Liposomal Drug Carrier Systems for Inhalation Treatment of Lung Cancer

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Dekan: **Prof. Dr. Kaspar Hegetschweiler**

Berichterstatter: **Prof. Dr. Claus-Michael Lehr**

Dr. Carsten Ehrhardt

Prof. Dr. Alexandra K. Kiemer

Die vorliegende Dissertation entstand unter der Betreuung von

Prof. Dr. Claus-Michael Lehr

in der Fachrichtung Biopharmazie und Pharmazeutische Technologie der Universität des Saarlandes

Bei Herrn Prof. Lehr möchte ich mich für die Überlassung des Themas und die wertvollen Anregungen und Diskussionen bedanken

Dedicated to:

Mum's spirit and dad
Brother and sister,
My beloved husband,
The heart who flourishes my life

Happiness keeps you sweet…
Trials keep you strong……...
Sorrows keep you human….
Failures keep you humble…
Success keeps you glowing…
But only God keeps you going.

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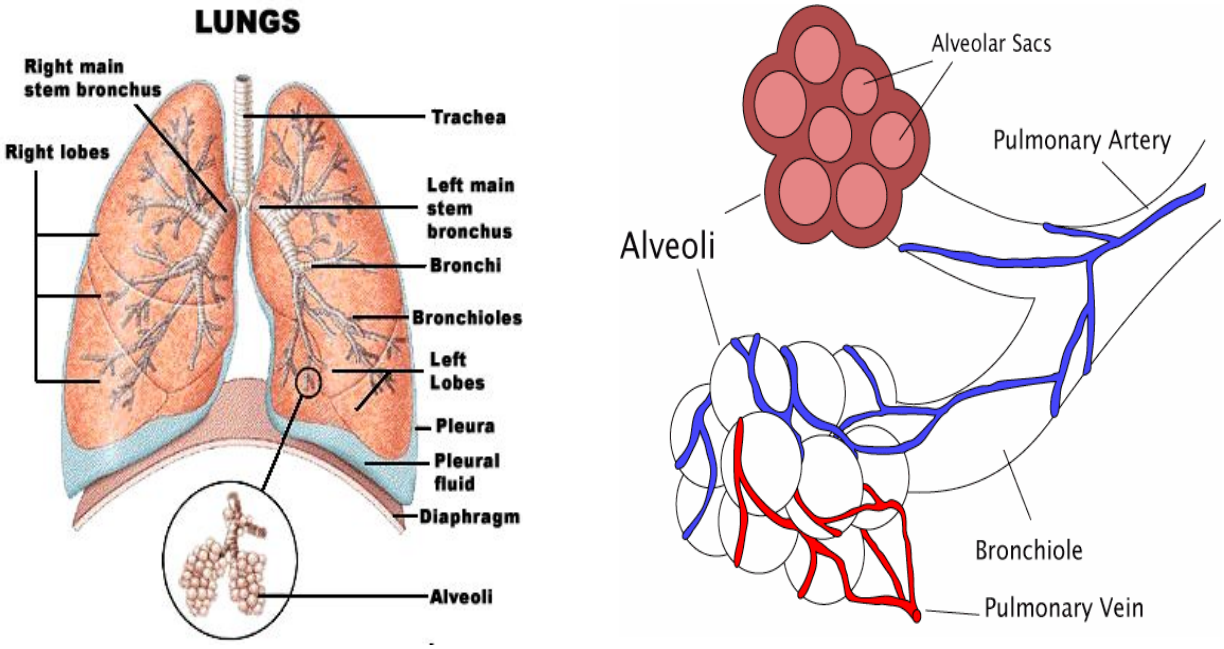
Chapter 1

General introduction

1.1 Lung anatomy

The lung is the body's organ of respiration. The trachea carries air into the body from the nose/mouth. It then splits into two bronchi which branch off into the left and right lungs. These primary bronchi branch into secondary bronchi, which in turn branch into tertiary bronchi which then become bronchioles. Each level of branching sees a decrease in diameter and an increase in the number of bronchioles. The bronchi can have diameters > 5 mm while terminal bronchiole diameter can be as small as 5-10 µm. Their diameter is controlled by bronchial smooth muscle which is under autonomic control. At the end of the terminal bronchioles are the alveoli. The alveoli are the site of gaseous exchange in the lung. Pulmonary capillaries surrounding the alveoli carry CO_2 and O_2 to and from the alveoli, respectively. The alveolar and micro capillary walls together make up what is known as the respiratory membrane. This consists of the alveolar epithelial lining (a layer of type I and type II alveolar cells and associated alveolar macrophages), an epithelial basement membrane, a capillary basement membrane and endothelial cells of the capillary (figure 1). Despite having several layers, the respiratory membrane is very thin (~ 0.5 µm). This allows for the rapid diffusion of O_2 and CO_2 across the membrane. It has been estimated that the lungs contain over 3×10^8 alveoli giving it a surface area of about 70-100 m^2 for the exchange of gases [1].

In air-breathing animals, respiratory anatomy has evolved in such a way as to actively thwart inhalation of putative airborne particulates.



A **B**

Figure 1: A) The anatomy of the human lung (taken from www.aduk.org.uk/gfx/lungs) **B)** Alveoli of the terminal bronchioles. They are covered in capillaries and it is here that gaseous exchange takes place (taken from http://en.wikipedia.org/wiki/Alveoli).

The epithelium of the airways is a continuous sheet of cells lining the luminal surface. The airway epithelium has at least four major types of cells, including basal cells, ciliated cells, goblet cells and Clara cells. On the surface of the epithelium of the proximal respiratory tract, ciliated cells predominate. Together with basal cells and a small percentage of goblet cells, they form a pseudostratified epithelium, in which ciliated cells occupy the majority of the luminal surface and basal cells are in contact with the basement membrane [2].

Starting with the trachea and ending in the alveolar sacs, the thickness of respiratory epithelium decreases gradually from 60 μm in the tracheobronchial epithelium to 0.2 μm in the alveolar region. Alveolar epithelial type I cells represent the principle cell type lining the surface of the alveoli. They are thin and broad representing 8.3% of the cell population within the human lung. The major functions of these cells, which cover 93% of the alveolar space, are to provide a surface for gas exchange and to serve as a permeability barrier.

Alveolar epithelial type II cells have a much smaller surface area per cell and they represent 16% of the total cells in the lung. They play a basic role in synthesis, secretion and recycling of surface-active material (lung surfactant), and they respond to alveolar injury by dividing, retaining morphological features of type II cells or differentiating into type I cells. Both type I and type II cells rest on the alveolar basement membrane that is nearly continuous. Epithelial cells are joined together by well-developed tight junctions. These junctions impart important permeability properties to the epithelial cell layer [3].

The alveolar region of the lung contains alveolar macrophages as well. These help in the clearance of inhaled particles. Alveolar macrophages are aid cells and serve well as primary defenders of the alveolar milieu [4].

1.2 Lung cancer

The thorax is a common site of involvement by primary and metastatic malignancy. The lung is unique among all organs in having a very high degree of exposure to the internal environment through the pulmonary blood flow and from the outside by exposure to air flow. Patients suffering from metastatic cancer have circulating cancer cells in their blood that are shed from the primary tumour. Hence, the lungs are continually exposed to these neoplastic cells. Fortunately, the natural defense mechanism in the lungs scavenge most, if not all, of the cells that are deposited in the pulmonary capillaries and interstitium. However, a few cells survive and proliferate into metastases. Thus, the lungs are a common site of metastatic disease [5].

Lung cancer, which includes cancer of the trachea and bronchi, is the third most common cause of death in the UK after heart disease and pneumonia. It is responsible for around a quarter of all cancer deaths. Pamela Mason reported that the mortality rate worldwide is highest in Scotland, closely followed by England and Wales. In England and Wales, lung cancer was responsible for nearly 29,000 deaths in 2002 with a male to female ratio of approximately two to one. The lung cancer incidence and death figures in UK are worse than other European and USA averages. The incidence number in 2002 in the world for men was 965,241 and for women was 386,891 [6].

Although the mortality rate for this disease has levelled off in men, it is still the most common cause of cancer death in this population group, and men account for 60% of all lung cancer cases. The incidence continues to arise in women, accounting for one in six of all cancer deaths. This is directly related to changes in smoking habits. In women, lung cancer is generally the second most common cause of cancer death after breast cancer. Risk increases with age - lung cancer is less common in people under the age of 40.

The prognosis in lung cancer patients is generally poor. About 80% of patients die within a year of diagnosis and only 5.5% are alive after five years. This is due to the speed with which the disease progresses and also to the nature of the patients, most of whom are older and often suffer from smoking related illnesses, including chronic obstructive pulmonary disease (COPD) and cardiovascular disease. Overall, patients with metastatic disease to the lung as well as those with locally advanced primary lung cancers, carry poor prognoses and are not usually amenable to curative therapy with surgery or with chemotherapy or radiation [5].

There are two main types of lung cancer based on the characteristics of the disease and its response to treatment. Non-small-cell lung carcinoma (NSCLC) accounts for 80% of all lung cancers. NSCLC is divided into:

1. Squamous carcinoma, which is the most common type, accounting for 35% of all lung cancer cases. The cells are usually well differentiated and locally spread. Widespread metastases occur relatively late.
2. Large-cell carcinoma, which accounts for 10% of all lung cancers. It is less well differentiated than the first type and metastasises earlier.
3. Adenocarcinoma, which accounts for approximately 27% of lung cancers. It arises from mucous glands and from scar tissues. Metastases is common to the brain and bones. It is the most common type of lung cancer associated with asbestos and is proportionally more common in non-smokers, women and older people.
4. Alveolar cell carcinoma, accounting for 1-2% of lung cancers.

The second major type is the small-cell lung carcinoma (SCLC), which accounts for 20% of all lung cancers. Arising from endocrine cells, these tumours secrete many polypeptide hormones. Some of these hormones provide feedback to the cancer cells and cause tumour growth. This type of tumour grows rapidly, taking approximately three years from initial malignant change to presentation.

Surgery is a treatment option in some patients with stage I or II NSCLC. Radiotherapy and chemotherapy can be offered.

Due to the importance of the lung for survival, it is often not possible to remove the tumours completely without dramatically reducing lung function. Thus, lung tumours are treated by radiation therapy or chemotherapy. Both types of treatment cause painful toxicity to the patient that may require a premature end of the therapy even though the tumour cells are successfully killed. The reason for this is that all cytotoxic drugs kill normal cells as well as cancerous cells, and this causes severe side effects. Furthermore, because of the blood circulation in the body, only a small fraction of the drug actually reaches the target tumour, and most of the drug acts on normal tissues or is rapidly eliminated. Therefore, to obtain a therapeutic effect, a relatively high dose of drug must be administered and usual drug formulations are used in a balance between killing the tumour (efficacy) and killing the patient (toxicity) [5-7].

Chemotherapy plays an important role in treating many patients with both NSCLC and SCLC. For patients with early-stage NSCLC, drugs can be used either following surgery (i.e., adjuvant chemotherapy) or before surgery is carried out (i.e., neoadjuvant chemotherapy). The goal of chemotherapy is to help "cure" the patient and improve long-term survival rates. Patients with SCLC and advanced NSCLC can also benefit from chemotherapy, with the aim of drug treatment being to prolong life, improve or maintain quality of life and control symptoms without causing unacceptable toxicity [7].

It has been reported that drug treatment plays an important part in the treatment of many patients with lung cancer and can help cure the disease. Where cancer is of the type or stage such that remission is unlikely to be achieved, chemotherapy can still prolong life, or improve or maintain a patient's quality of life. The development of biological agents targeting tumour cells might potentially result in these aims being achieved with less toxicity to patients [7-9].

One way to reduce the toxicity of the drug to the normal tissue is to direct the drug to the tumour cells with a drug delivery system. This is similar in concept to the use of a cruise missile to destroy only a military target while leaving surrounding buildings intact. Targeted drug delivery to lung tumours may prove to be the most efficacious and economical means by

which to treat lung cancer, since small groups of tumour cells that have spread away from the main tumour and are too few in number to be detected, can be exposed to a high level of drug by these "smart bombs". The bomb in this research is called a liposome. The liposome is a microscopic balloon, smaller than a red blood cell, formed from lipids. Cytotoxic drugs can be carried inside the liposome, where they do not come in contact with the cells. The lipids can be broken down by metabolism inside the body and the drug is then released. The technology for encapsulating drugs in liposomes has been thoroughly worked out by many laboratories around the world [10].

A standard treatment method for patients with extensive-stage SCLC is combination chemotherapy, with or without prophylactic cranial irradiation (PCI). Extensive SCLC has been associated with an untreated median survival of only a few months. The use of combination chemotherapy, such as: 1-etoposide with cisplatin or carboplatin, 2-doxorubicin (DOX) and cyclophosphamide with etoposide or vincristine, and 3-cisplatin, DOX, cyclophosphamide and etoposide, is associated with a response rate of over 50% and a median survival of 8-12 months. The use of adjunctive radiation therapy does not help in extending survival in extensive disease [9].

1.3 Doxorubicin

The treatment of cancer with medication (as opposed to surgery or radiation) is especially helpful when the cancer in question is not localised to one body area. Using medication allows body blood vessels to carry the medication to even remote or otherwise inaccessible areas. This form of treatment is called "chemotherapy".

In order for chemotherapy to be effective, the medications must destroy tumour cells and spare the normal body cells that may be adjacent. This is accomplished by using medications that affect cell activities that go on predominantly in cancer cells but not in normal cells. Most chemotherapy agents focus on the rapid cell division that characterises the spread of cancer cells.

DOX is anthracycline cytostatic antibiotic and is used against a variety of malignant solid tumours. It is clinically proven to be an effective anti-neoplastic against a number of malignancies in the treatment of cancers, including breast, ovarian, bladder and lung cancer, as well as non-Hodgkin's lymphoma, Hodgkin's disease and sarcoma. However, its toxicities and low therapeutic index have limited its use.

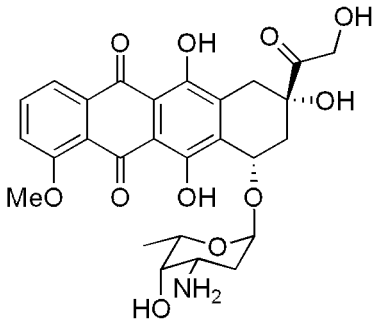


Figure 2: Chemical structure of doxorubicin ($C_{27}H_{29}NO_{11}$).

One of the most common brand names of DOX is Adriamycin®. DOX is a type of anti-cancer drug called an "anthracycline glycoside". It works by impairing DNA synthesis. One way in which DOX works is by binding to the cancer cells' DNA (the genetic code) and blocking an important enzyme (topo-isomerase II). This causes DNA to get tangled up and the cancer cells cannot divide and grow.

DOX is a red (as consequence of being highly conjugated compound (figure 2)) powder, which can be used as solution for injection into a vein (intravenously) or infused through a fine tube, inserted into a vein (cannula) or may be used through a central line. It is given slowly over a 10 minute in an IV drip rather than as a single quick shot. Chemotherapy is usually given as a course of several cycles of treatment. The treatment plan for DOX depends on which cancer one is having treatment for.

DOX is a very serious anti-cancer medication with definite potential to do great harm as well as great good. Myelosuppression occurs in 60-80% of patients, and it is the dose limiting toxicity in most patients. The cytotoxic effects of DOX are thought to be related to intercalation with nucleotide bases and cell membrane lipid binding activity. The intercalation inhibits nucleotide replication of DNA and RNA. DOX also interacts with topo-isomerase II that forms breaks in DNA complexes, thus stopping the synthesis of DNA and RNA. Cellular membrane binding of DOX can alter a variety of cellular functions.

The reduction of DOX by cellular enzymes results in the production of free hydroxyl radicals ($OH^{\cdot}$). The cardiac tissue does not have the enzymes to counteract the oxidative free radical build-up, and therefore is more prone to cellular damage and cardiotoxicity. This might result in irreversible myocardial infarction or fatal congestive heart failure, which may occur years after therapy has been discontinued. Because of the cumulative cellular damage, there is a maximum lifetime dose of DOX. Its dose-limiting toxicity is myocardial damage although mucositis, stomatitis, and myelosuppression are other well-recognised side effects.

The toxicities have led to rational development of delivery systems with an improved therapeutic index, less cardiotoxicity, and more selectivity for tumour tissue [10-12].

1.4 Liposomal drug delivery systems

Since Bangham's original description of bilayered phospholipid (PL) vesicles in 1965, liposomes have received much attention as transporters of pharmacological agents. These vesicles, ranging in size from 0.025 μm to greater than 20 μm, are composed of single or multiple PL membranes surrounding an aqueous compartment. According to their hydrophilic or hydrophobic tendencies, drugs can be entrapped in the aqueous or membrane phases, respectively. The preclinical evaluation of their use in the treatment of malignant disease has generated a considerable body of literature [13].

Liposomes are liquid crystals formed when naturally occurring PLs are equilibrated with excess water or aqueous salt solution. Some examples of the PLs commonly used in liposome production include lecithin, cholesterol (Chol), dipalmitoyl phosphatidylglycerol (DPPG), and hydrogenated soy-bean phosphatidylcholine (HSPC). The liquid crystals formed usually consist of two or more bimolecular lipid layers, or lamellae, which are separated by aqueous layers. These types of systems are referred to as multilamellar liposomes [8]. Water-soluble drugs can be entrapped in the liposomes within the aqueous core and aqueous layers while lipid-soluble drugs can be solubilised within the hydrocarbon interiors of the lipid bilayers. Molecules with both hydrophilic and lipophilic character are arranged in stable conformations within the liposome (figure 3A).

The net charge of a liposome can be varied by incorporation of lipids with negative or positive charges. For example, a long-chain amine will give positively charged vesicles, and diacetyl phosphate will give negatively charged vesicles. Positively charged liposomes have been used experimentally as carriers for anionic DNA [9].

Liposomes have been either employed as carriers of chemotherapeutic agents or biological response modifiers. Most of the chemotherapeutic agents used have been DOX or cisplatin derivatives. Muramyl peptides have been used as macrophage activators to invoke anti-tumour biologic responses in the host. Phase I and II trials using liposomes in cancer patients consistently demonstrate their safety. Encapsulation is often associated with a favourable effect on the therapeutic index, which is usually attributable to a reduction in toxicity of the active compound. Low grade fever and mild fatigue are the most common toxicities related specifically to the lipid constituents. Although objective tumour responses and stable disease

during treatment have been observed in early studies, further clinical trials are required to define the role of liposomal anti-tumour therapy in medical oncology.

The incorporation of drugs into liposomes has several theoretical advantages. They protect their contents from interaction with plasma components, while favourably altering the pharmacokinetics and biodistribution of the free compound. For example, preclinical data demonstrated that DOX encapsulated in liposomes was significantly less cardiotoxic than the free compound and the lower toxicity correlated with lower cardiac levels of the drug. The reduced toxicity may be associated with a slower release of DOX, since it has been shown that continuous infusion DOX is less cardiotoxic to humans. Since liposomes do not readily penetrate biological membranes, they can be used for the controlled release of drugs within body cavities such as the pleural, peritoneal or intrathecal spaces.

Although simple PL membranes will naturally target to the reticuloendothelial system (RES), manipulations of the liposome surface can be used for organ-specific or tumour-specific targeting.

By altering their physical parameters such as size, electrostatic charge, PL profile, and membrane characteristics, liposomes can be engineered to efficiently encapsulate different types of drugs and effectively transport them within the circulation. Each drug encapsulated in a liposome must be regarded as a unique pharmacologic entity dependent upon the components of the liposome and the conditions under which drug encapsulation takes place. Small changes in liposomal preparation or composition can have profound effects on drug bioavailability, activity, and toxicity.

Since liposomes produced by standard methods are sequestered primarily by the RES, they can be used to target therapy directly to malignant disease of the liver and spleen or activate macrophages for immunomodulation. By manipulating the physical properties of vesicles, for example, using small unilamellar vesicles composed of uncharged lipids, the RES can be avoided, circulation time increased, and tumour-targeting augmented.

There are two types of commonly used liposomes:

Multilamellar vesicles (MLVs; l-5 μm) and unilamellar vesicles (UVs; 0.05-0.2 μm). Because MLVs are composed of concentric layers of PLs, the aqueous compartment is reduced, allowing for better encapsulation of lipophilic drugs. These drugs associate with the inner and outer PL membranes.

When MLVs are subjected to sonication or extrusion through filters, UVs are formed. Small UVs (SUVs) measure less than 0.1 μm; large UVs (LUVs) measure between 0.1 and 0.25 μm. Generally, UVs are better suited for delivery of hydrophilic drugs. Since large liposomes are

rapidly sequestered by the RES, SUVs are employed to increase liposome circulation time. In general, the smaller the vesicle, the longer the circulation time. Small vesicles are utilised for slow drug release in the circulation and for non-RES targeting.

Animal data suggest also that liposomal DOX is less myelosuppressive than the free drug and is less likely to cause soft-tissue necrosis.

Gabizon *et al.* reported a phase I clinical trial in which liposome-encapsulated DOX was used to treat 32 patients with metastatic cancer after failure of conventional chemotherapy. The liposomes were composed of phosphatidylcholine (PC), phosphatidylglycerol (PG), Chol, and α-tocopherol succinate in a molar ratio of 7:3:4:0.2, respectively. The mean size was in the range of 0.3-0.5 μm. Liposomes were infused at a concentration of 0.5-2.0 mg/ml, at a rate of 2-3 ml/min through a peripheral vein. Interestingly, gastrointestinal toxicity was mild without the use of prophylactic antiemetics. Fever was the most common toxicity (37%), whereas stomatitis and myelosupression were the dose-limiting toxicities. The maximum tolerated dose (MTD) was 120 mg/kg. Of the 18 patients evaluated for anti-tumour effect, one partial response and five marginal responses were observed. The duration of response was only 3 to 8 months. All responders had primary or metastatic hepatic involvement; two patients had been previously treated with intravenous DOX. Gabizon concluded that liposomal DOX can be safely administered at higher dose levels than the free drug and that phase II studies starting at a dose of 100 mg/kg are indicated [11].

Excluding cardiotoxicity, toxicities in all of the clinical trials utilising intravenously administered liposome-encapsulated doxorubicin (LED) parallel those of free DOX. Myelosuppression was dose-limiting and all investigators noted a significant incidence of nausea, vomiting and alopecia.

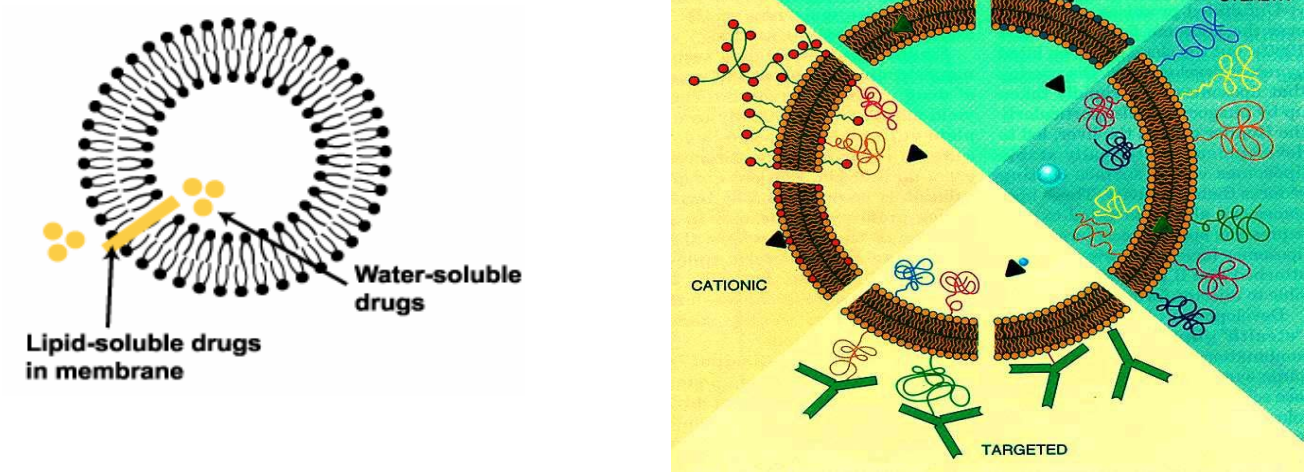
Macrophages recognise liposomes as foreign particles through a process known as opsonisation. The liposomes are "labelled" by the RES by attaching antibody molecules. These "labels" are recognised by the macrophages and the liposomes are taken up and destroyed. A surface modification to the liposomes by attaching polyethylene glycol (PEG) chains enables the liposomes to resist opsonisation, thus allowing them to escape macrophage uptake and extend their residence time in the blood to hours or days. These "PEGylated" liposomes are also known as Stealth® liposomes (figure 3B) [14].

In some instances it may be of therapeutic benefit to have liposomes taken up by macrophages (e.g., macrophage located microbial or viral diseases) and this concept of passive targeting of macrophages offers possible therapeutic opportunities.

It is also possible to make liposomes site-specific by covalently attaching 'homing devices' to the outside bilayers. Attaching receptor-specific ligands to the surface of liposomes and the idea of immunoliposomes where antibodies or antibody fragments are bound to the surface of the liposome, have been investigated. Here, the aim is active targeting to a desired site, not just passive targeting of macrophages (e.g. immunoliposomal DOX targeted to human CD34(+) leukaemia cells) These ideas are summarised below in figure 3B [12].

Liposomes possess the ability to encapsulate drugs, proteins, enzymes and nucleic acid and these liposomal systems have been used intravenously, orally and intramuscularly to decrease toxicity, increase specific uptake of drug and to control drug release.

They may also be useful in pulmonary drug delivery systems from a delivery, targeting, sustained release and toxicity-limiting point of view. Administration to the lung directly can deliver the drug to the site of action and therefore avoid stability problems of IV delivery, in which liposomes may release their drug content in the blood before reaching the target site. Free drug in solution is generally absorbed rapidly from the airways to the circulation. By retaining the drug in liposomes, it may be possible to increase the drugs contact time at the active site and retard its absorption into the bloodstream. This would mean a lower dosage requirement and a reduction in systemic toxicity associated with high levels of the drug in systemic circulation [8].



A **B**

Figure 3: Diagrammatic representation of a liposome in which the bilayers of polar PLs alternate with aqueous compartments **A:** Possibility of entrapment of different drugs in liposomes (taken from http://blog.case.edu), **B:** Liposomes can be modified with different molecules either for targeting or for stabilisation (taken from www.unizh.ch/onkwww/lipos.htm).

1.5 TfR-based targeting for anti-cancer therapeutics

An attractive strategy to enhance the therapeutic index of drugs is to specifically deliver these agents to the defined target cells, thereby keeping them away from healthy cells, which are also sensitive to the toxic effects of the drugs. This would allow for more effective treatment achieved with a better tolerance.

Many attempts are being made to explore the potential of specific and target-oriented delivery systems. Examples include polymer drug delivery systems based on liposomes, and ligand-receptor combinations. The last case has received major attention in the past few years due to the potential of non-immunogenic, site-specific targeting to ligand specific biosites of the naturally existing ligands and their receptors. The best-characterised and efficient cellular mechanism of uptake of transferrin (Tf) has been exploited for the delivery of anticancer drugs, proteins, and therapeutic genes into primarily proliferating malignant cells that overexpress transferrin receptors (TfR).

TfR-targeted therapy has emerged as an interesting drug-delivery tool with dual functionality. For example, targeting of TfR can lead to delivery of therapeutic agents into tissues of choice or across epithelial barriers of choice. The seemingly contradictory effects can be achieved by focusing the targeting strategies toward different aspects of TfR-related biology.

One of the best explored avenues for TfR-based drug targeting strategies involves the use of anti-cancer based therapeutics conjugated to Tf (or to TfR recognising antibody) to preferentially direct the drug towards TfR rich cancer cells. High levels of TfR expression have been demonstrated in many tumours and significantly, studies have also shown that the TfR are expressed more abundantly in malignant tissues than in their normal counterparts [15]. TfR are also more abundantly expressed in rapidly dividing cells than in quiescent cells because of their pivotal role in iron uptake and the absolute requirement for iron in rapid cell proliferation [16]. Therefore, TfR expressed on cancer cells have been seen as a suitable target for the delivery of therapeutics by receptor-mediated endocytosis.

Many anti-cancer agents have been considered for conjugation to Tf by varying methods, including direct chemical linkage, liposomal packaging of toxin and linkage to Tf, conjugation of DNA/polylysine complexes to Tf, and conjugation of liposome/DNA complexes to Tf. A great variety of cytotoxic agents have been conjugated to Tf and investigated as potential anticancer therapeutics. Some of the more notable examples have been methotrexate, DOX, cisplatin, ricin A, daunorubicin, and toxin CRM107 [17]. Conjugation of these toxins to Tf has the dual benefits of reducing toxicity in undesired tissues while increasing the targeting efficiency to the cancerous cells. Of special note is that

conjugation to Tf significantly enhances the effectiveness of these agents in many multi drug-resistant cell lines. For example, Tf-DOX was 5–10 times more effective than DOX- control in killing DOX-resistant cell lines, whereas in DOX-sensitive cell lines, the conjugate was only four to five times more effective than the control. The exact mechanism by which Tf conjugation allows DOX to effectively bypass the drug-resistance machinery of the resistant cell lines is not fully understood; however, it is conceivable that internalisation of the Tf-DOX conjugate allows for sequestration into the endocytic pathway, away from drug efflux proteins (such as PGP and/or MDR), which normally reside at the plasma membrane [18].

A variation on the theme of using toxins conjugated to Tf has been investigated to increase efficiency of uptake at the cellular surface. This approach relies on the formulation of the toxin in liposomal delivery vehicles to further enhance the efficiency of cellular uptake.

For example, when Tf-liposome therapy was combined with conventional radiation therapy, complete tumour regression was observed in human prostate cancer and the treatment group showed no signs of relapse up to six months later [17]. Further developments in Tf-based targeting of anti-cancer drugs will most likely play important roles in anti-cancer therapy.

Cells take up iron by using a variety of mechanisms. In higher organisms, one principal pathway of cellular iron acquisition is by the receptor-mediated uptake of Tf-bound iron, which is one of the best understood processes in cell biology. Figure 4 shows the current model of iron uptake from Tf via TfR-mediated endocytosis. Briefly, the process is triggered by the binding of Fe_2-Tf to a specific cell-surface TfR. After endocytosis via clathrin-coated pits, which bud from the plasma membrane as membrane bound vesicles or endosomes, the Fe_2-Tf·TfR complex is routed into the endosomal compartment. Upon maturation and loss of the clathrin coat, the endosome becomes competent to pump protons in a process energised by ATPase, and the endosomal lumen is rapidly acidified to a pH of about 5.5. At this pH, the binding of iron to Tf is weakened, leading to iron release from the protein. The free Fe^{3+} released to endosomes is reduced to Fe^{2+} on the *cis*-side of the endosomal membrane probably mediated by oxidoreductase. Fe^{2+} is subsequently transported out of the Tf cycle endosome by the divalent metal transporter DMT1, i.e., from the endosomal membrane to the cytosol. Once in the cytosol, iron is utilised as a cofactor for aconitase, the cytochromes, RNA reductase, and haeme, or stored as ferritin. After release of iron into the endosome, the resultant apo-Tf·TfR complex is then recruited through exocytic vesicles back to the cell surface. At extracellular physiological pH, apo-Tf dissociates from its receptor due to its low affinity at pH 7.4, and released into the circulation to be reutilised. ATP-mediated energy is necessary for sustaining TfR-mediated endocytosis and recycling.

It has been shown that the number of receptors displayed on the cell surface is proportional to iron uptake and that iron deficiency induces TfR gene expression, which implies the significance of TfR in iron uptake. Furthermore, surface display of TfR is affected by its total cellular concentration, as well as its distribution and rate of recycling between the cell surface and cell interior. The efficiency of TfR function is also influenced by other proteins, including SFT (stimulator of iron transport) which stimulates iron uptake by both Tf and non-Tf pathways [19].

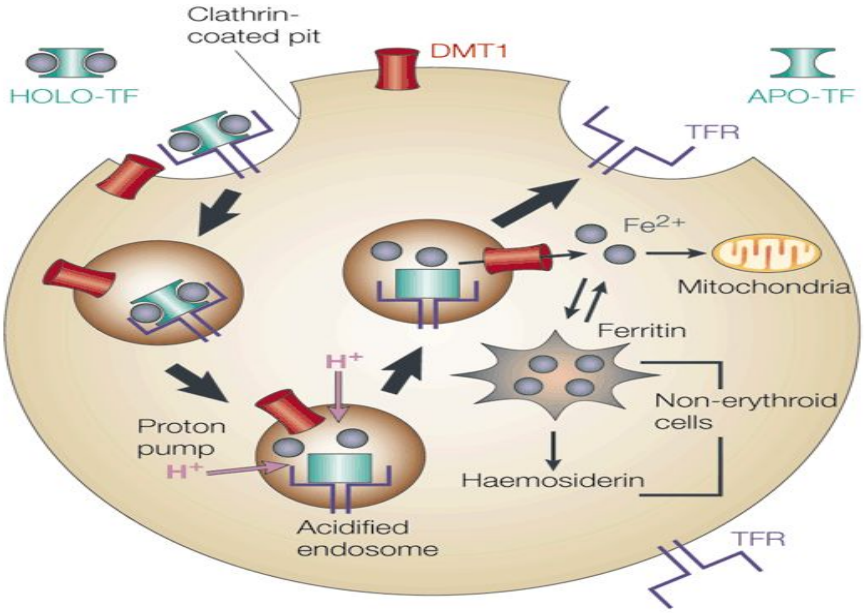


Figure 4: The transferrin cycle. HOLOTRANSFERRIN (HOLO-TF) binds to transferrin receptors on the cell surface. The complexes localise to clathrin-coated pits, which invaginate to initiate endocytosis. Specialised endosomes form, and become acidified through the action of a proton pump. Acidification leads to protein conformational changes that release iron from transferrin. Acidification also enables proton-coupled iron transport out of the endosomes through the activity of the divalent metal transporter 1 protein (DMT1). Subsequently, APOTRANSFERRIN (APO-TF) and the transferrin receptor both return to the cell surface, where they dissociate at neutral pH. Both proteins participate in further rounds of iron delivery. In non-erythroid cells, iron is stored as ferritin and haemosiderin [taken from 16].

