1. INTRODUCTION

Liposomes are artificial membranes, in most cases composed of phospholipids like phosphatidylcholines (PC), phosphatidylethanolamines (PE) and phosphatidylserines (PS), enclosing an aqueous compartment. They form spontaneously when phospholipids are placed in an aqueous environment, because of their dual preference to solvents, which was first described by Bangham and Horne in 1962 [1]. Amphiphilic lipids consist of one lipophilic part that is soluble in nonpolar solvents and a hydrophilic part soluble in polar solvents. Liposomes can be classified according to their structural properties or to their preparation method [2,3].

Table 1: Overview of commercially available liposomal products [4]

trade name (FDA approval) product/drug substances	type of dosage form	phospholipids and drug/lipid ratio	liposome type particle size
Abelcet® (1995) Amphotericin B	liposomal suspension	DMPC:DMPG 7:3 molar ratio drug/lipid 1:1 molar ratio	MLV < 5 µm
AmBisome® (2001) Amphotericin B	freeze-dried liposomes	213 mg hyd. soy phsophatidylcholine 84 mg distearoylphosphatidylglycerol 52 mg cholesterol, 0.64 mg α tocopherol drug/lipid 1:1 [w/w]	SUV < 100 nm
DaunoXome® (1996) Daunorubicin citrate	liposomal suspension	distearylphospatidylcholine/ cholesterol, 2:1 molar ratio lipid/drug 18.7:1 [w/w]	SUV ~ 45 nm
Doxil® (1999) Doxorubicin liposomes	liposomal suspension ready to use	3.19 ml/ml MPEG-DSPE 9.58 mg/ml HSPC 3.19 mg/ml cholesterol drug/lipid 1:6 [w/w]	LUV 100 nm
Myocet® (2006) Doxorubicin liposomes	liposomal suspension	Egg-Pc/cholesterol 1:1 molar ratio drug/lipid 1:1 molar	OLV 180 nm
Visudyne® (2001) Verteporfin® for injection Benzoprophyrin liposomes	freeze-dried liposomes	Egg phosphatidyl glycerol Dimyristoyl phosphatidylcholine Ascorbyl palmitate and butylated hydroxytoluene, 15 mg DS/vial DS/PL 1:7.5-15 weight ration	SUV ~ < 100nm
Junovan® (expected 2007) Muramyltripeptidephosphatidyl-ethanolamine (MTP-PE)	freeze-dried	POPC/DOPS 7:3 [w/w] DS/lipid 1:250 [w/w] tert. butanol	MLV 2-5 µm

An advantage, making liposomes an efficient drug delivery system, is the possibility that both hydrophilic and lipophilic molecules can be entrapped either into the aqueous core or into the lipid bilayer [5]. Due to their structure and the employed lipid molecules, liposomes are biocompatible, biodegradable and relatively non-toxic [6]. The application

of liposomes as drug carriers and pharmaceutical products depends on their colloidal stability, chemical composition, microencapsulating and surface properties. A classification of liposomal products ranges from drug-dosage forms [7] over cosmetic formulations [8] to diagnostics [9] and various applications in the food industry [10]. In drug delivery applications they have been extensively investigated for the delivery of anti-tumor substances [11], antimicrobial agents for treatments of bacterial [12], viral [13] and parasitic induced diseases [14], as well as for the use as immunological adjuvants for vaccines [15]. Other studies demonstrated the entrapment of genes [16] and DNA [17] into liposomal systems. Currently, many clinical studies for liposomal preparations are under investigations and several products have already entered the market (Tab 1).

2. LIPOSOMAL PREPARATION

Since the introduction of liposomes as drug delivery vehicles in the 1970s a comprehensive knowledge on strategies improving stability and on interaction characteristics between drugs and lipid membranes has been gained. The cosmetic industry was the first to launch a product containing liposomes in 1986. It took almost ten more years until the first pharmaceutical product Abelcet® reached the market in 1995. Although the successful development of many products has been achieved and extensive investigations have been made over decades, the production and stabilization of liposomal formulations for long term storage is still a major concern.

