODUCTION

1.1 NANOCAR

This research internship is connected to the NANOCAR project. NANOCAR signifies 'NANObody-nanoparticle CARrier platforms to visualize dysfunctional endothelium and asymptomatic arterial thrombosis'. The endothelial cells form the barrier between the bloodstream and tissue. They are essential for maintaining the physiological functions of the healthy vascular wall. Endothelial dysfunction is a major risk for atherosclerotic disease and local thrombus formation, which can lead to myocardial infarction, stroke or embolism.

The visualization of the location and extent of dysfunctional endothelial cells may be a breakthrough in early diagnosis of cardiovascular disease. Another purpose is improving the assessment of therapeutic efficacy. Liposomes, which selectively recognize dysfunctional endothelial cells or superficial thrombi by coupling nanobodies, will carry contrast agents for imaging. Heavy chain-only antibodies, produced by *Camelidae*-species (camels, alpacas, llamas), are functional antibodies composed of only two identical heavy chains (Nelson 2010) and are applicable in humans. A nanobody is the antigen-binding variable domain of a heavy chain-only antibody.

A further challenge is to translate this idea to site directed delivery of antithrombotic drugs (Application form NANOCAR 2017).

1.2 LIPOSOMES

1.2.1 Composition

Liposomes are lipid-based vesicles and consist of an aqueous core within a lipid bilayer. These spherical particles have sizes ranging from 30 nm to several micrometers (Akbarzadeh, et al. 2013). They are reported for the first time in May 1964 by Alec D. Bangham and his colleagues. The spontaneously forming lipid vesicles were described as 'smectic mesophases' (Madni, et al. 2014). A smectic phase can be defined as a mesophase with mechanical properties similar to those of soaps (de Gennes and Prost 1993).

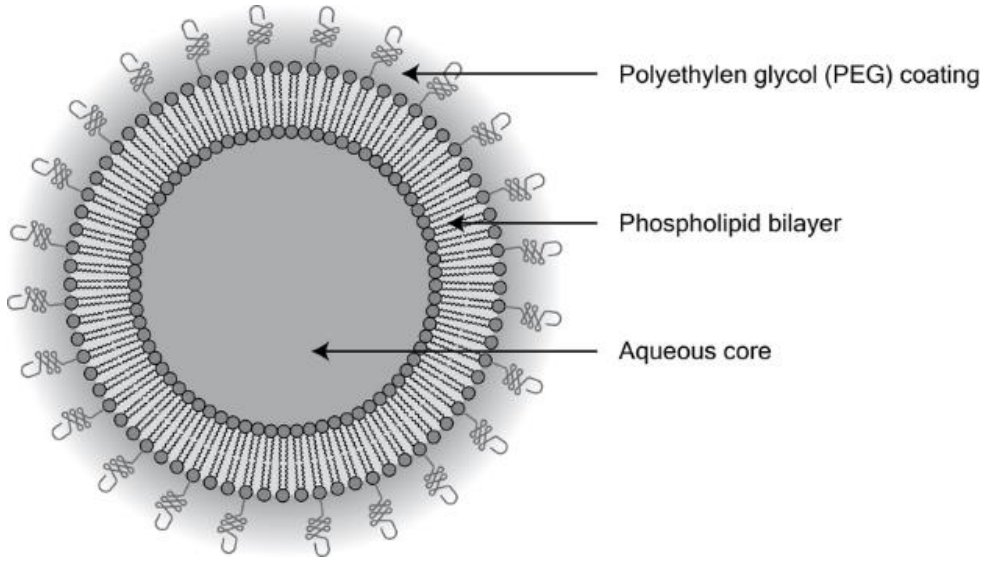

Figure 1.1: Schematic diagram of PEGylated liposome (Kubeček, et al. 2015).

Each phospholipid consists of a hydrophilic head and two hydrophobic tails. The tails, composed of hydrocarbon chains, show affinity for each other through hydrophobic interactions, while the hydrophilic parts are directed towards the aqueous phase (Figure 1.1) (El-Sherbiny, Elkholi and Yacoub 2014). A commonly used lipid is DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine), which is a saturated, zwitterionic lipid, as shown in Figure 1.2. However, charged lipids may be included in the bilayer of a liposome for a number of reasons. One of the main reasons is the increase in surface charge density, which prevents close approach of liposomes and in turn aggregation or fusion. (Philippot and Schuber 1994).

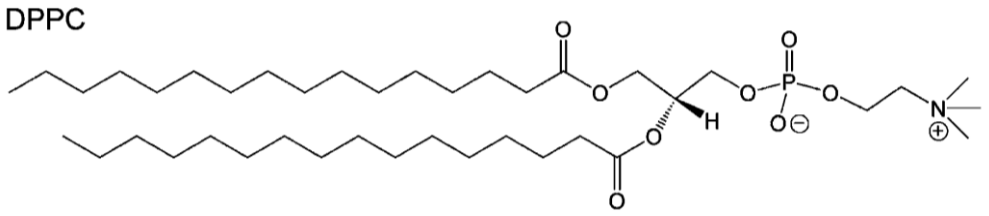

Figure 1.2: The chemical structure of DPPC (Wu, et al. 2014).

A further major structural component in the bilayer of a liposome is cholesterol. This molecule has an important role in balancing fluidity and rigidity of the bilayer. It also increases impermeability by repairing defects in the packing of the phospholipid molecules. These properties result from its ability to order chains of fluid phase lipids, which leads to increased bilayer thickness and a smaller partial molecular volume and area. Furthermore, cholesterol has the ability to cause positional (regular

distances between lipids) disordering of gel phase lipids while still maintaining a high degree of orientational (lipids point in the same direction) ordering. In this way, cholesterol reduces the fluidity above the phase transition temperature, while it increases the fluidity below the phase transition temperature (Philippot and Schuber 1994). The high cholesterol content state characterized by low positional order but high orientational order is termed the liquid ordered (L_o) phase. Pure DPPC is in an ordered and tightly packed state of carbon chains below its pretransition temperature of 34°C (beginning of melting process). This gel phase changes to the L_o phase with increasing cholesterol content (Luckhurst and Veracini 1994, Mills, et al. 2009).

A further stabilizing property of cholesterol is due to increased separation between choline head groups, reduced hydrogen bonding strengths and electrostatic interaction. This stabilizes the bilayer and lowers its permeability to water and other molecules (Chibowski and Szczes 2016).

An important drawback of liposomes applying as a drug carrier, is their short half-life. Phagocytosis by the reticuloendothelial system, an essential component of the immune system, results in a relatively rapid rate of clearance from the circulatory system. PEGylation, a chemical modification of the surface of the liposome with polyethylene glycol (PEG) (Figure 1.1, p. 1), leads to a significant decrease in the uptake of liposomes by the reticuloendothelial system. This is supposedly due to the reduced opsonization of the liposome surface by proteins that mediate macrophage recognition as a result of steric stabilization. The flexible polyethylene glycol chains sterically hinder approach and docking of the opsonins. This enhances their circulation time, which improves their targeting capabilities (see 1.2.2). More benefits of PEGylation include high water solubility, reduced cytotoxicity, decreased immunogenicity and high stability due to steric repulsion, which prevents fusion and disruption of the liposomes (El-Sherbiny, Elkholi and Yacoub 2014). PEG-coated liposomes or ‘stealth liposomes’ are obtained by incorporating PEG-lipid conjugates (phospholipids with a PEG polymer coupled to the hydrophilic head) during the preparation. Usually these are incorporated at concentrations between 3 to 9 mol% of total lipid. Alternative methods involve physically adsorbing the polymer onto the surface of the vesicles or covalently attaching reactive groups onto the surface of preformed liposomes (Immordino, Dosio and Cattel 2006). Cholesterol is a possible anchor for PEGylation as well (Zhao, et al. 2007).

In conclusion, the stability, rigidity, fluidity and charge of the bilayer is determined by the choice of bilayer components.

1.2.2 Purposes

Liposomes were initially used as a model for biological membranes because of the obvious analogy in terms of structure. However, scientists began to realize that this cell-like structure could serve as a carrier for potent drugs. The encapsulation of a therapeutic agent was described for the first time in 1971. The last two decades, liposomes have developed into one of the most studied drug delivery systems. Liposomes are able to accommodate hydrophilic as well as lipophilic drugs. A hydrophilic substance can be solubilized in the aqueous core, while a lipophilic substance will be located inside the lipid bilayer. The encapsulation of a hydrophilic drug may increase its penetration through lipophilic biological membranes, while the low solubility of a lipophilic drug in the aqueous body fluids can be enhanced by liposomal encapsulation.

The major benefits of liposomes as a drug carrier include sustained action, enhanced bioavailability, high cellular uptake and targeted delivery of therapeutic agents. Currently, there are liposome-based drug delivery systems developed for a wide range of therapeutic purposes: cancer chemotherapy, vaccines, gene therapy, antimicrobials, sensitive macromolecules, topical drug delivery, diagnostic techniques, etc. (Madni, et al. 2014).

One of the main issues of the use of liposomes as a carrier system is limited specificity of delivery. Actively targeted liposomes are liposomes which are covalently coupled with antibody(fragments) to their surface. These targeting ligands are attached in order to induce specific interaction between ligand and target receptor, cell or tissue. Immunoliposomes that are targeted by antibody(fragments) are meant to enhance the safety of a potent therapeutic agent by limiting its exposure to target sites and consequently protect non-target tissues against toxic effects. When its site of action is intracellular, the therapeutic agent must gain entry to the cell. The liposomal content could leak out of the vesicles after the association and enter the cells as free drugs, the bilayer could fuse with the cell membrane with entry of contents into the cytoplasm or the contents could enter via cellular endocytosis. The

concept of targeting is further applicable for diagnostic imaging (Huang, Kennel and Huang 1983, Madni, et al. 2014).

1.2.3 Preparation

Liposomes have self-assembling properties due to the amphipathic (both hydrophobic and hydrophilic) characteristics of phospholipids. During the preparation, change in polarity forces the components to organize into the most thermodynamically and energetically favourable structure. I.e. introducing an aqueous phase to (an organic solvent containing) a mixture of lipids will lead to association of hydrophobic sections into spherical bilayers (Akbarzadeh, et al. 2013). This paper focusses on two methods for the preparation of liposomes: thin-film hydration and microfluidics-based manufacturing.

The thin-film hydration method is the conventional and most common technique for liposome preparation. The mixture of lipids is dissolved in an organic solvent. The solution is kept in a rotary evaporator and a dry thin lipid layer is observed against the wall of the flask. The liposomes are formed by hydrating the thin lipid film in an aqueous medium (see Figure 1.4, p.8) (Ghanbarzadeh, Valizadeh and Zakeri-Milani 2013).

Liposomes prepared by thin-film hydration, are generally multilamellar and have a broad size distribution. However, the particle size is critical for distribution of the liposomes to the site of action. Small liposomes (around 100 nm) have the advantage of increased circulation time and better uptake in target cells. Large liposomes are mostly unsuitable for use because of rapid clearance by the reticuloendothelial system. In order to reduce the particle size, thin-film hydration is followed by extrusion. The vesicles are forced through filters with a certain pore size, obtaining unilamellar liposomes with a uniform diameter (Nagayasu, Uchiyama and Kiwada 1999, Deshantri 2017). Another possibility to reduce the particle size is to keep the suspension in a sonicator bath. Sonication agitates the particles, by transforming sound waves into mechanical energy (Ghanbarzadeh, Valizadeh and Zakeri-Milani 2013, Garcia-Vaquero, et al. 2017).

Microfluidic technology has emerged as an alternative to bulk methods for synthesizing nanoparticles. Figure 1.3 demonstrates the principle of the

NanoAssemblr™ system. The system controls a microfluidic mixing cartridge, containing a Y-junction chip with two inlets, each connected to a syringe. As the lipid mixture that is solubilized in organic solvent diffuses into the aqueous phase, the lipids become less soluble and self-assemble into planar lipid bilayer discs. To reduce the exposure of the hydrophobic lipid chain to the hydrophilic phase, the lipid discs bend and close into spherical liposomes (Ran, Middelberg and Zhao 2016). A mixer based on patterns of grooves on the floor of the channel produces a chaotic flow and is referred as the staggered herringbone mixer (Stroock, et al. 2002).