1.6 Inhalation therapy

The lung's anatomy and its transport function between the external environment and the systemic circulation are perfectly matched. The alveolar capillary membrane is only two cells in thickness and extends over a vast area (70-100 m^2). The physiological mechanisms controlling airway function and pulmonary hemodynamics also strive to produce optimal opportunity for external/internal exchanges to occur. Thus, the lungs represent a significant portal between the external and internal environments. Inhalation therapy, as the name suggests, involves the delivery of drugs to the respiratory tract for either local or systemic effects. The most common use of inhalation therapy is for the treatment or prophylaxis of respiratory conditions, such as bronchial asthma, COPD and cystic fibrosis.

It is not surprising; therefore, that illicit or traditional drug delivery to the systemic tissues has been achieved via lung inhalation. By contrast few pharmaceutical agents are currently available for systemic delivery via the inhalation route.

Patients are dependent on inhalation of some kind of aerosol, generated either from a liquid drug solution in a nebuliser, a dry powder inhaler (DPI), or a pressurised metered dose aerosol. In all instances, these devices produce a mist containing particles of varying sizes. Large 20-μm particles have a high degree of inertia and are deposited in the throat (to be swallowed). The smallest particles (diameter 0.2-2.0 μm) constitute "the respirable fraction" and are carried in inspired air into the respiratory bronchioles and alveoli. Between these two extremes is a broad range of particles whose fate depends heavily on the behaviour of the patient. *In vitro* tests routinely show small deviations in the pattern of particle deposition into artificial "lung" systems, whereas *in vivo* studies produce a much wider variation. However, some generalisations are possible. Deposition into the alveoli is small with, for example, only approximately 10% of the administered material is getting to the terminal airways from a pressurised metered dose aerosol. By contrast, approximately 80% are deposited into the upper airways. The fate of this airway-deposed material may be: removal from the lung via mucociliary clearance, metabolism within the airway mucosa, or absorption through the mucosa into the bronchial circulation. Thus, we might expect an inhaled pharmaceutical agent, provided by a pressurised metered dose inhaler (pMDI), to have a pharmacokinetic distribution which favours pharmacological action on the airways rather than systemic activity [20].

The delivery of drugs to the lungs by inhalation has been investigated extensively and a large number of formulations are now in use. Formulations are usually powders for inhalation, which may or may not be pressurised, or nebulised solutions from which the drug is inhaled

as a mist. There are a number of preparations for inhalation which are the subject of an individual monograph in the Pharmacopoeias. Examples include powders for inhalation such as sodium cromoglicate powder for inhalation, pressurised inhalations such as beclomethasone pressurised inhalation, and nebuliser solutions such as salbutamol nebuliser solution. These are all examples of drugs acting locally to treat diseased lung states.

There are many advantages associated with inhalation therapy. The delivery of a drug directly to its site of action can result in a much quicker onset of action, compared with parenteral or oral delivery. This may be highly desirable, for example when delivering bronchodilating drugs for the treatment of asthma. It may be possible to administer smaller doses locally, compared with parenteral or oral routes, thereby reducing the incidence of adverse side effects. Drugs with poor oral bioavailability may benefit from direct pulmonary delivery. For example, sodium cromoglicate is a mast-cell-stabilising anti-allergic agent, active at the lungs, which is poorly absorbed orally. And isoprenaline is a sympathomimetic bronchodilator, which is rapidly metabolised when given orally. In addition, first-pass metabolism of the drug in the liver can be avoided by pulmonary delivery. The lung is also an effective gateway for the delivery of drugs to the systemic circulation due to its large surface area with thin absorption barriers and abundance of capillaries. It also has less degradative enzyme activity than other routes; e.g., the gut, meaning it is a more stable environment to peptide- and protein-based drugs [21].

Although the inhalation route has many advantages it also has some disadvantages. Only a small percentage of the dose leaving a delivery device will actually reach its intended target in the lower respiratory system. The delivered drug quantity depends on the patient's ability to use the inhaler system correctly and/or control of their breathing [8]. Some drug is not inhaled correctly, while some has too small particle size and is breathed out. If the target sites are the smaller bronchioles and alveoli, particles which are too large may not be able to reach them. The optimal particle size for penetration to these peripheral pulmonary regions is 0.5–5 μm.

Some drugs with beneficial local effects may have toxic systemic effects and their absorption from the lungs would therefore be undesired. (e.g., cytotoxic anti-cancer drugs). Pulmonary delivery of drugs is complicated by: (1) the need for training patients to coordinate breathing and inhaling of aerosols; (2) rapid absorption of most drugs, necessitating frequent dosing which often is responsible for systemic side effects; (3) poor aqueous solubility of drugs which may cause local irritation and inflammation in the airways or prevent the use of aerosols entirely; and (4) poor cytosolic penetration of drug to treat intracellular pathogens. Another therapeutically undesirable aspect of pulmonary drug delivery is rapid absorption of

most drugs from the lung, necessitating frequent dosing, e.g., of bronchodilators and corticosteroids [22].

For these reasons, novel ways of delivering drugs to the pulmonary system are actively being considered and researched. One approach which has been investigated is the use of liposomes as carriers in inhaled systems.

1.7 Liposomes for inhalation

Topical modes of application of liposomes, including the pulmonary route, have been explored for tissue-selective, and potentially cell-targeted drug delivery.

Liposomes are believed to alleviate some of the problems encountered with conventional aerosol delivery due to their ability to: (1) serve as a solubilisation matrix for poorly soluble agents; (2) act as a pulmonary sustained release reservoir; and (3) facilitate intracellular delivery of drugs, specifically to alveolar macrophages. Consequently, liposomes may provide a means to prevent local irritation of lung tissue and reduce pulmonary toxicity, prolong local therapeutic drug levels, and generate high intracellular drug concentrations. Cumulatively, this would result in reduced systemic spill-over and an increase in apparent drug efficacy.

There are many disease states which could potentially benefit from treatment with aerosolised liposome-encapsulated drugs. A number of possible therapies employing inhaled liposomes have been investigated. The majority of studies focus on the potential for targeting of the lung with drugs for local effect.

A film of surfactant lies at the interface between the alveolar subphase fluid and gas phases. It is a stable film that reduces surface tension at the interface, thus stabilising the air spaces and facilitating gaseous exchange. This pulmonary surfactant system is made up of PLs (synthesised in alveolar epithelial type II cells) which are combined with other surfactant components such as proteins and other lipid components, of which cholesterol (Chol) is the most abundant (approximately 10% by mass), the other neutral lipids occurring in trace amounts only (table 1) [23].

In the normal lung, surfactant clearance and surfactant release balance to maintain a stable surface film. Situations do exist where this balance is not maintained and this can lead to alveolar collapse and fluid accumulation in the lungs. This condition is most common in very premature babies, many of which do not have the ability to produce pulmonary surfactant. Certain mixtures of exogenous PLs have shown the ability to adsorb from the alveolar subphase and spread to a monolayer which reduces surface tension. Liposome suspensions of

these PLs have been tested for activity as surfactant replacements in pre-term animals [24, 25] and humans [26]. The studies showed that the replacement therapy was successful and in the last one none of the treated babies died, compared with 8 of the controls.

The use of inhaled liposomes for the treatment of cancer in the lungs has been investigated. Juliano *et al.* carried out much of the work in this area in the late 70s and early 80s.

The first study demonstrated the effect of liposomal encapsulation on the pharmacokinetic behaviour of anti-tumour drugs. Cytosine Arabinose (Ara-C), vinblastine, actinomycin D and daunomycin all showed enhanced retention in the bloodstream and tissues [27].

Table 1. Surfactant composition in bronchoalveolar lavage fluid from healthy persons (Griese 1999)

Component (content or activity)	
Total protein mg/mL	(0.04±0.15)
Total PL mg/mL	(0.01±0.13)
PL class (% total)	
PC	(53.1±83.8)
PG	(8.3±27.4)
Phosphatidylinositol	(1.2±13.5)
Phosphatidylethanolamine	(0.3±21.0)
Phosphatidylserine	(0.0±5.7)
Sphingomyelin	(0.8±8.3)
Lysophosphatidylcholine	(0.0±4.5)
Surfactant proteins (SP) mg/mL	
SP-A	(0.8±15.0)
SP-B	(0.7±15.3)
SP-D	(0.9±1.3)

In another study, they attempted to use this idea of enhanced retention to obtain localised, organ-specific action of anti-tumour drugs. Again, Ara-C was used. They reported that liposomes instilled into the trachea of rats became widely distributed into the air spaces in the lungs and that, while free Ara-C rapidly escaped the lung after administration, liposomal Ara-C remained in the lung for long periods and only slowly redistributed to the blood and urine. They also reported that a dose of liposomal Ara-C effectively inhibited DNA synthesis in the lung but not elsewhere in the body (i.e., intestine and bone marrow), while the same dose of

free Ara-C caused DNA synthesis inhibition at these remote sites. They went on to suggest that this approach may be useful in treating small pulmonary metastases or secondary metastases that may occur, for example, in advanced breast cancer. This approach would not be effective in the treatment of large lung tumours as they often block the airways, making inhaled delivery difficult. Although the trial focused on Ara-C, mainly because of its well known and predictable pharmacology, the authors suggest that another anti-metabolite drug with known activity against lung tumours (5-fluorouracil) might be better suited for clinical use. They cite unpublished evidence that this is indeed the case [28].

Another anticancer drug which has been investigated for possible use as an inhaled liposome preparation is interleukin-2 (IL-2), a lymphokine which stimulates the proliferation of T-lymphocytes and thus amplifies immune response to an antigen. It also acts on B-lymphocytes, and modulates interferon-gamma production and natural killer cell activation. It has proven *in vitro* anti-tumour activity but systemic *in vivo* toxicity has been a problem. Khanna *et al.* reviewed the use of IL-2 liposomes inhalation therapy in dogs in two separate studies [29, 30]. The first study confirmed an increased leukocyte cell count after inhalation of IL-2 liposomes versus inhalation of free IL-2 [29]. The second study reported that pet dogs with naturally occurring pulmonary metastases and primary lung carcinomas accepted inhalation treatments with IL-2 liposomes easily. It was concluded that nontoxic and effective treatment of pulmonary metastases of osteosarcoma is possible with nebulised IL-2 liposomes [30].

A phase I clinical trial of nebulised IL-2 liposomes in 2000 reported that no significant toxicity was observed in nine patients with pulmonary metastases (three cohorts of three patients; each cohort at a different dose). It was concluded that the delivery of IL-2 liposomes by inhalation is well tolerated but that further studies were required to determine its efficacy as a viable anti-cancer therapy [31].

The lung is also a common site for infection by bacteria and fungi. Local delivery of bacterial and fungal antibiotics is another possible use for inhalation therapy, and liposomes have also been studied in this context.

A study in 2000 compared aerosol characteristics and *in vivo* deposition of liposomal and non-liposomal amphotericin B. Amphotericin B is a polyene antifungal antibiotic effective against a wide range of fungal infections including invasive pulmonary aspergillosis. The results showed that liposomal amphotericin B (AmBisome®) was more stable towards nebulisation than non-liposomal amphotericin B. However, although it was effective, aerosol

concentrations showed no advantage over non-liposomal amphotericin B, i.e., the delivered dose was the same for both [31].

A recent study (2004) investigated the design of liposomal aerosols for delivery of rifampicin to alveolar macrophages using the "active targeting" approach. Alveolar macrophages are the densest site of tuberculosis (TB) infection. PC and Chol-based liposomes were used. They were modified by imparting negative charge to the surface or by coating them with alveolar macrophage-specific ligands. *In vitro* airway penetration was 1.5–1.8 times higher for the liposome encapsulated drug than for the plain drug aerosol. The study concluded that encapsulating anti-TB drugs in surface-altered liposomes and delivering them to the respiratory tract as an inhaled aerosol will improve chemotherapy versus pulmonary TB [32].

Liposomes have also been investigated for their possible use in inhaled bronchodilator preparations. These preparations are used in the management of reversible airway obstruction in asthma and in some COPD patients [33].

Sympathomimetic amines act as bronchodilators by stimulating β_2-adrenergic receptors found on bronchial smooth muscle. They do, however, possess some β_1-adrenergic activity. Stimulation of β_1-adrenoreceptors in the cardiovascular system can cause tachycardia. Liposomal systems may offer a way to deliver these drugs to the bronchioles for prolonged local effect while retarding their entry into the systemic circulation and thereby reducing systemic side effects such as tachycardia.

The ability of metaproterenol sulphate (a β_2-agonist) to protect anaesthetised, mechanically ventilated dogs against acetylcholine-induced bronchoconstriction was investigated by Cabezas *et al.* in 1971 [34]. Free and liposome-encapsulated metaproterenol were used. The parameters measured were the protection from acetylcholine challenge and the increase in heart rate. It was found that the protection offered by the encapsulated drug was at least equivalent to that of the free drug. More importantly, it was found that there were substantially smaller increases in heart rate when metaproterenol was encapsulated within liposomes. It was concluded that liposomes offered a useful way to reduce the systemic side effects associated with inhaled sympathomimetic amines.

Some prostaglandins (e.g., PGE_1 and PGE_2) have been shown to have bronchodilating effects beneficial to asthmatic patients. However, irritation of the upper respiratory tract and aggravation of respiratory function have been reported. Mizushima *et al.* investigated the irritant effect of aerosolised PGE_1 versus aerosolised liposomal PGE_1 in a study in 1983 [35]. They found the irritant effect of lipo-PGE_1 aerosol on the upper respiratory tract in man to be

10 times weaker than that of non-lipo-PGE_1. Their bronchodilating actions were similar. Lipo-PGE_1 would be the preferred option for clinical use.

Budesonide (BUD) is a corticosteroid used in the prophylactic management of asthma. Systemic side effects are a problem with these inhaled corticosteroids. These include impaired tissue repair and immune function and increased susceptibility to infection. Liposomal encapsulation of BUD controls the release of the drug, thus maintaining therapeutic concentrations in the lung while reducing the amount of drug in circulation and thereby reducing systemic toxicity. Joshi and Misra (2001) outlined the production and stabilisation of liposomal budesonide for DPI [36]. Small multilamellar liposomal vesicles (SMLVs) loaded with BUD were successfully prepared and stabilised by lyophilisation in DPI formulations with a reasonable shelf-life. Their findings demonstrate the possibility of delivering this liposomally entrapped BUD to terminal bronchioles.

Their ability to retard absorption of drug from the lungs implies that liposomes may also have a use in the sustained release of drugs into the systemic circulation. One interesting study investigated the use of inhaled liposome-encapsulated fentanyl as a post-operative analgesic [37]. Inhalation therapy using non-liposomal fentanyl provided satisfactory but brief post-operative pain relief. Using a mixture of free and liposome-encapsulated fentanyl (FLEF) led to rapid onset of analgesia, due to the free drug in formulation, followed by the controlled release of the drug from liposomes at therapeutic levels over 24 hours. The study demonstrated several advantages of inhaled liposomal fentanyl over other routes of opioid drug delivery in providing pain relief. Inhaled therapy is simple and noninvasive. It avoids the oral route which is not ideal in post-operative patients who may be nauseous and could vomit. It also avoids first pass metabolism of the drug. The plasma concentrations of drug were maintained at a higher therapeutic level than those from IV administration over 24 hours. The initial peak in plasma fentanyl levels present in IV administration was avoided.

The systems suitable for the delivery of liposomes to the respiratory tract have been extensively reviewed by Niven *et al.* Particular emphasis was put on the factors influencing the nebulisation of liposomes in three articles from 1990-1992 [8, 38, 39]. Niven and Schreier in 1994 [40] and Desai *et al.* in 2002 [41] reviewed the delivery of liposomes in dry powder form.

Niven noted that, if a drug was to leak rapidly from liposomes while being aerosolised, then the effectiveness of the preparation would be reduced or lost completely. In his studies he used various liposomes which encapsulated 5,6-carboxyfluorescein (CF). The level of encapsulated CF before and after nebulisation was used to determine ability to resist leakage.

His first paper described the effects of nebulisation on a range of liposomes carrying a neutral, net positive or net negative charge. The results showed that for all liposome compositions tested there was a release of encapsulated CF to the surrounding buffer solution during nebulisation and, furthermore, the lipid composition of liposomes had a definite effect on their ability to retain drug during nebulisation. He concluded that these effects should be seriously considered when formulating a drug-liposome preparation for inhalation after aerosolisation [8].

In the second study, Niven investigated the effect that altering the size of liposomes would have on the release of encapsulated drug during nebulisation. The results showed a clear relationship between liposome size and percentage of CF lost during nebulisation. The smallest liposomes (0.2 μm diameter) showed the least CF loss (~7.9%) while, unextruded, referred to as the largest liposomes (> 5 μm diameter) showed the greatest loss (~76.8%). A suggestion to explain these findings was that the first several surrounding bilayers are more "tightly" packed than subsequent layers and thus present a rate-limiting barrier to the release of encapsulated solute. In addition, the outer bilayers are more exposed to the effects of nebulisation than the inner ones. The collision nebuliser used in the study produced aerosol particles with a mass median aerodynamic diameter MMAD of ~1.2 μm. It was suggested that, in general, if vesicles are nebulised for short periods and prepared significantly smaller than the mean size of the aerosol droplets, the majority of solute may be retained inside the vesicle. The conclusions of this study were that the release of drug from the liposomes is biphasic in nature and that smaller liposomes release less solute during nebulisation. It is important when considering liposome size, therefore, to reach a balance which minimises the unwanted release of solute from liposomes during nebulisation without compromising their entrapment efficiency (i.e., deliverable dose) [8, 38].

In the third paper, the effects of operating conditions and local environment on the stability of liposomes were investigated. These effects included those of nebuliser jet air pressure, buffer pH, buffer osmotic strength and temperature on the stability of the liposomes. The study concluded that to minimise leakage of CF from liposomes of HSPC:DPPG at a 9:1 ratio, a) the pressure should be kept to a minimum; b) the pH should be high enough to ensure ionisation of the CF or other anionic drug; c) the buffer medium should be hypertonic; and d) the temperature should not be greater than the gel-to-liquid crystalline phase transition [39].

The 1994 study by Schreier and Niven described the formulation of lyophilised liposome cakes, their micronisation and aerosolisation using a DPI. The *in vivo* distribution of the powders when aerosolised in a silicone elastomer throat attached to an Anderson cascade

impactor was studied. The results demonstrated that milled micronised liposome powders can be effectively aerosolised at a fixed flow rate [40].

Desai *et al.* (2002) investigated the effect of lyophilisation and jet-milling on liposome integrity. They reported that lyophilisation resulted in considerable leakage of a model drug at lower concentrations of lactose, and jet-milling further augmented the leakage for all the lyophilised formulations. In an attempt to overcome these problems, they investigated the feasibility of formulating PL-based powders that result in spontaneous formation of liposomes in an aqueous environment. Lyophilisation was not involved in their production, and jet-milling, when not preceded by lyophilisation, did not reduce encapsulation efficiency. Some practical examples were tested (e.g. ciprofloxacin) and it was found that jet-milled PL-based powder formulations showed high encapsulation efficiencies (~95%) compared with a high amount of leakage (> 50%) observed due to jet-milling of lyophilised liposome formulations [41].

Abu-Dahab *et al.* (2001) demonstrated how using lectin-functionalised liposomal preparations were able to enhance cell association to human alveolar cell line (A549 cells) and primary human alveolar cells. (Lectins have been classed as second generation bioadhesives due to their ability to recognise and bind to exposed carbohydrate residues on glycoproteins, which are found on the surface of epithelial cells.) It was also demonstrated that these lectin-functionalised liposomal preparations are stable towards nebulisation [42].

As these studies suggest, we are gaining more and more insight into the formulation issues associated with liposomes for inhalation. The safety issues associated with inhaled liposomes have also been investigated in some detailed literature [43].

1.8 Fate of inhaled particles

The fate of inhaled particulates in the respiratory tract depends upon the dynamic interactions of three factors: (1) the physical characteristics of the aerosol; (2) the function and anatomy of the respiratory tract in health and disease; and (3) the diverse particle clearance mechanisms operant in the lung. An extensive literature exists on the theoretical and experimental aspects of each of these factors as they apply to aerosol inhalation in general [2].

Several dynamic clearance mechanisms actively purge the lung of deposited particles. Swallowing, expectoration and coughing constitute the first sequence of clearance mechanisms operant in the naso/oropharynx and tracheobronchial tree. A major clearance mechanism for inhaled particulate aerosols, including liposomes, is the "mucociliary escalator". It consists of ciliated epithelial cells reaching from the naso/oropharynx and the

upper tracheobronchial region down to the most peripheral terminal bronchioles. Incessant beating of the cilia, in concert with mucus secreted by goblet cells, contributes to an extremely efficient clearance mechanism. The major clearance mechanism in the alveolar regions of the lung is uptake by pulmonary alveolar macrophages.

As such, the respiratory tract is organised as a sequential filtering system consisting of the naso/oropharynx, the tracheobronchial tree, and the pulmonary parenchyma. Aerosol particles < 100 μm generally do not enter the respiratory tract and are trapped in the naso/oropharynx, whereas particles < 40 μm can deposit in the upper parts of the tracheobronchial tree. Particles must generally have an average aerodynamic diameter < 5 μm in order to reach the alveolar space. In addition to anatomical constraints, physical mechanisms govern aerosol particle deposition in the respiratory tract. A major physical determinant is inertial impaction, which occurs more in the naso/oropharynx and upper tracheobronchial regions with increased airflow velocity. In general, the faster the respiratory rate, and the larger the inhaled particle, the more likely inertial impaction occurs. Sedimentation is operational in the more peripheral regions of the tracheobronchial tree (small airways) and in the pulmonary parenchyma, where air velocity is relatively low. Very small (sub-micron size) aerosol particles travel essentially unhindered to the alveolar region as their deposition is governed by diffusion, i.e., random motion while being suspended in the surrounding gas phase. This process occurs both during inspiration and expiration [22].

Liposomes, unlike other inhaled particulates reaching the alveoli, are also cleared via incorporation into the surfactant PL pool, where processing, uptake, and recycling of endogenous (and liposomal) PLs by alveolar type II cells take place.

Despite the fact that targeting of liposomes to specific cells in the lung appears to be an attainable goal, a large fraction of lipid delivered to the lung will eventually be removed from the respiratory tract by clearance either via the mucociliary escalator or via incorporation into the surfactant pool as well as by uptake into alveolar macrophages.

Since mucociliary clearance is highest in the central airways, relative liposome distribution in the central vs. peripheral lung spaces will determine the rate of removal of liposomes and, thus, the duration of drug action. Furthermore, since mucociliary clearance may be impaired in the diseased lung, elimination data extrapolated from studies in healthy human volunteers will have to be verified in the diseased target population.

Much is known about the pulmonary fate of exogenous PL, mainly due to the work with artificial lung surfactant employed to treat respiratory distress syndrome in newborns.

Overall, the rate of removal of exogenous PLs was found to be within the range of endogenous surfactant turnover time of 2-7 h. One may conclude that exogenous PL delivered via aerosol will most likely associate with the surfactant pool and not disturb the physiologic processes associated with surfactant turnover.

As mentioned above, inhaled particles are dealt with by the lung in different ways depending on the region of lung which receives the deposition. Large particles impacting in the oropharynx are swallowed soon after inhalation. Medium-sized particles deposit within the tracheal-bronchial tree and are swept out of the lung by mucociliary clearance. Finally, the smaller particles which enter the alveoli are either engulfed by the macrophages of the pulmonary RES, or remain suspended in the air to be exhaled with the next breath. Liposomes seem to be handled by the lungs in a similar way. The fate of alveolar liposomes is more controversial.

The absorption of intact liposomes across the alveolar-capillary membrane is also possible. Detailed studies, however, of the fate of radiolabelled liposome components show that the overall kinetics for the movement of liposome PLs and Chol from the alveoli, to the lung tissue and hence into the circulation, were similar to but a little delayed compared with non-liposome surfactants. These data suggest that the liposomes break down within the alveoli and that the lipid constituents enter the surfactant pool to be absorbed and re-utilised [20]. These kinetic studies of liposome materials suggest that the inhaled lipids are dealt with as constituents of the surfactant pool. As such there seems little possibility of accumulation within the lungs to provoke unwanted side effects. In addition, extensive studies of the long-term effects of inhaled liposomes have not revealed any alteration in histopathology in the primate, or alteration in pulmonary function in the guinea pig.

The PLs used in liposome production are naturally occurring PLs. Lipids account for nearly 20% of the total dry tissue weight of the lung. Some are associated with the pulmonary surfactant system while the rest are associated with membrane, transport and energy metabolism systems. It has been shown that exogenous PLs administered to the lungs readily associate with the large pool of endogenous lipids with no apparent physiological or pathological disturbance of lung function.

In one study performed by Oyarzun *et al.* (1980), the instillation of radiolabelled liposomes into lungs of rabbits in amounts ~10% of total alveolar surfactant pool was investigated. Results showed it caused no unwanted pulmonary responses; i.e., arterial blood gases and pH, lung appearance, protein content and cell constituency were comparable to normal animals [44]. In another study, by Myers *et al.* (1993), the pulmonary effects of chronic exposure to

liposome aerosols in mice were investigated. They found that chronic exposure produced no untoward effects on survival, histopathology and macrophage function in the mice [45].

The same group reported on the effects of liposome aerosol inhalation on pulmonary function in healthy human volunteers and concluded that inhalation of small particle PC liposomes had no deleterious effects on pulmonary function in these individuals [45].

It also appears unlikely that phospholipase-mediated hydrolysis of liposome components happens to any great extent at the respiratory epithelium, which means that reactive or potentially irritating free fatty acids or lysophosphatides should not be released from liposomes administered by inhalation.

These studies all conclude that liposomes themselves offer little inherent threat to the pulmonary function of a patient when administered by inhalation and can therefore be considered as a safe and viable option for aerosol delivery.

1.9 Conclusions:

Liposomes offer many advantages as drug carriers to the lungs. They can accommodate a wide variety of drugs or macromolecules within their multilamellar structure including proteins, enzymes, chemotherapy and nucleic acids. They are stable for long periods *in vivo,* but nonetheless they are eventually fully catabolised.

They can be used to passively target macrophages while modifications to their surface structure can help them avoid opsonisation (e.g., attaching PEG chains to their surface) or actively target certain sites in the body (e.g., attaching receptor specific ligands).

They have been shown to be useful in the delivery of drugs for local effect in the lungs including the delivery of exogenous pulmonary surfactant to pre-mature babies; the delivery of anti-cancer drugs to treat pulmonary metastases; the delivery of anti-fungal and anti-bacterial antibiotics to treat pulmonary infection; the delivery of bronchodilators for symptomatic relief in asthma and COPD patients; and the delivery of corticosteroids for prophylactic treatment of these conditions.

They have been shown to be effective in sustained release preparations which use the lung as a depot for delivery to the systemic circulation (e.g., in post-operative analgesia).

Liposomes have been successfully formulated for inhalation as nebulised solutions and as lyophilised or jet-milled dry powders for inhalation.

Novel ways of formulating PL-based powders that result in spontaneous liposome formation in aqueous environments have been investigated for delivering liposomes for dry powder inhalation in an attempt to reduce drug leakage associated with lyophilisation and jet-milling.

Studies on the stability of liposomes towards nebulisation have shown that their stability depends on their lipid composition and size, as well as on environmental conditions, such as temperature, air pressure, buffer pH, and osmotic strength.

Safety studies have shown that liposome-aerosols offer little inherent threat to the pulmonary function of patients when administered by inhalation and can therefore be considered to be a safe and viable option for drug delivery.

Many opportunities exist for the use of liposomes in inhalation therapy and many formulations have shown promise in clinical trials. Further work is required to refine and develop these formulations before they can be considered as viable therapies or alternative therapies from efficacy, safety and economic points of view.

The direct exposure of the lung to the outside environment together with the easy accessibility make novel and innovative therapies possible for delivering of therapeutic agents directly to the lungs. This targeted therapy can be exploited for the therapy of lung cancer and

pulmonary metastatic disease because of such obvious advantages as low systemic toxicity, high pulmonary concentration, increased lymphatic absorption, and easy administration by the patient (e.g., at home).

There has been a significant amount of work done over the past three decades investigating this approach, mostly by means of animal studies. There have also been some human trials with encouraging results. However, most of the research has focused on strategies for immunomodulation. More recently, there has been increasing interest in using gene therapies, as well as in direct delivery of chemotherapeutic agents to the lung.

We propose to turn the liposome into a "smart bomb" by attaching homing molecules to a lipid anchor and including these in the liposome.

The address we will use is called the transferrin protein (Tf). Tumour cells often have receptors on their surface, designated CD71 receptors that stick to bind Tf. The problem for using Tf to target the tumour is that all nucleated cells in the body, such as red blood cells, erythroid cells, and brain cells, express TfR [46]. Thus, the Tf can only be used to target the liposome exclusively to the tumour cells if these cells significantly over-express the surface marker CD71 s, which is exactly the case in tumour cells.

In order to enhance the amount of drug delivered by Tf, these can be entrapped in Tf-conjugated liposomes, instead of coupling the drugs directly to Tf.

In all studies employing Tf-labelled liposomes for parenteral application, the half life of these liposomes was a critical point. PEG-modified liposomes were used to decrease the clearance from circulation by the RES [47].

1.10 Setting objectives

The promise of an enhanced therapeutic effect on lung cancer using Tf-modified liposomal aerosols led us to the studies discussed in this thesis. The specific objectives of this PhD project were:

1. To investigate pulmonary epithelial cell types (both healthy and cancerous) for (over)-expression of TfR.
2. To design Tf-conjugated-liposomes using different modification protocols, as well as techniques to confirm this conjugation step.
3. To encapsulate doxorubicin in liposomal drug carriers and to assess the binding-uptake properties of the liposomes in healthy and tumour cell types. Furthermore, to determine the cytotoxicity of these systems.
4. To study the nebulisation of liposomal formulations conjugated with Tf by air-jet and ultrasonic nebulisers, as well as the stability of the nebulised systems. Furthermore, to assess the stability of the systems after incubation in lung surfactant.

1.11 References

[1] B Myers, The respiratory system, in L Shaw (Ed.) Anatomy and physiology, Nelson Thorens Ltd., Cheltenham, U.K., (2005) 148--165.

[2] A B Thompson, R A Robbins, D J Romberger, J H Sisson, J R Spurzem, H Teschler, S I Renards, Immunological function of the pulmonary epithelium, Eur Respir J. 8 (1995) 127--149.

[3] K C Stone, R R Mercer, B A Freeman, L Y Chang, J D Crapo, Distribution of lung cell numbers and volumes between alveolar and nonalveolar tissues, Am Rev Respir Dis. 146(2) (1992) 454--456.

[4] I Kopzik, S Haung, J D Paulauskik, J J Godleski, Particle opsonisation and lung macrophage cytokine response, J Immunol. 151 (1993) (2753--2759).

[5] R D Rao, S N Markovic, P M Anderson, Aerosol therapy for malignancy involving the lungs, Curr Cancer Drug Targets. 3(4) (2003) 239--250.

[6] P Mason, Lung cancer - the disease and non-drug treatment, Hospital Pharmacist. 12 (2005) 129--135.

[7] K Todd, Lung cancer - the role of chemotherapy, Hospital Pharmacist. 12 (2005) 137--143.

[8] R W Niven, H Schreier, Nebulization of liposomes. I. Effects of lipid composition, Pharm Res. 7(11) (1990) 1127--1133.

[9] AT Florence, D Attwood, Chapter 6 in Physicochemical principles of pharmacy, MacMillan. (1998) 81--100.

[10] D B Fenske, N Mauer, P R Cullis, Encapsulation of weakly-basic drugs, antisense oligunucleotides, and plasmid DNA with large unilammelar vesicles for drug delivery application, in V.P. Torchilin, V. Weissig (Eds.) Liposomes: A practical approach 2nd ed., Oxford University Press, Oxford, U.K., (2003) 167--191.

[11] A Gabizon, T Peretz, A Sulkes, S Amselem, R Ben-Yosef, N Ben-Baruch, R Catane, S Biran, Y Barenholz, Systemic administration of doxorubicin-containing liposomes in cancer patients: a phase I study, Eur J Cancer Clin Oncol. 25(12) (1989)1795--1803.

[12] C Carrion, M A de Madariaga, J C Domingo, In vitro cytotoxic study of immunoliposomal doxorubicin targeted to human CD34+ leukemic cells, Life Sci. 75(3) (2004) 313--328.