Particularly the industrial manufacturing of liposomes at a large scale is very challenging, as the production process is complex and consists of several steps. The procedure is based on several production steps, which are varying in complexity and are generally well described. Figure 1 provides an overview on a standard large scale production process for liposomes. As a first step the compounds will be dissolved in an appropriate solvent depending on the properties of the used lipids and drugs. The residual solvent concentration in the formulation must be reduced by an elimination step before continuing the processing of the liposomal formulation. A variety of hydration and homogenization steps can be used. Finally, the sterilization step and the stabilization are important to achieve a stable product. For invasive administration in humans by the parenteral route a sterilization of the liposomal formulations is essential. The most common method is sterile filtration using a 0.2 µm membrane [18]. Also gamma-irradiation [19], heat sterilization of the end product by autoclaving at 121°C [20] or steam sterilization [21] has been used for the sterilization of liposomes.

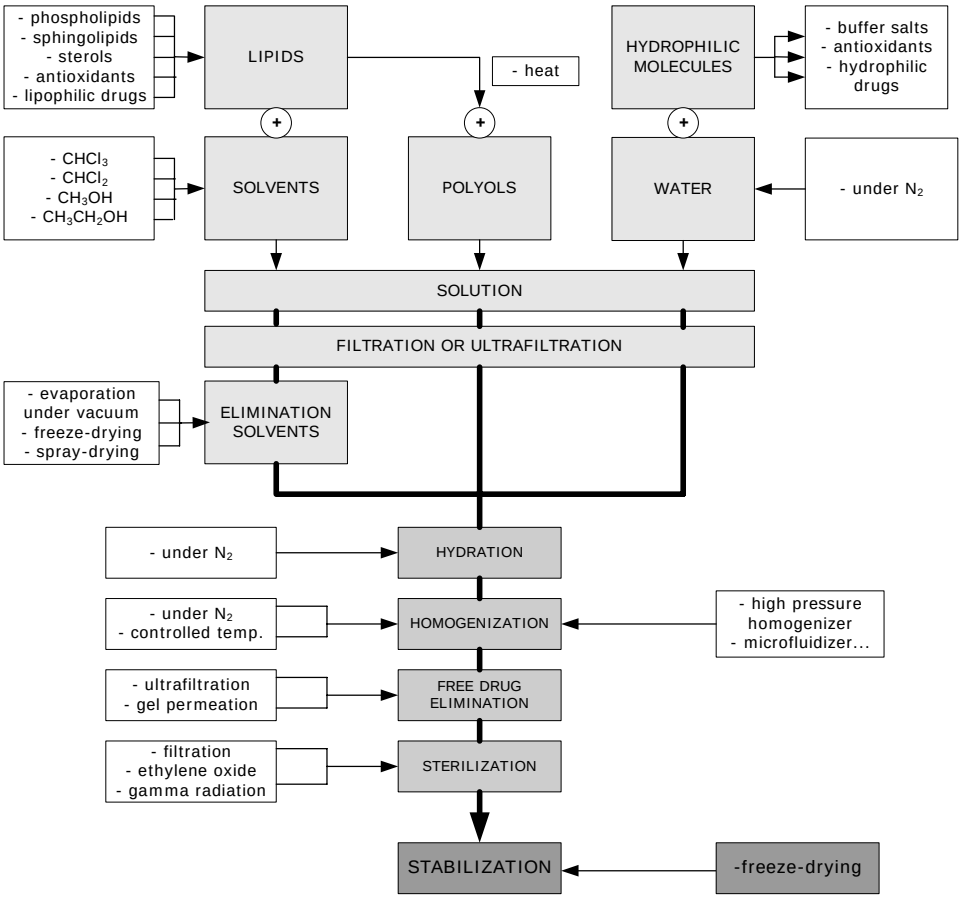

Figure 1: Schematic diagram for a large-scale liposome facility. Adopted from Redziniak et al. 1995 [22].

During the last two decades many of these process steps were further investigated and optimized. One approach described in literature is the introduction of new components or solvents for the preparation of solutions during the production of liposomes [23]. Organic solvents are often employed in liposomal production processes to solubilize either the lipids or the drug. The elimination of such organic solvents and the removal of non-encapsulated drugs are often required for the purification of the final product. In several cases this represents the limiting factor for scalability of the process. Additionally, to avoid stability problems like sedimentation or leakage of liposomes over the storage time the manufacturing with uniform liposomal size distributions by homogenization is particularly important [24]. For certain applications it is necessary to achieve certain liposome sizes e.g. multi-lamellar (MLV), small uni-lamellar (SUVs), large uni-lamellar (LUVs) or multi-vesicular vesicles (MVVs). The effect of liposome size can be important in respect of drug loading [25] or the circulation time accumulation behavior after application to the patient [26].