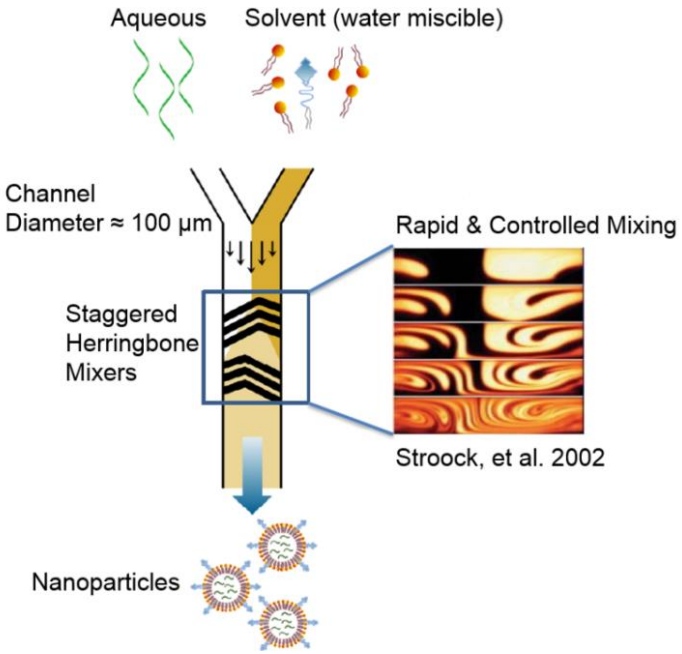

Figure 1.3: Representation of microfluidic chip for NanoAssemblr™ (Ramsay 2015).

Microfluidics-based manufacturing provides a suitable method to prepare small and nearly-monodisperse liposomes. Properties as particle size can be altered by adjusting operating variables such as volumetric flow rate ratio (FRR), at which components are pumped through the cartridge, mixing ratio, lipid composition, etc. (Precision Nanosystems n.d.).

With several liposomal formulations on the market, and even more in clinical trials, new techniques for production of liposomes are considered. Opposed to conventional laboratory methods, they should allow scale-up, better reproducibility and process control (Koynova and Tenchov 2015).

1.3 LOADING STRATEGIES

A lipid bilayer, with its thickness of approximately 5 nm, is a barrier for many substances, including ions, charged molecules and larger non-charged water-soluble molecules. Consequently, many hydrophilic substances can be encapsulated inside the aqueous core of a liposome (Gubernator 2011). There are several strategies to load drugs into the liposomes, some of them are described below.

The most common encapsulation method is based on thin-film hydration. A part of the aqueous solution containing water-soluble substances is passively enclosed. With this method, a large amount of the drug remains unencapsulated in the external aqueous medium.

The following methods improve the encapsulation efficiency of hydrophilic substances and are illustrated in Figure 1.4.

The ‘freezing-and-thawing technique’ uses repeated cycles of freezing and thawing a liposome suspension and produces physical disruption of the bilayers because of the formation of ice crystals. This allows better drug penetration and increase in liposome volume (Gubernator 2011). ‘Dehydration-rehydration vesicles’ are prepared by hydration of a lipid film with an aqueous solution, followed by lyophilization (removing water through a freeze-drying process) along with the drug and redispersion of the powder in an aqueous solution. This method allows higher encapsulation as well. ‘Reverse-phase evaporation’ consists of forming an emulsion of an organic phase, which contains the lipid, and an aqueous phase, which contains the drug. The organic solvent is subsequently removed by evaporation, resulting in large liposomes (200-500 nm) with a substantial fraction of the aqueous phase entrapped (Szoka and Papahadjopoulos 1978, Eloy, et al. 2014).

Active loading of liposomes, also referred as ‘remote loading’, is a technique to encapsulate drugs with high efficiency. A pH gradient is generated by preparing liposomes in a certain pH, followed by shifting the pH of the external medium using size exclusion chromatography. The principle behind this pH gradient is that the uncharged drug diffuses into the liposome and becomes protonated or deprotonated. The charged drug is entrapped and can no longer permeate through the bilayer. This method requires that the drug molecule is an amphipathic weak base or acid, and

has a logD at pH 7 in the range of -2.5 to 2. The logD value is related to the logP value. For an ionizable compound, the distribution coefficient D must be taken into account. It is defined as the ratio of the concentrations of both the ionized and unionized molecules in the n-octanol and aqueous phase at a determined pH value (Eloy, et al. 2014, Andrés, et al. 2015). A further approach of creating a pH gradient consists of precipitating the drug molecules, which have a low solubility in a certain pH range or form insoluble complexes with particular ions (Gubernator 2011).

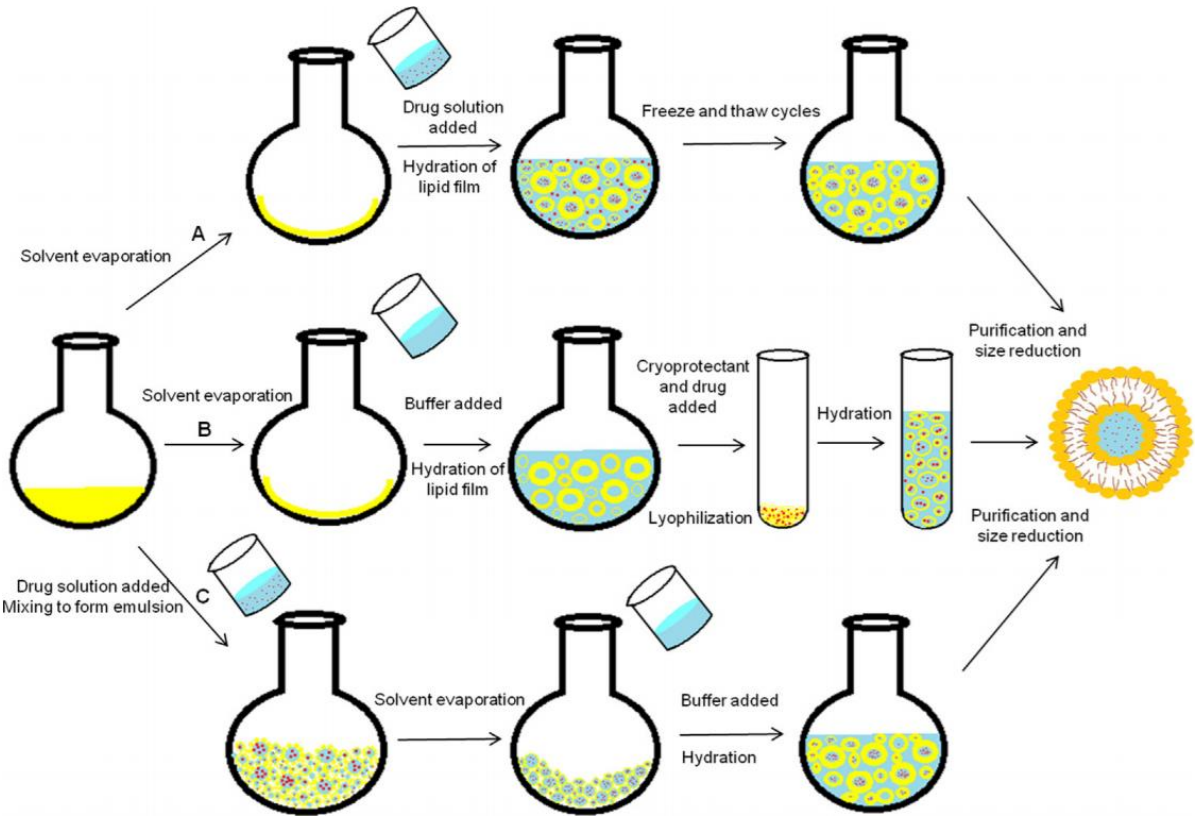

Figure 1.4: Hydrophilic drug encapsulation by thin-film hydration followed by (A) freeze–thaw cycles, (B) dehydration–rehydration of preformed empty liposomes and (C) reverse-phase evaporation (Eloy, et al. 2014).

1.4 EPTIFIBATIDE

Activation of the glycoprotein (GP) IIb/IIIa receptor (an integrin receptor) on the platelet membrane induces the binding of fibrinogen and is the final stage of platelet aggregation. Platelet aggregates form the structural basis for an occlusive thrombus, the major causes of ischemia. Eptifibatide (Integrilin®) is a cyclic heptapeptide and a non-immunogenic, potent and rapidly reversible inhibitor of GPIIb/IIIa. Inhibition of GPIIb/IIIa has emerged as a therapeutic strategy for management of acute ischemic coronary syndromes, such as non-Q-wave myocardial infarction or unstable angina,

and acute ischemic complications of percutaneous coronary interventions (Phillips and Scarborough 1997, Tcheng and O'Shea 2002, Gecommentarieerd Geneesmiddelenrepertorium 2017).

As shown in Figure 1.5, eptifibatide contains a homologous sequence as the RGD (arginine - glycine - aspartic acid) tripeptide present in fibrinogen. However, a difference between arginine and the arginine derivate ('homoarginine') in eptifibatide is noticed: introducing one more carbon atom gives the amino acid lysine-like characteristics. In fact, eptifibatide is based on a peptide sequence found in snake venom, which inhibits GPIIb/IIIa with higher affinity than other integrins. Instead of RGD, this peptide contains a KGD sequence, with lysine replacing arginine. Due to this particular sequence, eptifibatide has a high specificity for GPIIb/IIIa, without affecting the binding properties of other integrins. An interchain disulfide bridge gives this peptide a ring structure, in order to impart resistance to proteolysis (Tcheng and O'Shea 2002, Dunlap and Huryn 2018).

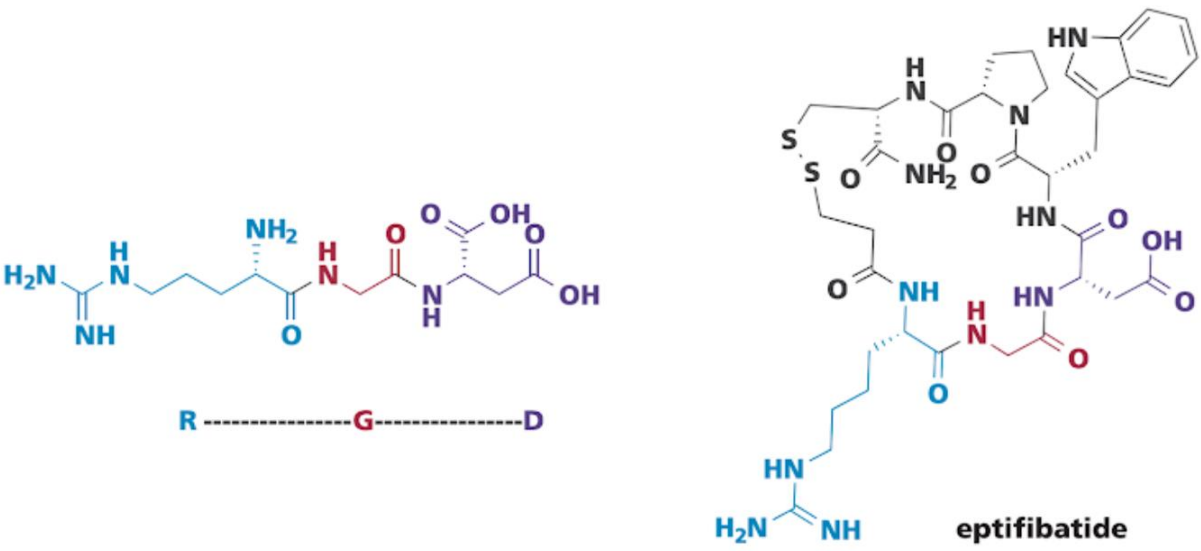

Figure 1.5: The chemical structure of RGD (left) and eptifibatide (right) (Dunlap and Huryn 2018).

Figure 1.6 shows a basic representation of the mechanism of GPIIb/GPIIIa mediated platelet aggregation. In response to disrupted vascular endothelium, physiologic platelet agonists such as adenosine diphosphate (ADP), epinephrine, collagen or thrombin, generate intracellular signals that convert the inactive GPIIb/IIIa on resting platelets to its active conformation. This exposes a high-affinity binding site on the extracellular domain of GPIIb/IIIa for ligands such as fibrinogen. In the

presence of calcium ions, fibrinogen and/or von Willebrand factor bind to active GPIIb/IIIa crosslinking adjacent platelets into aggregates (Gresele, et al. 2012).

By blocking the extracellular domain ligand binding site, eptifibatide interferes with the ligand binding to GPIIb/IIIa. This inhibits the platelet aggregation, as illustrated in Figure 1.6 (b). Aside from eptifibatide, there are two other categories of GPIIb/IIIa antagonists approved for clinical use. Abciximab, a monoclonal antibody, is directed against the active conformation of GPIIb/IIIa and tirofiban, a small molecular weight compound, competes with fibrinogen binding (Wu, Matijevic-Aleksic and Dahlback 2005, Gresele, et al. 2012).