[13] R Banerjee, Liposomes: application in medicine, J Biomater Appl. 16(1) (2001) 3--21.

[14] T M Allen, C Hansen, Pharmacokinetic of stealth versus conventional liposomes: effect of dose, Biochem Biophys Acta. 1068(2) (1991) 133--141.

[15] E J Lee, E J Oh, Y J Park, H K Lee, B K Kim, Soluble transferrin receptor (sTfR), ferritin, and sTfR/Log ferritin index in anemic patients with nonhematologic malignancy and chronic inflammation, Clin Chem. 48(7) (2002) 1118--1121.

[16] L Hongyan, Z M Qian, Transferrin/transferrin receptor-mediated drug delivery, Med Res Rev. 22(3) (2002) 225--250.

[17] A Widera, F Norouziyan, W C Shen, Mechanism of TfR-mediated transcytosis and sorting in epithelial cells and applications toward drug delivery, Adv Drug Deliv Rev. 55 (2003) 1439--1466.

[18] M Singh, H Atwal, R Micetich, Transferrin directed delivery of adriamycin to human cells, Anticancer Res. 18(3A) (1998) 1423--147.

[19] Z M Qian, L Hongyan, S Hongzhe, H Kwokping, Targeted drug delivery via the transferrin receptor-mediated nndocytosis pathway, Pharmacol Rev. 54(4) (2002) 561--587.

[20] Mc Calden, A Thomas, Particulate systems for drug delivery to the lung, Adv Drug Deliv Rev. 5(3) (1990) 253--263.

[21] K M G Taylor, G Taylor, I W Kellaway, J Stevens, the stability of liposomes to nebulization, Int J Pharm. 58(1) (1990) 57--61.

[22] H Schreier, Gonzalez-Rothi, R J Stecenko, A Arlene, Pulmonary delivery of liposomes, J Control Release. 24(1-3) (1993) 209--223.

[23] M Griese, Pulmonary surfactant in health and human lung diseases: state of the art, Eur Respire J. 13 (1999) 1455--1476.

[24] V Bender, M Ali, M Amon, E Diefenbach, N Manolios, T Cell antigen receptor peptide-lipid membrane interactions using surface plasmon resonance, J Biol Chem. 279(52) (2004) 54002--54007.

[25] M Obladen, D Popp, C Scholl, H Schwarz, F Jahnig, Studies on lung surfactant replacement in respiratory distress syndrome. Rapid film formation from binary mixed liposomes, Biochemica Biophysica Acta. 735(2) (1983) 215--224.

[26] C J Morley, A D Bangham, N Miller, J A Davis, Dry artificial lung surfactant and its effect on very premature babies, Lancet. 1(8211) (1981) 64--68.

[27] R L Juliano, D Stamp, Pharmacokinetics of liposome-encapsulated anti-tumour drugs. studies with vinblastine, actinomycin D, cytosine arabinoside, and daunomycin, Biochem Pharmacol. 27(1) (1978) 21--27.

[28] R L Juliano, H N McCullough, Controlled delivery of an antitumour drug: localized action of liposome encapsulated cytosine arabinoside administered via the respiratory system, J Pharmacol Exp Ther. 214(2) (1980) 381--387.

[29] C Khanna, D E Hasz, J S Klausner, P M Anderson, Aerosol delivery of interleukin 2 liposomes is nontoxic and biologically effective: canine studies, Clin Cancer Res. 2(4) (1996) 721--734.

[30] C Khanna, P M Anderson, D E Hasz, E Katsanis, M Neville, J S Klausner, Interleukin-2 liposome inhalation therapy is safe and effective for dogs with spontaneous pulmonary metastases, Cancer. 79(7) (1997) 1409--1421.

[31] E J Ruijgrok, A G Vulto, E W M van Etten, Aerosol delivery of amphotericin B desoxycholate (Fungizone) and liposomal amphotericin B (AmBisome): aerosol characteristics and in-vivo amphotericin B deposition in rats, J Pharm Pharmacol. 52(6) (2000) 619--627.

[32] S P Vyas, M E Kannan, S Jain, V Mishra, P Singh, Design of liposomal aerosols for improved delivery of rifampicin to alveolar macrophages, Inter J of Pharm. 269(1) (2004) 37--49.

[33] B Muller, H Garn, R Hochscheid, Impaired recycling of the surfactant-like liposomes in type II pneumocytes from injured lungs, Thorax. 58 (2003) 127--134.

[34] G A Cabezas, P D Graf, J A Nadel, Sympathetic versus parasympathetic nervous regulation of airways in dogs, J Appl Physiol. 31(5) (1971) 651--655.

[35] Y Mizushima, K Hoshi, H Aihara, M Kurachi, Inhibition of bronchoconstriction by aerosol of a lipid emulsion containing prostaglandin E1, J Pharm Pharmacol. 35(6) (1983) 397.

[36] M R Joshi, A Misra, Liposomal budesonide for dry powder inhaler: preparation and stabilization, AAPS Pharm Sci Tech. 2(4) (2001) 25.

[37] O R Hung, S C Whynot, J R Varvel, S L Shafer, M Mezei, Pharmacokinetics of inhaled lLiposome-encapsulated fentanyl, Anesthesiology. 83(2) (1995) 277--284.

[38] R W Niven, M Speer, H Schreier, Nebulization of liposomes. II. The effects of size and modeling of solute release profiles, Pharm Res. 8(2) (1991) 217--221.

[39] R W Niven, T M Carvajal, H Schreier, Nebulization of liposomes. III. The effects of operating conditions and local environment, Pharm Res. 9(4) (1992) 515--520.

[40] L Garcia-Contreras, A J Hickey, Pharmaceutical and biotechnological aerosols for cystic fibrosis therapy, Adv Drug Deliv Rev. 54(11) (2002) 1491--1504.

[41] T R Desai, J P Wong, R E W Hancock, W H Finlay, A novel approach to the pulmonary delivery of liposomes in dry powder form to eliminate the deleterious effects of milling, J Pharm Sci. 91(2) (2002) 482--491.

[42] R Abu-Dahab, U F Schafer, C M Lehr, Lectin-functionalized liposomes for pulmonary drug delivery: effect of nebulization on stability and bioadhesion, Eur J Pharm Sci. 14(1) (2001) 37--46.

[43] G Strom, D J A Crommelin, Liposomes: que vadis?, PSTT. 1(1) 1998 19--31.

[44] M J Oyarzum, A J Clements, A Baritussio, Ventilation enhances pulmonary alveolar clearance of radioactive dipalmitoyl phosphatidylcholine in liposomes, Am Rev Respir Dis. 121(4) (1980) 709--721.

[45] M Myers, Pulmonary effect of chronic exposure to liposome aerosols in mice, Exp lung res. 19(1) (1993) 1--19.

[46] D A Thomas, M A Myers, B Wichert, H Schreier, R J Gonzalez-Rothi, Acute effects of liposome aerosol inhalation on pulmonary function in healthy human volunteers, Chest. 99(5) (1991) 1268--1270.

[47] D A Thomas, M A Myers, B Wichert, H Schreier, R J Gonzalez-Rothi, Immunological evaluation of the new stable ultrasound contrast agent LK565: a phase one clinical trial, Cardiovasc Ultrasound. 2(1) (2004) 16.

[48] E Wagner, D Curiel, M Cotten, Delivery of drugs, proteins and genes into cells using transferrin as a ligand for receptor-mediated endocytosis, Adv Drug Deliv Rev. 14(1) (1994) 113--135.

Chapter 2

Expression pattern of CD71 in respiratory epithelial cells

Parts of this chapter have been submitted for publication: Samah Anabousi, Udo Bakowsky, Marc Schneider, Hanno Huwer, Claus-Michael Lehr, Carsten Ehrhardt, *In vitro* assessment of transferrin-conjugated liposomes as drug delivery systems for the inhalation therapy of lung cancer.

2.1 Abstract

Introduction Targeted drug delivery is a current issue of research in drug delivery. The expression of transferrin receptors (TfR, CD71) is significantly elevated in many cancers, and thus the TfR is a promising candidate for tumour-specific drug delivery.

Purpose In this study, the extent of TfR expression levels in different lung cancer cell lines was assessed using fluorescence-activated cell sorting (FACS). Additional studies were carried out using a model of human alveolar epithelial cell in primary culture (hAEpC).

We studied the localisation of TfR expression in different lung epithelial cell types (i.e., bronchial epithelial cells and alveolar epithelial cells) by confocal laser scanning microscopy (CLSM).

Results TfR were found to be expressed to a significantly higher extent in bronchial epithelial cells compared with their alveolar counterparts. In addition, cells of cancerous origin (i.e., A549 cell line) showed an expected higher TfR expression level than normal alveolar epithelial type II cells in primary culture. CLSM revealed TfR to be located primarily at basolateral sites of cells with the exception of cells undergoing mitotic proliferation, which also showed TfR in their luminal surfaces, due to loss in cell polarity.

2.2 Introduction

2.2.1 Transferrin/transferrin receptors in tumours

Tf is a plasma protein that functions as the major iron transporter protein. Many *in vitro* studies have demonstrated that plasma Tf, which is synthesised in the liver, is essential for cellular proliferation. Thus, a number of cell lines grown continuously in defined (serum-free) media all demonstrate that Tf, along with several hormones and smaller organic compounds, is required for cellular proliferation. Most studies have supported the notion that Tf functions to maintain cellular proliferation by providing iron. These studies concluded that Tf acts as a promoter of cell growth, based on its transport of iron in plasma. It could be hypothesised, therefore, that specialised cellular proliferation *in vivo*, of tissues that are not well vascularised, might be limited by insufficient delivery of Tf-bound iron from plasma. There is good evidence, however, that under certain conditions some tissues synthesise Tf, permitting specialised proliferation. One example, the Sertoli cell of the testes, demonstrates synthesises of Tf to provide iron to proliferating spermatocytes [1].

The Tf that is produced in the lung cancer cells acts as an autocrine promoter of cellular proliferation, in a similar fashion to a number of other growth factors synthesised by malignant cells. That autocrine secretion of Tf promotes cell growth is supported by the fact that Tf synthesis markedly increases shortly before and during the period when the cells are entering active phases of the cell cycle, a time when it appears that Tf, and particularly iron, is necessary in order for cell division to proceed [2].

An increase in Tf synthesis appears to precede increased expression of TfR, and this latter event is clearly important in maintaining cellular proliferation. In a study performed by Vostrejs *et al.* (1988), experiments were carried out to examine the effects of various agents that would affect iron metabolism and cellular proliferation of NCI-H5 10 cells, which is a small-cell lung cancer line. At low plating density, growth of these cells is dependent on added Tf, while at higher plating density, the addition of Tf-iron to the media is associated with a more rapid initial rate of proliferation, in contrast to cells grown in Tf-free medium. The authors concluded that in normal cells Tf synthesis is required for proliferation, but appears to be not an absolute requirement. On the other hand, this is not the case in the highly proliferating cells as in tumours, in which the Tf synthesis is necessary for furthering their proliferation [3].

There was a clear observation of a preferential response to Tf in certain cell lines of the rat mammary adenocarcinoma. TfR expression of those cells correlated with metastatic capability

[4]. Others have also found that TfR expression in neoplasms associates with tumour stage, progression, or predicted survival [5]. In a series of immunohistological studies, a correlation of this nature was seen in melanomas, breast carcinomas, bladder cell transitional cell carcinomas, a maxillary cancer, and non small-cell lung cancer [6].

Tumour cell responses to factors that regulate growth, motility, adhesion, and other cellular responses in the normal organ environment, have been shown to be involved in successful metastasis to particular organ sites. Tf, along with the synthesis of other autocrine promoters of cell growth, may permit tumour cell growth *in vivo* in areas not well vascularised. Synthesis of this important plasma growth factor may explain why SCLC has an extremely short doubling time for a tumour even without much vascularisation [3]. One may hypothesise that specific agents that would affect iron metabolism, including monoclonal antibodies against Tf or TfR, as well as liposomal drug carrier carrying anticancer drugs labelled with Tf, may provide new strategies in the treatment of small-cell lung cancers.

2.2.2 Role of transferrin

Tf exerts a proliferative effect on cells in culture by supplying iron for key synthesis processes required for cell growth. Tf binds two atoms of iron, one at each of two sites located at the amino terminal and carboxy terminal domains of the protein.

Iron-replete Tf interacts with a cell surface TfR, a homodimeric disulfide-linked glycoprotein of Mr 190,000. The receptor ligand complex is internalised in structures that mature into acidic endosomes, the low pH of which causes the release of the bound iron from the complex. Iron is then translocated to areas of need, such as sites of synthesis of mitochondrial electron transport proteins and ribonucleotide reductase. The latter enzyme consists of two non-identical R1 and R2 subunits; the iron saturated state of R2 is required for activity. Maintenance of the function of this enzyme is essential for the synthesis of DNA and the proliferation of cells [7].

2.2.3 Transferrin in the lung

The large surface area of the lower respiratory tract presents a challenge to pulmonary host defense. In contrast to the upper airways, the epithelium of alveoli is devoid of the protective mucus barrier and the ciliated cells that enable the constant clearance of foreign material in the upper airways. Whereas alveolar macrophages may provide a formidable first line of defense against pathogenic organisms, of particular concern is the presence of excessive levels of free iron in the surface fluid lining the alveolar epithelium in the distal lung.

Increased levels of free iron in the distal lung (as a consequence of pathological conditions such as smoking or chronic inhalation of metallic dusts) have been associated with tissue injury and fibrosis, primarily attributable to the ability of iron to catalyse the formation of highly reactive hydroxyl radicals [8].

Excessive levels of free iron in the lower lung have also been associated with facilitating the growth of intracellular mycobacteria. The task of sequestering free iron lies with the iron-binding proteins, of which Tf is the primary constituent in the surface fluid lining the airspaces of the lower lung. Tf is an 80-kDa glycoprotein that is responsible for transporting iron, via its two ferric-binding sites, throughout the vasculature. In addition to this primary role, Tf has been implicated as the major source of antioxidant capacity in the lung. Indeed, the levels of Tf, as a percentage of total protein, detected in bronchoalveolar lavage fluid have been found to be high (4.0%–5.6%) compared with values for plasma (3.0%). The presence of such high levels of apo-Tf in surface fluid lining the alveolar epithelium suggests that there may be a specialised transport mechanism for Tf from the systemic circulation to the alveolar fluid. The tight epithelial barrier of the alveoli excludes a passive paracellular transport route for the large Tf molecule, consistent with the hypothesis that the transport of Tf into, and possibly out of, alveolar lining fluid might be regulated by an active transcellular transport process. Endocytosis of Tf by the TfR and subsequent transcytosis into alveolar lining fluid would be a likely mechanism for this process. However, the extent and nature of TfR distribution among the cells of the rat alveolar epithelium remain unknown.

In a study performed by Wiedera *et al.* it was demonstrated that alveolar type II epithelial cells express significant TfR, and that synthesis of TfR is down regulated upon transdifferentiation from type II to type I cell-like phenotypes. Exposure of alveolar epithelial cells cultures to keratinocyte growth factor (KGF) results in retention of TfR, indicating that the maintenance of type II cell phenotype results in the retention of TfR synthesis. In addition, the synthesised TfR is limited exclusively to the basolateral surface of monolayers of alveolar epithelial cells. This indicates that alveolar type II cells are probably involved in the transcytotic transport of Tf between plasma and alveolar lining fluid [6].

2.2.4 Transferrin receptor and lung cancer

The lungs are the common site of both metastasis and 1° neoplasia. The average lung cancer mortality rate is 90% and this is the leading cause of cancer-related deaths in both men and women.

One reason for the poor survival rate is that traditional methods of treatment, such as surgical resection, radiation and chemotherapy, have failed to eradicate lung cancer. Systemic chemotherapy has been employed with little success due to development of toxic side effects of the anticancer drugs, which are given by the oral or intravenous route of administration.

In cancer therapy, the major goal is preferential destruction of malignant cells, while sparing normal tissues. Although most anti-cancer drugs could be chemically modified to accumulate in tumour tissues, their selectivity is not optimal, which will result in cytotoxicity to normal structures. Targeted drug delivery could be an alternative for tackling these problems giving enhanced accumulation in tumour cells' [9].

An attractive strategy to enhance the therapeutic index of drugs is to specifically deliver these agents to the defined target cells, thereby, keeping them away from healthy cells, which are also sensitive to the toxic effects of the drugs. This would allow for more effective treatment along with better tolerance. Many attempts are being made to explore the potentials of specific and target-based drug delivery systems, among them liposome-based delivery systems, and ligand-receptor-mediated delivery systems [10, 11]. TfR have been widely explored for receptor-mediated delivery of anticancer agents and in enhancing the transport of drugs across the blood brain barrier and more recently, across the epithelial cells.

In polarised cells, Tf can be transcytosed from the apical to basolateral membrane. This mechanism is exploited in TfR-mediated drug delivery across the blood-brain barrier as well as across the epithelial barrier of the small intestine. TfRs are present in high density in human GI epithelium, and Tf can resist tryptic and chymotryptic degradation. Shen and co-workers were the first to realise the potential of TfR-mediated transport in improving oral delivery of therapeutic agents. They conjugated Tf to insulin via disulfide linkages and demonstrated that the conjugation of insulin to Tf resulted in a 5- to 15-fold increase in the transport of insulin across the Caco-2 cell monolayer. As pointed out earlier, since this enhancement is specific for the conjugated protein and takes place without causing the tight junctions to open even momentarily, this approach is most desirable in terms of toxicity and damage to the epithelium [12].

In a study done by Whitney *et al.* 1996, the authors were aiming to define the frequency of expression of transferrin receptor in lung cancer specimens and to gather preliminary data regarding the prognostic value of this tumor-related antigen. By studying the tissue immunoreactivity with a murine monoclonal antibody directed against transferrin receptor in patients with non small-cell lung cancer who underwent surgical resection during the period from January, 1988, to May, 1991, they concluded that transferrin receptor is expressed in the

majority of lung cancers and the presence of transferrin receptor in NSCLC may be an indicator of poorer prognosis in certain groups of patients [4].

In lung cancer, lymphoma and breast cancer, it has been shown that the expression of TfR correlates with tumour differentiation, probably implying some prognostic value. A soluble form of TfR (sTfR) in human serum has been shown to be proportional to the density of cellular TfR. This utility idea was used by measuring sTfR in the serum and bronchoalveolar lavage (BAL) fluid of patients with lung cancer and patients with chronic obstructive pulmonary disease in a study performed in 1997 by Dowlati *et al.* BAL fluid was centrifuged to separate the supernatant from the cellular component. Cells were lysed in a detergent and cell-associated TfR was measured by enzyme-linked immunosorbent assay (ELISA). The results have shown that BAL supernatant and cell-associated TfR were able to detect lung cancer with a sensitivity of 91%, a specificity of 59%, and positive and negative predictive values of 81% and 71%, respectively. The authors concluded that BAL cell-associated TfR may help in the differential diagnosis of lung cancer vs. pneumonia [13].

In the search for tumour-related antigens with survival predictive value, previous studies have yielded varied conclusions regarding the expression of the TfR in lung cancer [14].

Drug-resistant tumour cells express a number of upregulated proteins. A study done by Kuvibidila *et al.* (2004) showed twice the concentration of TfR on the surfaces of drug-resistant SCLC cells as compared to sensitive cells, an observation which is consistent with similar data obtained by the same methods on drug-resistant leukaemia cells [15]. Because the expression of TfR is up-regulated by iron need, TfR levels increase with increased cell proliferation, while decreasing when cell proliferation is reduced.

In another study, with the preliminary data regarding the prognostic value of this tumour-related antigen, tissue immunoreactivity was studied with a routine monoclonal antibody against TfR in patients with NSCLC who underwent surgical resection at the Medical Center Hospital of Vermont during the period from January 1988 to May 1991. The results show that normal lung tissues did not stain for TfR; however, 13 of 17 (76%) adenocarcinomas, 13 of 14 (93%) squamous cell carcinomas, and the one large cell carcinoma stained positively for TfR. Straining for TfR was graded according to pattern and intensity. The study conclusions were that TfR is expressed in the majority of lung cancers and that the presence of TfR in NSCLC may be an indicator of poorer prognosis in certain groups of patients [16].

The goal of this study is to measure the levels and locations of TfR expression in different lung epithelial cell types (i.e., bronchial epithelial cells and alveolar epithelial cells) by means FACS and CLSM

2.3 Materials and methods

2.3.1 Cell culture conditions

Primary culture of human pneumocytes

hAEpC II were isolated from human non-tumour lung tissue, which was obtained from patients undergoing lung resection. The use of human material for isolation of primary cells was reviewed and approved by the respective local Ethical Committee (State Medical Board of Registration, Saarland). Isolation was performed according to a protocol previously described by Elbert *et al.* (1999) [17], with a slight modification of the enzymatic digestion. Briefly, the chopped tissue was digested using a combination of 150 mg trypsin type I (T-8003, Sigma, Deisenhofen, Germany) and 0.641 mg elastase (LS022795, CellSystems, St. Katharinen, Germany) in 30 ml balanced salt solution (BSS; 137 mM NaCl, 5.0 mM KCl, 0.7 mM Na_2HPO_4 7 H_2O, 10 mM HEPES (N-[2-hydroxy-ethyl]piperazine-N´-[2-ethanesulfonic acid]), 5.5 mM glucose, penicillin (100 units/ml) and streptomycin (100 µg/ml), pH 7.4) for 40 min at 37°C. The AEpC II cell population was purified from the crude cell mixture using a combination of differential cell attachment, centrifugation with a percoll density gradient, and cell sorting with magnetic beads (anti-HEA (EpCAM) MicroBeads, Miltenyi Biotec, Bergisch Gladbach, Germany) [18]. The average yield of hAEpC cells was 0.8×10^6 cells/g tissue (n = 19) with a purity of > 90% determined by staining cells for alkaline phosphatase. Purified hAEpC II cells were then seeded at a density of 300,000 cells/cm^2 on collagen/fibronectin-coated polyester filter inserts (Transwell Clear, 12 mm in diameter, 3460, Corning, Bodenheim, Germany) using saline-adenine-glucose-mannitol medium (SAGM) (Cambrex Bio Science, Verviers, Belgium) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and with addition of low serum (1% FBS) in order to suppress fibroblasts. After approximately eight days in culture, the hAEpC exhibited a morphology similar to that of alveolar type I epithelial cells [19, 20].

A549 cell line

The A549 cell line has been a popular model for human alveolar adenocarcinoma [21]. A549 cells were obtained from BioWhittaker (Walkerville, MD, USA) via Boehringer Ingelheim Bioproducts (Ingelheim, Germany) and used at passage numbers 92-95 in this study. A549 cells were cultured in RPMI-1640 medium (Sigma) supplemented with 10% FBS at 37°C in a humidified atmosphere containing 5% CO_2. Cells were plated in 25 ml culture flasks and polyester filter inserts (Transwell Clear, 24 mm in diameter, 3450, Cornin, Bodenheim,

Germany) and allowed to grow till confluency (~ 5-7 days after seeding). The medium was changed every other day.

Calu-3 cell line

Calu-3 is a human bronchial epithelial cell line isolated from an adenocarcinoma of the lung [22]. This cell line has been shown to exhibit serious cell properties and to form confluent monolayers of mixed phenotype, including ciliated and secreting cells. Calu-3 cells were purchased from ATCC (Manassas, VA, USA) and used at passage numbers 70-73. The cells were grown in Eagle's minimum essential medium (EMEM) supplemented with 10% FBS, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 µg/ml streptomycin and 100 U/ml penicillin) at 37°C in a humidified atmosphere containing 5% CO_2. Cells were plated in 75 ml culture flasks and polyester filter inserts (Transwell Clear, 24 mm in diameter, 3450, Corning, Bodenheim, Germany) at a density of 10^5 cells/cm^2. The medium was changed every other day.

16HBE14o- cell line

The 16HBE14o- cell line was a gift from Dieter C. Gruenert (California Pacific Medical Center, San Francisco, CA, USA). This continuous cell line was generated by transformation of normal bronchial epithelial cells obtained from a one-year-old heart-lung transplant patient [22, 23]. Transformation was accomplished with SV40 large T antigen using the replication-defective pSVori$^-$ plasmid. Passages 2.48-52 were used in this study. Cells were seeded in tissue culture flasks and polyester filter inserts (Transwell Clear, 24 mm in diameter, 3450, Corning, Bodenheim, Germany) at a density of 10^5 cells/cm^2 and grown in EMEM supplemented with 10% FBS, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin G, at 37°C in a 5% CO_2 incubator. The medium was changed every other day.

2.3.2 FACS analysis

Freshly isolated hAEpC II cells were washed in phosphate-buffered saline (PBS; 129 mM NaCl, 2.5 mM KCl, 7.4 mM $Na_2HPO_4 \times 7\ H_2O$, 1.3 mM KH_2PO_4; pH 7.4) after the isolation procedure and were then prepared for flow cytometry. hAEpC I cells cultured to day 8 post seeding, were detached from the culture plastics using Accutase (Chemicon, Hampshire, UK) followed by washing with PBS and prepared for flow cytometry as described below. A549, 16HBE14o- and Calu-3 cells were also detached from the culture plastics by using the same

procedure after reaching confluency (see above). Mouse monoclonal anti-transferrin receptor antibody (CBL137, Chemicon, Hofheim, Germany) at a 1:50 dilution was incubated with the cells for 60 min at 37°C. For the isotypic control, mouse IgG1 (Sigma) was used. Following the incubation, cells were twice re-suspended in PBS and then incubated with a 1:100 dilution of FITC-labelled goat anti-mouse $F(ab')_2$ fragments (DakoCytomation, Hamburg, Germany) for 20 min at 37°C. After washing, cells were analysed by flow cytometry using a FACSCalibur (BD, Heidelberg, Germany) with CellQuest software. Samples were analysed using excitation at 488 nm. Forward scatter was used to gate the cell subset of interest and eliminate debris, dead cells and cell aggregates.

Five thousands cells were counted in triplicate for each cell type investigated. To exclude any autofluorescence from the cells, instrument settings were adjusted to favour of using untreated cells. To quantify TfR and to prove the reproducibility in different experiments, TfR-positive cells were gated accordingly and the mean FL-1 in the gated population was compared in two preparations.

2.3.3 Immunofluorescence confocal laser scanning microscopy

Mouse monoclonal anti-TfR antibody (clone: RVS-10, Chemicon) was diluted 1:100 in PBS containing 1% (w/v) BSA. Mouse IgG1κ (MOPC-21, Sigma) was used as isotypic control. Transwell-grown respiratory epithelial cells were stained on days 5 to 8 after seeding. All cells were fixed for 10 min with 2% (w/v) paraformaldehyde (PFA) and blocked for 10 min in 50 mM NH_4Cl, followed by permeabilisation for 8 min with 0.1% (w/v) Triton X-100. After 60 min incubation with 100 µl dilution of the primary antibody, the cell layers were washed three times with PBS before incubation with 100 µl of a 1:100 dilution of Alexa Fluor 488-labelled goat anti-mouse $F(ab')_2$ fragment (Invitrogen, Karlsruhe, Germany) in PBS containing 1% (w/v) BSA. One µg/ml of propidium iodide was then added for counterstaining cell nuclei. After 30 min incubation, the specimens were washed three times with PBS and embedded in FluorSave anti-fade medium (Calbiochem, Bad Soden, Germany). Images were obtained with a confocal laser scanning microscope (CLSM, MRC-1024, Bio-Rad, Hemel Hempstead, U.K.) with the instrument settings adjusted so that no positive signal was observed in the channel corresponding to green fluorescence of the isotypic controls.

2.4 Results

2.4.1 FACS

In the FACS experiments, five different types of respiratory epithelial cells were chosen, originating from two different areas of the human lung, the bronchial and the alveolar tissue.

The bronchial area was represented by two continous cell lines, Calu-3 (cancerous) and 16HBE14o- (immortalised normal bronchial epithelial cells).

The alveolar area was represented by the A549 cell line (cancerous), and human alveolar epithelial cells in primary culture. Three preparations of each cell type were used in duplicate. The first one was only purified and washed with buffer without further manipulation and used as control for estimation of gate area by setting forward-scatter as well as side-scatter. The second one was incubated with isotypic-Ab in order to allow the subtraction of non-specific interaction; while the last one was incubated with TfR-Ab. Results were shown as dot plots (figure 5). As seen in figure 5, sample of the gained data shows increased forward scatter signal which was observed in case of the bronchial cell types suggesting a larger cell size. All the other samples were gated the same (data not shown).

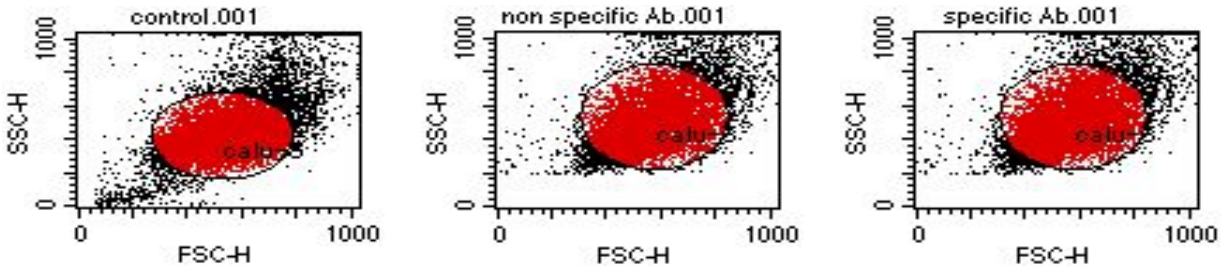


Figure 5: Dot plot of the selected region for events for the Calu-3 cells incubated with 1. No Ab as control, 2. Non specific Ab as negative control and 3. Specific Anti.TfR antibody.

Dot plot data were then analysed and converted to mono-histograms or overlaid-histograms. The comparison of these histograms led us to the following observations:

1- In case of hAEpC II, there is only little difference between the control and specific Ab histograms, implying that the TfR are not present in this cell type (figure 6).

2- In case of hAEpC I, there was no difference between the dot plots and histograms, of control and cells incubated with Tf antibody, further confirming the role of TfR as a proliferation marker (figure 7).

3- In the A549 cell line, TfR seems to be expressed at a higher level, compared to hAEpC II. This finding supports literature data that a higher expression level for TfR can be found in cancer cells (figure 6).

4- Both studied bronchial cell lines showed a clear difference between the control and cells associated with the anti TfR-Ab, which proves significant expression of TfR in these cell lines (figure 6).

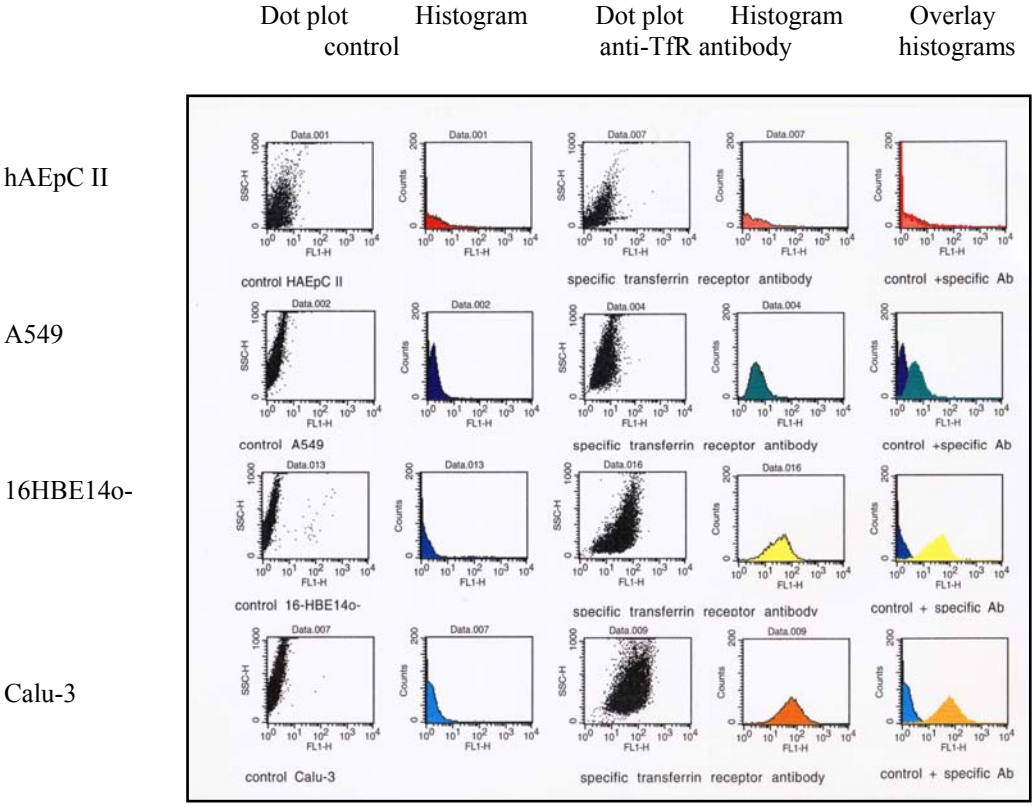


Figure 6: Dot plots and histograms of the all cell lines and hAEpC II that were used in the experiments. The first column represents the dot plots of the used samples incubated with no antibody. The second column represents the histograms of the same samples. Column 3 represents the dot plots are depicted the cells incubated with specific anti-TfR-antibody. In column 4 the histograms of these dot plots are shown. Column 5 shows overlay-histograms of the two histograms of each sample.