A simplification of the homogenization method has been another optimization goal within the last years. Therefore, preparation techniques were optimized for example the use of supercritical gases as solvents [27]. Based on the ethanol injection technique new approaches to control the local lipid concentration at the injection point were developed which result in spontaneous formation of homogenous liposomes [28]. Furthermore, new techniques like the jet disperser were introduced to optimize the homogenization step [29].

Another issue, which has to be overcome, is the prevention of physical or chemical reactions occurring during manufacturing or long term storage. Chemical stability is directly dependent on the composition of the liposomes. Lipid peroxidation and hydrolysis are the most common processes of chemical degradation. Physical processes can be the loss of bilayer compounds due to desorption, leakage of entrapped material, vesicle fusion and aggregation. A possible approach to stabilize the liposomal formulations is the removal of water and the production of a dried formulation. Only freeze-drying was used so far as dehydration process for approved products on the market.

3. LIPOSOMAL FORMULATIONS CONTAINING PACLITAXEL

Paclitaxel is an antineoplastic drug used in the treatment of breast and ovarian cancer [30] derived from the bark of Taxus brevifolia a natural complex diterpene with an extended side chain necessary for activity on C13 [31]. The major concern of Paclitaxel in formulation development is its low solubility of 1 µg/ml in aqueous media, which makes its formulation challenging [32]. The commercially available formulation Taxol® contains 6 mg Paclitaxel, dissolved in 527 mg of the surfactant Cremophor® EL (polyethoxylated castor oil) and is finally filled up with water-free ethanol ad 1 ml [33]. Cremophor® EL and ethanol are used to improve the solubility of Paclitaxel. A further dilution step with a physiological solution is necessary as described by Straubinger et al. (1995) prior to administration, which often results in a Paclitaxel precipitation [34]. To circumvent irritations for the patient by precipitated material, a filter is placed between the infusion bag and the injection port. Additionally, serious side affects are known for Paclitaxel in combination with Cremophor® EL because of an increased toxicity and hypersensitivity reactions [35]. Studies to overcome this drawback by using the solubility enhancer Pluronic®, a block co-polymer [36], or by the preparation of emulsions [37] revealed no advantages with respect to precipitation and crystallization. The approach of an oral administration of Paclitaxel (Paxene®) failed, due to the low absorption and the reduced bioavailability [38]. As an alternative to Cremophor® EL based formulations,

liposomal preparations were developed to overcome some of these drawbacks which are summarized by Haas (2005) [39] and Holvoet et al. (2007) [40].
The formulation EndoTAG®-1, which is used for our studies is based on cationic liposomes developed as delivery system for neovascular targeting of Paclitaxel inserted into the lipid bilayer [41]. For this formulation the solubility and loading approach was extensively discussed by Gruber in 2004 [42]. The lipid complex is composed of the two lipids N-[1-(2.3-Dioleoyloxy)propyl]-N,N,N,-trimethylammonium chloride (DOTAP-Cl) and 1,2-Dioleoyl-sn-glycero-3-phosphatidyl choline (DOPC). DOTAP-Cl is a synthetic lipid with a positively charged headgroup, whereby DOPC is a zwitterionic natural phospholipids. The mechanism of action in the neovascular cancer therapy of the so called vascular-disrupting agents (VDA) can be described as a specific targeting of blood vessels [43]. In this way the tumor tissue compartment and the newly formed endothelial tumor vasculature are affected. This lead to thrombus formation with a subsequent occlusion of the tumor blood vessel, which may cause a reduction or even a collapse of such vessels [44]. The consequence is a reduced or even hindered tumor supply with nutrients and oxygen [45]. The lipids used for EndoTAG®-1 exhibit an overall positive surface charge of the liposome. Due to the negatively charged surface of proliferating or activated endothelial cells in the tumor blood vessels and the positive charge of the liposomes a certain targeting effect of the Paclitaxel loaded liposomes can be achieved [46]. This specific charged phospholipid headgroup of the liposomes itself interacts through electrostatic interactions with negative charges of phospholipid headgroups, which are preferentially expressed on the tumor endothelial cells [47]. The intracellular uptake may occur via endocytosis and/or membrane fusion, but it is not yet fully understood as described by Michaelis and Haas (2007). To approve the unique mode of action, clinical phase II studies are currently carried out [48].