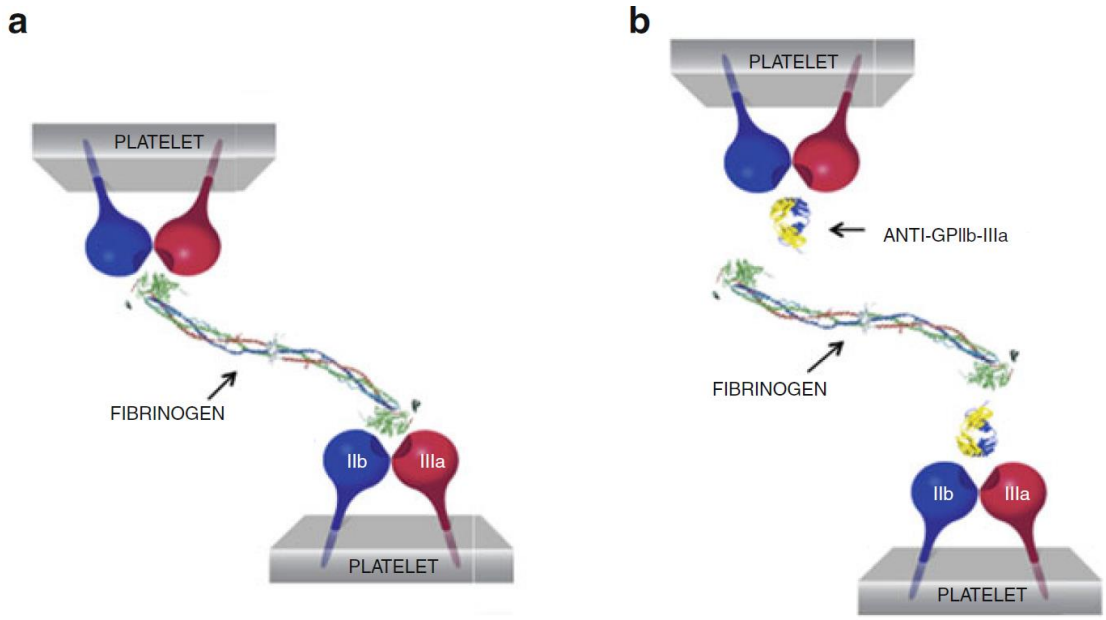

Figure 1.6: The mechanism of GPIIb/GPIIIa mediated platelet aggregation. (a) Fibrinogen binds to GPIIb/IIIa in its active conformation. (b) GPIIb/IIIa antagonists prevent ligand binding to active GPIIb/IIIa (Gresele, et al. 2012).

All three categories of GPIIb/IIIa inhibitors have a narrow therapeutic range. Bleeding complications are a major limitation of administration (Wu, Matijevic-Aleksic and Dahlback 2005). Particular patient populations are known to be at increased risk of bleeding tendencies. Female, renal insufficiency and age above 75 are independent risks of major bleeding, aside from use of GPIIb/IIIa antagonists. In conclusion, safety must be weighed against efficacy when eptifibatide is considered as antithrombotic therapy (Saab, Ionescu and Schweiger 2012).

The XLogP3-AA value of eptifibatide, a computed LogP estimation (represents the logarithm of the partition coefficient P, which is defined as the ratio of the solute concentrations between n-octanol and water), is equal to -2.4 (PubChem 2005, Cheng, et al. 2007). The ACDLogD value at pH 7.4, an estimated logD value, is equal to -4.4 (ChEMBL n.d.). This indicates a hydrophilic character. Eptifibatide contains two functional groups which are ionizable in neutral milieu. The guanidine group on the homoarginine residue is an extremely strong base, with a pK_a of approximately 12.48. The carboxylic acid group on the aspartic acid residue has a pK_a of 3.87 (see Figure 1.5, p.9). Consequently, the eptifibatide molecule is generally ionized at both the guanidine group (protonated) and the carboxylic acid group (deprotonated) (Zhao and Yalkowsky 2001).

Eptifibatide has a short half-life of approximately 2.5 hours (Ho and Gibaldi 2003), due to inactivation and elimination by renal filtration and enzymatic degradation. To compare, the circulation half-life of DPPC-based liposomes is in the order of dozens of hours, regardless of PEGylation (Molloy, et al. 2017). The efficiency of eptifibatide could be enhanced through protection by liposomes and targeted delivery to the site of thrombus using nanobodies (Bardania, et al. 2017). Furthermore, liposomal encapsulation with targeted delivery has the ability to improve the selectivity and prevent side effects such as severe bleeding by limiting accumulation in non-targeted tissues (Bardania, et al. 2017).

2 OBJECTIVES

Eptifibatide is an intravenous antiplatelet agent administered for the treatment of thrombosis. Encapsulation in a carrier increases its half-life and reduces the accumulation in non-targeted tissues, by the coupling of targeting ligands, specific to dysfunctional endothelial cells or superficial thrombi. A lower dose is administered, but a higher amount of eptifibatide is achieved at the site of action. This will reduce the risk of bleeding tendencies and increase the efficacy, compared to systemic antiplatelet therapies. Encapsulation of this drug through preparation via thin-layer hydration is described in literature. This paper will evaluate the passive loading efficiency when the liposomes are prepared via microfluidic technology, an advanced method which is much more cost- and time-effective than thin-layer hydration.

The encapsulation efficiency will be assessed by preparation of different batches via the NanoAssemblr™. A further objective is to establish a protocol for purification of the batches, in order to remove the untrapped eptifibatide. If the batch doesn't contain free drug molecules, the drug delivery will be more specific, which is intended. The next challenge is verifying if the suspension doesn't contain free eptifibatide. The external medium is analysed by centrifugal precipitation and centrifugal filtration. The thin layer batches are made for comparing. In order to measure the quantities of eptifibatide in the batches, an HPLC method needs to be developed. The method must be able to quantify low concentrations and it should allow the escape of drug molecules out of the liposomes. The functionality of the eptifibatide-loaded liposomes is verified in aggregation tests.

Different batches are made: eptifibatide solubilized in H_2O, HBS and ethanol. Additionally, a lower concentrated batch and a thin-film hydration batch are prepared. The liposome content is determined with both a phosphate and a cholesterol assay. This leads to a minor objective: evaluation of the correlation between the assays.

3 MATERIALS AND METHODS

3.1 PREPARATION OF LIPOSOMES

3.1.1 NanoAssemblr™

Liposomes are prepared using the NanoAssemblr™ Benchtop system and cartridge (Precision Nanosystems, Vancouver, Canada). The software enables the control of the operating variables (Table 3.1). The lipid mixture is made of DPPC, DSPE-mPEG-2000 and cholesterol. The lipids are dissolved in 5 mL ethanol (Merck Millipore, Darmstadt, Germany) for a concentration of 20 mM.

Table 3.2 shows the composition of the lipid mixture. Liposomes are prepared at RT and stored at 4°C.

Table 3.1: Operating variables of the NanoAssemblr™ for the preparation of liposomes

FRR (aqueous:ethanol)	3:1
Total flow rate	12 mL/min
Total volume	0.9 mL
Start waste volume	0.1 mL
End waste volume	0.05 mL

Table 3.2: Composition of 20 mM lipid mixture

Lipid	MW (g/mol)	% each lipid	Concentration (mM)	Weight (mg)
DPPC[a]	734.04	61.7	12.34	45.3
DSPE-mPEG-2000[b]	2805.5	5.0	1.00	14.0
Cholesterol[c]	386.65	33.3	6.67	12.9

[a] DPPC: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (Nanocs, Boston, USA)
[b] DSPE-mPEG-2000: 1,2-distearoyl-phosphatidylethanolamine-methyl-polyethyleneglycol-2000 (Nanocs, Boston, USA)
[c] (Sigma-Aldrich, Steinheim, Germany)

With the aim of comparing the loading efficiency of eptifibatide, different batches are made. A solution of 25 mg/mL eptifibatide (MCE, Monmouth Junction, USA) in 25% DMSO (Merck Millipore, Darmstadt, Germany) is prepared and stored at -80°C. DMSO enhances the solubility of the drug. This solution is diluted in either the aqueous phase or the ethanol phase (Tabel 3.3).

Tabel 3.3: Solutions for preparation of batches

	Aqueous phase	Ethanol phase
Batch 1	5 mg/mL eptifibatide in H_2O	Lipid mixture
Batch 2	5 mg/mL eptifibatide in HBS[a]	Lipid mixture
Batch 3	HBS[a]	5 mg/mL eptifibatide in lipid mixture
Batch 4	1 mg/mL eptifibatide in H_2O	Lipid mixture

[a] HBS: HEPES-buffered saline (10 mM HEPES (VWR Chemicals, Leuven, Belgium), 150 mM NaCl, pH 7.4)

3.1.2 Thin-film hydration (TFH)

A round-bottom flask with 3 mL lipid mix (same composition as represented in Table 3.2, p.13) is attached to the Rotavapor R-300, with vacuum pump V-700 (Buchi, Flawil, Switzerland), in order to create an atmosphere of approximately 300 mbar. A lipid film is formed by evaporation. The solvent is heated in a water bath of 55°C to accelerate this process. When the solvent is evaporated, the flask is placed under nitrogen gas (approximately 20 bar) (Linde Gas, Schiedam, The Netherlands) in order to remove trace amounts of solvent. The lipid film is rehydrated in 1.5 mL HBS containing 5 mg/mL eptifibatide. Heating in a water bath or sonication can be used to stimulate the hydration. Multilamellar lipid vesicles are formed with a particle size of approximately 2 to 3 µm. With the aim of preparing smaller unilamellar liposomes, extrusion (with a Gastight syringe, Hamilton Company, Reno, USA) through filters (Whatman®, Little Chalfont, UK) with a pore size of 0.2 (4 times) and 0.05 µm (4 times) is performed.

3.1.3 Isolation and purification

3.1.3.1 Gel filtration chromatography

The principle of gel filtration chromatography (a type of size exclusion chromatography) is used to separate the liposomes from free eptifibatide. (Macro)molecules move through a bed of porous beads, diffusing into the beads dependent on size and shape. For these batches PD MiniTrap™ columns containing 2.1 mL of Sephadex™ G-25 resin were applied (GE Healthcare, Little Chalfont, UK). Sephadex resins are produced by cross-linking dextran with epichlorohydrin. The degree of cross-linking determines the exclusion limit M_r, for this column equal to

5000 Da. Larger molecules, for example liposomes, will elute sooner than smaller molecules, which can permeate the beads (Millner 1999). Free eptifibatide, with a MW of 832 Da, should be separated in this way (Zhao and Yalkowsky 2001).

The different batches of liposomes have volumes of approximately 0.7 mL. However, the column is intended for sample volumes up to 0.5 mL, so two columns are used for purification of one batch. The elution is performed, following a gravity protocol, in several steps of adding water or HBS, depending on the aqueous phase of the batch in question.

The cholesterol content in the eluates is determined to differentiate the eluates with the highest amount of liposomes. This assay shows that the liposomes come out of the column between 0.2 mL and 0.8 mL eluting buffer (water or HBS), so this fraction is collected.

3.1.3.2 Dialysis

To be sure the possible remaining amounts of free eptifibatide are excluded, the batches are subjected to dialysis against HBS. The batch is injected through the membrane of a Slide-A-Lyzer™ Dialysis Cassette (Thermo Scientific, Rockford, USA) with a MWCO of 20 kDa and a maximum sample volume of 3 mL. Removing air of out the cassette to compress the membrane windows will ensure the sample contacts the greatest surface area. The passive diffusion of free eptifibatide takes place over night at 4°C, in a 4 L cup with magnetic stirrer.

3.1.3.3 Verifying purification efficiency

One of the main challenges is to find a technique to analyse the solution apart from the liposomes. Quantifying this solution can give information about the purification efficiency and leakage of eptifibatide out of the liposomes.

The first approach involves making a precipitate of liposomes with the Centrifuge 5427 R (Eppendorf, Hamburg, Germany), by applying a centrifugal force of 20817 x g for a certain time. The acceleration due to gravity, denoted by g, is equal to 8.9 m/s^2 (Sircar 2008). The force which is set in the centrifuge, is expressed in the acceleration it causes, in relation to the gravitational acceleration g (units g), also referred as RCF (relative centrifugal force).

Not the whole batch, but a small section is subjected to the centrifuge, because the impact of this force on the liposomes and leakage of the drug is unidentified. Time of centrifuging is dependent on the volume of the suspension subjected to the force, accordingly the distance to travel by the liposomes to the bottom of the tube. The supernatant is isolated very cautiously with a micropipette, avoiding suction of the pellet. Different procedures are attempted to obtain a clean (i.e. without liposomes) supernatant. Two of them are given in Table 3.4 below.

Table 3.4: Centrifugation procedures

	Start volume	Centrifugation variables	Volume supernatant isolated	Centrifugation variables of supernatant	Volume supernatant isolated
1	100 µL	2 hr, 20817 x g	80 µL	1 hr, 20817 x g	60 µL
2	100 µL	1 hr, 20817 x g	35 µL		

The second approach involves ultrafiltration of the liposomes (a section of the batch). The liposomes are retained and the filtrate is tested for eptifibatide, which should pass through the filter if it is present in the external medium. This is performed with two different centrifugal devices: Pierce® Concentrator, with a MWCO of 100 kDa (Thermo Scientific, Rockford, USA) and Nanosep®, with a MWCO of 30 kDa (Pall Gelman Laboratory, Ann Arbor, USA). After spinning 100 µL, subjected to the ultrafilters, for 20 minutes at 20817 x g and 15 minutes at 14000 x g respectively, the filtrate is isolated.