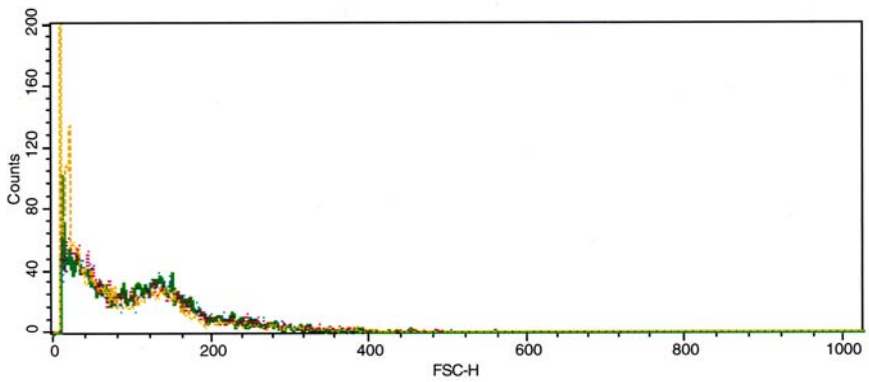


Figure 7: Overlay histogram of control and sample of hAEpC I.

For comparison, all histograms are overlaid in the graph shown in figures 8, as well as the mean values for TfR signal in comparison to the isotypic control are depicted in the bar chart in figure 9. Both graphs indicate that the expression of TfR on the cells surfaces is significantly higher in Calu-3 > 16-HBE14o- > A549 which is then undetected in hAEpC.

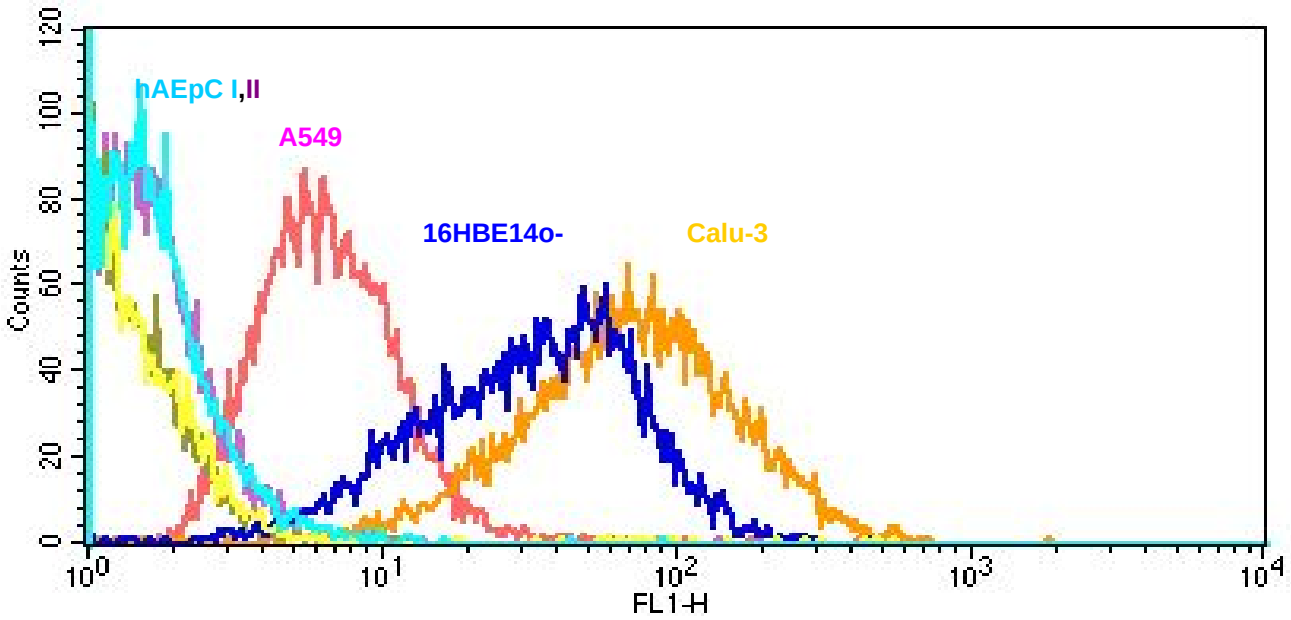


Figure 8: Flow cytometry of transferrin receptor (TfR, CD71) expression in different pulmonary epithelial cell types. Freshly isolated alveolar type II cells and type I-like cells after 8 days in primary culture reveal a low signal for TfR. Signals for alveolar A549 cells are higher, followed by 16HBE14o- and Calu-3 bronchial epithelial cells.

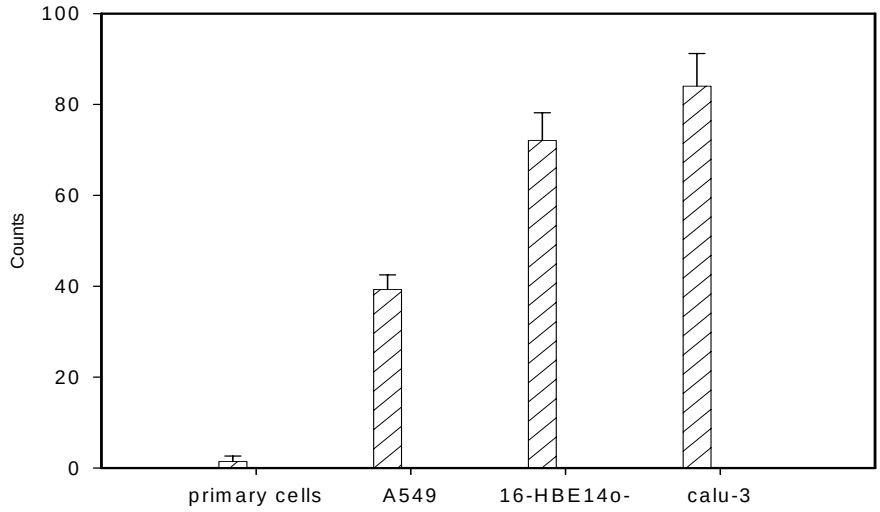


Figure 9: Mean value of TfR signal in comparison to isotypic control

2.4.2 Transferrin receptor localisation

By immuno-CLSM the localisation of TfR was assessed in the different cell types. In general, all cell types showed TfR molecules located predominantly at their basolateral membranes (figure 10A). Cells undergoing mitotic proliferation at the time of fixation showed an additional strong signal for TfR on their apical aspect as well due to the loss of their polarization (figure 10B).

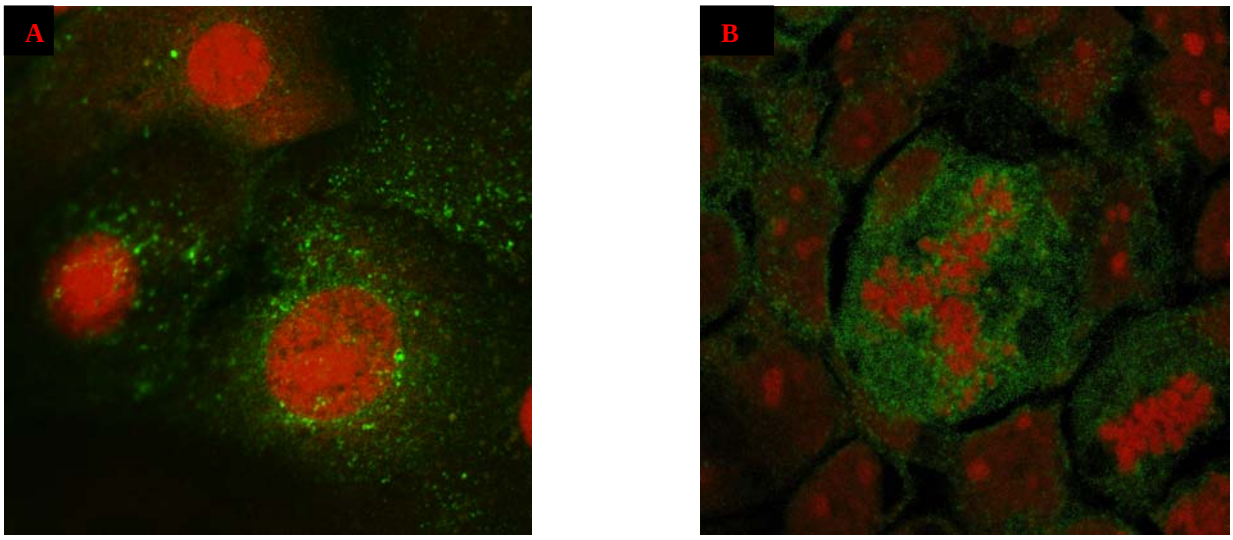


Figure 10: Localisation of TfR by immuno-confocal laser scanning microscopy. **A)** Human alveolar epithelial cells after eight days of primary culture show a type I cell-like phenotype with TfR (green) located predominantly at their basal aspect. **B)** Calu-3 bronchial epithelial cells undergoing mitotic proliferation at the time of fixation showed an additional strong signal for TfR on their apical membranes. Cell nuclei were counterstained with propidium iodide. Scale bars represent micrometers.

2.5 Discussion

Flow cytometry of TfR expression in freshly isolated hAEpC II cells, as well as in hAEpC I-like cells after eight days in primary culture reveal very low signals not significantly different from the control values (figure 8). By flow cytometry we also revealed that TfR expression levels in A549 cells, which were derived from an adenocarcinoma of the lung show properties similar to those of AEpC II [24], were much higher than corresponding levels for normal pneumocytes. These findings are comparable to results in the literature, reporting that TfR expression in non proliferating cells is low or frequently undetectable. In a parallel study performed using rat alveolar epithelial cells (rAEpC) in primary culture (Widera *et al.*, 2003), data showed that TfR expression is down regulated upon transdifferentiation to the type I-like phenotype and the TfR expression might be specific for the hAEpC II phenotype. However, in our study both types showed no significant difference from the control cells [6].

The signal was higher for the two bronchial epithelial cell lines, Calu-3 and 16HBE14o-, with no significant difference between them (figure 8). Again, this result is comparable to data mentioned in literature, which indicate that TfR is expressed on rapidly dividing cells with 10,000 to 100,000 molecules per cell; commonly found on tumour cells or cell lines in culture [6]. According to the results above, we can conclude that the expression level of TfR in the cancerous cells in both alveolar and bronchial area in lung, is higher than in normal tissues. In addition, TfR were found to be expressed in bronchial tissues rather than in alveolar one (figure 9). Furthermore, CLSM of the cells (hAEpC, A549, Calu-3, 16HBE14o-) in culture proved the basolateral localisation of TfR in all cells, as well as apical localisation in cancerous cells. Consequently, application of the proposed chemotherapy by inhalation will probably enhance the uptake of the drug by the cancerous cells, while sparing the normal cells (figure 11).

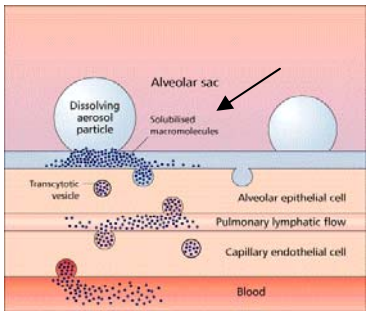


Figure 11: The arrow indicates the application side of the inhaled therapy which is faced by the apical side of the alveolar epithelial cells (taken from http://upload.lung.ch).

With the aid of these interesting findings, in which we have investigated the expression level of our target receptor, we can move further in the project to develop targeted drug delivery systems for local aerosol therapy of lung cancer, since we believe that such therapy could further increase the effectiveness of anti-tumour treatment while systemic side effects might be further reduced.

2.6 References

[1] P G Cavanaugh, L B Jia, Y Y Zou, G L Nicolson, Transferrin receptor overexpression enhances transferrin responsiveness and the metastatic growth of a rat mammary adenocarcinoma cell line, Breast Cancer Res Treat. 56(3) (1999) 201--215.

[2] D N Carney, A F Gazdar, G Bepler, J G Guccion, P J Marangos, T W Moody, M H Zweig, J D Minna, Establishment and identification of small cell lung cancer cell lines having classic and variant features, Cancer Res. 45(6) (1985) 2913--2923.

[3] M Vostrejs, P L Moran, P A Seligman, Transferrin synthesis by small cell lung cancer cells acts as an autocrine regulator of cellular proliferation, J Clin Invest. 82(1) (1998) 331--339.

[4] G L Nicolson, Paracrine and autocrine growth mechanisms in tumour metastasis to specific sites with particular emphasis on brain and lung metastases, Cancer Metastasis Rev. 12(3-4) (1993) 325--343.

[5] J F Whitney, J M Clarck, T W Griffin, S Gautsam, K O Leslie, Transferrin receptor expression in non small-cell lung cancer. Histopathologic and clinical correlates, Cancer. 76(1) (1995) 20--25.

[6] A Widera, K Beloussow, K J Kim, E D Crandall, W C Shen, Phenotype-dependent synthesis of transferrin receptor in rat alveolar epithelial cell monolayers, Cell Tiss Research. 312(3) (2003) 313--.318.

[7] L Hongyan, Z M Qian, Transferrin/transferrin mediated drug delivery, Med Res Rev. 22(3) (2002) 225--250.

[8] K R Smith, A E Aust, Mobilisation of iron from urban particulate leads to generation of reactive oxygen species *in vitro* and induction of ferritin synthesis in human lung epithelial cells, Chem Res Toxicol. 10(7) (1997) 828--834.

[9] A Gijsens, A Derycke, L Missianen, D DeVos, J Huwyler, A Eberle, P De Witte, Targeting of the photocytotoxic compound AIPcS4 to Hela Cells by transferrin conjugated PEG-Liposomes, Int J Cancer. 101 (2002) 78--85.

[10] Z M Qian, L Hongyan, S Hongzhe, H Kwokping, Targeted drug delivery via the transferrin receptor-mediated nndocytosis pathway, Pharmacol Rev. 54(4) (2002) 561--587.

[11] H Iinuma, K Maruyama, K Okinaga, K Sasaki, T Sekine, O Ishida, N Ogiwara, K Johkura, Y Yonemura, Intracellular targeting therapy of cisplatin-encapsulated transferrin-polyethylene glycol liposome on peritoneal dissemination of gastric cancer, Inter J Cancer. 99(1) (2002) 130--137.

[12] D Shad, W C Shen, Transcellular delivery of an insulin-transferrin conjugate in enterocyte-like Caco-2 cells, J Pharm Sci. 85(12) (1996) 1306--1311.

[13] A Dowlati, M Loo, T Bury, G Fillet, Y Beguin, Soluble and cell-associated transferrin receptor in lung cancer, Br J Cancer. 75(12) (1997) 1802--1806.

[14] D Sadava, T Phillips, C Lin, S E Kane, Transferrin overcomes drug resistance to artemisinin in human small-cell lung carcinoma cells, Cancer Letters. 179(2) (2002) 151--156.

[15] S Kuvibidila, T Gauthier, R P Warrier, W Rayford, Increased levels of serum transferrin receptor and serum transferrin receptor/log ferritin ratios in men with prostate cancer and the implications for body-iron stores, J Lab Clin Med. 144(4) (2004)176--182.

[16] J F Whitney, J M Clark, T W GriBin, S Gautam, K O Leslie, Transferrin receptor expression in nonsmall cell lung cancer: Histopathologic and clinical correlates, Cancer. 76 (1995) 20--25.

[17] K J Elbert, U F Schaefer, H J Schaefers, K J Kim, V H Lee, C M Lehr, Monolayers of human alveolar epithelial cells in primary culture for pulmonary drug absorption and transport studies, Pharm Res. 16 (1999) 601--608.

[18] C Ehrhardt, K J Kim, C M Lehr, Isolation and culture of human alveolar epithelial cells, Methods Mol Med. 107 (2005) 207--216

[19] S Fuchs, A J Hollins, M Laue, U F Schaefer, K Roemer, M Gumbleton, C M Lehr, Differentiation of human alveolar epithelial cells in primary culture: morphological characterization and synthesis of caveolin-1 and surfactant protein-C, Cell Tissue Res. 311 (2003) 31--45.

[20] N Demling, C Ehrhardt, M Kasper, M Laue, L Knels, E P Rieber, Promotion of cell adherence and spreading: a novel function of RAGE the highly selective differentiation marker of human alveolar epithelial type I cells, Cell Tissue Res. 323(3) (2006) 475--88.

[21] K A Foster, C G Oster, M M Mayer, M L Avery, K L Audus, Characterization of the A549 cell line as a type II pulmonary epithelial cell model for drug metabolism, Exp Cell Res. 243(1998) 359--366.

[22] J Fogh, G Trempe, Human Tumour Cells *In Vitro*, (1975) Plenum Press, New York. 115--159.

[23] A L Cozens, M J Yezzi, K Kunzelmann,T Ohrui, L Chin, K Eng, E Finkbeiner, J H Widdicombe, D C Gruenert, CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells, Am J Respir Cell Mol Biol. 10(1) (1994). 38--47.

[24] M Lieber, B Smith, A Szakal, W Nelson-Ress, G Todaro, A continuous tumour-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells, Int J Cancer. 17 (1976) 62--70.

Chapter 3

Tf conjugation to liposomal surfaces

Parts of this chapter have been published. Samah Anabousi, Michael Laue, Claus-Michael Lehr, Udo Bakowsky, Carsten Ehrhardt (2005) *In vitro* assessment of transferrin-conjugated liposomes as drug delivery systems for the inhalation therapy of lung cancer. *European Journal of Pharmaceutics and Biopharmaceutics,* **60**(2), 295-303.

3.1 Abstract

Introduction Site-specific delivery of drugs and therapeutics can significantly reduce drug toxicity and increase the therapeutic effect. Tf is a suitable ligand for conjugation to drug delivery systems to achieve site-specific targeting, due to its specific binding to TfR, expressed on several cell types of therapeutic interest.

TfRs have been reported to be highly expressed on the surfaces of tumour cells and the well characterised and efficient mechanism of internalisation of Tf has been exploited for the delivery of anticancer drugs, proteins, and therapeutic genes into primariy proliferating malignant cells. Liposomes are effective vehicles for drugs, genes and vaccines and can be easily modified with proteins, antibodies, and other appropriate ligands, resulting in attractive formulations for targeted drug delivery.

Purpose In this study, we employed atomic force microscopy (AFM) and transmission electron microscopy (TEM) to confirm the achieved conjugation of Tf to liposomes by three different coupling methods. In addition, the conventional assay for quantification of protein amount, bicinchoninic acid (BCA), and PL content (according to Steward) were performed.

Results AFM and TEM were able to display Tf-molecules on the liposomal surfaces and can be routinely used to obtain additional visual information on the protein-drug carrier conjugation in a fast and reliable manner.

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3.2 Introduction

3.2.1 Advantages of ligand-targeted liposomes

Anticancer drug therapy is fraught with systemic toxicities resulting from cytotoxicity towards normal cells. Cancer cells share many common features with the normal host cells from which they originate, so finding unique targets against which anticancer drugs can be selectively directed is difficult. Many anticancer drugs have a marginal selectivity for malignant cells because they target the reproductive apparatus in cells having high proliferation rates. However, anticancer drugs having this mechanism of action naturally result in high toxicities against rapidly dividing normal cells, for example, hair follicles, germ cells and haematopoeitic cells, leading to dose-limiting side effects like mucositis, stomatitis, alopecia and reproductive effects. The side effects associated with chemotherapy limit the dose or cumulative doses that can be administered to patients, which can lead to relapse of the tumour and often to the development of drug-resistance. The medical community has sought alternative therapies that improve selective toxicities against cancer cells, i.e., therapies that increase efficacy and/or decrease toxicity, resulting in an increase in the therapeutic indices of the anticancer drugs. Many anticancer drugs, following intravenous administration, have large volumes of distribution resulting from their rapid uptake into all the tissues of the body. One successful approach has been to use drug carriers like liposomes to alter the pharmacokinetics and biodistribution of anticancer drugs. In general, liposome encapsulation of drugs results in (sometimes dramatic) reductions in their volume of distribution and significant increases within tumour accumulation [1].

Liposomes (PL bilayer vesicles) are the most advanced of the particulate drug carriers and are now considered to be a mainstream drug delivery technology. Both classical and Stealth® liposomes rely on "passive" targeting to increase the localisation of anticancer drugs to solid tumours. Growing solid tumours, as well as regions of infection and inflammation, have capillaries with increased permeability as a result of the disease process (e.g., tumour angiogenesis). Pore diameters in these capillaries can range from 100 to 800 nm. Drug-containing liposomes that have diameters in the range of approximately 60–150 nm are small enough to extravasate from the blood into the tumour interstitial space through these pores. Normal tissues contain capillaries with tight junctions that are impermeable to liposomes and other particles of this diameter. This differential accumulation of liposomal drugs in tumour tissues relative to normal cells is the basis for the increased tumour specificity for the

liposomal drugs relative to free (non liposomal) drugs. In addition, tumours lack lymphatic drainage and therefore, there is low clearance of the extravasated liposomes from tumours.
Passive targeting can result in increases in drug concentrations in solid tumours of several-fold relative to those obtained with free drugs. The mechanism of action of the liposomal drugs is thought to result from sustained release of drug from the liposomes and diffusion of the released drug throughout the tumour interstitial fluid, with subsequent uptake of the released drug by tumour cells.
In attempts to increase the specificity of interaction of liposomal drugs with target cells and to increase the amount of drug delivered to these cells, recent efforts in the liposome field have been focusing on the development of ligand-targeted liposomes (LTLs). These liposomes utilise targeting moieties coupled to the liposome surface to selectively deliver the drug-liposome package to the desired site of action (active targeting) [2].
While ligands can be readily attached to the surface of either classical or Stealth® liposomes, ligand-targeted Stealth® liposomes have clear pharmacokinetic advantages over ligand-targeted classical liposomes for *in vivo* applications, and the former are used almost exclusively for applications involving active targeting. The main advantages of LTLs are that: Relatively few ligand molecules per liposome (10–20) are required to selectively deliver high payloads of drugs to target cells via the mechanism of receptor-mediated internalisation. Unlike other delivery systems such as drug-ligand conjugates or ligandotoxins, which deliver few molecules of drug or toxin (<10) per ligand molecule, LTLs can be exploited to deliver thousands of molecules of drug using few tens of molecules of ligands on the liposome surface [3].

3.2.2 Choice of target receptor

It was the German bacteriologist Paul Ehrlich who in late nineteenth century, coined the term "magic bullet", meaning a chemical that travels through the body and selectively kills diseased cells without harming neighbouring healthy ones. Drug delivery with "magic bullets" to cells, tissues or organs is now medically described as "active targeting". With the availability of suitable ligands, this approach takes advantage of relatively abundant expression of a particular receptor on the target cell relative to non-target cells [4,5].
Targeting moieties may include antibody molecules or fragments thereof, low molecular weight compounds, naturally occurring or synthetic ligands like peptides, carbohydrates, glycoproteins, or receptor ligands; that is essentially any molecule that selectively recognises

and binds to target antigens or receptors over-expressed or selectively expressed on cancer cells can serve as a targeting moiety.

3.2.3 Internalisation

The ability of the target cell to internalise LTLs is an important selection criterion in choosing a targeting ligand. If the ligand triggers receptor-mediated internalisation of the entire liposome–drug package into the cell interior, then arguably more drug will be delivered to the target cells. This mechanism should work well for drugs like DOX or methotrexate that escape degradation by lysosomal enzymes and low pH. However, for drugs like cytosine arabinoside (ara-C) that are acid labile and/or do not survive lysosomal degradation, liposomal targeting to internalising epitopes will be less efficacious.

If non-internalising ligands are used, liposome contents will be released over time at or near the cell surface, and (a portion of) the released drug will enter the cell by passive diffusion or other normal transport mechanisms. Although increased concentrations of drug may be achieved at the cell surface by this mechanism, it can be argued that, in the dynamic *in vivo* environment (e.g., plasma or lymph), the rate of diffusion and redistribution of the released drug away from the cell will exceed the rate at which the drug enters the cell, particularly for drugs like DOX, which have a large volume of distribution.

Targeting to non-internalising epitopes might be efficacious in solid tumours through the bystander effect, in which cells lacking the target epitope can be killed by drug released at the surface of neighbouring cells having bound liposomes. Internalisation of ligands into the target cells is also required for other targeted therapeutics, such as immunotoxins and antibody–drug conjugates. Internalisation of ligand-targeted therapeutics depends on various factors such as type of receptor or epitope, antigen/receptor density, antibody valency, and rate of internalization and re-expression of the target epitope [6-10].

3.2.4 Chemical linkage

Various therapeutic agents have been chemically linked to Tf. Several studies have been reported to link DOX with Tf via the formation of a Schiff base. Glutaraldehyde has frequently been used for this purpose. Briefly, certain amounts of Tf and DOX both dissolved in 150 mM NaCl were directly mixed and followed by the addition of glutaraldehyde (in 150 mM NaCl) drop wise. The coupling procedure was stopped by the addition of ethanolamine. The conjugate was subjected subsequently to purification and characterisation. The conjugates prepared in such a way were found to exhibit cytotoxicity.

Although direct coupling methods are easy to carry out, they have some disadvantages in that polymeric products are likely to be formed during the preparation, and the resulting conjugates are poorly defined chemically with respect to the link between drugs and carrier proteins. A new coupling approach has been attempted, in which the stability of the bond between the Tf and the drug can be finely tuned. This was achieved by synthesis of the first derivatising the drug with a spacer group, such as a maleimide spacer, and then attaching the drug derivative to the carrier protein (e.g., Tf). In this way, the bond between the drug and the spacer can act as a cleavage site, allowing the drug to be released inside the cells [11].

A liposomal carrier system, which was produced by using SUVs made of pure PLs chemically cross-linked to human Tf, liposome-entrapped α-interferon (α-IFN) when conjugated with Tf-polylysine (Tf-PLL) exhibited the antiproliferative effect against murine bladder tumour cell MBT2. Cell uptake of Tf-PLL-liposome was markedly enhanced in a dose-dependent manner. There was also a strong correlation between anti-proliferative activity and uptake of liposome by the tumour cells, indicating that Tf-PLL-liposome promotes intracellular delivery of α-IFN and enhances the effect of α-IFN against MBT2 cell growth [12].

Tf-pendant type immunoliposome (TF-PEG-ILP) was shown to have a higher uptake in K-562 cells *in vitro* compared with non-targeted liposomes. The TF-PEG-ILP, examined in the B16 melanoma-bearing mice, exhibited a prolonged circulation time, a low liver uptake and concomitantly high accumulation into the tumour tissue and a longer residence time. Liposomes conjugated with anti-TfR have also been used for specific drug delivery [12]. A liposome-immoblised anti-Tac (a monoclonal antibody directed against the IL-2 receptor) and anti-TfR (a monoclonal antibody directed against TfR) were compared for specific binding, internalisation, and intracellular drug delivery to adult T-cell leukaemia (ATL). The authors found that drug-containing liposomes conjugated to anti-Tac were more than tenfold times more effective in inhibiting growth of ATL cells than the nonspecific control conjugates. Anti-Tac conjugates caused minimal growth inhibition of Molt-4 cells over the concentration range effective against the ATL cells. Anti-TFR-coupled liposomes gave better growth inhibition of HUT-102 and MT-1 cells (40- to 60-fold) than anti-Tac conjugates. Both anti-Tac-directed and anti-TFR-directed liposomes are effective for intracellular drug delivery to ATL cells and may represent a useful method of treatment in this disease. [13].

3.2.5 Techniques for coupling ligands to liposomes

A variety of techniques for coupling targeting ligands to liposomes have been described. In general, coupling methods for the formation of LTLs should be simple, fast, efficient, and reproducible, yielding stable, non-toxic bonds. The biological properties of the ligands, e.g., target recognition and binding efficiency, should not be substantially altered by attachment to the lipids. The LTLs should have stabilities and circulation half-lives long enough to allow them to reach and interact with the target cells. Further, the coupling reaction should not alter the drug loading efficiency and drug release rates in a negative way [8].

Several methods have been developed for coupling water-soluble proteins to the surfaces of liposomes in attempts to prepare stable liposomes with high binding-affinity for cell membranes. Such proteoliposomes may serve as efficient carriers for transfer of drugs, enzymes and nucleic acids into cells.

A particular useful protein-liposome coupling procedure involves covalent modification of functional groups on the proteins with lipid residues, imparting to the proteins increased hydrophobicity and affinity for liposomal membranes. Using this type of approach to prepare homogeneous liposomes with defined properties, it is essential to understand in more detail how lipid-modified proteins interact with each other in aqueous solution and incorporate into liposomal membranes [7].

A number of possibilities for covalent attachment of ligands to sterically-stabilised liposomes exist. Ligands can be linked to polar headgroups of PLs forming sterically stabilised liposomes (S-liposomes) (figure 12A). Alternatively, the attachment site can be positioned at the end of the polymer chain using some of the liposomes-grafted PEG chains as macromolecular spacers between the antibody and the liposomes surface (figure 12B).

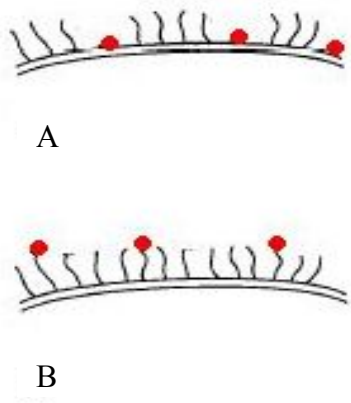


Figure 12: Different approaches for coupling of proteins to sterically stabilised liposomes.

3.2.6 Coupling strategies

Initially, ligands were coupled to PL headgroups, e.g., phosphatidylethanolamine, at the surface of Stealth® liposomes, but the steric barrier imparted by the PEG polymer resulted in low coupling efficiencies and interfered with target binding of the LTLs, particularly when higher concentrations of PEG with high molecular weights were present. To avoid this, ligands were coupled to the PEG terminus. This strategy avoids masking of the ligands by the PEG layer, and provides perfect accessibility to the ligand molecules for their target cells. Several end-functionalised derivatives of PEG have been synthesised for coupling antibodies to the PEG-terminus [8].

One of the commonly used coupling groups is malemide (Mal)-PEG. Mal-PEG coupling methods rely on the formation of thioether bonds between proteins and liposomes, which results in efficient formation of a stable bond. In this method, protein molecules are thiolated using 2-iminothiolane (Traut's reagent) and reacted with maleimide groups on the PEG-termini. A disadvantage with this method is that the free thiol groups may react among themselves to produce disulphide bonds leading to cross-linking of proteins molecules or proteioliposomes, causing unwanted aggregation and too rapid clearance from circulation. However, the presence of PEG serves to inhibit aggregation to a large extent. Another problem is that the random introduction of thiol groups in the protein molecule may interfere with the biological properties of the molecule, leading to interference with the binding of the protein to its receptor, receptor activation and/or endocytosis.

The other covalent bond which can be used is the amide bond, which could be established to attach the protein to the liposomal surface. *N*-glutaryl PE or DSPE-PEG-COOH are used as a membrane anchors for coupling of Tf to the liposome surface. In this method, the free amino groups of proteins are linked to the carboxylic carrier in the presence or absence of water-soluble carbodiimide and using N-hydroxysulfosuccinimide as a carboxyl activation agent. The advantage of this method is that it is not necessary to modify the protein prior to the coupling reaction.

In a different approach, Stealth® liposomes can be converted into LTLs by a versatile 'post insertion technique'. Ligands are coupled to end-functionalised groups in PEG micelles and the ligand-PEG conjugates are then transferred in a simple incubation step from the micelles into the outer monolayer of pre-formed, drug-loaded liposomes. This method allows a combinatorial approach to the design of targeted liposomes that minimises manufacturing complexity, allowing a variety of ligands to be inserted into a variety of pre-formed liposomes containing a variety of drugs. This allows the ligand-targeted therapeutics to be tailored to

patient's disease profile without the need for separate manufacturing procedures for each ligand/drug combination. Also, since the conditions for the successful insertion of the ligand is now decoupled from the conditions for the preparation of and loading of drug into liposomes, conditions can be optimised for independently drug loading and ligand insertion. Targeted liposomes prepared using the post-insertion approach have been shown to have *in vitro* drug leakage rates, cell association and cytotoxicity profiles and therapeutic efficacies comparable to those of liposomes prepared by conventional coupling procedures like the Mal-PEG coupling method [9].

3.2.7 Transmission electron microscopy

An electron microscope visualizes the structural information carried by scattered electrons. In a fixed-beam transmission electron microscope, scattered electrons emerging from the irradiated sample are collected over a narrow solid angle and focused by the objective lens onto the image plane as seen in figure 13 A. Here, elastically scattered electrons interfere with the unscattered electrons to produce a phase-contrast image, whereas the inelastically scattered electrons generate a diffuse background image that can, in some microscopes, be eliminated by an energy filter. In the transmission electron microscope (TEM), which was first introduced in the late sixties, the objective lens focuses the beam onto an atomic-scale sample volume. All scattered electrons can then be collected by a variety of detectors placed behind the specimen and their information exploited to the fullest extent.