4. STABILIZING OF LIPOSOMAL FORMULATION

In early studies up to the 1990s typically large multi-lamellar vesicles in the micrometer range were employed. More recent investigations revealed an advantage of homogenous uni-lamellar vesicles in the size range of 50 to 200 nm. Liposome stability decreases with increasing size and shows an optimum at a size between 80 and 200 nm. The selection of an appropriate size is always a compromise between loading efficiency, which increases with liposome size, and the resulting decline in stability [49]. Physical and chemical stability of liposomes can be strengthened by choosing a homogenous size, the optimum encapsulation efficiency and by the addition of diverse excipients like antioxidants (e.g. alpha- or gamma tocopherhol) or chelating agents (e.g. like ethylenediaminetetraacetic

acid (EDTA) or diethylene triamine pentaacetic acid (DTPA)). Furthermore, by the optimization of the size and the formulation conditions, liquid formulations can be stable for several years like DauXome®, Doxil® or Myocet®. With the addition of appropriate cryoprotectants and lyoprotectants, liquid formulations can also be frozen or lyophilized to enhance the stability. As already mentioned, freeze-drying is exclusively employed as dehydration process for industrial manufacturing and stabilization of approved liposomal products up to the present. Freeze-drying (FD) is extensively discussed as a standard method to stabilize liposomal formulations [50,51,52] and will be discussed more in depth in chapter 2 of this thesis. Besides the commercial Paclitaxel containing liposomal formulations AmBisome® and Visudyne® are freeze-dried. Other techniques like spray-drying (SD) of liposomal products have been demonstrated to be feasible in several publications for regular manufacturing of dried liposomal formulations especially for the stabilization of lipophilic drugs [53,54,55,56].

The current manufacturing process of the Paclitaxel loaded liposomal formulation EndoTAG®-1 was described by Michaelis and Haas (2007). The formulation comprises 3 mol% Paclitaxel in a 10 mM DOTAP-Cl/DOPC lipid matrix dissolved in ethanol. Polydisperse liposomes are formed during the ethanol injection of the Paclitaxel containing lipid stock solutions into an aqueous trehalose phase. The size of the liposomes is adjusted to 200 nm using consecutive extrusion steps through a membrane with pore size of 0.2 µm followed by a sterile filtration step. However, the liquid formulation is not suitable for long term storage over several months due to the crystallization tendency of Paclitaxel. To achieve sufficient storage stability and to avoid Paclitaxel crystallization the formulation is finally lyophilized. This process was developed by Gruber (2004) to achieve a stable product for at least two years.

The major goal of this thesis was to overcome the limitations related to the liposome formation process and the freeze-drying cycle during the preparation of the EndoTAG®-1 formulation. Several extrusion cycles are necessary to achieve homogenous liposomes. The blocking of the membrane is a frequent problem related to the extrusion process especially at large scale and can only be overcome by an exchange of the blocked membrane. With filling volumes of 25 ml in a single vial the freeze-drying process takes about one week. For the application to the patient a large volume of the EndoTAG®-1 needs to be injected and therefore, several vials are combined to achieve the application volume. Instead of the classical lyophilized cake, free flowable bulk material would be beneficial to simplify the dosing for the patient. A scale-up of the so far used production process is related to tremendous difficulties. Due to the mentioned obstacles in the current production process of the EndoTAG®-1 formulation, alternative large scale

production methods for liposome formation and drying of bulk particles should be investigated.