3.2 CHARACTERIZATION OF LIPOSOMES

3.2.1 Quantification

3.2.1.1 Phospholipid content

A phosphate assay (Rouser, Fkeischer and Yamamoto 1970) is applied in order to quantify the phospholipid content in the liposome batches. This assay is based on colorimetry to determine the amount of inorganic phosphate. After degradation of the phospholipids to inorganic phosphate with perchloric acid, phosphate reacts with molybdate forming phosphomolybdic acid (1). This is reduced to a blue complex by ascorbic acid (2) (Nagul, et al. 2015).

(1) PO_4^{3-} + 12 (MoO_4^{2-}) + 27 H^+ → $H_3PO_4(MoO_3)_{12}$ + 12 H_2O

(2) $H_3PMo(VI)_{12}O_{40}$ + reductant → $[H_4PMo(VI)_8Mo(V)_4O_{40}]^{3-}$ (blue)

The intensity of this blue colour is proportional to the concentration of phospholipids in the liposome suspension and is measured at a wavelength of 797 nm (based on the law of Lambert-Beer, see formula 3.1).

$$A(\lambda) = a(\lambda) \cdot l \cdot C \qquad (3.1)$$

Where $A(\lambda)$: absorbance at a specific wavelength λ

$a(\lambda)$: specific absorption coefficient at wavelength λ ($L.g^{-1}.cm^{-1}$)

l: cell path length (cm)

C: concentration of the sample ($g.L^{-1}$)

A 0.5 mM stock phosphate solution (6.99 mg $NaH_2PO_4.H_2O$ (Merck, Darmstadt, Germany) dissolved in 100 mL aq. dest.) is added in duplicate quantities of 0, 20, 40, 60, 80, 100, 120 and 160 μL to glass tubes in order to prepare the standards. An appropriate volume of the liposome batches containing approximately 50 nmol phospholipids is added in duplicate to glass tubes as well. These are placed in a heating block (ThermoChem, Santa Rosa, California) and evaporation takes place at 180°C until dryness is observed. 0.3 mL perchloric acid (Sigma, Steinheim, Germany) is added to the tubes which are mixed, covered with marbles and placed in a heating block at 180° for at least 45 min. When the tubes are cooled down, 1.0 mL aq. dest., 0.5 mL 1.25% hexa-ammoniummolybdate solution (Merck, Darmstadt, Germany) and 0.5 mL fresh 5% w/v ascorbic acid solution in aq. dest. (Merck, Darmstadt, Germany) are added to the tubes. The tubes are mixed, covered with marbles and placed in a waterbath at boiling point (Julabo, GmbH, Seelbach, Germany) for 5 min, after which they are cooled down to room temperature using a cold water bath.

300 μl of each sample is transferred to a 96 well plate (Greiner Bio-one, Solingen, Germany) and Spectramax M2e (Molecular Devices, San Jose, USA) is applied in order to measure the absorbance at 797 nm. The absorbance is plotted against the phosphate concentration of the standards and a calibration curve is generated. Linear regression is applied to calculate the amount of phosphate in the

samples, from which the phospholipid amount is determined (Deshantri 2017, Deschepper 2017).

3.2.1.2 Cholesterol content

The cholesterol assay is easier and faster to perform than the phosphate assay. That's the reason why this assay is introduced: to evaluate the presence of liposomes rapidly, for example in the eluted fractions after gel filtration chromatography. The assay is based on determination of cholesterol after enzymatic oxidation by cholesterol oxidase (1). The colorimetric indicator is a quinoneimine, which is measured at a wavelength of 500 nm (see formula 3.1, p. 17). This quinoneimine is generated from 4-aminoantipyrine, phenol and hydrogen peroxide under catalytic action of peroxidase (Trinder's reaction) (2).

$$\text{(1) cholesterol} + O_2 \xrightarrow{cholesterol\ oxidase} \text{cholesterol-3-one} + H_2O_2$$

$$\text{(2) } 2\ H_2O_2 + \text{4-aminoantipyrine} + \text{phenol} \xrightarrow{peroxidase} \text{quinoneimine} + 4\ H_2O$$

A calibration curve is set up by diluting the cholesterol stock solution with a concentration of 5.2 mM (200 mg/dL). 11 standards are prepared with the following concentrations: 5.2, 4, 2.97, 2.6, 2, 1.49, 1.04, 0.52, 0.26, 0.13 and 0 mM. 10 µL of the standard/sample is added to a well of a blank assay plate, in duplicate. 190 µL of the reagent (Diasys, Holzheim, Germany) is added to the wells with a multichannel pipet, attempting to start the reactions nearly simultaneously. The reagent includes a buffer (pH 6.7), phenol (5 mM), 4-aminotipyrine (0.3 mM), cholesterol esterase (≥200 U/L, is responsible for the hydrolysis of cholesterol esters, if needed), cholesterol oxidase (≥100 U/L) and peroxidase (≥3000 U/L). The plate is incubated at RT for 15 minutes under agitation and measured with Spectramax M2e (Diasys 2012).

3.2.2 Particle size

To determine the size distribution of the liposomes batches, a nanoparticle tracking analysis (NTA) using the NanoSight NS 500 (Malvern, Worcestershire, UK) is performed. This is an individual particle visualization technique in which a part of the sample is illuminated by a laser beam. To have a good number of about 10 to 100 particles in view of the laser beam, the liposomes are first diluted in ultrapure water in an appropriate amount, for example $1/10^4$.

When particles in the size range of 10 to 2000 nm pass through the beam, the light is scattered in a way that is dependent on their size and refractive index (Tian, et al. 2016). This scattered light is captured by a microscope with a charge-coupled device (CCD) camera attached to it. Three 30-second videos at a speed of 30 frames per second are recorded from a different frame and subsequently analysed by the NTA software. This software tracks and analyses the Brownian motion of the particles from the observed light. After calculating the average distance travelled by each particle in the frame in function of time, the sphere-equivalent hydrodynamic diameter of the observed particles is determined from its average velocity using the Stokes-Einstein equation (see formula 3.2). As a result, the size distribution of the liposomes is obtained (van Oirschot - Hermans and van Dommelen 2013, Malvern 2016, Deschepper 2017).

$$d(h) = \frac{kT}{3\pi\eta D} \qquad (3.2)$$

Where $d(h)$: sphere-equivalent hydrodynamic diameter

k: Boltzmann constant ($m^2.kg.s^{-2}.K^{-1}$)

T: temperature (K)

η: viscosity ($kg.m^{-1}.s^{-1}$)

D: diffusion coefficient ($m^2.s^{-1}$)

3.3 QUANTIFICATION OF EPTIFIBATIDE

Quantification of eptifibatide in the liposome suspensions is achieved with High-Performance Liquid Chromatography (also referred as High-Pressure Liquid Chromatography or HPLC), using a 2795 Alliance system (Waters Corporation, Milford, USA). Detection is performed by ultraviolet (UV) absorption. Eptifibatide shows maximum absorbance at 219 and 275 nm. The wavelength of 275 nm is preferred since it prevents interference with trifluoroacetic acid (TFA), a component present in the mobile phase (Savadkouhi, et al. 2016). The intensity of the signal is determined by the area under the curve (AUC) and is proportional to the concentration of eptifibatide present in the sample.

The chromatographic separation is performed using a C18 column (Atlantis® dc18, Waters Corporation, Milford, USA). This inert, silica-based, reversed-phase

column is modified with octadecyl carbon chains (C18), and is applied for retention of polar components. The column has a length of 100 mm, an internal diameter of 3 mm, a particle size of 3 µm and a pore size of 100 Å. The analysis is performed using isocratic elution, i.e. the composition of the mobile phase is kept constant, and at ambient temperature (25°C). The flow rate is set at 0.5 mL/min, the injection volume is 35 µL and each run lasts 5 minutes.

The mobile phase is composed of ultrapure water and acetonitrile (Sigma-Aldrich, Steinheim, Germany) at the ratio of 68:32 (v/v), with 0.1% (v/v) TFA (Sigma-Aldrich, Steinheim, Germany), according to Savadkouhi, et al. TFA is often used in the mobile phase as a buffer component to lower the pH for separation of peptides and proteins. More important in this case, TFA also serves as an ion-pairing reagent. Ion-pairing reagents are added to improve retention of ionic and highly polar substances on a reversed-phase column, by acting as a counter ion to form a neutral-like substance (Gooding and Regnier 2002). Acetonitrile (CH_3CN), a polar organic solvent, has a low absorbance at short UV wavelengths and is responsible for liposome bursting. Disruption of the bilayer is necessary to make sure eptifibatide can escape out of liposomes for interaction with the stationary phase. Acetonitrile has an amphipathic character, due to its capability of accepting hydrogen bonds and its hydrophobic methyl group. This leads to insertion into the lipid bilayer, which causes bursting of the liposomes (Yoshida, et al. 2014).

To investigate which percentage of acetonitrile is needed for disruption of the liposomes, different ratios of water and acetonitrile are tested with one particular batch, prepared via microfluidic-based manufacturing. The effect of higher ratios of acetonitrile on the measured amount of eptifibatide is evaluated. 30 µL of the suspension is diluted and mixed in 15 µL water:acetonitrile with the following ratios: 68:32, 60:40, 50:50, 40:60, 30:70, 20:80 and 10:90. The obtained peak areas are compared in order to select the ratio that gives the highest amount of eptifibatide.

12 standard solutions are composed in order to set up a calibration curve. The first standard is made by diluting the eptifibatide stock solution of 25 mg/mL 100 times, using the mobile phase as diluent. A predilution is made for accurate pipetting. A serial dilution is prepared with dilution factor 2. Each sample is diluted with dilution factor 1.5 (30 µL liposomes + 15 µL mobile phase).

3.4 FUNCTIONALITY ASSAY

The functionality of the eptifibatide loaded liposomes is tested with an aggregometer (model 700, Chrono-log Corporation, Havertown, USA), which measures platelet aggregation. Platelet-rich plasma (PRP) is incubated in the aggregometer and stirred at 900 revolutions per minute at 37 ºC. The light transmission through the troubled suspension is measured. After addition of an agonist platelet aggregation takes place. Because of the formation of aggregates, the suspension becomes clear which causes an increase in light transmission through the cuvette. These optical changes are recorded and expressed as percentages, relative to a blanc.

PRP is isolated from whole blood by centrifugal acceleration of 160 x g for a period of 15 minutes (Rotina 380, Hettich, Tuttlingen, Germany). After separation of PRP from the red blood cells, the blood is subjected to the centrifuge again, with a force of 2000 x g for 10 minutes. Platelet-poor plasma (PPP) is isolated and used as a blanc. To obtain reliable aggregation results, the platelet count in PRP should be in the range of $200 \cdot 10^9$ to $250 \cdot 10^9$/L. This is verified with the Cell-Dyn Emerald Haematology Analyzer (Abbott, Abbott Park, USA) and if required, PRP is diluted with PPP. The experiments are performed as soon as possible after PRP collection (approximately within 3 hours), since platelets reactivity toward agonists significantly decreases during storage (Bausset, et al. 2012).

As an agonist, a thrombin receptor activating peptide (TRAP, Bachem, Bubendorf, Switzerland) is used. This is a small synthetic peptide, capable of reproducing several effects of thrombin. This agonist induces platelet aggregation (Cicala, et al. 1999). However, the presence of eptifibatide will inhibit this process. In this way, the functionality of the drug loaded liposomes is evaluated in plasma. The required amount of TRAP is determined before the experiments with liposomes, because of the individual differences of the blood donors. This is accomplished by testing different concentrations of TRAP, and selecting the lowest concentration that gives maximum aggregation. The required concentration in plasma is in the order of 5 µM. In every run, a positive control (only TRAP) is included, to make sure the aggregation takes place without inhibitor.

The liposomes are tested in two ways: with SDS (sodium dodecyl sulphate, Sigma-Aldrich, Steinheim, Germany) and without SDS. Mixing the liposomes with SDS (0.25%) will lead to disruption of the bilayer, which allows release of the drug. Theoretically, the liposomes without SDS may not show inhibition. The cuvettes are prepared by pipetting 300 µL PRP and an appropriate amount of liposomes (in the order of 20 µL), whether or not mixed with SDS. After setting the baseline to determine the difference in light transmission between PRP and PPP, the aggregation is started by adding the agonist (Koekman 2013).

4 RESULTS

The following results are related to the liposome batches made with the NanoAssemblr™ on May 2, 2018 and on May 14, 2018 and will be referred as 180502 and 180514, respectively. Batch 4 is only made once (on May 2, 2018), because of poor results. The thin-film hydration batch is made on April 16, 2018 and will be referred as 180416, TFH.