An image is generated simply by stepping the focused beam along a sampling grid. Therefore, the image may be considered as a large collection of individual scattering experiments. Various types of signals that can be discriminated by scattering angle and energy-loss transfer convey different structural and chemical information and may be captured simultaneously in different channels. This sequential and controlled acquisition of information lends itself to quantitative analyses that are difficult to realize with other instruments. In addition, as there is no limitation on the solid angle and the energy-loss interval over which the scattered electrons may be collected; 60-100% of them contribute to the image. This provides a unique opportunity to image beam-sensitive biological macromolecules at low dose [14, 15].

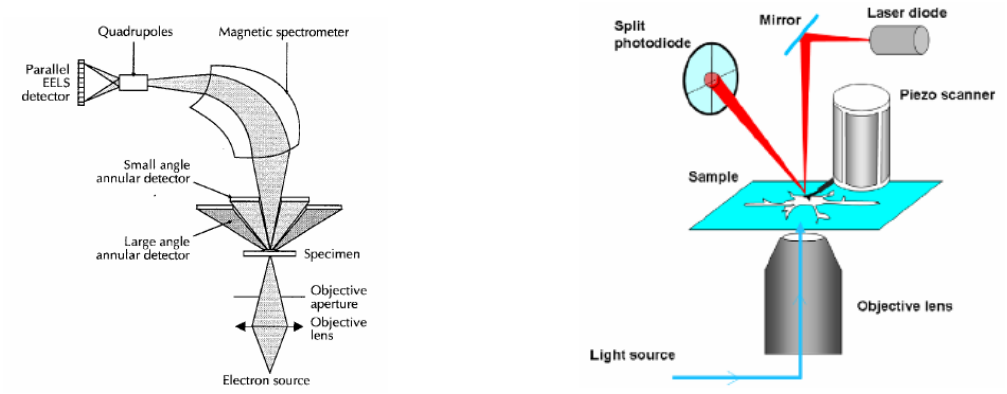
3.2.8 Atomic force microscopy

AFM belongs to the broad family of scanning probe microscopes in which a proximal probe is exploited for investigating properties of surfaces with subnanometer resolution. At the beginning the emphasis was mainly on the improved imaging resolution compared to that of

optical microscopy, but, soon after, it became clear that AFM was much more than just a high-resolution microscope. The possibilities of spectroscopic analysis, surface modification and molecular manipulation gave rise to a real breakthrough in the realm of AFM use. In biological applications, the most appealing advantage of the AFM as a high-resolution microscope in comparison with other techniques such as TEM is that it allows measurements of native biological samples in physiological-like conditions avoiding complex sample preparation procedures and artefacts connected with them. The use of mild imaging conditions opened the way to dynamic studies in which conformational changes and molecular interactions could be followed in real time at the single-molecule level.

The set of samples of biological interest studied by AFM ranges nowadays from the smallest biomolecules, such as phospholipids, proteins, DNA, RNA, to subcellular structures (e.g., membranes), all the way up on scale to living cells and tissues. Not only structural properties can be investigated, but also mechanical or chemical and functional properties are the focus of many AFM applications.

As illustrated in figure 13 B, the AFM works by scanning, in a raster fashion, a very tiny tip mounted at the end of a flexible micro-cantilever in gentle contact with the sample. This relative motion is performed with sub-Ångström accuracy by a piezoelectric actuator (usually a tube, sometimes a tripod). Interacting with the sample the cantilever deflects and the tip–sample interaction can be monitored with high resolution exploiting a laser beam impinging on the back of the cantilever. The beam is reflected towards a split photo-detector giving a sort of optical lever which amplifies cantilever deflections. In almost all operating modes, a feedback circuit, connected to the cantilever deflection sensor and to the sample vertical piezo element, keeps tip–sample interaction at a fixed value by controlling the tip–sample distance. The amount of feedback signal, measured at each scanning point of a 2D matrix, is used to form a 3D reconstruction of the sample topography which is then displayed as an image [16, 17].



A **B**

Figure 13: Schematic diagram of A) TEM (taken from 14) B) AFM (taken from 16).

In work reported here, three different methods of linking Tf to DSPC/Chol liposomes were used in a comparative manner [10,18-22]. The goal of this study has been to visualise the actual protein binding, using two independent microscopic methods, i.e., AFM and TEM. AFM is a surface analytical method that can generate nano-scale topographic images by scanning a fine silicon tip across a surface. For its ability to provide high-resolution imaging under physiological, non-destructive conditions without the necessity of previous fixation of the sample, AFM has developed into a powerful tool for studying structural details. TEM is widely used to image structures near the atomic level. While the maximum resolution is lower in TEM than in AFM, the contrast and image formation is much better understood in TEM than in AFM. Thus, these two imaging methods may provide complementary information and support each other.

3.3 Materials and methods

3.3.1 Materials

Reagents were obtained from the following sources; 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-glutaryl (*N*-glutaryl-PE), 1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol) 2000] (DSPE-PEG_{2000}-COOH) were purchased from Avanti Polar Lipids (Alabaster, AL). N-(3-Dimethylaminopropyl)-N´-ethylcarbodiimide hydrochloride (EDC), human holo-transferrin, 2-iminothiolane (Traut's reagent), Sepharose CL-4B, goat anti-human transferrin antibody and the BCA kit for protein determination were from Sigma (Seelze, Germany). Chol was obtained from Riedel-de Haën (Seelze, Germany). Sulpho-N-hydroxysuccinimide (S-NHS) was obtained from Perbio Science (Bonn, Germany). 1,2-Distearoyl-*sn*-glycero-3-phosphocholine-(polyethylene glycol) 2000-maleimide (DSPE-PEG_{2000}-MAL) was from Nektar (Huntsville, AL). Centrisart-10 and -20 (molecular weight cut off: 10 kDa and 20 kDa, respectively) concentrators were from Vivascience (Hannover, Germany).

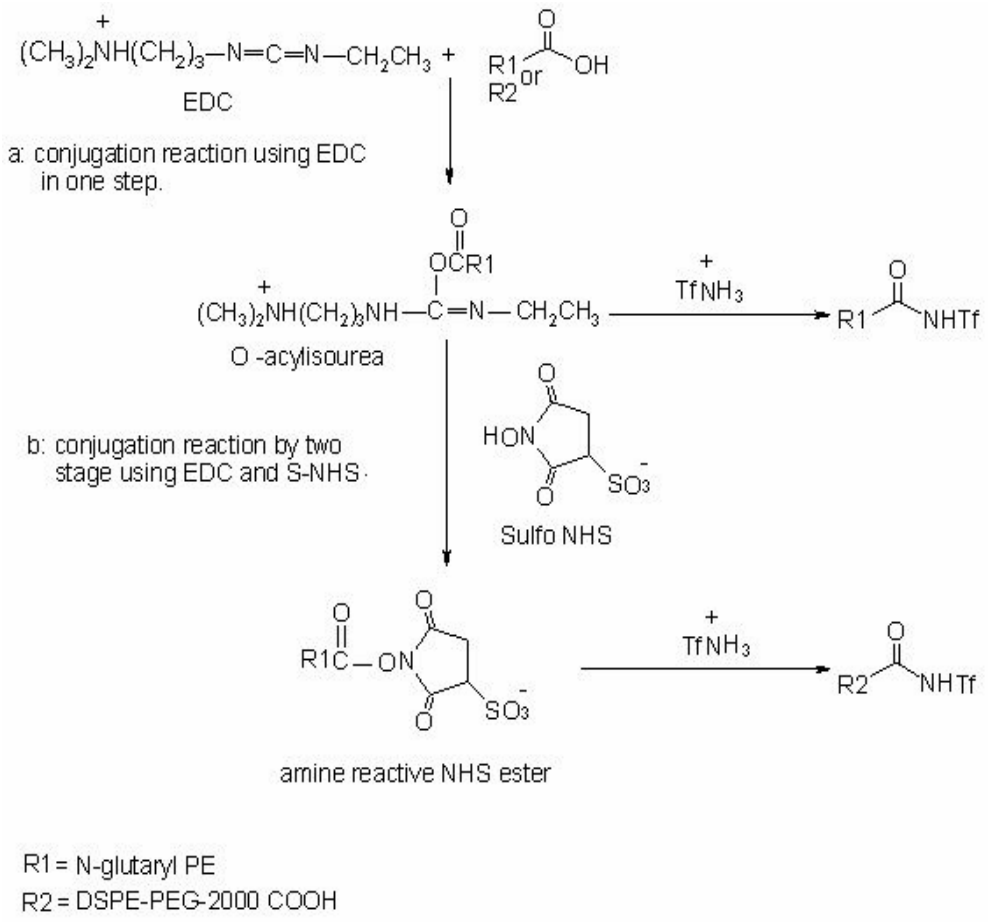
3.3.2 Liposome preparation

The liposomes were prepared from Chol, DSPC and the corresponding linker lipid at the following molar ratios: DSPC:Chol:linker (6:3:0.6 mol%). Briefly, a mixture of PL and Chol in chloroform:methanol (2:1) was dried to a thin lipid film in a rotary evaporator (Büchi, Essen, Germany). The solution to be encapsulated was then added to result in a final lipid concentration of 10 mg lipid/ml corresponding buffer. After vortexing, the sample was incubated for 10 min at a temperature above the transition temperature of the lipids (53°C for DSPC:chol) in a cabinet drier [23]. Unilamellar liposomes were prepared by extruding the resulting multilamellar vesicles eleven times through a 200-nm polycarbonate membrane, followed by extrusion eleven times through a 100-nm membrane using a Liposofast Basic Device (Avestin, Mannheim, Germany).

3.3.3 Coupling of transferrin to the liposomes

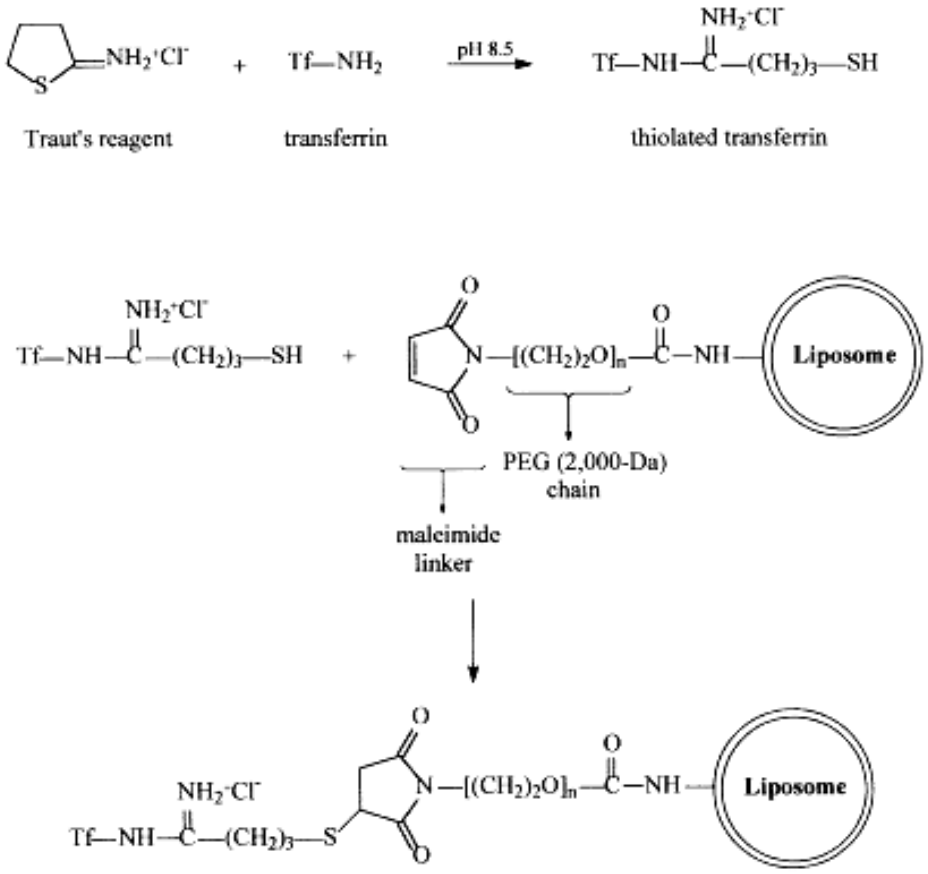
In the first two methods, an amide bound between *N*-glutaryl-PE or DSPE-PEG_{2000}-COOH as linker lipid is formed to link Tf to the liposome surface. In both methods, a bond between free amino groups of the protein and carboxylic groups of the linker is formed in the presence of a water-soluble carbodiimide (Scheme 1). The difference, however, is that method 2 uses

sulpho-N-hydroxysuccinimide (S-NHS) in addition to N-(3-dimethylaminopropyl)-N´-ethylcarbodiimide hydrochloride (EDC). S-NHS is a water soluble analogue to NHS which is used to modify carboxyl groups to amine-reactive NHS esters. This is accomplished by mixing the NHS with a carboxyl containing molecule and a dehydrating agent, EDC, which reacts with the carboxyl group first and forms an amine-reactive intermediate, an O-acylisourea.



Scheme 1: The expected coupling reaction of transferrin to the surface of liposomes using *N*-glutaryl PE and DSPE-PEG_{2000}-COOH as linker lipids.

Method 3 relies on the formation of thioether bonds between the protein and the liposome. Here, the Tf molecules are thiolated using Traut's reagent and react with maleimide groups on the PEG-terminus (scheme 2).



Scheme 2: The expected coupling reaction of transferrin to the surface of liposomes

Method 1: N-glutaryl-PE. 2 mg EDC were added per 1 µmol of lipid in PBS and incubated for 6 h at room temperature. Excess EDC was then removed by ultrafiltration using a Centrisart-10 concentrator. In the next step, 125 µg Tf/µmol PL was added and incubated overnight at room temperature. Free Tf was separated from the liposomes by passing the liposome suspension through a Sepharose CL-4B gel [18,19].

Method 2: DSPE-PEG$_{2000}$-COOH. The lipids were re-suspended in an appropriate amount of 400 mM citrate/5 mM phosphate buffer (pH 4.0). Then, 1 ml PBS (pH 7.5) and 360 µl of both EDC (0.25 M in H_2O) and S-NHS (0.25 M in H_2O) were added per 10 µmol of lipid. The mixture was allowed to incubate for 10 min at room temperature, before adjusting to pH 7.5 with 1 M NaOH. 125 µg Tf/µmol PL was added and gently stirred for 8 h at 4°C. Unbound

protein was removed by passing the liposome suspension through a Sepharose CL-4B gel column [20].

Method 3: DSPE-PEG$_{2000}$-MAL. The lipid mixture was hydrated with PBS (pH 7.4). Then, 125 µg Tf/µmol PL (in PBS) was added to 2 ml borate-EDTA buffer (0.15 M Na borate, 0.1 mM EDTA, pH 8.5) containing 400 nmol of fresh Traut's reagent. This mixture was incubated in the dark for 60 min on a rotational shaker (at 110 rpm). The thiolated Tf was concentrated by ultrafiltration (Centrisart-20) to a volume of 0.2 ml, diluted with 2 ml PBS (pH 8.0) and concentrated again to 0.2 ml. The Tf was immediately added to the liposomes and left to react overnight at room temperature without further agitation. Lastly, the transferrin-conjugated liposomes were separated from free Tf by Sepharose CL-4B gel filtration [20,21].

3.3.4 Photon correlation spectroscopy

Liposomal size determination of Tf-conjugated and plain liposomes was carried out using a Zetasizer 3000 HS (Malvern Instruments, Herrenberg, Germany) equipped with a photon correlation spectroscopy unit. The scattered light was detected at a scattering angle of 90°. Measurements were performed at 25°C. For all measurements, samples were diluted 50-fold in distilled water to obtain comparable viscosities. PCS gives information about the mean diameter of the bulk liposome population and the width of the diameter distribution via the polydispersity index (PI). Mean values and standard deviation were calculated from at least three determinations.

3.3.5 ζ-potential measurements

The ζ-potential measurements of the Tf-conjugated and plain liposomes were carried out in the standard capillary electrophoresis cell of a Zetasizer 3000 HS at pH 7.4 in the presence of NaCl to adjust the conductivity to 50 µS/cm. Measurements were performed at 25°C with automatic duration. The instrument was routinely calibrated with a –50 mV latex standard (Malvern). The electrostatic mobility was converted into the ζ-potential using the Helmholtz-Smoluchowski equation. The mean values and standard deviation were calculated from three independent measurements (three runs each).

3.3.6 Phospholipid concentration

PL concentration was assayed according to Stewart's protocol [24]. Briefly, the standard curve was obtained by adding 2 ml of ferrothiocyanate reagent to different concentrations of DSPC, ranging from 0 to 0.5 mg/ml. The test tubes content was vortexed vigorously for 15

sec, centrifuged at 1000 rpm for 5 min and the lower layers were removed with a Pasteur pipette. Absorbance of the formed complex was assessed using a plate reader (Cytofluor II, PerSeptive Biosystems, Wiesbaden, Germany) at a wavelength of 485 nm. Liposome samples were treated similarly after dehydrating the aliquots of liposome suspension (100µl) under a nitrogen stream and re-dissolving them in 2 ml of chloroform.

3.3.7 Protein assay and Tf-binding efficacy

The average amount of transferrin conjugated to the liposome (i.e., amount of PL) was quantified as described by Derycke *et al.* [22]. 100 µl of liposome suspension were added to 400 µl of methanol. The mixture was vortexed and centrifuged (10 sec at 9,000 g). Then, 200 µl of chloroform were added and the sample was vortexed and centrifuged again (10 sec at 9,000 g). For phase separation, 300 µl of water were added and the sample was vortexed again and centrifuged for 1 min at 9,000 g. The upper phase was carefully removed and discarded. Three hundred µl of methanol were added to the chloroform phase and the interphase with the precipitated protein. The sample was mixed and centrifuged to pellet the protein (2 min at 9,000 g). The supernatant was removed and the protein pellet was dried under a stream of air. The pellet was then dissolved in 20 µl of PBS (pH 7.4) and the concentration was determined with BCA protein assay using pure holo-transferrin as standard. The coupling efficiency was calculated as µg Tf/µmol PL.

3.3.8 Transmission electron microscopy

The coupling of transferrin to liposomes was assessed by transmission electron microscopy using two approaches: negative staining and immuno electron microscopy.

For negative staining, a formvar-coated copper grid (300 mesh, hexagonal fields) was placed on 15 - 30 µl droplets of liposome suspensions for 2-3 min at room temperature. To improve adhesion of the liposomes on the formvar film, grids were pre-treated with glow discharge in the argon plasma of a sputter coater for 2 min. After adhesion of liposomes, grids were washed on four droplets of MilliQ water before they were placed on a drop of uranyl acetate (2%) for 2 min. Finally, grids were dried at room temperature after removing the excess liquid.

Immuno electron microscopy was done with liposome suspensions after filtration through a centrifugal filter (molecular weight cut off 100 kDa; Microcon, Millipore, Schwalbach, Germany) in order to reduce the amount of free unbound transferrin. Droplets (10-15 µl) of liposomal suspension were put on parafilm and brought in contact with a copper grid for 2-3

min (see above). Grids were then washed twice with PBS and pre-treated for 2 min with PBS/glycine (50 mM) followed by TBS/BSA-C (0.1% acetylated bovine serum albumin in Tris-buffered saline; Aurion, Wageningen, The Netherlands) for 2 min to block non specific binding. The anti-transferrin antibody was diluted at 1:20 in TBS/BSA-C and applied for 15 min at room temperature, followed by washing twice with TBS/BSA-C. Liposomes were finally negatively stained with uranyl acetate (see above).

Transmission electron microscopy was performed with a Tecnai 12 Biotwin (FEI Co., Eindhoven, The Netherlands) using 120 kV acceleration voltage. Images were recorded with a CCD camera (Megaview III; Soft Imaging Systems, Münster, Germany) at a resolution of at least 1376X1032 pixels.

3.3.9 Atomic force microscopy

The liposomal formulations with and without Tf modification were prepared as described above and diluted in ultrapure water (MilliQ, 18.4 MΩ, pH 5.5). Not later than one hour after preparation, the liposomes were directly transferred onto a silicon chip by dipping it into the liposome suspension. Atomic force microscopy was performed on a Digital Nanoscope IV Bioscope (Veeco Instruments, Santa Barbara, CA). The microscope was vibration-damped. Commercial pyramidal Si_3N_4 tips (NCH-W, Veeco Instruments) on a cantilever with a length of 125 µm, a resonance frequency of about 220 kHz and a nominal force constant of 36 N/m were used. All measurements were performed in tapping mode to avoid damage of the sample surface. The scan speed was proportional to the scan size and the scan frequency was between 0.5 and 1.5 Hz. Images were obtained by displaying the amplitude signal of the cantilever in the trace direction, and the height signal in the retrace direction, both signals being simultaneously recorded as described previously [25]. The results were visualised either in height or in amplitude mode.

3.3.10 Statistical analysis

Results are expressed as mean ± S.D. Significance ($P < 0.05$) of differences in the size, polydispersity (PI) and zetapotential values from several ($n \geq 3$) data groups were determined by one-way analysis of variances (ANOVA), followed by Neumann-Keuls-Student post-hoc tests.

3.4 Results

3.4.1 Characterisation of liposomes

The physical properties of the liposomes before and after coupling of Tf are given in tables 2 and 3. The size of the liposomes before conjugation of Tf was in the range of 120 nm for method 1 (*N*-glutaryl-PE) and 150 nm for method 2 (DSPE-PEG_{2000}-COOH), while liposomes prepared according to method 3 (DSPE-PEG_{2000}-MAL) were of a size around 165 nm. After the addition of Tf, the average sizes increased between 5 to 10 nm for all samples under investigation, with the lowest increase (i.e., ~ 3 nm) for method 3 and increases of ~ 7, 6 nm for methods 1 and 2, respectively. The PI did not show any significant alteration, indicating that the stability of the liposomes was not negatively affected by any of the methods.

DSPC is slightly negatively charged at pH 7.4, and this imparted a negative charge on the surface of blank liposomes Values for the ζ-potential were between – 20 mV and – 40 mV for the Tf-free liposomes. In case of *N*-glutaryl-PE (method 1) and DSPE-PEG_{2000}-COOH (method 2), the conjugation of negatively-charged Tf (IP 5.9) resulted in a significant decrease of the ζ-potential, while no significant change could be observed for liposomes prepared according to method 3 (DSPE-PEG_{2000}-MAL).

Table 2. Size and polydispersity index (PI) of liposomes before and after coupling of transferrin determined optically (values represent the mean ± SD of three batches).

Liposome composition	Before addition of transferrin		After addition of transferrin	
	Size (nm)	PI	Size (nm)	PI
DSPC:chol:*N*-glutaryl-PE	121.40±5.57	0.250±0.03	129.00±1.66	0.230±0.01
DSPC:chol:DSPE-PEG_{2000}-COOH	148.43±2.97	0.197±0.02	154.80±0.85	0.157±0.01
DSPC:chol:DSPE-PEG_{2000}-MAL	166.00±10.41	0.133±0.02	169.33±8.76	0.147±0.01

Table 3. ζ-potential of liposomes before and after coupling of transferrin at pH 7.4 (values represent the mean ± SD of three batches).

Liposome composition	Before coupling (mV)	After coupling (mV)
DSPC:chol:*N*-glutaryl-PE	-31.67±1.53	-42.33±2.08
DSPC:chol:DSPE-PEG_{2000}-COOH	-20.67±1.15	-32.33±2.08
DSPC:chol:DSPE-PEG_{2000}-MAL	-21.67±1.15	-21.33±1.53

3.4.2 Tf-binding efficacy

The amounts of Tf (assessed by the BCS assay) in correlation with the amount of total PL in the different formulations are given in table 4. While the amount of PL was comparable between all three methods, there were significant differences in the protein content of the samples. Method 2 resulted in 108.76 µg Tf / µmol PL, method 1 in 84.93 µg Tf / µmol PL, and method 3 exhibited the lowest protein content with 11.87 µg Tf / µmol PL.

Table 4. Transferrin amount, PL amount and coupling efficiency using three different linker lipids (values represent the mean ± SD of three batches).

Liposome composition	Tf amount (µg/ml)	PL amount (mg/ml)	Coupling efficiency (µg Tf/µmol PL)
DSPC:chol:*N*-glutaryl-PE	840.57±43.43	8.26±0.20	84.93
DSPC:chol:DSPE-PEG_{2000}-COOH	1075.69±35.59	7.44±0.22	108.76
DSPC:chol:DSPE-PEG_{2000}-MAL	124.82±12.45	8.35±0.10	11.87

3.4.3 Transmission electron microscopy

The different preparations of liposomes were visualised by transmission electron microscopy after negative staining with uranyl acetate. Transferrin-conjugated liposomes revealed a particulate surface coating, which was absent in the corresponding unconjugated liposome preparations (figure 14 A, B). Surface particles on transferrin-conjugated liposomes showed a maximal length of about 10 ± 3.6 nm (n = 50). The space between the transferrin-conjugated

liposomes was also filled with particulate material, while in preparations of unconjugated liposomes, the background between the liposomes appeared to be unstructured. The degree of particulate decoration varied among the three coupling methods. Liposomes produced according to method 2 showed a higher density of particles on their surfaces compared to liposomes produced according to methods 1 and 3 (data not shown).

In order to investigate whether the particulate decoration found on the conjugated liposomes was due to binding of transferrin, specific antibodies raised against human transferrin were added before the negative staining procedure. Liposome suspensions were filtered with a centrifugal filter to remove unbound protein or reagents prior to the measurement. As a result, the transferrin-conjugated liposomes were clearly decorated by fluffy dark structures. In some cases these structures were arranged on the liposome surface like spokes on a hub (figure 14 C). Non conjugated liposomes only occasionally showed a slight decoration with similar structures at their surface (figure 14 D). The different decoration of conjugated and non conjugated liposomes is indicative of antibody complexes in conjugated liposomes. Taken together, these data suggest that transferrin molecules were exposed on the surface of transferrin-conjugated liposomes.

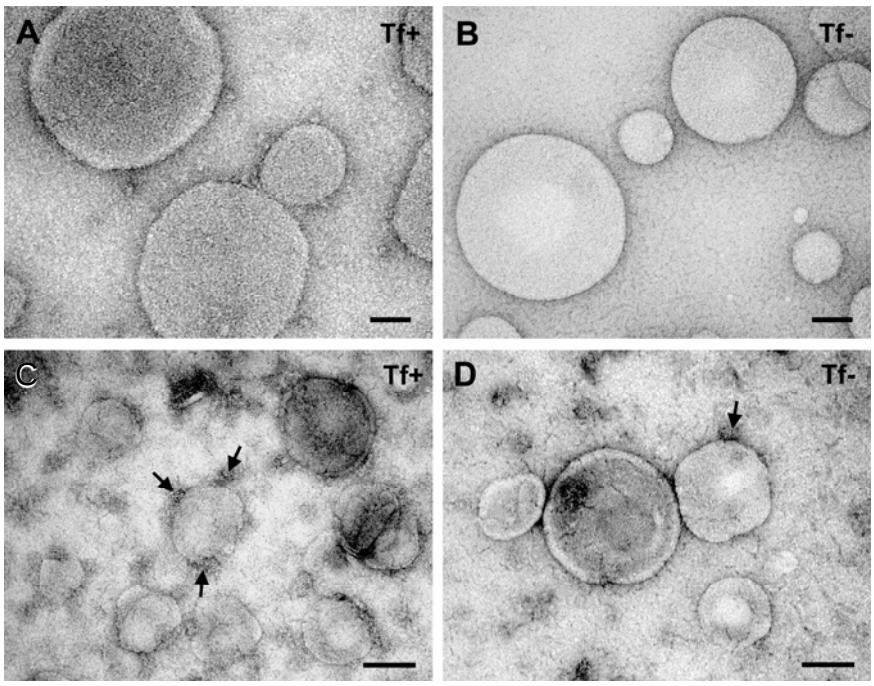


Figure 14: TEM of liposomes prepared with DSPE-PEG_{2000}-COOH (method 2) Bar = 50 nm.. **A, B** Liposomes after negative staining with uranyl acetate. Liposomes that have been conjugated with transferrin (A, Tf+) display small particles on their surface, while control preparations (B, Tf-) appear to be smooth and undecorated. **C, D** Liposomes immunolabelled with anti-transferrin antibody. The conjugated liposomes (**C**, Tf+) reveal a fluffy darkly stained decoration (arrows). In the control (**D**, Tf-) only a few liposomes are slightly decorated. Note the background between the liposomes is decorated equally in both preparations.

3.4.4 Atomic force microscopy

Atomic force microscopy was employed to visualise all liposomal formulations under wet conditions and to confirm the particle size and morphology measurements performed by PCS and TEM. For size determination, all visible particles within a representative scan area were individually evaluated. The obtained data can generally deviate from the results of PCS measurements, because of an interaction of the soft and flexible liposomes with the surface of the silicon wafer. However, the individual particle evaluation allowed excluding such artefacts from further analysis.

The plain liposome formulations using *N*-glutaryl-PE, DSPE-PEG_{2000}-COOH, and DSPE-PEG_{2000}-MAL showed average vesicle sizes of 165 ± 16 nm (n = 45), 157 ± 12 nm (n = 57), and 172 ± 9 nm (n = 29), respectively. PCS produced respective values of 121 nm, 148 nm, and 166 nm (table 2). The covalent coupling of Tf to the liposome surface led to an increase in diameter between 5 and 15% (192 ± 22 nm for *N*-glutaryl-PE, 168 ± 17 nm for DSPE-PEG_{2000}-COOH, and 180 ± 12 nm for DSPE-PEG_{2000}-MAL). In the case of both plain and modified liposomes produced according to method 1, the vesicle morphology was relatively unstable on the silicon support, resulting in a liposome spreading into flat rafts with sizes between 300 nm and 900 nm (data not shown). The incorporation of PEGylated lipids into the liposomes induced a steric stabilisation in the other two formulations with liposomes found to be round and of spherical shape (figure 15).

The surface of plain liposomes was always smooth and no structures could be observed (figure 15 A, B). When Tf was linked to the liposomes, small globular structures, localised on the liposomal surface became visible, exhibiting the highest rate of appearance in liposomes produced according method 2 (figure 15 C, D). These particles had a size of 10.53 ± 1.45 nm (n = 27), comparable to data assessed by TEM. From the molecular weight, the size of a single Tf molecule can be calculated to be 4-5 nm [26]. The determined size was slightly larger, indicating the formation of associates comprising of two or three Tf molecules.

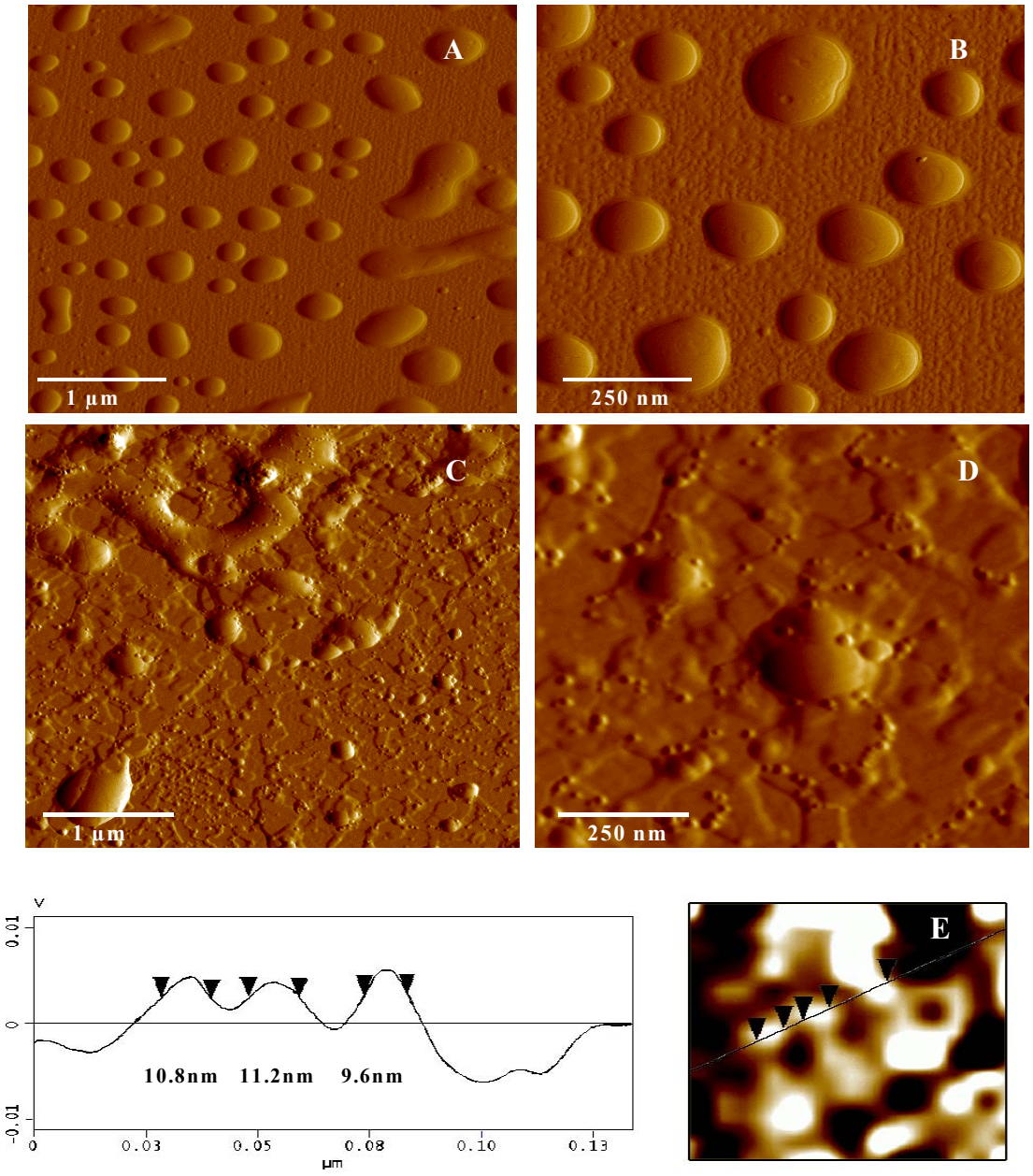


Figure 15: AFM images of liposomes prepared with DSPE-PEG_{2000}-COOH. **A, B** Plain liposomal formulations. The liposomes show a smooth surface morphology. **C, D** Liposomes covalently modified with Tf. Small globular structures are visible at the surface. **E** Line scan of liposome surface. The globular structures were measured to be 10.53 ± 1.45 nm in diameter.