5. OBJECTIVE OF THE THESIS

In this thesis alternative techniques that can be scaled up to industrial scale for the drying and stabilization of the liposomal Paclitaxel formulation EndoTAG®-1 should be explored and compared for their ability to achieve an ideal stabilizing effect. The primary goal was to identify possible techniques to preserve the physical and chemical stability of the liposomes and to study the effect of process parameters on the product without performing major changes in the formulation. Furthermore, we focused on particulate drying processes to benefit from the advantages of the free flowing bulk material and flexibility in dosing. The combination of the engineering part, designing new processes with the product quality after the drying was the major task. Several drying techniques were included into the thesis, all with the same liquid formulations but with different approaches to achieve a dry product (Fig 2). Furthermore, to overcome the complex preparation of liposomes a new technique should be developed that allows the formation of homogeneous liposomes within a single process step. With such a process some of the preparation steps described in figure 1 could be replaced and the complexity of an industrial process could be reduced.

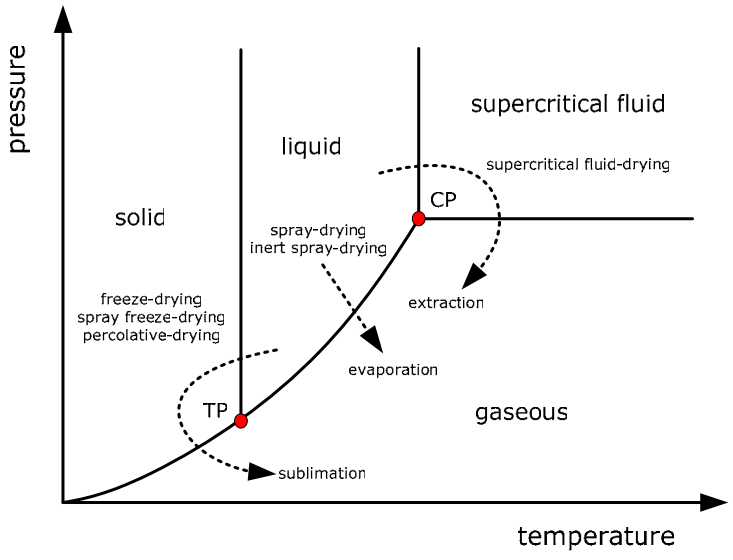

Figure 2: Schematic pressure-temperature diagram with the triple point (TP) and the critical point (CP) and the different phases. The investigated drying pathways from the liquid phase over solid (freeze-drying, spray freeze-drying, percolative vacuum-drying) and supercritical phase (supercritical fluid-drying) and the direct evaporation (spray-drying and inert spray-drying) are shown.

To gain further information on the existing formulation and production process, the freeze-drying (FD) process should be studied and optimized in **chapter 2**. Although freeze-drying is the accepted production technique the process is very time consuming. Therefore, we wanted to increase the efficiency of the lyophilization process by different approaches. One approach was to shorten the drying process by optimizing the sublimation rate. Furthermore, the approach to increase the filling volume for an enhanced productivity was to be tested. Finally, the cake geometry should be varied to investigate the heat and mass transfer properties.

In **chapter 3** spray freeze-drying (SFD) should be evaluated as a method to stabilize liposomes in free flowable dry granules. As no SFD equipment is available at our department, suitable set-ups should be planned and technically implemented, which allow the controlled production of different particle sizes. The impact of process conditions on the particle properties like size and morphology, as well as the liposomal integrity and the drying speed compared to the conventional freeze-drying process should be studied.

In **chapter 4** a new drying method the percolative vacuum-drying (PVD), which combines several aspects of spray freeze-drying, vacuum-drying and atmospheric freeze-drying should be tested as an approach to further enhance the drying process, with special focus on a decrease in drying time. The first task was the implementation of a technical set-up for this non standard process. Generally, the limitations for the sublimation of water are the relatively low driving force of heat and mass transfer resulting in very time consuming processes. The idea was to overcome these limitations and to enhance the sublimation rate by the introduction of a percolation gas streaming through the particle layer. The process parameter should varied to gain deeper knowledge of this new drying-method

Processes like spray-drying (SD) require high drying temperatures but result in a rather quick evaporation of the aqueous content. Therefore, in **chapter 5** such a fast technique should be tested for the manufacturing of small and dry particles from the liposomal formulations. Variations in the solid content and process parameters, like liquid flow rate, nozzle type or temperature and the related physico-chemical properties of the dried powder, as well as the liposomal integrity should be evaluated. These experiments should give insight into the overall preservation of the liposomal integrity during spray drying. An important goal was to perform first studies with Paclitaxel loaded liposomes to test the integrity of Paclitaxel formulations after SD.