4.1 ISOLATION AND PURIFICATION

In order to detect in which fraction the liposomes eluate from the gel filtration column, the cholesterol content is determined in the different fractions. This assay shows that the liposomes come out of the column between 0.2 mL and 0.8 mL (indicated as 600 µL in Table 4.1) eluting buffer (water or HBS), so this fraction is collected. The flow-through indicates the fraction collected after sample application, and has a volume of 500 µL. These results are obtained after optimization of the separation.

Table 4.1: Absorbance at 500 nm of quinoneimine, an indicator proportional to the cholesterol content. The eluted fractions are sequentially represented (from left to right) (batch 180502).

Volume	Flow-through	200 µL	600 µL	200 µL	200 µL
Batch 1	0.0581	0.0604	0.2097	0.0774	0.0651
Batch 2	0.0585	0.0621	0.1921	0.0754	0.0602
Batch 3	0.0623	0.0617	0.1904	0.0782	0.0641
Batch 4	0.0604	0.0626	0.1548	0.0762	0.0640

Linear regression ($y=0.1658x+0.0559$, $R^2=0.9991$) gives the following concentrations of cholesterol in the fractions between 0.2 and 0.8 mL: 0.9287 mM, 0.8223 mM, 0.8121 mM and 0.5978 mM for batch 1, 2, 3 and 4 respectively. The concentrations measured in the other fractions are generally 10 times lower.

Figure 4.1 plots the concentration eptifibatide, determined with HPLC, in function of the buffer volume eluted from the column. This graph is obtained during the optimization of the purification protocol. The separation is performed by adding buffer (in this case water since the batch is prepared with water) in steps of 250 µL.

Every flow-through after buffer application is isolated and quantified. Furthermore, the liposome recovery of this procedure is determined during optimization by comparing the amount of liposomes applied for separation and the amount of isolated liposomes. These are calculated by measuring the phospholipid concentration of the start suspension and the isolated suspension, and multiplying these concentrations with the corresponding volumes (the volume applied for separation and the isolated volume respectively). This gives a recovery of generally 90%.

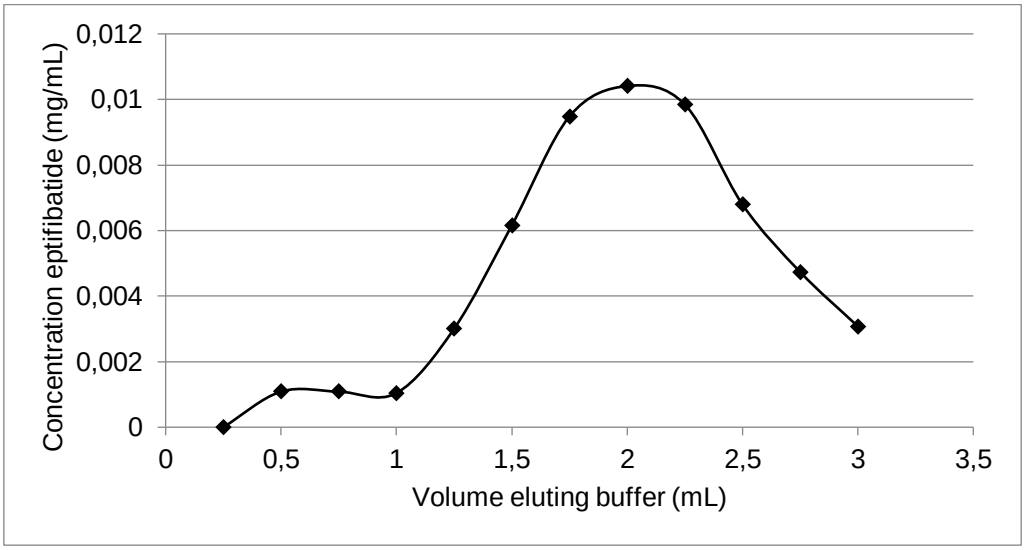

Figure 4.1: Concentration eptifibatide in function of buffer volume eluted from the column.

4.2 CHARACTERIZATION OF LIPOSOMES

4.2.1 Quantification

Table 4.2: Phospholipid and cholesterol concentration of the liposome samples.

Batch		Phospholipid concentration (mM)	Cholesterol concentration (mM)	Ratio
180416	TFH	1.2789		
180502	1	0.7462	0.6107	1.22
	2	0.7944	0.6225	1.28
	3	0.5826	0.4460	1.33
	4	0.8480	0.6373	1.31
180514	1	0.7306	0.5475	1.33

	2	0.7438	0.5855	1.27
	3	0.5450	0.4231	1.28
			Mean ± SD	1.29 ± 0.04

A liposome suspension made via thin-layer hydration is generally much more concentrated (in the range of 15 to 20 mM), but dilation occurred during gel filtration chromatography, because only 80 µL left over from this batch (and sample volume of the column is equal to 500 µL).

4.2.2 Particle size

Table 4.3: Modal liposomal diameter (nm) of eptifibatide-loaded liposomes

Batch		Modal liposomal diameter (nm)
180416	TFH	139.1
180502	1	79.0
	2	73.5
	3	128.8
	4	70.1
180514	1	71.9
	2	64.7
	3	105.5

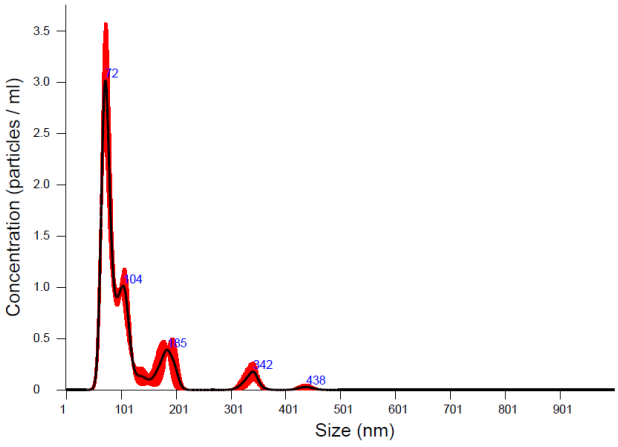

Figure 4.3: Size distribution of 180514 batch 1

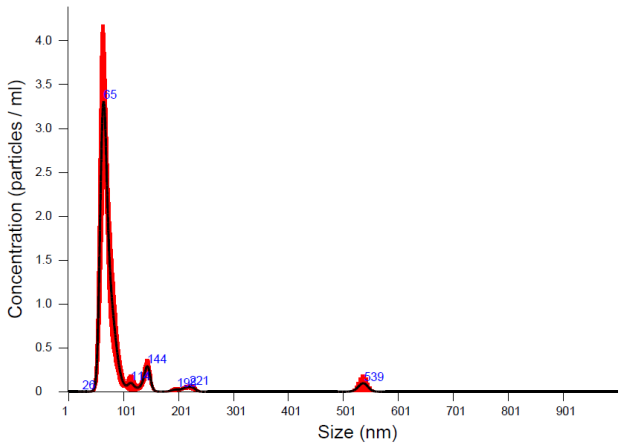

Figure 4.2: Size distribution of 180514 batch 2

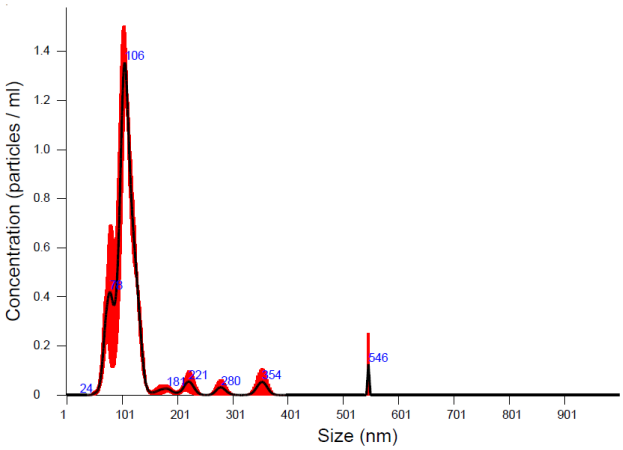

Figure 4.4: Size distribution of 180514 batch 3

4.3 QUANTIFICATION OF EPTIFIBATIDE

Two analyses will be described, of which the first one has no quantification. Something went wrong with the preparation/detection of the standards, as a result of which no calibration curve could be generated. Even though, the obtained results from the samples can be valuable. The dilution factor is set at 1.5 for every run. The first runs are diluted with different concentrations of acetonitrile (ACN), but still in the same way as the other samples (dilution factor 1.5). Flow-throughs (=filtrates) are obtained by filtration using Pierce® Concentrator (see 3.1.3.3). Supernatants are isolated after centrifugation procedure 1 (see Table 3.4). A part of the batch is not subjected to dialysis, in order to evaluate the removal of unencapsulated eptifibatide due to this osmotic-based procedure. The retention time (t_r) can be defined as the time from injection of the sample to the time of compound elution (taken at the maximum of the peak). The AUC is expressed in arbitrary units (AU). The AUC values are compared with the concentration of phospholipids ('C' in Table 4.4 and Table 4.5), and the ratio is the hypothetical AUC for a phospholipid concentration of 1 mM.

Table 4.4: HPLC analysis 1

	Sample description	Batch	t_r (min)	AUC (AU)	C (mM)	Ratio
1	H2O:ACN 68:32	180502 1	2,652	10113		
2	H2O:ACN 60:40		2,633	9493		
3	H2O:ACN 50:50		2,632	7746		

4	H2O:ACN 40:60			2,619	8330		
5	H2O:ACN 30:70			2,608	8768		
6	H2O:ACN 20:80			2,599	7826		
7	H2O:ACN 10:90			2,588	7444		
8	Liposomes	180502	1	2,629	10588	0,7462	14189
9			2	2,620	10744	0,7944	13524
10			3	2,618	13961	0,5826	23963
11			4	2,604	2292	0,8480	2703
12		180514	1	2,615	10715	0,7306	14666
13			2	2,608	10174	0,7438	13678
14			3	2,610	9550	0,5450	17523
15	Flow-through	180502	1	no peak			
16			2	2,621	57476		
17			3	2,610	53728		
18			4	2,598	63550		
19		180514	1	2,601	60764		
20			2	2,623	27377		
21			3	2,611	48558		
22	Supernatant	180514	1	2,647	92915		
23			2	no peak			
24			3	no peak			
25	Before dialysis	180514	1	2,665	32226	0,9848	32725
26			2	2,599	36159	0,7583	47683
27			3	2,600	17922	0,7335	24434

For the second HPLC analysis, a calibration curve is generated. As an illustration, two chromatograms of the standards are shown below (Figure 4.5 and Figure 4.6), especially the highest standard and the lowest standard, 0.25 mg/mL and 0.00012207 mg/mL respectively. Two chromatograms of the samples are represented as well (Figure 4.7 and Figure 4.8), run 4 and run 12 (Table 4.5). Two

calibration curves are shown: the original (Figure 4.9), including twelve standards, and the one which is used for quantification of the samples (Figure 4.10), including the eight lowest standards. This last calibration curve is applied because it is more representative (and will give more accurate values) for the samples (the concentrations of eptifibatide in the samples are relatively low, in this way they fall within the range of these standards). Flow-throughs are obtained by filtration using Nanosep® (see 3.1.3.3). Supernatants are isolated after centrifugation procedure 2 (see Table 3.4). The dilution factor is equal to 1.5 for every run.