3.5 Discussion

It has been the goal of this study to visualise the binding of a protein, transferrin, to a colloidal drug carrier system using two independent microscopic methods, AFM and TEM. To achieve this, ligand-modified DSPC/Chol liposomes were prepared according to three previously published methods and investigated with regard to their physico-chemical properties and efficacy of Tf-modification.

AFM and TEM were able to detect Tf at the liposomal surface on the molecular level in a fast and reproducible manner. Both microscopic techniques can deliver semi-quantitative visual information on the actual functionalisation of nano-scale drug carriers with protein or antibody molecules. While the use of AFM does not require any fixation or preparation of the sample prior the measurement, the advantage of TEM is the possibility to reach a higher level of specificity when using antibodies raised against the protein under investigation.

Intriguingly, the three chosen conjugation reactions resulted in significant differences with regard to their coupling efficacy. This became apparent using both the conventional characterisation methods such as the BCA assay and the assessment of ζ-potential, but also by employing the newly introduced microscopic methods. Method 2, using DSPE-PEG_{2000}-COOH as linker lipid, exhibited the highest amount of bound Tf, while method 3, using DSPE-PEG_{2000}-MAL, still showed significant amounts of bound Tf on the liposomal surfaces, but in comparison on a three-times lower scale. A possible explanation of this phenomenon is that in method 3, free thiol groups may react among themselves to produce disulphide bonds leading to cross linking of protein molecules before the actual conjugation to the linker. In addition, the random introduction of thiol groups in the Tf molecule may interfere with the biological properties of the molecule resulting in a lower affinity to its receptor [27]. The higher efficacy of method 2, in comparison to method 1, might be attributed to a stabilisation of the intermediate of this reaction by the additionally used S-NHS [28, 29].

In summmary, it can be concluded that the microscopic methods introduced herein, AFM and TEM, are able to present fast and reliable complementary visual information on the protein-modification of colloidal drug carriers. When used in addition to conventional techniques, they can significantly enhance the process of characterisation of new systems for advanced drug delivery.

3.6 References

[1] A Widera, F Norouziyan, W C Shen, Mechanism of TfR-mediated transcytosis and sorting in epithelial cells and applications toward drug delivery, Adv Drug Deliv Rev. 55(11) (2003) 1439--1466.

[2] P Afzelius, E J Demant, G H Hansen, P B Jensen, Covalent modification of serum transferrin with phospholipid and incorporation into liposomal membranes, Biochim Biophys Acta. 979(2) (1989) 231--238.

[3] W P Faulk, B L His, P J Stevens, Transferrin and transferrin receptors in carcinoma of the breast, Lancet. 2(8191) (1980) 390--392.

[4] S Y Joo, J S Kim, Enhancement of gene transfer to cervical cancer cells using transferrin-conjugated liposomes, Drug Dev Ind Pharm. 28(8) (2002) 1023--1031.

[5] K Maruyama, O Ishida, T Takizawa, K Moribe, Possibility of active targeting to tumour tissues with liposomes, Adv Drug Deliv Rev. 40(1-2) (1999) 89--102.

[6] E Wagner, D Curiel, M Cotten, Delivery of drugs, proteins and genes into cells using transferrin as a ligand for receptor-mediated endocytosis, Adv Drug Deliv Rev. 14(1) (1994) 113--135.

[7] C Tros de Ilarduya, M A Arangoa, M J Moreno-Aliaga, N Duzgunes, Enhanced gene delivery in vitro and in vivo by improved transferrin-lipoplexes, Biochim Biophys Acta. 1561(2) (2002) 209--221.

[8] V P Torchilin, Drug targeting, Eur J Pharm Sci. 11(2) (2000) S81--91.

[9] K Shimizu, K Tamagawa, N Takahashi, K Takayama, Y Maitani, Stability and antitumour effects of all-trans retinoic acid-loaded liposomes contained sterylglucoside mixture, Int J Pharm. 258(1-2) (2003) 45--53.

[10] G Bendas, A Krause, U Bakowsky, J Vogel, U Rothe, Targetability of novel immunoliposomes prepared by a new antibody conjugation technique, Int J Pharm. 181(1) (1999) 79--93.

[11] M Singh, H Atwal, R Micetich, Transferrin directed delivery of adriamycin to human cells, Anticancer Res. 18(A) (1998):1423--1427.

[12] Q Z Ming, L Hongyan, S Hongzhe, H Kwokping, Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway, Phramacol Rev. 54(4) (2002) 561--587.

[13] K M Hege, D L Daleke, T A Waldmann, K K Matthay, Comparison of anti-Tac and anti-transferrin receptor-conjugated liposomes for specific drug delivery to adult T-cell leukaemia, Blodd. 74(6) (1989) 2043--2052.

[14] N D Browning, E M James, K Kishida, I Arslan, J P Buban, J A Zaborac, S J Pennycook, Y Xin, G Duscher, Scanning transmission electron microscopy: an experimental tool for atomic interface science, Rev Adv Mat Sci. 1(1) (2000) 1--26

[15] A Engel, C Colliex, Application of scanning transmission electron microscopy to the study of biological structure, Curr Opin Biotechnol. 4(4) (1993) 403--411.

[16] A Alessandrini, P Facci, AFM: a versatile tool in biophysics, Meas Sci Technol. 16(6) (2005) R65--R92.

[17] L Haggerty, A M Lenhoff, STM and AFM in biotechnology, Biotechnol Prog. 9 (1993) 1--11.

[18] P Sarti, P Ginobbi, I D'Agostino, G Arancia, E Lendaro, A Molinari, R Ippoliti, G Citro, Liposomal targeting of leukaemia HL60 cells induced by transferrin-receptor endocytosis, Biotechnol Appl Biochem. 24(3) (1996) 269--276.

[19] I Ezpeleta, J M Irache, S Stainmesse, C Chabenat, J Gueguen, A M Orecchioni, Preparation of lectin-vicilin nanoparticle conjugates using the carbodiimide coupling technique, Int J Pharm. 142(2) (1996) 227--233.

[20] O Ishida, K Maruyama, H Tanakashi, M Iwatsuru, K Sasaki, M Eriguchi, H Yanagie, Liposomes bearing polyethyleneglycol-coupled transferrin with intracellular targeting property to the solid tumours in vivo, Pharm Res. 18(7) (2001) 1042--1048.

[21] A Gijsens, A Derycke, L Missiaen, D de Vos, J Huwyler, A Eberle, P de Witte, Targeting of the photocytotoxic compound AlPcS4 to Hela cells by transferrin conjugated PEG-liposomes, Int J Cancer. 101(1) (2002) 78--85.

[22] A S Derycke, P A de Witte, Transferrin-mediated targeting of hypericin embedded in sterically stabilized PEG-liposomes, Int J Oncol. 20(1) (2002) 181--187.

[23] T M Koyama, C R Stevens, E J Borda, K J Grobe, D A Cleary, Characterizing the gel to liquid crystal transition in lipid-bilayer model systems. Chem Educator. 4(1) (1999) 12--15.

[24] N Zuidam, R de Vrueh, D J A Crommelin, Characterization of Liposomes, in V.P. Torchilin, V. Weissig (Eds.) Liposomes: A practical approach 2nd ed., Oxford University Press, Oxford, U.K., (2003) 31--78.

[25] V Oberle, U Bakowsky, I S Zuhorn, D Hoekstra, Lipoplex formation under equilibrium conditions reveals a three-step mechanism, Biophys J. 79(3) (2000) 1447--1454.

[26] U Bakowsky, W Rettig, G Bendas, J Vogel, H Bakowsky, C Harnagea, U Rothe, Characterization of the interactions between various hexadecylmannoside-phospholipid

model membranes with the lectin Concanavalin A, Phys Chem Chem Phys. 2(20) (2000), 4609--4614.

[27] P Sapra, T M Allen, Ligand-targeted liposomal anticancer drugs, Prog Lipid Res. 42(5) (2003) 439--462.

[28] Z Grabarek, J Gergely, Zero-length crosslinking procedure with the use of active esters, Anal Biochem. 185(1) (1990) 131--135.

[29] J V Staros, R W Wright, D M Swingle, Enhancement by N-hydroxysulfosuccinimide of water-soluble carbodiimide-mediated coupling reactions, Anal Biochem. 156(1) (1986) 220--222.

Chapter 4

Binding and uptake of liposomal formulations

Parts of this chapter have been submitted for publication: Samah Anabousi, Udo Bakowsky, Marc Schneider, Hanno Huwer, Claus-Michael Lehr, Carsten Ehrhardt, *In vitro* assessment of transferrin-conjugated liposomes as drug delivery systems for the inhalation therapy of lung cancer.

4.1 Abstract

Introduction Many tumour cells show a phenotype with an increased expression level of TfR, CD71. Therefore, TfR appears to be an excellent candidate target for tumour-specific drug delivery.

Purpose The objectives of this work were to assess levels of uptake and cytotoxicity of liposomal formulations with Tf modification *in vitro*, using lung cell culture models.

Results The observed higher expression levels of TfR correlated well with enhanced uptake of the Tf-conjugated liposomes into the cancerous cell types, as well as with increased levels of cytotoxicity, when studies were conducted using doxorubicin-filled liposomes. The liposomal uptake was temperature-dependent and inhibitable by excess free Tf.

4.2 Introduction

4.2.1 Drug resistance in cancerous tissues

Drug resistance continues to be a major challenge in cancer treatment. Intrinsic or acquired drug resistance occurs frequently in most cancers, and often involves resistance to multiple agents simultaneously (multidrug resistance, MDR). A number of mechanisms for drug resistance have been described. These include: overexpressed drug export pumps, caused e.g., by P-glycoprotein (PGP) and multidrug-resistance protein (MRP); decreased drug uptake, such as altered folate carriers; inactivation of drugs, such as via glutathione-mediated reduction; overexpression of target enzymes, such as upregulated thymidylate synthase; altered drug targets, such as topoisomerase II; increased DNA repair capacity; reduced ability to undergo apoptosis; and many others.

Among these mechanisms, the role of PGP in multidrug resistance has been one of the most intensively studied. PGP, encoded by the *MDR1* gene, is a member of the ABC (ATP-Binding Cassette) transport protein family and is frequently overexpressed in the MDR phenotype. Other membrane-bound transporters capable of mediating drug efflux include multidrug-resistance protein MRP and other related proteins. These proteins actively transport a variety of heterocyclic substrates, including cytotoxic drugs such as anthracyclines, vinca alkaloids, mitoxantrone, paclitaxel, and others, out of the cell. Alternatively they can sequester drug substances into other cellular compartments.

Specific inhibitors of these resistance mechanisms have been widely pursued as a means to restore drug sensitivity. Although still actively under investigation, specific resistance inhibitors have yet to gain registration for clinical use. Progress towards therapeutic success has been hampered by such issues as inadequate specificity, both predictable and unforeseen toxicities, uncertainly about the true prevalence and contribution of the known resistance mechanisms, paucity of predictive assays to identify tumours dependent upon particular mechanisms, and multiplicity and redundancy of resistance mechanisms [1].

4.2.2 Overcoming drug resistance by liposomes

An alternative strategy for overcoming drug resistance is based on new drug delivery systems to achieve selective drug accumulation in tumour tissues, tumour cells, or even compartments of tumour cells. Liposomal carriers have become clinically accepted in cancer treatment, and as such comprise examples of delivery systems that can enhance the utility of anticancer drugs. For example, long-circulating liposomes and other macromolecular carriers can increase drug

deposition in solid tumours, which may help to overcome drug resistance. Other liposome strategies include modifications for controlled release, which may increase drug bioavailability; and ligand-targeted liposomes, such as immunoliposomes or Tf-conjugated liposomes, which can internalise into tumour cells for intracellular drug delivery and maximal drug efficacy.

Liposomes are membrane-bound vesicles capable of packaging drugs for various delivery applications. For cancer treatment, a number of distinct liposome classes have emerged, based on structural features and associated pharmacologic strategies for delivery. Thus far, the most clinically successful liposomal drugs for cancer treatment have been SUVs, which consist of a single PL bilayer enclosing an inner aqueous compartment for drug encapsulation. The pharmacokinetics and biodistribution of liposomes depend on properties such as size, surface charge, and membrane composition [2].

Current liposomal delivery systems can potentially provide pharmacologic advantages over free drug, including providing a means to overcome drug resistance. Liposome delivery can yield increases in the area-under-the-concentration-versus-time-curve (AUC) in plasma and tumour by 60 to 600-fold or greater. At this level, liposomal delivery may overcome the activity of multidrug transporter systems, perhaps even in highly resistant tumours. It should be noted, however, that actual plasma and tumour drug concentrations reflect total drug (free and liposome-encapsulated) in these two compartments [3].

In order to create agents capable of targeting drug carriers to tumour cells, we and others have studied approaches for ligand-targeted liposomes, which combine Tf and liposome technologies. This system can be designed for specific recognition of a target antigen followed by receptor-mediated endocytosis. In principle, the therapeutic index can be increased by targeting liposomes and their contents directly into tumour cells, and this may be useful especially for resistant cancers.

Evidence for this targeting includes studies with targeted liposomes containing either growth factor ligands or antibodies directed against growth factor receptors. For example, folate targeting has been pursued using liposomes conjugated to folic acid, which were rapidly internalised into tumour cells via the folate receptor. *In vivo* studies in a resistant murine lung carcinoma tumour model (M109R-HiFR) showed superior efficacy of folate-targeted liposomal DOX as compared with free drug or non-targeted liposomal DOX. The TfR has also been targeted by immunoliposomes. Anti-Tf immunoliposomes were used to deliver DOX in a DOX-resistant clone of human leukaemia cells (K562/ADR) [4]. Immunoliposome delivery was associated with minimal drug efflux and nearly equivalent DOX levels in resistant K562/ADR cells as compared with sensitive parental K562 cells. In contrast, free DOX was

efficiently effluxed and present at 45-fold lower drug levels in the resistant clone. In separate studies, anti-TfR immunoliposomes containing mAb OX26 were used to deliver radiolabelled digoxin in immortalised RBE4 rat brain capillary endothelial cells. Cellular uptake of digoxin was 25-fold higher using immunoliposomes, and was unaffected by the PGP inhibitor ritonavir [5].

4.2.3 Liposomal doxorubicin as anti-neoplastic

DOX is an effective therapy for many cancers. Unfortunately, the free form is delivered to normal tissue as well as to cancerous tissues resulting in a great number of toxicities. Reduction of DOX by cellular enzymes results in build-up of oxidative free radicals. Cardiac tissue does not produce enzymes that degrade these oxidative free radicals. Therefore, cardiotoxicity may result in many patients and results in a maximum lifetime dose that severely limits usefulness.

The development of PEG-liposomal delivery of DOX has improved the pharmacokinetic/pharmacodynamic profile of the drug. The PEG-liposomal DOX formulation increases the half-life, maximum concentration, and area under the curve. This results in a higher therapeutic index for DOX.

In a study performed by Eavarone *et al.* (2000) using free- and liposomal-loaded DOX in C6 gliomal cell model, the authors show that DOX encapsulation within liposomes lacking PEG chains allowed a 292% increase in uptake by glioma compared to liposomes of similar composition containing PEG. While PEG chains appear sterically unsuited for tumour uptake, the incorporation of PEG into the liposome membrane has been shown to increase circulation times dramatically and to decrease drug leakage during circulation. Ligands were coupled to the distal ends of the PEG chains in an attempt to increase uptake through receptor-mediated targeting while maintaining PEG stability. Tf was used as a targeting agent for glioma TfR, and albumin was used as a similar molecular weight protein control. Tf-coupled liposomes showed a 412% increase over non-carboxylated PEG-liposomal uptake and a 104% increase over albumin-coupled liposomal uptake, indicating that the increased efficacy of these liposomes is due to TfR targeting rather than to PEG modification or the addition of non-specific proteins [6].

4.2.4 Loading of liposomes with drugs

The main medical and pharmaceutical application of liposomes is delivery of drugs and other bioactive agents for therapy or for *in vivo* diagnostics. Such applications will require sufficient loading of the drug into the liposomes. Classical liposomes, first described by Bangham, are

made up of amphiphilic PLs and cholesterol which, upon hydration, self-associate to form bilayers surrounding an aqueous interior. Hydrophilic drugs can be entrapped in the aqueous interior and hydrophobic drugs can be associated with the bilayer.

Amphiphilic drugs that are weak bases or weak acids can also be loaded into the liposome interior using remote loading methods like the ammonium sulphate method for DOX or the pH gradient method for vincristine. A number of loading strategies are currently available (figure 16). The selection of optimal loading procedure should be based on a scientific rationale [7].

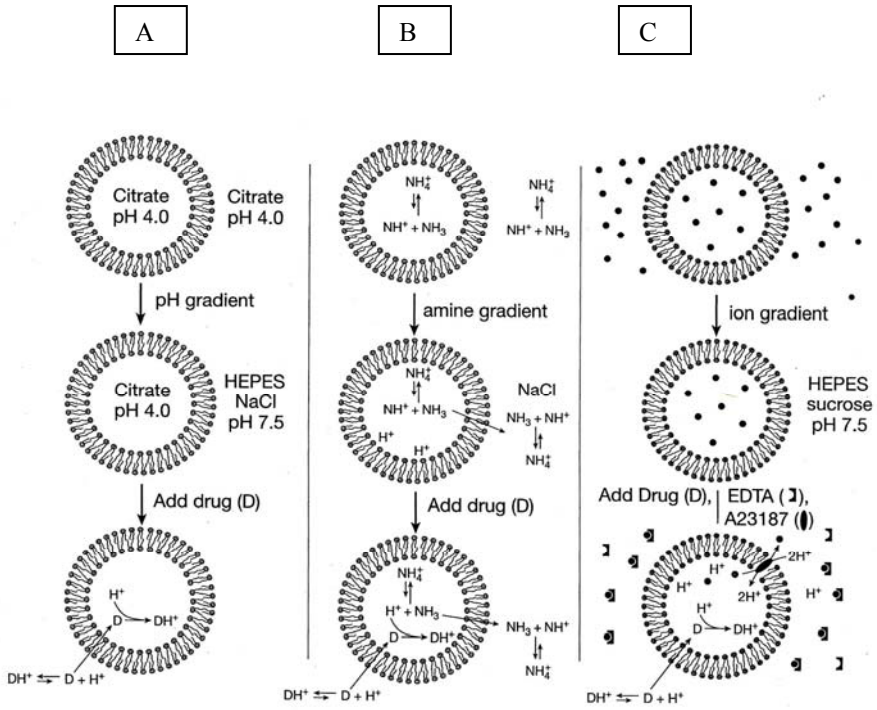


Figure 16: Diagramatic representation of drug uptake in response to transmembrane pH gradients. A: the standard pH gradient method. B: Transmembrane gradient of ammonium sulphate. C: Ionophores

The various agents can be classified into three groups based on their oil/water and octanol/water partition coefficients (K_p). The first group(group I) includes hydrophilic agents with very low oil/water and octanol/water K_p. The second group (group II) includes amphiphatic agents of low oil/water K_p but variable, and in some cases even high, octanol/water K_p, which is controlled by the pH and, to a lesser extent, by the ionic strength of the medium. Good examples of this group are the anthracyclines, to which DOX belongs. The third group (group III) includes hydrophobic agents having high oil/water and octanol/water K_p. These agents of group III are associated with the liposome bilayer(s) although their loading efficiency is rather low. Agents of group I do not interact with the liposome bilayer, and their

encapsulation is dependent on, and limited mainly by, the vesicle trapped volume. The smaller the liposome size, the lower the trapped volume and, therefore, the encapsulation rate.

Drugs of group II offer a better chance to obtain loading at a dose needed for therapy in humans, even for small liposomes. This is explained either by the high affinity of the drug for the liposomal membrane, or by its ability to be remotely loaded, reaching a very high concentration in the intraliposomal aqueous phase. Some of the amphipathic weak bases of group II can diffuse through the liposome bilayer as unprotonated species which then become trapped in the intraliposomal aqueous phase due to a proton gradient which shifts then to group I. Therefore, they cannot cross the lipid bilayer and accumulate in the intraliposomal aqueous phase [8].

Amphiphathic amines, such as catecholamines, can be loaded by a pH gradient. Liposomes should be prepared in acidic medium, and the pH gradient created by elevating the pH of the extraliposomal acidic medium. This concept was extensively used and it has been demonstrated that liposomes loaded by such an approach, when compared with use of the free drug, have lowered toxicity and improved efficacy. Liposomes containing DOX which was remotely loaded through a pH gradient have already been used in clinical trials. Deamer and Harang demonstrated that a pH gradient can be created by a photochemical reaction. However, application of this approach for drug delivery is questionable, since it depends on liposomes containing ferrocyanide [9].

According to Haran *et al.* the remote loading approach could be achieved by creating an ammonium sulfate gradient ($[(NH_4)_2SO_4]_{lip}>[(NH_4)_2SO_4]_{med}$). This is used as the driving force for loading of amphipathic weak bases such as anthracyclines into liposomes.

Small liposomes loaded by an ammonium sulphate gradient with either DOX or epirubicin were used extensively in animal studies involving rodents and dogs and also in clinical trials in humans. In all cases, liposomes loaded by this method show high stability of DOX during storage and during circulation *in vivo*, as well as better tumour localisation, lower toxicity and higher efficacy compared to either use of the free drug or the drug loaded into conventional liposomes [10].

4.3 Materials and methods

4.3.1 Cell culture conditions

Primary culture of human pneumocytes

Fresh human type II alveolar epithelial cells (hAEpC II) were isolated from non-tumour lung tissue which was obtained from patients undergoing lung resection as described previously in chapter II. For the uptake and binding study, the cells were seeded at a density of 300,000 cells/cm^2 on collagen/fibronectin-coated polyester filter inserts (Transwell Clear, 12 mm in diameter, 3460, Cornin, Bodenheim, Germany) using SAGM (Cambrex Bio Science, Verviers, Belgium) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and with addition of low serum (1% FBS) in order to suppress fibroblasts. Cells were grown either for three days (hAEpC II) or for eight days (hAEpC I). For the cytotoxicity study, same procedure was followed with seeding of the cells on 96-well plates.

A549 cell line

In this study, passage numbers 92-95 were used. A549 cells were cultured, as previously described, in RPMI-1640 medium (Sigma) supplemented with 10% FBS at 37°C in a humidified atmosphere containing 5% CO_2. Cells were plated in 25 ml culture flasks, polyester filter inserts (Transwell Clear, 24 mm in diameter, 3450, Corning, Bodenheim, Germany) or 96-well plates at a density of 10^5 cells/cm^2 to confluency. The medium was changed every other day.

Calu-3 cell line

Passage numbers 70-73 were used. The cells were grown in EMEM supplemented with 10% FBS, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 µg/ml streptomycin and 100 U/ml penicillin) at 37°C in a humidified atmosphere containing 5% CO_2. Cells were plated in 75 ml culture flasks, polyester filter inserts (Transwell Clear, 24 mm in diameter, 3450, Corning, Bodenheim, Germany) or on 96-well plates at a density of 10^5 cells/cm^2 till confluency. The medium was changed every other day.

16HBE14o- cell line

Passages 2.48-52 were used in this study. Cells were seeded in tissue culture flasks, polyester filter inserts (Transwell Clear, 24 mm in diameter, 3450, Corning, Bodenheim, Germany) and 96-well plates at a density of 10^5 cells /cm^2 till confluency and grown in EMEM supplemented

with 10% FBS, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin G, at 37°C in a 5% CO_2 incubator. The medium was changed every other day.

4.3.2 Liposome preparation

Liposomes were prepared using a slightly modified protocol according to our previously published method which is described in chapter III [11]. Briefly, liposomes were prepared from DSPC, Chol and the linker lipid DSPE-PEG_{2000}-COOH at the ratio 6:3:0.6 mol%. For PEGylated liposomes phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] ($_{M}PEG_{2000}$-DSPE) was added at 0.6 mol%. All lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA). PL and Chol in chloroform:methanol (2:1) was dried to a thin lipid film in a rotary evaporator (Büchi, Essen, Germany). The lipids were re-suspended in an appropriate amount of 400 mM citrate/5 mM phosphate buffer (pH 4.0) containing the fluorescent dye calcein at 50 mM resulting in a final lipid concentration of 10 mg lipid/ml. After vortexing, the sample was incubated for 10 min at a temperature above the transition temperature of the used lipids (53°C for DSPC:chol) in a cabinet drier. Unilamellar liposomes were prepared by extruding the resulting multilamellar vesicles eleven times through a 200-nm polycarbonate membrane, followed by extrusion eleven times through a 100-nm membrane using a Liposofast Basic device (Avestin, Mannheim, Germany).

For cytotoxicity studies DOX was encapsulated into the liposomes using the ammonium sulphate gradient method [8, 12]. Briefly, unilamellar liposomes were initially formed in buffer containing ammonium sulphate (250 mM final concentration) as described above. Non-entrapped ammonium sulphate was removed by ultracentrifugation at 30,000×*g* for 60 min at 4°C. Subsequently, liposome pellets were re-suspended using PBS containing 1.2 mM $(NH_4)_2SO_4$ and 0.1 mg/ml DOX. Liposomes were then incubated at 60°C for 24 h, ultracentrifuged again to remove free DOX and re-suspended in PBS.

Conjugation of Tf to the liposomal surfaces was achieved by adding 1 ml PBS (pH 7.5) and 360 µl of both EDC (0.25 M in H_2O) and S-NHS (0.25 M in H_2O) per 10 µmol of lipid. The mixture was allowed to incubate for 10 min at room temperature, before adjusting to pH 7.5 with 1 M NaOH. 125 µg Tf/µmol PL was added and gently stirred for 8 h at 4°C. Unbound protein was removed by passing the liposome suspension through a Sepharose CL-4B gel column. Mean particle size of the liposomal suspension was determined by dynamic light scattering. PLs concentration was determined by the colorimetric method and the amount of protein was determined by the BCA assay [11]. Encapsulation efficacy of DOX was assayed

with a fluorescence plate reader (Cytofluor II, PerSeptive Biosystems, Wiesbaden, Germany) at excitation and emission wavelengths of 485 and 530 nm, respectively.

4.3.3 Uptake studies

Studies of uptake of the liposomal formulations were conducted in 96-well plates using cell layers grown to confluence after 5-8 days, depending on cell type. Four different types of liposomes were tested in this assay: plain DSPC:chol liposomes without any functionalisation, PEGylated DSPC:chol liposomes, Tf-conjugated DSPC:chol liposomes, and PEGgylated Tf-conjugated DSPC:chol liposomes. The cells were washed with PBS followed by incubation with the liposomal formulation (final PL concentration 100 μM) for 30, 60, 90 or 120 min at 37°C. The same setup was used for experiments carried out at 4°C and in the presence of free Tf (50 μg/ml) in PBS. For each time point and condition, three wells were measured. After incubation, cells were washed 3 times with ice cold PBS and surface-bound liposomes were removed using pronase (1 mg/ml), before assessing total fluorescence (corresponding to internalised liposomal calcein) with a Cytofluor II fluorescence plate reader at excitation and emission wavelengths of 485 and 530 nm, respectively.

4.3.4 Cytotoxicity assay

Respiratory epithelial cells were plated in quadruplicate in 96-well plates and left alone for 24 h to adhere, before exposing them to increasing concentrations of liposomal DOX (0.003–5.7μM) for 2 h at 37°C. Four wells for untreated cells and medium were also prepared. After the incubation period, cell layers were washed three times with PBS and 100 μl of fresh culture medium were added per well, before incubation was continued for 60 h at 37°C. Cell viability was determined with a colourimetric assay, Cell Proliferation Reagent WST-1 (Roche Diagnostics, Germany). The test is based on the cleavage of the tetrazolium salt WST-1 in formazan by mitochondrial dehydrogenases in viable cells [13]. The formazan dye was quantified by a scanning multiwell spectrophotometer (TECAN, Crailsheim, Germany) by measuring the absorbance of the dye at 440 nm. After the incubation period, Cell Proliferation Reagent WST-1 was added (10 μl/well) and the absorbance measured after 24h.

4.3.5 Statistical analysis

Data are presented as mean ± standard deviation (n), where n is the number of observations. Differences among group means were determined by one-way analysis of variance followed by post-hoc Newman-Keuls-Student procedures and $p < 0.05$ taken as the level of significance.

4.4 Results

4.4.1 Characterisation of liposomes

Physicochemical properties of the different liposomal preparations are given in table 5. The ammonium sulphate gradient method for encapsulation of DOX resulted in an average encapsulation efficiency of 96.7±5.0%.

Table 5. Size, polydispersity index (PI) and zetapotential of liposomes before and after coupling of Tf (values represent the mean ± SD of three batches).

	Before addition of Tf		**After**	
	Size (nm)	Zetapotential (mV)	Size (nm)	Zetapotential (mV)
DSPC	120.50 ± 4.57	-40.67 ± 1.53	129.00±1.66	-20.88 ± 2.08
DSPC 5% PEG	126.40 ± 5.57	-32.31 ± 1.35	135.80±2.76	-19.12 ± 2.34

4.4.2 Uptake studies

In our experiments, the cellular uptake of liposomes was determined by measuring the fluorescence activity of calcein which was previously entrapped in the liposomes. Calcein is a very hydrophilic dye that normally does not cross cellular membranes [14]. Uptake of calcein into epithelial cells was found to be increasing over time for up to 2 h incubation. In figure 17 A, rates of calcein uptake into Calu-3 bronchial epithelial cells are depicted as an example for all other cell types which showed similar tendencies. Tf-conjugated liposomes showed significantly higher rates of uptake than their unmodified counterparts. Addition of PEG into the liposomal membranes had only a slight effect on calcein uptake. When carried out at 4°C, experiments resulted in a significantly lower calcein signal after 2 h incubation compared to values achieved at 37°C for both Tf-modified liposomal formulations. An even stronger inhibition of uptake could be observed when large amounts of free Tf were present in the buffer during the incubation period. The effects of temperature decrease and Tf competition were much less pronounced for the unmodified formulations (figure 17A). A comparison of all cell types used in this study regarding their level of calcein uptake after 2 h of incubation at 37°C showed the lowest calcein accumulation in hAEpC II and hAEpC I-like primary cells (figure 17B). Signals from the three continuous growing cell lines were all significantly higher with the highest values for uptake into A549 cells (figure 17B).

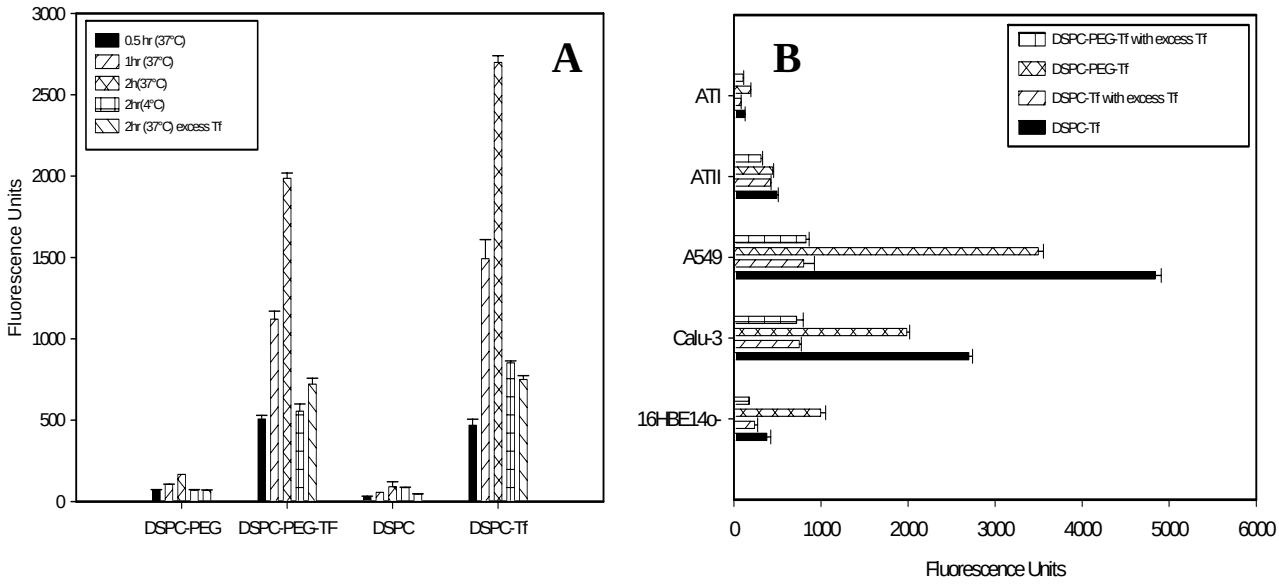


Figure 17: A) Uptake of calcein into Calu-3 bronchial epithelial cells from four different liposomal preparations. Plain liposomes (DSPC), PEGylated liposomes (DSPC-PEG), plain liposomes with Tf (DSPC-Tf)and PEGylated Tf-modified liposomes (DSPC-PEG-Tf). The uptake of calcein increased gradually with time and decreased drastically when experiments were carried out at 4°C and in the presence of excess free Tf. **B)** Uptake of calcein into A549, Calu-3, 16HBE14o-, hAEpC II and hAEpC I-like cells from four different liposomal preparations. The lowest calcein accumulation was found in hAEpC II and hAEpC I-like primary cells. Signals from the three continuous growing cell lines were all significantly higher with the highest values for uptake into A549 cells. Each data point represents the mean ± SD for $n = 3$.