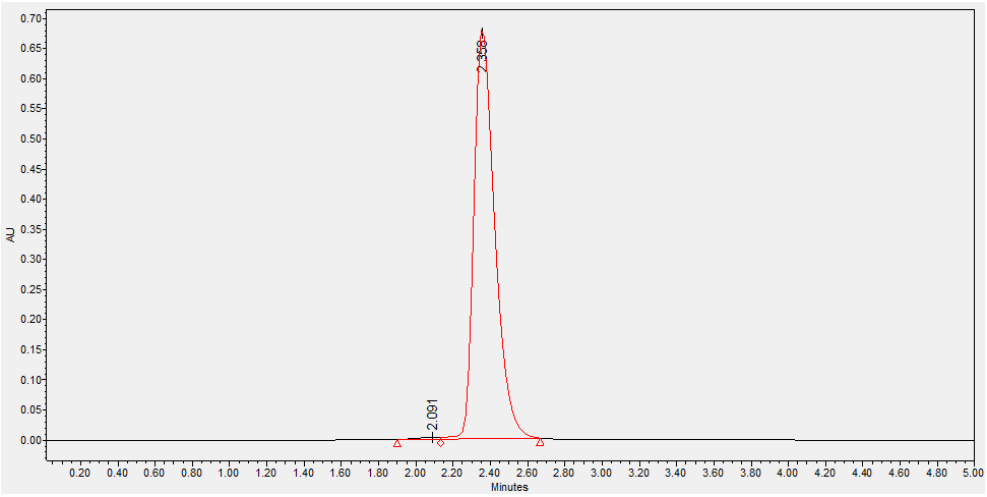

Figure 4.5: Chromatogram of standard 1

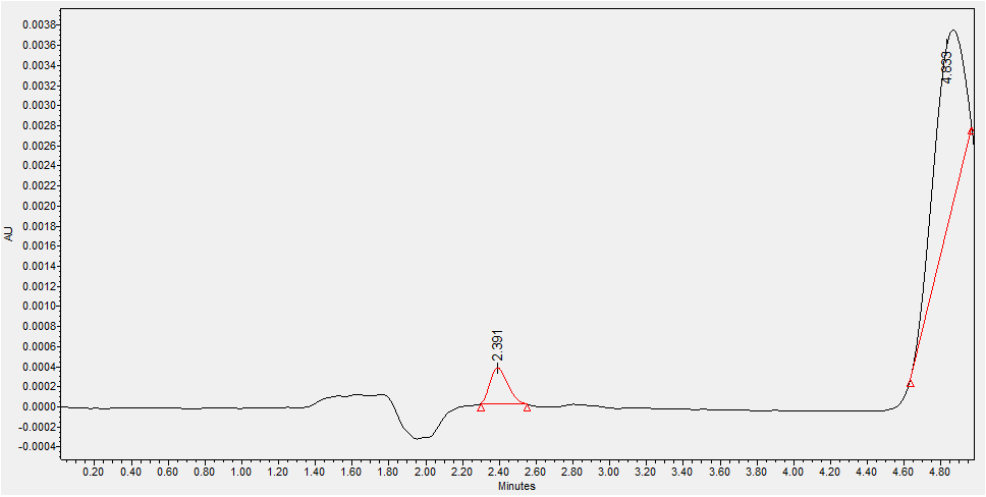

Figure 4.6: Chromatogram of standard 12

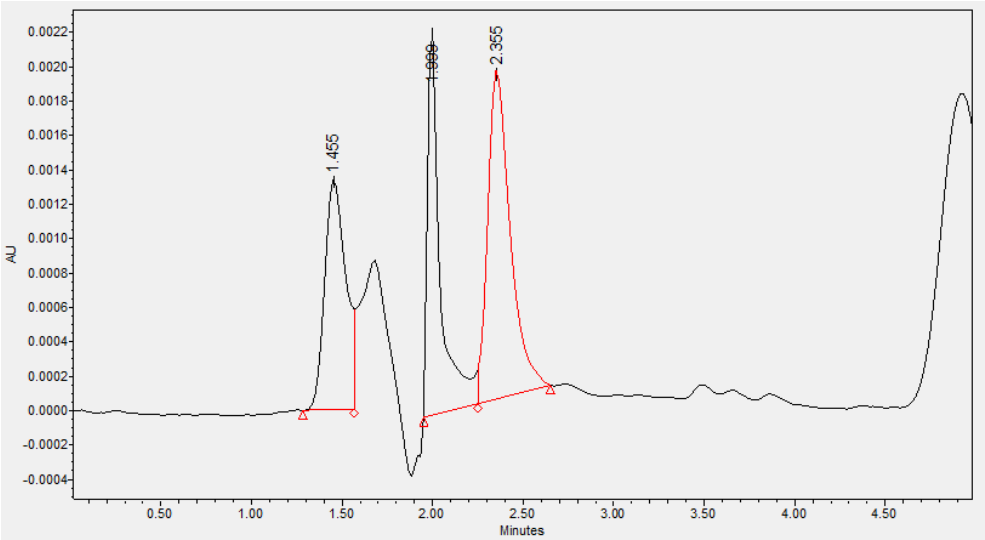

Figure 4.7: Chromatogram of 180502 batch 3

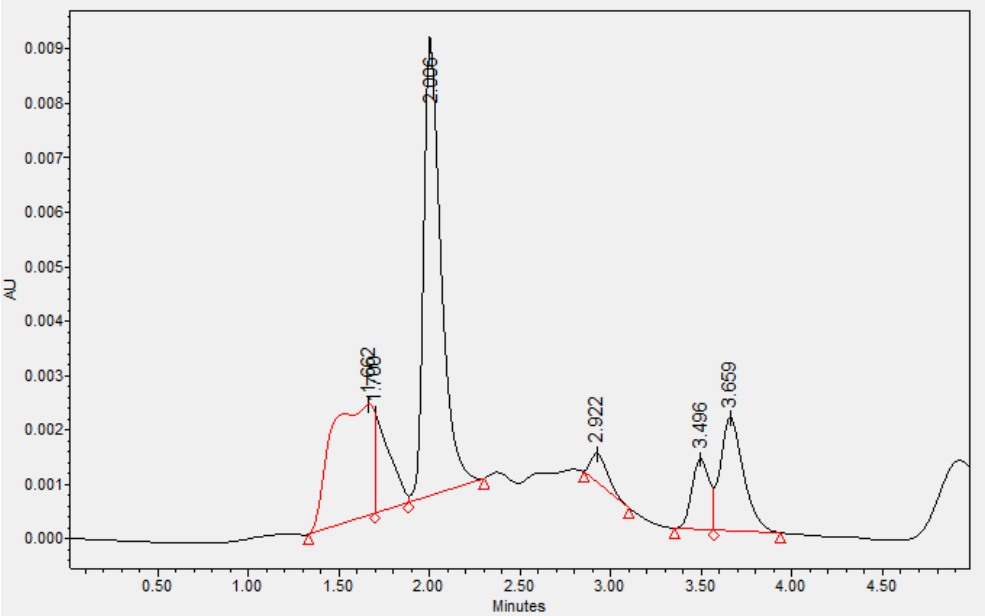

Figure 4.8: Chromatogram of the flow-through of 180502 batch 3

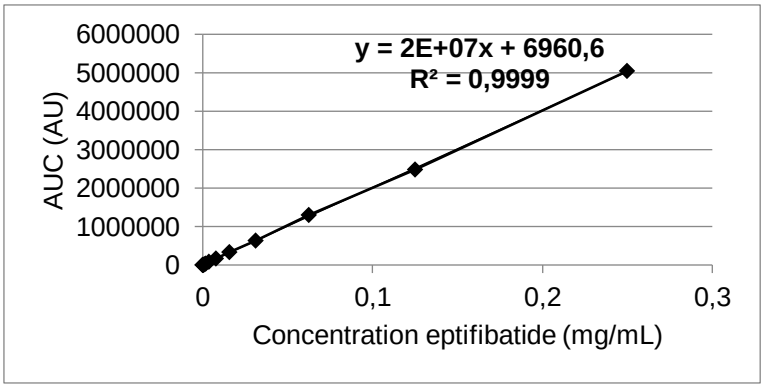

Figure 4.9: Calibration curve eptifibatide (0.25 mg/mL-0,00012207 mg/mL)

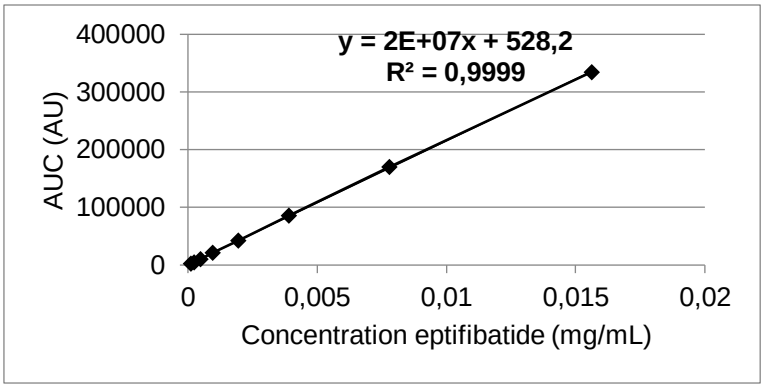

Figure 4.10: Calibration curve eptifibatide (0,015625 mg/mL-0,00012207 mg/mL)

Table 4.5: HPLC analysis 2

	Sample description	Batch		t_r (min)	AUC (AU)	C (mM)	Ratio
1	Liposomes	180416	TFH	2,365	338299	1,2789	264514
2		180502	1	2,353	42362	0,7462	56773
3			2	2,352	18283	0,7944	23014
4			3	2,350	15655	0,5826	26869
5			4	2,355	6603	0,8480	7787
6		180514	1	2,353	11693	0,7306	16006
7			2	2,351	10212	0,7438	13729
8			3	2,354	9145	0,5450	16781
9	Flow-through	180416	TFH	2,352	70863		
10		180502	1	2,338	12599		
11			2	2,346	5868		
12			3	no peak			
13			4	no peak			
14		180514	1	no peak			
15			2	no peak			
16			3	no peak			
17	Supernatant	180514	1	2,354	2010		
18			2	2,358	2104		
19			3	2,374	2135		

Linear regression (y=2E+07x+528.2, R^2=0.9999) is applied in order to calculate the concentrations of eptifibatide present in the batches. The dilution is taken into account: every concentration is multiplied by 1.5. The encapsulation efficiency (EE) is calculated via equation 4.1, assuming that the external medium doesn't contain free drug molecules.

$$EE\ (\%) = \frac{C_{eptifibatide} \cdot V_{batch}}{m_{eptifibatide}} \cdot 100 \qquad (4.1)$$

Where $C_{eptifibatide}$ (mg/mL): concentration measured via HPLC

V_{batch} (mL): original volume of the purified batch

$m_{eptifibatide}$ (mg): amount of eptifibatide used in preparation

Table 4.6: $C_{eptifibatide}$ (µg/mL) (calculated via calibration curve Figure 4.10), EE (%) (calculated via equation 4.1) and the corrected $C_{eptifibatide}$ (µg/mL) (calculated via calibration curve Figure 4.10, using the ratios in Table 4.5).

Batch		$C_{eptifibatide}$ (µg/mL)	$m_{eptifibatide}$(mg)	EE (%)[b]	$C_{eptifibatide}$ corrected for $C_{phospholipid}$ (µg/mL)
180416	TFH	23,5984	0,40[a]	2,9498	18,4435
180502	1	2,9231	2,75	0,1276	3,9299
	2	1,2408	2,75	0,0541	1,5713
	3	1,0572	1,00	0,1269	1,8407
	4	0,4248	0,55	0,0927	0,5075
180514	1	0,7804	2,75	0,0341	1,0817
	2	0,6769	2,75	0,0295	0,9227
	3	0,6024	1,00	0,0722	1,1359

[a] 7.50 mg is applied for preparation of 1.50 mL liposomes, so 0.40 mg is present in 0.08 mL liposomes (which is the volume applied in gel filtration chromatography).

[b] The EE is determined regarding the volumes obtained after gel filtration chromatography (for the TFH batch 0.5 mL, for the other batches 1.2 mL). The little dilution by dialysis is not taken into account, but is insignificant.

4.4 FUNCTIONALITY ASSAY

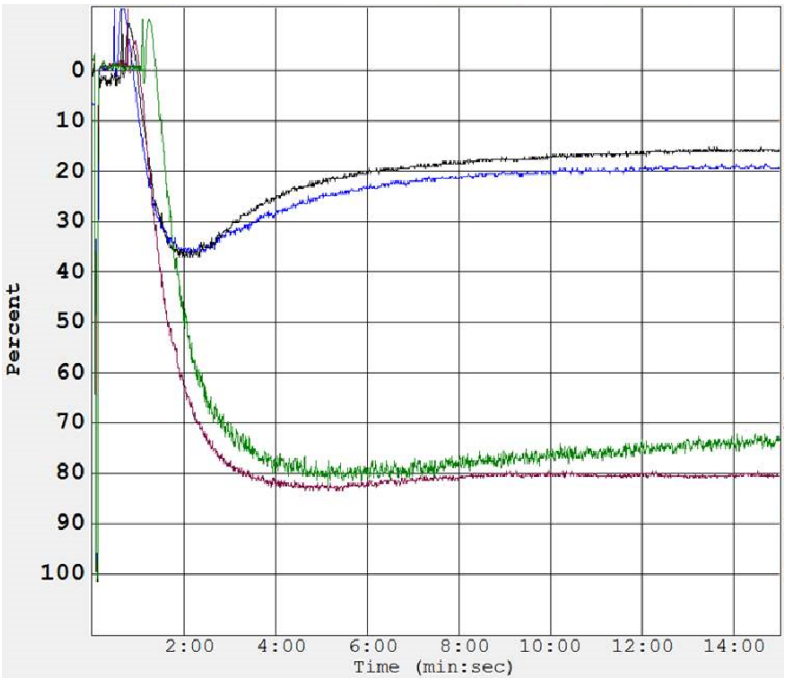

Figure 4.11: Aggregation graph (180502 batch 1 + SDS, 180502 batch 2 + SDS, 180502 batch 4 + SDS, control)

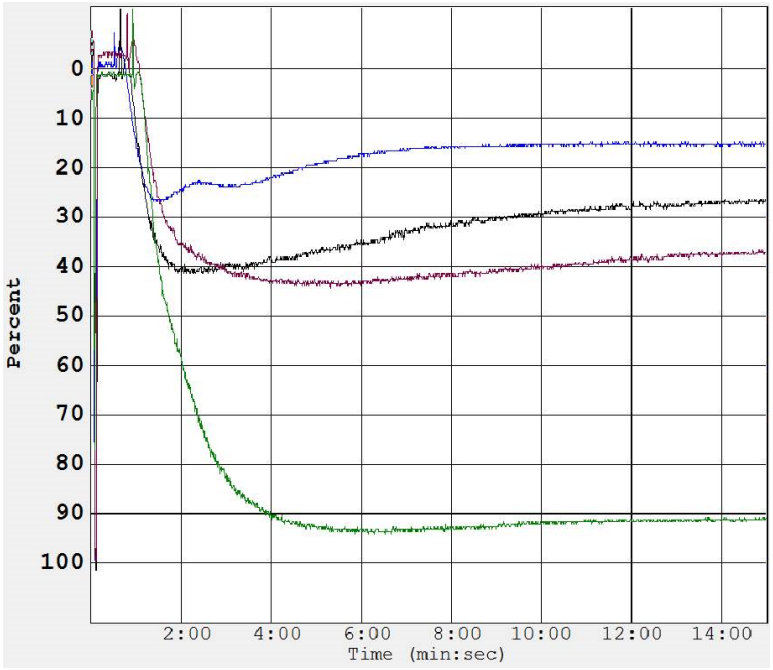

Figure 4.12: Aggregation graph (180502 batch 3 + SDS, 180514 batch 1 + SDS, 180514 batch 2 + SDS, control)

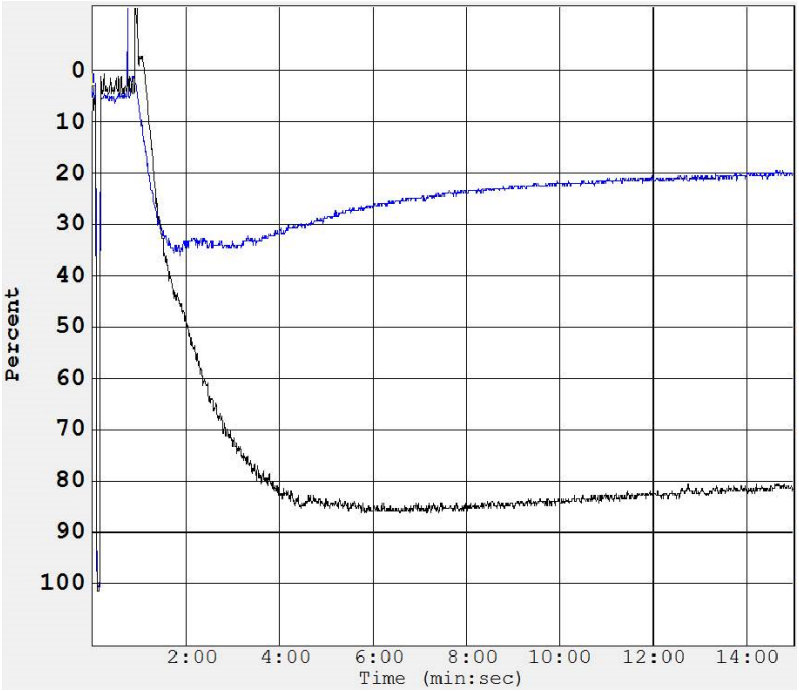

Figure 4.13: Aggregation graph (180514 batch 3 + SDS, control)

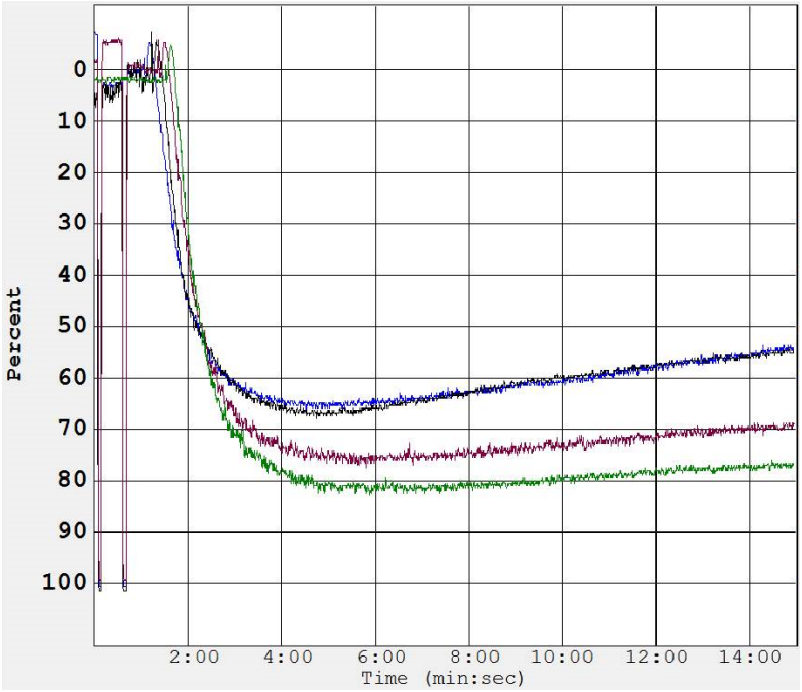

Figure 4.14: Aggregation graph (180514 batch 1 without SDS, 180514 batch 2 without SDS, 180514 batch 3 without SDS, control)

5 DISCUSSION

5.1 PREPARATION OF LIPOSOMES

Extrusion, a particle size reducing procedure subsequent to thin-film hydration, leaded to substantial loss of liposomes. This is due to the excessively high pressure of forcing the liposomes through the 0.05 µm filter. When the liposomes aren't able to pass through the pores, they find a way out and leak out of the extruder. The 0.2 µm filter should probably be followed by a 0.1 µm filter (instead of a 0.05 µm filter).

Batch 4, which is prepared with 1 mg/mL eptifibatide in the aqueous phase, is only made once, with the aim of evaluating the EE compared to the batches prepared with 5 mg/mL eptifibatide. The decision of excluding this batch is made because of the small peaks (and as a result low AUC values) obtained in HPLC, as a result of which it becomes difficult to maintain the accuracy. The noise present in the chromatogram restricts the determination of the AUC.

In literature, the EE is determined by formula 5.1 (Bardania, et al. 2017). In this essay, formula 4.1 on p. 31 is applied, because of the purification procedures, which don't allow the use of formula 5.1.

$$EE\ (\%) = \frac{C_{initial} - C_{free}}{C_{initial}} \cdot 100 \qquad (5.1)$$

Where $C_{initial}$: represents the total drug used for loading

C_{free}: represents the non-entrapped drug present in the hydration medium, separated by centrifugation

A major concern is the purification (removal of the non-entrapped drug) of the eptifibatide-loaded liposomes. The first approach involved filtration in an ultracentrifuge (not in detail described because of direct rejection of this procedure). The principle consists of retention of the nanoparticles while the free drug molecules can pass through the filter. During extensive ultracentrifugation, addition of buffer and resuspension of the liposomes took place systematically. However, the final filtrate fraction gave a high signal for eptifibatide. The reason for these unexpected results is unknown. Possibly ultracentrifugation has an impact on the liposomes or the liposome's content, causing leakage of the drug. Another hypothesis is that the liposomes obstruct the pores of the filter, through which the drug isn't capable to pass

the filter during the procedure and appears in the final filtrate fraction. When these batches are subjected to dialysis, a nearly clean external medium is observed, which introduces a promising purification method.

However, when new batches are purified via dialysis, it seems that this single procedure is insufficient. Eptifibatide is observed in the external medium, possibly due to saturation of the dialysis membrane by liposomes, which prohibits the passive diffusion of the free drug. This leads to the introduction of separation via gel filtration chromatography, which allows the removal of an extensive amount of free eptifibatide. Figure 4.1 (p. 24) illustrates this with HPLC analysis of the eluting fractions: the eptifibatide-loaded liposomes elute from the column, while a substantial amount of free eptifibatide retains and elutes subsequently (starting from a volume of 1 mL buffer). This technique has a recovery of generally 90% (amount of liposomes isolated after separation versus amount of liposomes applied), which is acceptable. Following gel filtration chromatography, the batches are subjected to dialysis, in order to remove remaining amounts of unencapsulated drug molecules. Efficiency of this method is verified by quantifying the batch before dialysis and after dialysis.

A further challenge is the analysis of the external medium. How is the extraliposomal solution tested/separated? The first approach involves centrifugation. However, settings such as centrifugal force or duration are difficult to establish. It's almost impossible to actually see if the supernatant is free from liposomes and it's difficult to estimate the velocity of precipitation, which is furthermore hard to find in literature. The formula for the sedimentation velocity (m/s) of a falling particle through a medium of significant density (Stokes's law) is generally written as (Pickover 2008):

$$v = \frac{2(\rho_p - \rho_f)\, g\, r^2}{9\mu} \qquad (5.2)$$

Where ρ_p: mass density of the particles (kg/m^3)

ρ_f: mass density of the fluid (kg/m^3)

g: centrifugal acceleration (m/s^2)

r: radius of the spherical particle (m/s)

μ: dynamic viscosity of the fluid (kg/m·s)

However, different aspects question the efficiency. First of all, centrifugation to sediment liposomes smaller than 100 nm is difficult, even at high (ultra)centrifugal forces. Secondly, the difference between the density of the liposomes and the aqueous medium is probably insufficient to permit quantitative sedimentation. Anyway, a high ultracentrifugal force and a long centrifugation time are required when the densities are closely matched, but due to these forces there is potential for bilayer disturbance, and as a consequence release of the drug (Wallace, et al. 2012). To avoid this, the batches are subjected to the centrifugal forces represented in Table 3.4 (p.16) and isolation of the supernatant is performed very cautiously, as close as possible to the surface of the solution. Different centrifugation procedures are tested, but most of the obtained supernatants gave unwanted results: detection of eptifibatide in HPLC and a positive signal in the cholesterol assay or with NTA. However, comparison of the eptifibatide concentration and the liposome amount present in the supernatant, gave the impression of adequate purification by gel filtration chromatography and dialysis.

The second approach involves centrifugal ultrafiltration through a semipermeable membrane. This technique is possibly problematic for suspensions with a high particle content due to clogging of the filter. Nevertheless, this method has advantages opposed to precipitation via centrifugation: separation occurs in the order of minutes and lower centrifugal forces can be applied, in order to maintain the particle integrity. Though, the operating forces in the filtration methods described on p. 16 are relatively high. Maybe these procedures could be done over again with lower, and consequently safer centrifugal forces (e.g. 2000 to 4000 x g). Both the Pierce® Concentrator and the Nanosep® come along with a protocol. The Pierce® Concentrator is performed with higher forces than prescribed in the protocol (maximum 15000 x g), which is considered as a misstep. Assuming that a force of 20817 x g doesn't cause damage, to the filter and/or to the liposomes, is probably inappropriate. Additionally, penetration of small liposomes (with a diameter up to 100 nm) through the 100 kDa MWCO membrane is conceivable (Wallace, et al. 2012). Analysis of the flow-through obtained after centrifugation, handling the Nanosep® filter devoted to its protocol, gives satisfying results (see 5.3).

5.2 CHARACTERIZATION OF LIPOSOMES

The lower liposome concentration of batch 3 (see Table 4.2 p. 24) is due to the addition of an aqueous eptifibatide solution to the lipid mixture, which results in a lower amount of phospholipids diffusing into the aqueous phase. Furthermore, an increase in particle size is observed in batch 3. This seems counterintuitive, because a decrease in phospholipid concentration usually leads to a decrease in particle size as well. The mechanism behind this statement explains the higher liposome size. As the FRR increases, mixing of the ethanol phase with the aqueous phase accelerates, through which the lipid and ethanol concentration reduce more rapidly. This shortens the liposome growth, by destabilization of the lipids in the aqueous medium. A lower FRR results in slower diffusion of the ethanol phase, which stabilizes the lipids and gives them more time to expand (Kastner, et al. 2015, Zook and Vreeland 2010). Translation of this principle to batch 3 leads to this hypothesis: introducing an aqueous phase (containing eptifibatide) to the lipid mixture in 100% ethanol, changes the polarity of the mixture. At this point, the lipids possibly have the opportunity to initiate hydrophobic interactions, in order to reduce the exposure of the hydrophobic chains to the medium. This could generate the basis for formation of larger liposomes.