4.4.3 Cytotoxicity assay

The effect of liposomal-DOX on the survival of the different respiratory cell types used in our study was assessed by the WST assay (figure 18). The cytotoxic effect was determined after 2 h of exposure to the liposomal formulation followed by washing and an additional 60 h of incubation in cell culture media. Alveolar epithelial cells in primary culture showed a low response with almost 70% surviving even the highest concentration of DOX used in this experiment (0.34 mg/ml). Survival of the other three cell types (i.e., A549, Calu-3 and 16HBE14o-) showed much stronger concentration dependency and, more importantly, significantly increased levels of toxicity. At 0.02 mg/ml DOX more than 50% of the tested cells were killed with increasing rates for higher DOX concentrations. When incubated with 0.34 mg/ml of free (i.e., not encapsulated) DOX, none of the cell types showed survival rates over 10% .

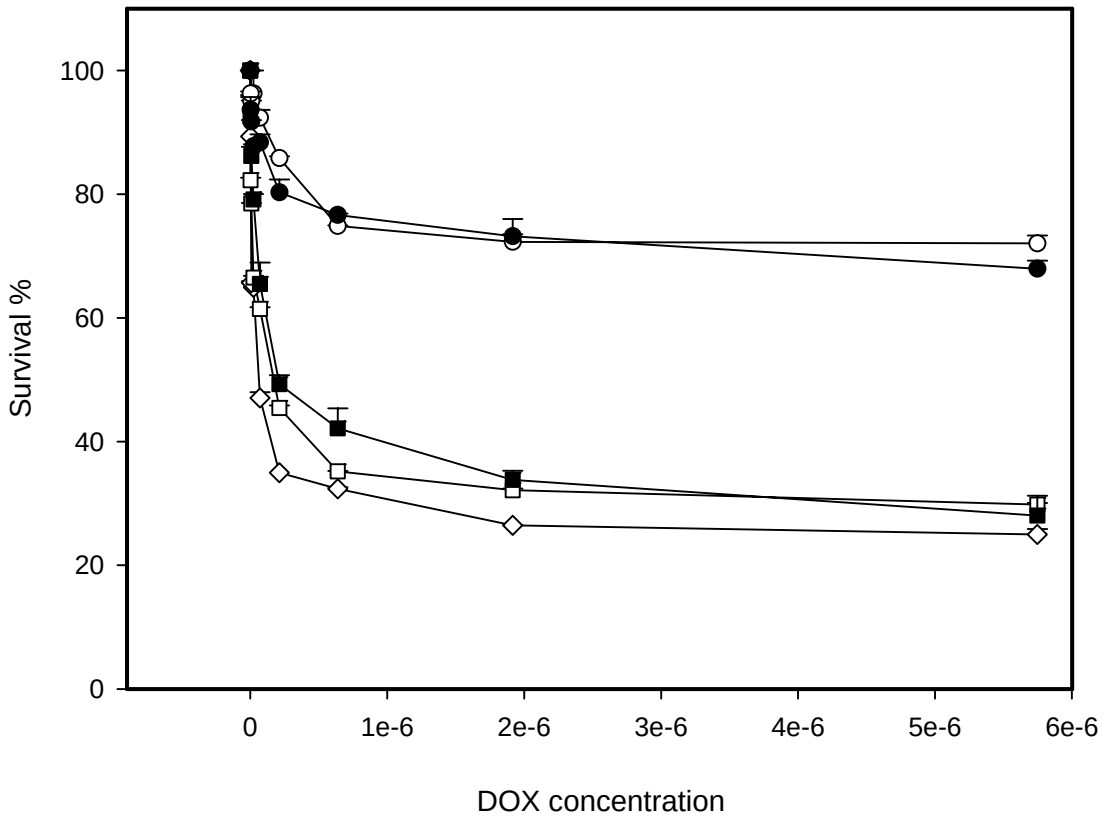


Figure 18: Survival of hAEpC II (○), hAEpC I-like (●), A549 (◊), 16HBE14o- (■), and Calu-3 cells (□) following treatment with DOX-loaded Tf-conjugated liposomes. Survival was determined after 2 h of liposome exposure followed by washing and an additional 60 h of incubation in cell culture media using WST-1 assay. Each data point represents the mean ± SD for $n = 3$.

4.5 Discussion

TfR has emerged as a potential ligand to enable drug targeting and delivery of therapeutic agents that would normally suffer from poor pharmacokinetic characteristics. TfR-directed targeting has enabled the efficient delivery of therapeutic agents to sites of interest, including the central nervous system and malignant tissues. In addition, by utilizing knowledge of the intracellular sorting and recycling pathways of TfR, one can maximize the transepithelial delivery of peptide-based therapeutics. For example, TfR-based strategies can achieve accumulation of the carried drug within targeted tissues or delivery of the therapeutic entity across tissues of interest. Further understanding of the intra-cellular events that govern the destiny of internalized TfR will result in ever increasing interest in TfR as a pharmaceutically relevant molecule.

The large surface area of the lower lung, together with its very thin air-blood barrier, provides excellent conditions for efficient gas exchange. The alveolar epithelium consists of two major cell types: the cuboidal type II cells, which produce lung surfactant and serve as precursors for the second very thin cell type I, which form about 93% of the alveolar surface.

Among several lung epithelial cell lines, obtained from broncho-alveolar carcinoma or by immortalisation, A549 cells are best characterized. These cells show some biochemical and morphological characteristics of type II alveolar epithelial cells. Microvilli and lamellar bodies serving as intracellular storage compartments for lung surfactant have been identified. A549 cells synthesize saturated PLs, the main component of lung surfactant. However, phosphatidylglycerols, which are specific for lung surfactant, are lacking. A549 cells as well as other lung epithelial cell line fail to develop substantial transepithelial electrical resistance, indicating the lack of a real epithelial barrier function to drug transport.

For primary culture, type II cells have been isolated from rats, rabbits, and recently from humans. Similar to the *in vivo* situation, these cells lose their type II cell characteristics. In contrast to A549 cells, alveolar epithelial cells cultured on permeable filter supports develop high transepithelial electrical resistance values indicating the formation of tight junctions.

Inhalational therapy for malignancy involving the lung is a quite new area of research; nevertheless, a number of studies using immune therapy with interleukins and interferons, and chemotherapy with aerosols of free 5-fluorouracil and 9-nitrocamptothecin, resulted in encouraging outcomes [15]. It is our aim to develop targeted drug delivery systems for local aerosol therapy of lung cancer, since we believe that such therapy could further increase the effectiveness of anti-tumour treatments while systemic side effects might be further reduced.

We investigated whether Tf-conjugated liposomes are suitable drug delivery systems for inhalational therapy of lung cancer. In comparison to unmodified liposomes, Tf-conjugates showed significantly higher uptake into respiratory epithelial cells of cancerous origin (i.e., A549, Calu-3 and 16HBE14o-), while in pneumocytes in primary culture uptake rates were much lower. These findings were in good agreement with levels of cytotoxicity of the formulations as determined by WST assay. Intriguingly, A549 cells showed a lower level of TfR expression than the bronchial *in vitro* models; nevertheless, uptake of Tf-modified liposomes into A549 cells was the highest of all investigated cell types. This initially surprising result is most likely caused by the lack of functional tight junctions in this cell line [16, 17]. Thus, transcellular diffusion is less restricted and the liposomes can be internalised not only by TfR of the apical but also the basal membranes. Uptake of liposomal calcein into cells was inhibitable both by low temperature and excess free Tf in the medium, strongly indicating that the uptake mechanism is an energy-driven, saturable process, as it has been described to be the case for TfR internalisation [18].

Receptor-mediated endocytosis pathways can be exploited for specific targeting of liposomes and intracellular delivery of liposome contents. Coupling liposomes to a ligand, that is directed towards an over-expressed receptor in cancer cells and that normally undergoes endocytosis, is a strategy that can improve selectivity and facilitate access of liposomes to the intracellular compartment.

In conclusion, a DSPE-PEG_{2000}-Tf conjugate was successfully synthesized as liposomes bearing an ammonium sulphate gradient for DOX loading. The conjugation of Tf to liposomes as a homing strategy resulted in increased uptake rates into tumour cells via TfR mediated endocytosis. Therefore, Tf-modified liposomes might be promising candidates for aerosol anticancer therapy in the lungs. However, further studies are necessary to investigate stability of these liposomal systems when undergoing nebulisation and when encountering lung fluids. As the next step, animal xenograft models of lung cancer should be employed to obtain important *in vivo* data.

Evidence for the mechanism of receptor-mediated targeting was further supported by competitive binding assays. The addition of Tf to the incubation medium dramatically decreased drug uptake within Tf-coupled liposomes. Competition by free Tf therefore may play a large role in the efficacy of this system *in vivo*.

These findings were supported in this part of the work by the fact that Tf-conjugated liposomes showed higher binding and uptake rates into cancer cells compared to their normal counterparts. Moreover, normal cells showed a higher resistance to the cytotoxic effect of

encapsulated DOX. Therefore, Tf-conjugated liposomes appear as promising drug delivery systems for an inhalational approach in lung cancer chemotherapy.

4.6 References

[1] C Mamot, D C Drummond, K Hong, D B Kirpotin, J W Park, Liposome-based approaches to overcome anticancer drug resistance, Drug Resist Update. 6(5) (2003) 271--279.

[2] A R Thierry, D Vige, S S Coughlin, J A Belli, A Dritschilo, A Rahman, Modulation of doxorubicin resistance in multidrug-resistant cells by liposomes, FASEB J. 7(6) (1993) 572--579

[3] K J Harrington, G Rowlinson-Busza, K N Syrigos, P S Uster, R G Vile, J S W Stewart, Pegylated liposomes have potential as vehicles for intratumoural and Subcutaneous drug delivery, Clin Cancer Res. 6(6) (2000) 2528--2537.

[4] D Goren, A T Horowitz, D Tzemach, M Tarshish, S Zalipsky, A Gabizon, Nuclear delivery of doxorubicin via folate-targeted liposomes with bypass of multidrug-resistance efflux pump, Clin Cancer Res. 6(5) (2000) 1949--1957.

[5] S Vandana, S K Jain, D V Kholi, Potential of transferrin and transferrin conjugates of liposomes in drug delivery and targeting, Am J Drug Deliv. 3(3) (2005) 155--170.

[6] D A Eavarone, X Yu, R V Bellamkonda, Targeted drug delivery to C6 glioma by transferrin-coupled liposomes, J Biomed Mater Res. 51(1) (2005) 10--14.

[7] D B Fenske, N Mauer, P R Cullis, Encapsulation of weakly-basic drugs, antisense oligunucleotides, and plasmid DNA with large unilammelar vesicles for drug delivery application, in V.P. Torchilin, V. Weissig (Eds.) Liposomes: A practical approach 2nd ed., Oxford University Press, Oxkord, U.K., (2003) 167--191.

[8] G Haran, R Cohen, L K Bar, Y Barenholz, Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases, Biochim Biophys Acta. 1151(2) (1993) 201--215.

[9] D W Deamer, E Harang, Light-dependent pH gradients are generated in liposomes containing ferrocyanide, Biosystems. 24(1) (1990) 1--4.

[10] D C Drummond, O Meyer, K Hong, D B Kirpotin, D Papahadjopoulos, Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors, Pharmacol Rev. 51(4) (1999) 691--744.

[11] S Anabousi, M Laue, C M Lehr, U Bakowsky, C Ehrhardt, Assessing transferrin modification of liposomes by atomic force microscopy and transmission electron microscopy, Eur J Pharm Biopharm. 60(2) (2005) 295--303.

[12] E Maurer-Spurej, K F Wong, N Maurer, D B Fenske, P R Cullis, Factors influencing uptake and retention of amino-containing drugs in large unilamellar vesicles exhibiting transmembrane pH gradients. Biochim Biophys Acta. 1416(1-2) (1991) 1--10.

[13] D A Scudiero, R H Shoemaker, K D Paull, A Monks, S Tierney, T H Nofziger, M J Currens, D Seniff, M R Boyd, Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumour cell lines, Cancer Res. 48(17) (1988) 4827--4833.

[14] P Saarinen-Savolainen, T Jarvinen, H Taipale, A Urtti, Method for evaluating drug release from liposomes in sink conditions, Int J Pharm. 159(1) (1997) 27--33.

[15] R D Rao, S N Marcovic, P M Anderson, Aerosol therapy for malignancy involving the lungs, Curr Cancer Drug Targets. 3(4) (2003) 239--250.

[16] K J Elbert, U F Schaefer, H J Schaefers, K J Kim, V H Lee, C M Lehr, Monolayers of human alveolar epithelial cells in primary culture for pulmonary drug absorption and transport studies, Pharm Res. 16(5) (1991) 601--608.

[17] B Forbes, C Ehrhardt, Human respiratory epithelial cell culture for drug delivery application, Eur J Pharm Biopharm. 60(2) (2005) 193--205.

[18] A Gijsens, A Derycke, L Missianen, D de Vos, J Huwyler, A Eberle, P de Witte, Targeting of the photocytotoxic compound AlPcS4 to Hela cells by transferrin conjugated PEG-Liposomes, Int J Cancer. 101(1) (2002) 78--85.

Chapter 5

Nebulisation of liposomal formulations

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5.1 Abstract

Introduction Oral inhalation of anticancer drugs or drug delivery systems is a novel therapeutic approach in the treatment of lung cancer and requires formulations which are sufficiently stabile during nebulisation and subsequent interaction with the surfactant lining of the lungs.

Purpose To assess the stability of plain and PEGylated transferrin-conjugated liposomes after nebulisation using two different nebulisers (the air-jet and ultrasonic types). Furthermore, to assess the integrity of the liposomal membranes after incubation in commercial lung surfactant solutions (Alveofact®).

Results All liposomal formulations showed no significant changes in their size after nebulisation, independent of the type of nebuliser and the liposomal formulation. However, PEGylation was of advantage when it came to interactions between liposomes and the surfactant lining of the lungs. PEGylated liposomes were significantly more stable and retained >80% of their drug load over 48 h, which is more than sufficient time for the drug carriers to be taken up by over expressed transferrin receptors of cancer cells in the lung. In conclusion, PEGylated and plain Tf-conjugated liposomes are stable enough to undergo nebulisation in the course of an inhalational therapy, but PEG-stabilisation results in a higher degree of membrane integrity in lung surfactant.

5.2 Introduction

5.2.1 Inhalation therapy

Aerosolised administration of drugs to the lung has been employed for many years to treat primarily localised disease states within the bronchi. Since this route of administration can deliver therapeutic agents to the diseased regions whilst reducing their distribution to the other organs, this is an excellent example of targeted drug therapy. Hence, a more favourable therapeutic index can be obtained for the treatment of lung diseases when drugs are administered by inhalation rather than by the oral route. Bronchodilators, anti-inflammatory agents, mucolytics, antiviral agents, anticancer agents and PLs protein mixtures for surfactant replacement therapy are all routinely given as aerosolised formulations.

The advantage of the aerosol mode of delivery is that the drug is deposited more uniformly over the respiratory tract, leading to local levels of the drug that may exceed the levels achieved by systemic administration.

Drug delivery systems, such as microparticles, nanoparticles or liposomes, may enhance the stability of substances applied to the lung by offering protection against aggressive environmental factors. Moreover, such schemes can exhibit controlled release properties.

Despite the advantages of inhalation delivery, there are also some related challenges to solve which are not associated with usual systemic delivery. The first major challenge is the atomisation of the drug formulation in a form suitable for inhalation. It is generally accepted that aerosol particles of 1–5 μm are required for deposition in the alveolar region of the lung, the region of the highest absorption. The primary factors influencing aerosol particle size and, ultimately, the site of aerosol deposition include the design of the inhalation device, as well as the physicochemical properties of the drug formulation.

In addition to the size of the individual particles, their concentration as well as surface characteristics both play an important role in determining the physicochemical properties of the suspension and subsequent behaviour during nebulisation. Early nebulisation studies showed a high tendency towards aggregation. Such occurrences may not only affect the properties of the suspension during nebulisation, but may also influence the overall dose effectively applied to the patient as well as the release kinetics of the formulation once in the alveolar region.

Aerosols are a highly desirable form of delivering medication to the lungs. Although they allow direct delivery of drug to target cells, aerosols can be inefficient. This is primarily due to anatomy and physiology of the airway, the breathing mechanism, operational factors

associated with the use of delivery devices, and factors inherent to device design. Crucial parameters are particle size, delivery efficiency and delivery rate [1].

Three systems for the administration of aerosolised medications that are widely used:

pMDI: the drug is either suspended or dissolved in a propellant and filled under pressure into a canister. Releasing a metered volume of the fluid causes the propellant to expand and evaporate rapidly, leaving the drug in the form of dry aerosol particles suitable for inhalation.

DPI: disperse small powder particles for inhalation into the lung. Shear forces generated by the patient's inhalation flow are used to deagglomerate the drug particles, which are commonly adhered to carrier particles.

Nebulisers: there are two types of medical nebulisers: the air-jet nebuliser, which is powered by compressed air, and the ultrasonic nebuliser, which derives the energy required to aerosolise drugs from high-frequency sound waves.

An inhalation system has to produce a particle-size distribution suitable for delivery to the lungs. Ideally, the diameter of the aerosol particles or droplets should be in the range of 1 μm to 5 μm (respirable fraction). Particles in this size range will be deposited primarily by sedimentation in the peripheral lung regions, the bronchioli and alveoli. Sedimentation is the major mechanism of deposition in the therapeutic use of aerosols.

5.2.2 Advantages of liposomes for inhalation

A significant disadvantage of many existing inhaled drugs is the relatively short duration of resultant clinical effects, and most medications administered in aerosol form require inhalation at least 3-4 times daily. This often leads to poor patient compliance with the therapeutic regime and increases the possibility of associated side effects. Recently a number of methods have been investigated as potential pulmonary sustained-release systems for short-acting drugs. These include the incorporation of drugs in liposomes and other biodegradable microspheres, the modification of chemical structure to produce either pro-drugs or drug conjugates with macromolecules, the use of sparingly soluble forms of the drug, and the preparation of magnesium hydroxide co-precipitates and complexes of the drug with cyclodextrins. Liposomes are one of the most extensively investigated systems for controlled delivery of drug to the lung, since they can be prepared with PLs endogenous to the lung as surfactants.

The development of liposomal formulations for aerosol delivery has expanded the potential for more effective utilisation of an array of potent and effective drugs. Liposomal aerosols in pulmonary therapy have many advantages including carrier suitability for lipophilic drugs,

sustained release, and prevention of local irritation, increased potency, reduced toxicity, and uniform deposition of locally active drugs. Liposomes can also be produced with a wide range of sizes and can incorporate both hydrophilic and lipophilic drugs. Many drugs have been incorporated into liposomes with a view to improving their pulmonary delivery, and some have been tested in animal and human subjects. These include, for example, cytotoxic agents, anti-asthma drugs, antimicrobial and antiviral compounds, antioxidant agents, and drugs with systemic actions [2].

Drug formulation plays an important role in producing an effective inhalable medication. Not only is it important to have a drug that is pharmacologically active, but it must also be efficiently delivered into the lungs to the appropriate site of action, and remain in the lungs until the desired pharmacological effect occurs. A drug designed to treat a systemic disease, such as insulin for diabetes, must be deposited in the lung periphery to ensure maximum systemic bioavailability. For gene therapy or antibiotic treatment in cystic fibrosis, prolonged residence of the drug in the lungs may be required and, to obtain the optimal therapeutic effect. Thus, a formulation that is retained in the lungs for the desired length of time, while avoiding the clearance mechanisms of the lung, may be necessary.

Sustained release from a therapeutic aerosol can prolong the residence of an administered drug in the airways or alveolar region, minimise the risk of adverse effects by decreasing the systemic absorption rate, and increase the likelihood of patient compliance by reducing dosing frequency. A sustained-release formulation must avoid the clearance mechanisms of the lung, including the mucociliary escalator of the conducting airways and macrophages in the alveolar region.

Liposomes have been studied for years, as a pulmonary drug delivery vehicle, and used as a means of delivering PLs to the alveolar surface for treatment of neonatal respiratory distress syndrome.

Liposomes are highly versatile drug carriers. They can be formed from a variety of lipids leading to a wide range of physiochemical properties that can alter the trapping efficiencies and release rates of the drug. The physiochemical properties, such as liposome size, bilayer fluidity, surface charge, as well as the method of preparation, affect their *in vivo* behaviour. The vesicle size and number of bilayers are critical parameters in determining the extent of drug encapsulation. Small liposomes (≤0.1 μm) are opsonised less rapidly and to a lesser extent than large liposomes (>0.1 μm). Small liposomes also have a slower release rate. Niven *et al.* demonstrated that 80 min after nebulisation of the liposomal preparation, large multilamellar vesicles (1-5 μm) lost 77% of their content while vesicles with a diameter of 0.2

μm lost only 8%. The preferred size range for clinical applications has been suggested to be 50–200 nm in diameter. Liposomes of this size would avoid phagocytosis by macrophages and still trap useful drug loads [3]. Design of drug carrying and targeted delivery systems using such small particles puts this branch of medicine within the regimen of nanotechnology.

Bilayer fluidity also influences the behaviour of liposomes. Lipids have a characteristic phase transition temperature (T_c). The T_c depends on the length and saturation of the fatty acid chains and can vary from 20°C to 90°C. The lipids exist in different physical states above and below this temperature. Below the T_c, the lipids are in a rigid, well-ordered arrangement (gel phase), and above the T_c in a liquid-crystalline state (fluid phase). The presence of high T_c lipids (T_c >37°C) makes the liposome bilayer less fluid at the physiological temperature and thus, less leaky. The fluidity of the bilayer appears also to influence the interaction of liposomes with macrophages. Liposomes composed with high T_c lipids having a lower uptake in the macrophages. Incorporation of cholesterol into the lipid bilayer affects the fluidity. At high concentrations (> 30 mol%), cholesterol can eliminate the phase transition, making the liposome more stable and less leaky.

The type and density of charge on the liposome surface are also important parameters. A negative charge decreases liposome aggregation and increases encapsulation efficiency, which also increasing liposome–cell interactions; however, charged liposomes may be cleared faster than neutral liposomes. Unlike anionic liposomes, cationic liposomes deliver their contents to cells by actual fusion with the cell membrane.

Liposomes, like other inhaled particles reaching the alveoli, are cleared by macrophages. Unlike other inhaled particles, the fate of liposomes can have a fate similar to that of endogenous lipids. The processing, uptake and recycling of liposomal PLs occurs through the same mechanism as that of endogenous surfactant via the alveolar type II cells. A search for a liposomal formulation that would evade recognition and uptake by the immune system and prolong its residence led to the development of liposomes with a polymer surface coating, such as PEG. The hydrophilic polymer coating attracts water to the liposome surface, preventing the association and binding of opsonins to the liposome, thereby inhibiting the body's molecular recognition processes of labelling the molecule as foreign for subsequent uptake and removal by macrophages, and subsequently increasing liposomal stability.

Among various aerosol delivery technologies, nebulisers have been extensively researched for the delivery of liposomes, since nebulisers are the most simple delivery devices for liposomes, unlike MDI or DPI. Liposomes may be delivered from nebulisers without further processing. Characteristics of aerosol produced by nebulisation will depend upon a number of

factors such as design of the nebuliser, operating conditions, local environment and aerosol output rate. For nebulisation of liposomal systems, the nature of the PL used for preparing the liposomal dispersion and its method of preparation will also play important roles in determining the nebulisation efficiency. In the last few years, many studies have been reported evaluating these various parameters for liposomal systems encapsulating therapeutic agents such as anti-inflammatories, antibiotics and bronchodilators. The liposomes used showed good nebulization efficiency, encapsulated delivery and lower leakage upon nebulization for all the drugs. The approach suggests a new direction for the respiratory delivery of liposomes by nebulization and opens new doors for future in vivo testing for the treatment of pulmonary diseases [4].

5.2.3 Inhalational Chemotherapeutic agents for treatment of lung cancer

In chemotherapy, cytotoxic drugs are used to kill cancerous cells. These drugs are currently administered intravenously or orally. Generally, combinations of chemotherapy and other drugs are used to mitigate adverse side effects, including skin, gastrointestinal, and bone marrow ailments. Such side effects are inevitable, because the drugs used are toxic to healthy cells as well as to cancer cells, and circulate throughout the body.

By delivering chemotherapeutic agents for lung cancer as a magnetically targeted aerosol, it may be possible to reduce adverse side effects by administering chemotherapy agents directly to the cancerous tissue [5, 6].

Aerosols provide a means to favourably alter the biodistribution of chemotherapeutic agents. This strategy holds promise as a unique way of delivering drug locally to the lung. The administration of chemotherapy directly to the lung also holds promise in the treatment of neoplasia in the lungs. New therapeutic agents, molecular targets and delivery approaches are needed to address the limited effectiveness of current treatment modalities for lung cancer. Among treatment avenues being explored as alternatives to systemic drug delivery, direct tumour targeted aerosolised delivery of chemotherapeutic agent, alone or in combination with other drugs seems a novel promising approach for the treatment of lung cancer.

Over the last few years, inhalation drug delivery for the treatment of lung cancer has received new attention from scientists. Recently, nebulised liposomal formulations of 9-nitrocamptothecin (9-NC) and paclitaxel have been studied in the treatment of lung cancer in animal models. Knight *et al.* tested the anticancer properties of liposomes containing campothcin and its analogue (9-NC) in different animal models. The drugs were formulated into liposomes with dilauroylphosphatidylcholine (DLPC). Aerosol treatment was associated

with significant reduction in the size of the implants. This effect was seen in all tumour histologies, and breast and lung cancer showed the best responsiveness. When the formulation was used also in a pulmonary metastases model created in animals, the treated animals had significantly fewer metastases in their lungs and lower overall lung weights when compared to the controls treated with empty liposomes or the untreated group [7].

In another study, this chemotherapy formulation was used for pulmonary metastatic disease in humans. It was administered to six patients with pulmonary metastases of tumours of different histologies. No significant toxicity was noted and three of the six patients had stabilisation of the disease. In the animal model, results showed that tumour surface areas were significantly smaller in cyclosporine-paclitaxel treated animals when compared to untreated control and paclitaxel or cyclosporine treated animals ($P < 0.01$) [6].

In another study, Wang *et al.* demonstrated that nebulised aerosol formulation of anticancer agent farnesol induces death of human lung cancer cells, H460 and A549, *in vitro*. Nearly 100% of lung cancer cytotoxicity was achieved by nebulisation of farnesol formulation using either Pari LC Star or LC plus nebulisers. The anticancer agent gemcitabine has been shown to inhibit the growth of primary osteosarcoma and osteosarcoma lung metastases. The response of LM7 and LM8 lung metastases to aerosol gemcitibine was dose dependent. The efficacy of aerosol *vs.* IP gemcitabine was compared using 0.5 mg/kg dose with the treatment initiated when primary tumour volume reached 130 mm^3. The number of micrometastases in the lung was significantly reduced only in mice receiving aerosol gemcitabine when compared to control and IV treated group [8]. Difluromethylornithine and 5-flurouracil are reported as effective chemo-preventive compounds against carcinogenesis of the upper respiratory tract by using aerosol delivery. Both compounds increased the percentage of animals free of tumours and prevented infiltrating squamous cell carcinoma by >50% [9]. Aerosolized delivery of chemopreventive agents, budesonide and isotretinoin, have been found to be effective in the chemoprevention of lung cancer in A/J mice [10].

5.2.4 Nebulisers

Nebulisers have been used for many years to treat asthma and other respiratory diseases. There are two basic types of nebuliser, air-jet and ultrasonic nebulisers. Nebulisers are probably the least efficient method of aerosol drug delivery. They are variable, slow, and expensive for basic use. Despite the above reservations, nebulisers are still typically the first-line therapy in acute asthma or COPD exacerbation where a large amount of medication is administered and variability is less of an issue. They require less cooperation and effort of an

ill patient. Further advantages of nebulisers are their ability to deliver high doses of drug to the lungs and the minimal coordination and effort required for inhalation in comparison to pMDIs or DPIs.

5.2.4.1 Air-jet nebulisers

The standard air-jet nebuliser applies basic Venturi principles to nebulise medication. A high velocity gas jet of oxygen or air is passed through a constriction and draws up liquid medication through a tube due to the relative vacuum. This produces an aerosol that is directed at the surfaces of the jet apparatus and is immediately shattered into small particles. These particles in turn are passed through the tubing to the patient. The large particles are left behind in the jet apparatus to be re-aerosolised.

With an air-jet nebuliser, aerosol particle size is directly proportional to compressed gas flow. It has been shown that more powerful systems that deliver 8 L/min of flow coupled with an intermittent (dosimetric) rather than continuous inhalation system produce more desirable particle size and less medication is lost to the environment.

Air-jet nebulisers are driven either by a portable compressor or from a central air supply. Essentially, a high-speed airflow through a narrow nozzle orifice entrains and disperses the liquid into droplets (primary generation) via a viscosity-induced instability. Droplet dispersion is improved by impact of droplets on a baffle structure and shear deformation into even smaller droplets (secondary generation). The resulting droplet size distribution still contains only a small fraction of respirable aerosol (droplets below 5 μm to 6 μm in size), but large droplets are recirculated within the nebuliser by means of secondary impaction structures and can eventually contribute to the respirable fraction.

This process is associated with evaporation effects that cause the gas phase to be nearly saturated with vapour, as well as decrease temperature within the nebuliser. A considerable part of the vapour arises from the larger recirculating droplets, thus increasing drug concentration in the remaining liquid (figure 19A).

For liposomal formulations, disruption of liposomes can occur due to mechanical stresses during nebulisation, possibly during primary generation and/or secondary generation, although such disruption is device-specific, it is most pronounced for large liposomes. More sophisticated nebulisers are breath-enhanced (e.g., Pari LC Plus and Pari LC Star), which entrain inhalation air in the droplet production region and produce aerosol at a higher rate during inspiration, but at a lower rate during expiration using a valve system. Due to this

effect, approximately 70% of the aerosol will be delivered to the patient during continuous nebulisation.

With air-jet nebulisers, all commercially available inhalation solutions and suspensions can be administered. Mechanical damage, which may cause denaturation of sensitive drug compounds (i.e., proteins and peptides) is minimised.

Enhanced delivery designs increase aerosol output by directing auxiliary air, entrained during inspiration, through the nebuliser, causing more of the generated aerosol to be swept out of the nebuliser and available for inhalation. Drug wastage during exhalation is reduced to the amount of aerosol produced by the jet air flow rate that exceeds the storage volume of the nebuliser. Adaptive aerosol delivery monitors a patient's breathing pattern in the first three breaths and then targets the aerosol delivery into the first 50% of each inhalation. This ensures that the aerosol is delivered to the patient during inspiration only, thereby eliminating drug loss during expiration that occurs with continuous output nebulisers.

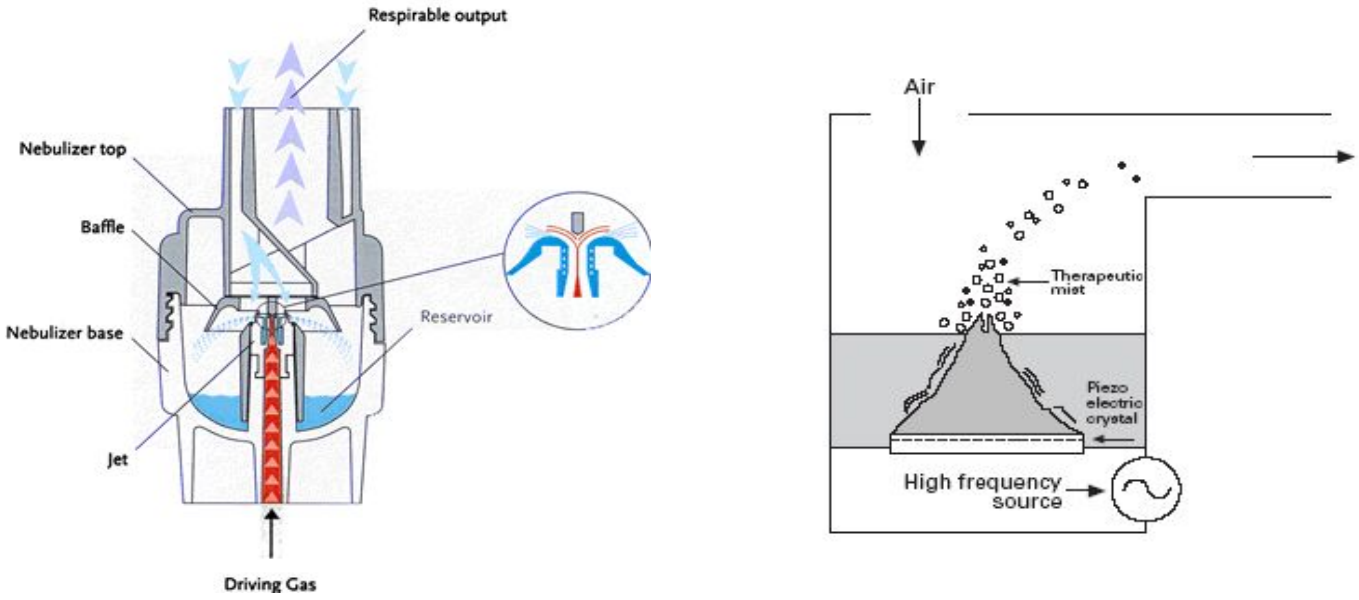

Figure 19: A) Air-jet nebuliser. **B)** Ultrasonic-nebuliser [taken from 11].