Anyhow, the liposome concentrations are relatively low (see Table 4.2 p. 24). This is due to the dilution through gel filtration chromatography. If a higher concentration is preferred, the batches could be concentrated by e.g. centrifugal filtration, until a certain volume of fluid is removed. Regarding the relatively small peaks obtained with NTA (see Figure 4.2, Figure 4.3 and Figure 4.4 p. 25), it may be concluded that the liposomes have a narrow size distribution and are, in other words, nearly-monodisperse.

As represented in Table 4.2 (p. 24), determination of the cholesterol content allows a decent estimation of the phospholipid concentration. Linear regression is applied in order to examine the correlation: $y=1.2453x+0.024$, $R^2=0.9564$, with x = the cholesterol concentration (mM) and y = the phospholipid concentration (mM). The ratios are given in Table 4.2 because of the assumption that the intercept, which is the y-coordinate where the curve crosses the y-axis, is close to zero (no detection of cholesterol should indicate absence of liposomes).

5.3 QUANTIFICATION OF EPTIFIBATIDE

The intercept in the calibration equation, should be theoretically close to zero. This is the case in the second calibration curve (includes the eight lowest standards, Figure 4.10 p. 30), compared to the first calibration curve. The calibration curve that includes the seven lowest standards, has an intercept of 9 and an R^2 of 1, but is better not applied because the detected signal of the thin-film hydration batch doesn't fall within the range of this curve.

Apparently, the set-up of the HPLC analysis succeeded. The calibration seems convenient and generally repeatable sample outcomes are obtained. However, some outcomes of e.g. flow-throughs or supernatants are certainly bizarre. Furthermore, a pattern is observed when high concentrated eptifibatide batches are injected onto the column. Especially run 1 and 9 in Table 4.5 (p.30) give a strong signal. The runs coming subsequent to these high concentrated runs give unexpected results. This insinuates that perhaps something went wrong in the HPLC system. Insufficient needle wash or remaining sample somewhere in the system seem to be possible explanations. Definitely, this needs to be clarified. To insure these unexpected results are due to the system and not to the samples, a high concentrated sample could be injected onto the column, followed by a couple of blanc solutions. Furthermore, a method validation should be performed, in accordance with the International Conference on Harmonization (ICH) guidelines. The following chromatographic parameters should be validated: specificity, linearity, sensitivity, precision, accuracy, detection limit and quantitation limit (ICH 2005, Savadkouhi, et al. 2016).

The liposomes should burst in an acetonitrile concentration of 32% (v/v). This is demonstrated by the analysis of different ratios of acetonitrile (see Table 4.4 p. 26), which shows that higher concentrations of this solvent don't result in higher eptifibatide amounts. This experiment is also performed earlier (but not reported), with higher concentrated batches (thin-layer hydration batches, not diluted by gel filtration chromatography) and even higher concentrations of acetonitrile (up to 1:5 dilution with 100% acetonitrile) but this didn't give differences as well. Instead the peaks got deformed with higher concentrations of the organic solvent. Besides, Yoshida, et al. (2014, 2018) reported that 20% (v/v) acetonitrile should be enough to

induce bursting of the liposomes. Intact liposomes probably won't retain on the column, because of its hydrophilic surface with steric repulsion of PEG molecules.

By interpreting the results in Table 4.4, Table 4.5 and Table 4.6, it seems that batch 3 (with eptifibatide solubilized in the lipid mixture) has a higher drug encapsulation than batch 1 and 2. This is possibly due to the larger liposomes obtained with this preparation method, resulting in a higher internal volume. The encapsulation efficiency for hydrophilic substances is proportional to the entrapped water volume of the liposomes (Bardania, Shojoasadati, et al. 2016). It is not certain that this is the only influence. Maybe the interaction (present in the ethanol phase) of the negatively charged head groups of the phospholipids and the positive charge of the exposed guanidine group in eptifibatide has an influence in the encapsulation. A further idea is that the liposome formation starts from the ethanol phase, by 'gradual' diffusion of the aqueous phase into the lipid mixture. In this way, the liposome formation goes out of a high concentration of eptifibatide instead of inversely (when preparation with eptifibatide solubilized in the aqueous phase).

Preparation of liposomes via thin-film hydration leads to an increased encapsulation as well. A higher eptifibatide concentration is observed compared with the batches prepared via microfluidic-based manufacturing (see 'Ratio' in Table 4.5). This is partially due to its liposome size, but this characteristic is probably not the only reason, because of the significant difference with batch 3, which also consists of larger liposomes. Apparently, passive encapsulation via TFH provides more (unidentified) advantages than only an increased particle size. During microfluidic-based preparation dilution of the 5 mg/mL eptifibatide solution with ethanol occurs, but this won't make the difference. Anyhow, the absolute amount of encapsulated eptifibatide in relation to the amount applied for preparation (EE) is definitely higher, which can be explained. Increasing the concentration of phospholipids leads to a higher amount of liposomes and as a consequence, an increased total internal volume (Bardania, Shojoasadati, et al. 2016). The ratio of the aqueous phase to the lipid mixture is for TFH equal to 1:2, while the ratio for microfluidic-based preparation is fixed at 3:1. This means that a relatively higher volume of the aqueous phase will be entrapped during TFH.

In batch 1 and 2, two different aqueous phases are used: aq. dest. and HBS, a HEPES-buffered saline, respectively. Buffer solutions are used to keep the pH nearly constant, in this case at pH 7.4. The pH of the medium can influence the charge of a drug molecule. A small neutral drug molecule is able to diffuse freely through the bilayer, while a larger polar or charged molecule is not able. E.g. glucose, which is a relatively large and polar molecule, isn't able to cross the cell membrane (Cooper 2000). In this way, it's imaginable that the zwitterionic eptifibatide molecule, which is way larger, stays usually encapsulated. However, it may be interesting to investigate the leakage in function of time. Whether the small difference between batch 1 and 2 (in favor of batch 1, observed in HPLC analysis 1 and 2, ignoring run 2 and 3 of HPLC analysis 2) is coincidentally or systematically, more research is required. Although the question is whether the small difference is worth the effort. A hypothetical reason for a lower concentration in the HBS batch is that HEPES molecules, which are zwitterions, and ions present in the buffer (Na^+ and Cl^-), act as counterions resulting in a more neutral-like peptide (Shockman and Wicken 1981). Still, the drug molecule is large and polar, so assuming that diffusion becomes all of a sudden easy is inappropriate. Although, low leakage during time and operations like vortexing is not excluded.

Batch 4 was introduced to compare the EE with the other batches, but rejected because of low signals. With the eptifibatide concentration five times lower during preparation, AUC values obtained in HPLC analysis 1 (Table 4.4) are also approximately five times lower. However, this is not the case in HPLC analysis 2 (Table 4.5). In this analysis, it's possible that the run still measures traces eptifibatide of the previous run, as already described, because batch 4 came before batch 3 in the actual run. Otherwise, further investigation is required (but definitely no priority) to notice if the EE is indeed higher with this lower eptifibatide concentration.

The high signals for the flow-throughs in Table 4.4 (p. 26) are bizarre, especially in contrast with run 15 (180502 batch 1), which doesn't give a signal. Assuming that something went wrong in the centrifugal filtration (using the Pierce® Concentrator) can explain the presence of a signal, but doesn't explain this extremely high signal. Analysis of the flow-throughs obtained with the Nanosep® filter, gives confirmative results (except for run 9, 10 and 11, Table 4.5): the absence of a peak ensures the purification efficiency. Free eptifibatide would definitely pass the 30 kDa

filter with its MW of 832 Da, supposing there are free molecules present in the liposome suspension. The signals of run 10 and 11 are possibly due to the high signal of run 9, the flow-through of the TFH batch. This batch is prepared on April 16, 2018, and the flow-through is isolated on May 18, 2018. During storage, leakage possibly occurred, resulting in a relatively high concentration of free eptifibatide.

The supernatants in HPLC analysis 1 give bizarre results as well. Run 22 (180514 batch 1) gives an inexplicable high signal, while the two other batches give no signal. In HPLC analysis 2, there is eptifibatide detected in the supernatants, but it's unknown whether this comes from leakage during centrifugation or from liposomes present in the supernatant. This could be verified by e. g. determination of the cholesterol content in the supernatants.

Table 4.4 represents the amounts of eptifibatide in the batch before dialysis. These values are significantly (approximately 3 times) higher than the batches subjected to dialysis, which signifies that dialysis is responsible for the removal of the remaining amounts of free eptifibatide. The batches subjected to dialysis are slightly diluted, but certainly not in the same order as the eptifibatide concentrations.

5.4 FUNCTIONALITY ASSAY

22.9 µL of the liposome suspension mixed with 3.3 µL 2% SDS results in a volume of 26.2 µL with 0.25% SDS, which is added to 300 µL PRP. Batches 1, 2 and 3 induce inhibition of aggregation with this ratio of 7:93 (v/v) sample:PRP, while batch 4 is not able to inhibit the aggregation. The last aggregation (Figure 4.14 p. 33) is performed without SDS. If the liposomes remain intact, they theoretically can't cause inhibition. Unluckily, the software of the aggregometer gave an error during this experiment, through which the runs got delayed. The platelets reactivity towards TRAP decreased, so the required amount of TRAP was determined again, which affects the reliability of this last run.

In these experiments, the release of eptifibatide is induced by the surfactant SDS (the applied concentration won't cause lysis of the platelets). However, SDS-induced destabilization of liposomes is not achievable *in vivo*, opposed to the following approaches that are able to improve drug release. 'Shear-sensitive nanocapsules' allow drug release along narrowed blood vessels, due to high shear

(Molloy, et al. 2017). ‘Magnetic liposomes’, liposomes with a magnetizing agent dissolved in the aqueous phase, release drugs by applying an altering magnetic field. ‘pH-sensitive liposomes’ are liposomes of which the bilayer is destabilized at lower pH values (Madni, et al. 2014). They release drugs near to tumors, infections and inflammation. These are just a few examples of the many techniques that are investigated in research.

5.5 FUTURE PLANS

First of all, the experiments described in this essay should be repeated, to ensure that the obtained results are consistent. Additionally, the stability of the eptifibatide-loaded liposomes should be investigated, by quantifying the leakage of the drug during storage.

The ambition of this project is to couple nanobodies to the surface of the drug-loaded liposomes, which selectively recognize dysfunctional endothelial cells or superficial thrombi. Now, the recommended dose of eptifibatide is 180 µg/kg administered as a bolus (rapid intravenous injection), followed by infusion of 1 µg/kg/min (Tcheng and O'Shea 2002). The dose of the therapeutic agent can be reduced through release of the drug at target sites, but further research is required to investigate which dose is effective with this type of drug delivery. A further purpose involves the loading of contrast agents for imaging, e.g. gadolinium.

To improve the encapsulation efficiency by preparation via microfluidics, the preparation could possibly be followed by freeze-thaw cycles. Optimization of the freezing-and-thawing technique after thin-film hydration with an eptifibatide solution is yet described in literature. Further attempts to improve the EE, irrespective of preparation via microfluidic technology, may be solubilizing the drug in the ethanol phase when preparing liposomes via thin-film hydration, or preparing liposomes via dehydration-rehydration. Reverse-phase evaporation seems like a less suitable method because of the large size of the liposomes. Remote loading via a pH gradient also seems inconvenient because eptifibatide is a zwitterion, and consequently not able to diffuse through the bilayer as an uncharged molecule.

6 CONCLUSIONS

The paper focused on the encapsulation efficiency of microfluidic-based preparation of liposomes, the purification of the batches and the set-up of an HPLC method.

The purification was a success. Most of the flow-throughs obtained after centrifugation, handling the Nanosep® filter devoted to its protocol, don't give a peak for eptifibatide, which means that the external medium doesn't contain free drug molecules. Both the gel filtration chromatography and the dialysis are responsible for the removal of a significant amount of free eptifibatide.

When liposomes are prepared via a microfluidic technique, with the drug solubilized in the ethanol phase, larger particle sizes are observed. This is probably due to the addition of an aqueous phase before the preparation starts. Larger liposomes result in a higher encapsulation volume, which can explain the increased drug encapsulation efficiency. The signal of the thin-film hydration batch (also larger liposomes) is much higher than the batches prepared via a microfluidic technique. The difference between the batches made in H_2O and HBS is marginal. Further research is required to secure an actual difference. Maybe the choice of buffer has an influence on the leakage, so experiments over time are required as well.

An additional achievement is that it may be concluded that the cholesterol content is a good predictor for the phospholipid concentration. The cholesterol assay is doable in less than half of the time required for performance of the phosphate assay.

Good fundamentals are achieved in de set-up of the HPLC method. Unfortunately, sometimes very strange results were attained, concluding that the method needs to be optimized and validated in order to get reproducible results. However, the ratio of 32:68 (v/v) acetonitrile:water present in the mobile phase should be enough for eruption of the bilayer.

Low concentrations are obtained, resulting in a substantial loss of the drug that is untrapped. Further research is definitely required to improve the encapsulation with the easy and fast preparation via microfluidics, and to find out what the required doses are in targeted drug delivery.

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