5.2.4.2 Ultrasonic nebulisers

With the ultrasonic nebuliser, a fountain is created in the nebuliser by the ultrasonic energy from a piezoelectric crystal vibrating at a high frequency (usually 1-3 MHz). The higher the frequency, the smaller the droplets produced. Droplets are thrown off from the fountain (figure 19B). The output is large so nebulisation is faster. This is particularly useful with larger volumes. Two disadvantages of the ultrasonic method are the relative expense of the equipment and the fact that large-sized particles with a suboptimal deposition profile are produced.

The physical properties of drug formulations may have an effect on nebulisation rates and particle size. The viscosity, ionic strength, osmolarity, pH and surface tension may prevent the nebulisation of some formulations. If the pH is too low, or if the solution is hyper- or hypoosmolar, the aerosol may induce bronchoconstriction, coughing and irritation of the lung mucosa. As well, high drug concentrations may decrease the drug output with some nebulisers; colomycin at concentrations >75 mg/ml foams in all nebulisers, especially ultrasonic ones, making aerosolization of the drug very inefficient if not impossible.

5.2.5 Lung surfactant

Lung surfactant is a complex mixture of lipids and proteins [12]. The lipids are the major surfactant component by weight, making about 85-90% of the whole isolated surfactant. Approximately 90% of this lipid fraction consists of a mixture of PLs. The remaining 10% is composed of other lipids, mainly chol. Nearly 70% of the fatty acids are saturated under normal conditions, the most common saturated acid being palmatic acid. 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) is the surfactant component which is predominantly responsible for the reduction of surface tension [12, 13].

5.2.6 Challenges

Through a reasonably well developed and proven technology, the pulmonary application via inhalation is still accompanied by several unique challenges. First of all, the formulations need to be converted into an aerosol that can be inhaled by the patient. Nebulisation by air-jet and ultrasound are the two predominant methods utilised therapeutically to aerosolise drug solutions [9]. Air-jet nebulisers operate with compressed air, transporting liquid through nozzles creating small aerosol droplets, whereas ultrasonic nebulisers use the piezoelectric effect to generate high-frequency acoustic energy which generates aerosol droplets by cavitation. Both types of nebulisers offer various advantages and disadvantages. Air-jet

nebulisation can affect liposomes as a result of the high shear forces applied during nebulisation, while ultrasonic energy is notorious for altering or damaging some aerosolised drug substances [10, 14]. Furthermore, ultrasonic nebulisers are usually more expensive and have not yet even been investigated for the aerosolisation of liposomes. A significant disadvantage of air-jet nebulisers is the lower output, which results in a prolonged exposure time of the formulation to shear forces.

The next challenge is the preparation of a liposomal formulation which maintains its integrity in the lung surfactant lining long enough to be internalised into the tumour. Lung surfactant is a complex mixture of lipids and proteins, and there is no guarantee that liposomes will survive intact for appropriate time, since they are composed of PLs which are natively present in lung surfactant [15, 16].

In this study, the effect of PEGylation on the stability of previously developed transferrin conjugated liposomal systems during nebulisation using two different types of nebulisers, air-jet and ultrasonic, was investigated. In addition, the stability of the liposomal systems in commercial lung surfactant was assessed. This was intended as an *in vitro* model for the situation where liposomes particles have successfully reached target cells in the lung.

5.3 Materials and methods

5.3.1 Liposome preparation

Liposomes were prepared using a slightly modified protocol according to our previously published method [17]. Briefly, liposomes were prepared from DSPC, Chol and the linker DSPE-PEG_{2000}-COOH at the ratio 6:3:0.6. For surfactant-stability studies, liposomes with DPPC were prepared using the same molar ratios as stated above. PEGylated liposomes were prepared by addition of phosphoethanolamine-*N*-[methoxy(polyethyleneglycol)-5000] ($_{M}PEG_{5000}$-DSPE) at 5 mol%. All lipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA). The mixture of PL and chol in chloroform:methanol (2:1) was dried to a thin lipid film in a rotary evaporator (Büchi, Essen, Germany). The lipids were re-suspended in an appropriate amount of 400 mM citrate/5 mM phosphate buffer (pH 4.0) containing the fluorescent dye calcein at 50 mM resulting in a final lipid concentration of 10 mg lipid/ml. After vortexing, the sample was incubated for 10 min at 60°C, a temperature above the transition temperature of the used lipids (53°C for DSPC:Chol) in a cabinet drier. Unilamellar liposomes were prepared by extruding the resulting multilamellar vesicles eleven times through a 200-nm polycarbonate membrane, followed by extrusion eleven times through a 100-nm membrane using a Liposofast Basic Device (Avestin, Mannheim, Germany).

Conjugation of Tf to the liposomal surfaces was achieved by adding 1 ml PBS (pH 7.5) and 360 µl of both EDC (0.25 M in H_2O) and S-NHS (0.25 M in H_2O) per 10 µmol of lipid. The mixture was allowed to incubate for 10 min at room temperature, before adjusting to pH 7.5 with 1 M NaOH. 125 µg Tf/µmol PL was added and gently stirred for 8 h at 4°C. Unbound protein was removed by passing the liposome suspension through a Sepharose CL-4B gel column (Sigma).

PL concentration was determined by the colorimetric method of and the amount of protein was determined by the BCA assay [17, 18]. Encapsulation efficacy of doxorubicin (DOX) was assayed with a fluorescence plate reader (Cytofluor II, PerSeptive Biosystems, Wiesbaden, Germany) at excitation and emission wavelengths of 485 and 530 nm, respectively.

5.3.2 Photon correlation spectroscopy

The mean particle size of the liposomal suspension was investigated by dynamic light scattering. Size determination of the liposomal formulations before and after nebulisation was carried out using a Zetasizer 3000 HS (Malvern Instruments, Herrenberg, Germany) equipped

with a photon correlation spectroscopy unit. The scattered light was detected at a scattering angle of 90°. Measurements were performed at 25°C. For all measurements, samples were diluted 50-fold in distilled water to obtain appropriate counts. PCS provides information about the mean diameter of the bulk population and the width of distribution via the polydispersity index (PI). Mean values and standard deviation were calculated from three determinations.

5.3.3 ζ-potential measurements

ζ-potential measurements of the liposomal formulation before and after nebulisation were carried out in the standard capillary electrophoresis cell of a Zetasizer 3000 HS at pH 7.4 in the presence of NaCl to adjust the conductivity to 50 µS/cm. Measurements were performed at 25°C with automatic duration. The instrument was routinely calibrated with a –50 mV latex standard (Malvern Instruments). The electrostatic mobility was converted into the ζ-potential using the Helmholtz-Smoluchowski equation [19]. The mean values and standard deviation were calculated from three independent measurements (3 runs each).

5.3.4 Atomic force microscopy

The liposomal formulations with and without Tf modification were prepared as described above and diluted in ultrapure water (MilliQ, 18.4 MΩ, pH 5.5). Approximately one hour after preparation, the liposomes were directly transferred onto a silicon chip by dipping the chip into the liposome suspension. Atomic force microscopy was performed on a vibration-damped Digital Nanoscope IV Bioscope (Veeco Instruments, Santa Barbara, CA). Commercial pyramidal Si_3N_4 tips (NCH-W, Veeco Instruments) on a 125 µm cantilever; a resonance frequency of about 220 kHz and a nominal force constant of 36 N/m were used. All measurements were performed in tapping mode to avoid damage of the sample surface. The scan speed was inversely proportional to the scan size and the scan frequency was between 0.5 and 1.5 Hz. Images were obtained by displaying the amplitude signal of the cantilever in the trace direction, and the height signal in the retrace direction, both signals being simultaneously recorded as described previously [20]. The results were visualised either in height or in amplitude mode.

5.3.5 Liposome membrane integrity after nebulisation

The effect of PEGylation on the stability of liposomes undergoing nebulisation was assessed in two different systems, an air-jet nebuliser Pari LC star (Pari, Starnberg, Germany) operated

with a Pari Boy compressor, and an ultrasonic nebuliser, Optineb (Nebutec, Elsenfeld, Germany), operated at a frequency of 2.4 MHz and 12 l/min air flow rate. The filling volumes of the nebulisers were 4 ml and the nebulisation to residual volume was performed in approximately 10-20 min. The nebulised samples were collected by aerosol deposition on a glass plate and subsequent collection in 1.5 ml Eppendorf tubes. All nebulisation studies were repeated in triplicate.

Integrity of the liposomal membranes after nebulisation was assessed by measuring calcein fluorescence with a fluorescence plate reader (Cytofluor II, PerSeptive Biosystems, Wiesbaden, Germany) at excitation and emission wavelengths of 485 and 530 nm, respectively, before and after liposomal disruption by Triton X-100 (1% final concentration). The values for integrity of the liposomes were determined from the equation

$$\%\,Integrity = \frac{F^{N}{}_{T} - F^{N}{}_{I}}{F_{T} - F_{I}} \times 100 \qquad \textit{eqn. 1}$$

where F_I and F^N_I are the calcein fluorescence intensities of each sample before, and F_T and F^N_T the calcein fluorescence intensities after nebulisation in the absence and presence of 1% Triton X-100, respectively. The values obtained after addition of Triton X-100 were corrected for dilution.

5.3.6 Liposome membrane integrity in lung surfactant

The membrane integrity of plain and PEGylated Tf-modified DPPC and DSPC liposomes after incubation in PBS (pH 7.4) or natural bovine surfactant extract (Alveofact®, Boehringer Ingelheim, Ingelheim, Germany) was evaluated by calculating the percentage retention of liposome-encapsulated calcein, as previously described [21]. Alveofact, a surfactant isolated from bovine lung lavage fluids, was used at a final concentration of 3 mg/ml in PBS (pH 7.4), to mimic conditions in the human respiratory system. Calcein was encapsulated in the liposomes at such a concentration that its ability to fluoresce was quenched, and thereby any fluorescence measured was due to calcein leakage and dilution in the exterior aqueous media. In brief, nine volumes of Alveofact solution or buffer were mixed with one volume of liposomal formulation (adjusted in order to have a final lipid concentration of 1 mg/ml in all cases) and samples were incubated at 37°C or 4°C for up to 48 h. At different time intervals, the latency and retention of calcein were estimated by measuring fluorescence of a 100 µl-sample with a fluorescence plate reader (Cytofluor II, PerSeptive Biosystems, Wiesbaden,

Germany) at excitation and emission wavelengths of 485 and 530 nm, respectively, before and after liposomal disruption by Triton X-100 (1% final concentration).

The values for calcein latency and retention were determined from the equations

$$\%Latency = \frac{F_T - F_I}{F_T} \times 100 \qquad eqn.\ 2$$

and

$$\%Retention = \frac{\%Latency_T - \%Latency_I}{\%Latency_T} \times 100 \qquad eqn.\ 3$$

where F_I and F_T are the calcein fluorescence intensities of each sample in the absence and presence of 1% Triton X-100, respectively. The values obtained after addition of Triton X-100 were corrected for dilution.

5.3.7 Statistical analysis

Data are presented as mean ± standard deviation (n), where n is the number of observations. Differences among group means were determined by one-way analysis of variance followed by post-hoc Newman-Keuls-Student procedures and $p < 0.05$ taken as the level of significance.

5.4 Results

5.4.1 Characterisation of liposomes

All different liposomal formulations had an initial size of 190 - 210 nm and a ζ-potential of ~ -20 mV (table 6). The initial calcein latency (%) of Tf-conjugated liposomes was measured immediately after separation of liposomal and free calcein (when liposomal fractions were eluted from the Sephadex column). Latency values were comparable for all investigated preparations at 62.38±6.13% (DPPC:chol-Tf), 61.38±1.42% (DSPC:chol-Tf), 66.30±0.42 (DPPC:chol-PEG-Tf), and 65.81±3.06 (DSPC:chol-PEG-Tf).

Table 6. Physico-chemical properties of liposomal formulations before and after nebulisation by air-jet or ultrasound assessed by photon correlation spectroscopy and zetasizer.

	Before nebulisation			Collected aerosol droplets after nebulisation			Reservoir content after nebulisation		
Air-jet nebuliser	Size (nm)	PI	ζ-potential (mV)	Size (nm)	PI	ζ-potential (mV)	Size (nm)	PI	ζ-potential (mV)
DSPC Tf	210.1	0.04	-20	222.1	0.06	-42	217.4	0.03	-58
DSPC 5% PEG Tf	189.1	0.08	-22	195.8	0.06	-19	192.5	0.07	-22
Ultrasonic nebuliser									
DSPC Tf	210.1	0.04	-20	214.5	0.06	-32	214.9	0.08	-46
DSPC 5% PEG Tf	189.1	0.08	-22	173.5	0.12	-22	187.4	0.11	-24

5.4.2 Stability after nebulisation

Liposomes prepared from DSPC:chol-Tf were used in this study either plain (i.e., non PEGylated) or PEGylated. Both formulation types were tested in an air-jet and an ultrasonic nebuliser. The integrity of the liposomes was assessed by means of calculating the loss of encapsulated calcein and by visualising morphological changes by AFM.

In tables 6 (PCS) and 7 (AFM), changes in size and ζ-potential are summarised. The size of both formulations in all devices changed only marginally, as did the ζ-potential in the case of

the PEGylated liposomes. Non-PEGylated liposomes showed a reduction in ζ-potential upon nebulisation. The integrity, i.e., calcein retention pre- vs. post-nebulisation, also showed no marked difference between the type of nebuliser or the formulation (figure 20). Integrity of all investigated samples was around 90%. Figure 21 shows the morphology of the liposomes before (figure 21A and 21D) and after (figure 21B, 21C, 21E and 21F) nebulisation. Generally, plain liposomes (figure 21A, 21B and 21C) as well as PEGylated liposomes (figure 21D, 21E and 21F) maintained their spherical morphology. However, PEGylation resulted in less fusion and a slightly rounder shape. With regards to the nebuliser technology, ultrasound produced less fusion (figure 21C and 21F) compared to air-jet nebulisation (figure 21B and 21E). Liposomes which were collected from the nebulisation chambers after nebulisation did not display altered morphology at all.

Table 7. Physico-chemical properties of liposomal formulations before and after nebulisation with air-jet or ultrasound assessed by atomic force microscopy.

	Before nebulisation		Collected aerosol droplets after nebulisation		Reservoir content after nebulisation	
Air-jet nebuliser	Size (nm)	Size range (nm)	Size (nm)	Size range (nm)	Size (nm)	Size range (nm)
DSPC Tf	192.3 ± 17	152 - 267	215.4 ± 34	83 - 401	203.1 ± 16	154 - 247
DSPC 5% PEG Tf	163.7 ± 18	105 - 187	167.5 ± 23	95 - 227	166.6 ± 12	113 - 184
Ultrasonic nebuliser						
DSPC Tf	192.3 ± 17	152 - 267	254.4 ± 45	127 - 344	199.7 ± 45	157 - 243
DSPC 5% PEG Tf	163.7 ± 18	105 - 187	145.3 ± 8	132 - 158	172.1 ± 16	122 - 198

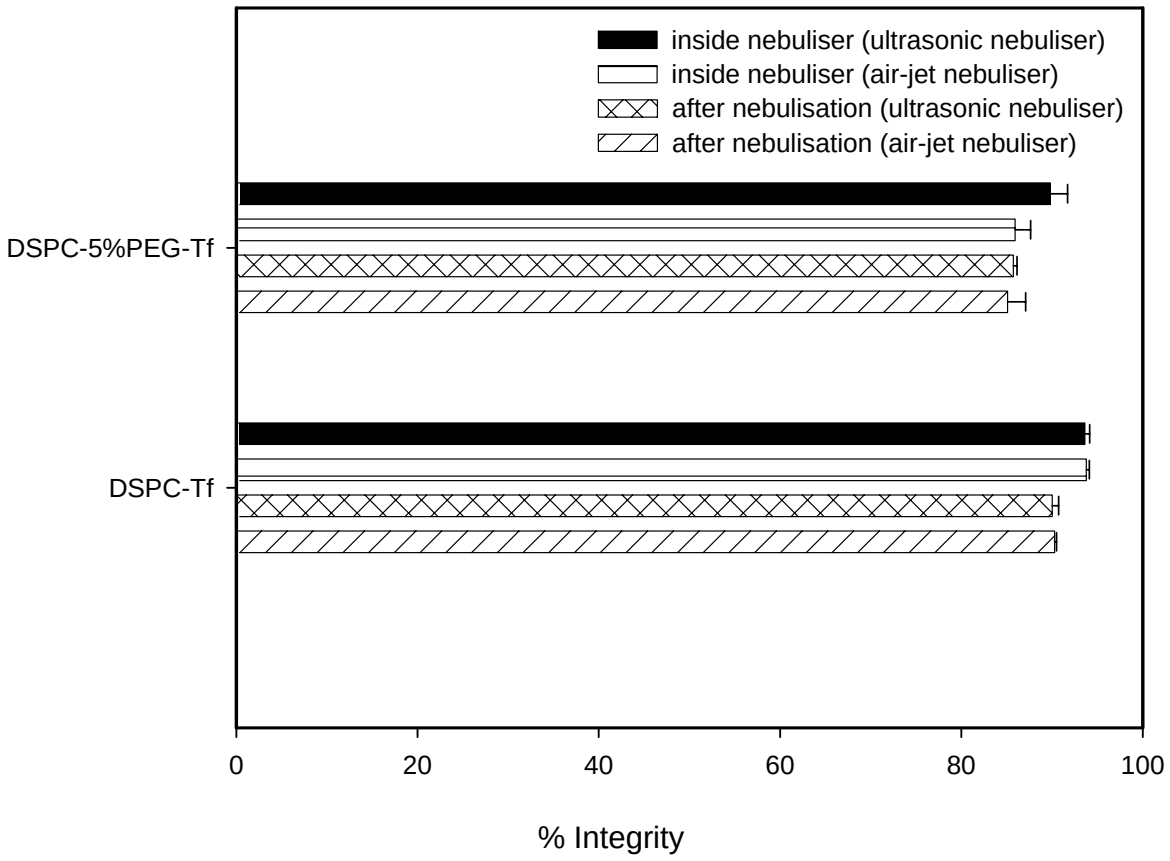

Figure 20: Integrity of liposomes during nebulisation. Liposomes were collected from droplets after nebulisation using an air-jet or ultrasound nebuliser or were taken from the nebulisation chamber of the respective device. The integrity of the liposomal membranes was calculated from the retention of calcein (initial value = 100%).

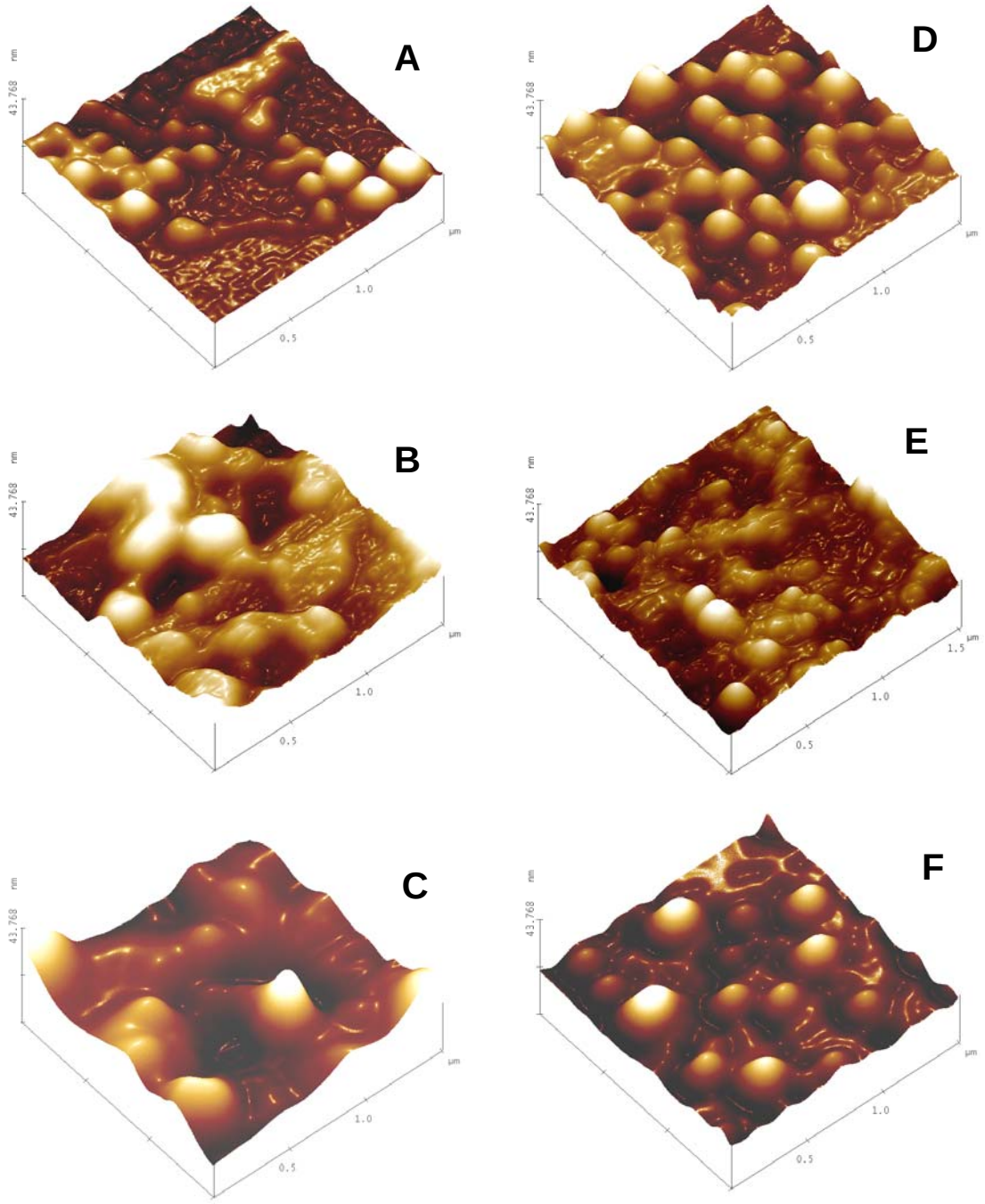

Figure 21: Atomic force microscopy images of surface-deposited liposomal formulations before and after nebulisation. A) DSPC:chol-Tf before nebulisation; B) DSPC:chol-Tf after air-jet nebulisation, C) DSPC:chol-Tf after ultrasonic nebulisation; D) DSPC:chol-PEG-Tf before nebulisation; E) DSPC:chol-PEG-Tf after air-jet nebulisation; F) DSPC:chol-PEG-Tf after ultrasonic nebulisation.

5.4.3 Stability in lung surfactant

Four different liposomal formulations were prepared and compared in terms of stability in lung surfactant solution: DPPC:chol-Tf, DPPC:chol-PEG-Tf, DSPC:chol-Tf, and DSPC:chol-PEG-Tf. As shown in figure 22 A and 22 B, membrane integrity of liposomes made from DSPC exhibited higher values than their counterparts prepared from DPPC, when incubated in the Alveolfact solution. Furthermore, the liposomal composition had a significant impact on the liposomal stability; PEGylated preparations were generally more stable than plain liposomes. Incubation at higher temperatures (i.e., 37°C (figure 22A, 22C) vs. 4°C (figure 22B, 22C)) resulted in higher retention values for the encapsulated calcein. All formulations showed almost no release of calcein in presence of buffer as incubation media either at 4°C or 37°C (figure 22 C and 22D).

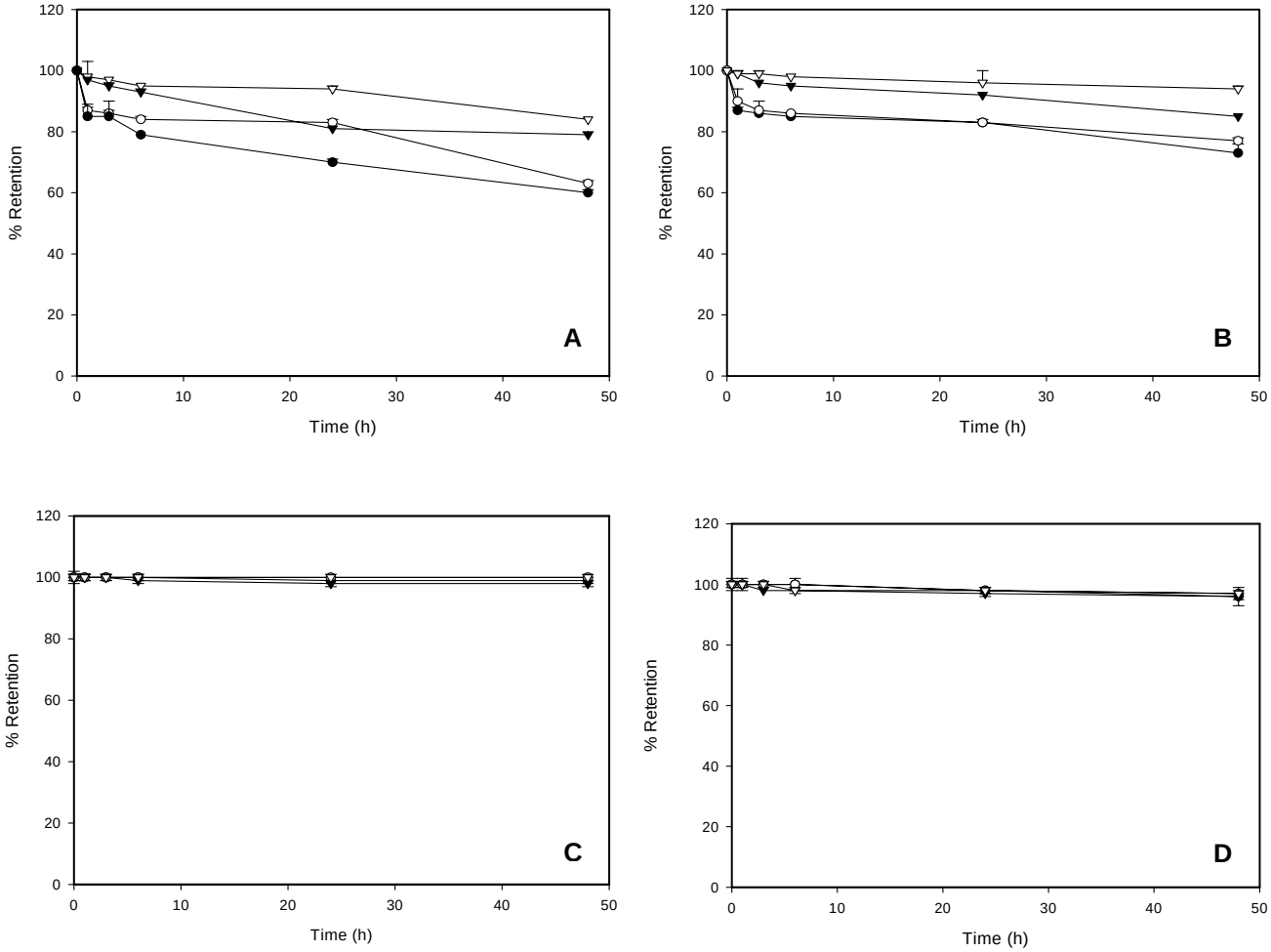

Figure 22: %Retention of liposomal content after incubation in Alveofact®. DPPC:chol-Tf (●), DPPC:chol-PEG-Tf (▼), DSPC:chol-Tf (○), and DSPC:chol-PEG-Tf (∇) liposomes were incubated for 48 hours at 37°C (A, B) or 4°C (C, D) and release of calcein from the vesicles was measured.

5.5 Discussion

Conventional approaches to lung cancer treatment, including traditional chemotherapy, have shown relatively limited success. Indeed, the overall 5-year survival rate of 14% has not changed for 50 years. Thus, it is clear that new therapies or new contributions of therapies are needed to create maximum anti-tumour response without damage of normal respiratory tract tissues.

Studies of liposomes for pulmonary drug delivery have shown that liposomes deposited in the peripheral airways are retained for prolonged periods of time. This may result in prolonged drug presence in the airways, localised drug action in the respiratory tract, and decreased incidence of systemic adverse effects. All reported studies of liposome delivery to the human lung have employed jet nebulisers. Nebulisers have thus far been preferred to the alternative systems for drug delivery, namely pMDIs and DPIs, because they are capable of delivering large dose volumes. Moreover, liposomes can be produced by conventional techniques and usually require no further processing except removal of unentrapped drug where appropriate. However, jet nebulisers, which use compressed gas to generate an aerosol from aqueous solutions or suspensions, may structurally damage some liposome formulations due to the shearing forces and recycling of liquid that occur within the nebuliser. The major determinants of liposome stability to jet nebulisation are the size of the liposomes relative to the size of the aerosolised droplets, and the air pressure employed to generate the aerosol. Ultrasonic nebulisers are less widely employed for drug delivery than jet nebulisers. With these devices, the energy necessary for the atomisation of liquids is generated by high frequency vibrations in a piezoelectric crystal, which produce a fountain of droplets in the nebuliser chamber, of which the smallest are expelled and inhaled by patients. Operation of ultrasonic nebulisers causes an increase in the liquid reservoir temperature by up to 15°C. This can cause chemical breakdown of heat-labile materials.

Targeting drug delivery into the lungs has become one of the most important aspects of systemic or local drug delivery systems. Consequently, in the last few years, techniques and new devices intended to deliver drugs into the lungs have been widely developed. Due to their inherent versatility, liposomes are ideal candidates for pulmonary drug delivery [22]. They offer many advantages including suitability of the carrier for lipophilic and hydrophilic drugs, superior sustained-release properties, and reduced local irritation and toxicity due to the usage of endogenous compounds [23, 24]. By engineering different parameters, formulations can be tailored to each specific therapeutic application. Our laboratories are particularly interested in inhalation therapy of malignancies involving the lung. This novel approach to treat lung

cancer holds promise as a means to avoid systemic toxicity while obtaining improved therapeutic effect [7]. The objective of the present study was to assess the effect of PEGylation of the liposomal membrane on stability during nebulisation and longevity after deposition in lung surfactant fluids. Insertion of PEGylated lipids into liposomal membranes (i.e., sterically-stabilised liposomes or Stealth® liposomes) has been reported to prolong longevity of circulating liposomes by a drastic reduction of opsonisation events [25, 26]. One of the major drawbacks of classical liposomes has been their rapid clearance from blood, due to adsorption of plasma proteins (opsonins) to the "naked" PL membrane, triggering recognition and uptake of the liposomes by the MPS, also referred to as the RES. A major advance in the field of liposomes came with the development of Stealth® liposomes, which utilize a surface coating of a hydrophilic carbohydrate or polymer, usually a lipid derivative of PEG, to help evade MPS recognition. The inclusion of PEG or other hydrophilic polymers extends the half-life of liposomes from less than a few minutes (classical liposomes) to several hours (Stealth® liposomes) and changes the pharmacokinetics of the liposomes from dose-dependent, saturable pharmacokinetics to dose-independent pharmacokinetics. To date, liposomes coupled to antibodies or antibody fragments, folate or Tf have been the most extensively researched LTLs.

Intriguingly, PEGylation has not had a great influence on the integrity during nebulisation. Both plain and PEGylated liposomes were equally stable in the air-jet and the ultrasonic nebuliser with retention values ~90%. However, AFM revealed less fusion and a slightly rounder morphology in case of the PEGylated liposomes. The choice of nebuliser made no significant difference in retention values, but again there was a slightly better morphology when using ultrasound instead of an air-jet.

We found that PEGylation significantly increased the stability of the liposomes in a lung surfactant assay. PEGylation results in Born-hydration repulsion caused by the establishment of a non-diffusive water layer around the liposomes, in addition to steric repulsion caused by the PEG-chains. This finding is in agreement with previous reports on the stabilising properties of PEG-lipids in liposomal formulations [25]. The choice of PL also had an effect on the membrane integrity in lung surfactant, as DPPC-containing liposomes were slightly less stable than those produced with DSPC. It can be speculated that the high content of DPPC in the lung surfactant results in more rapid disintegration of liposomes prepared from the homologous lipid.

In conclusion, PEGylated and plain Tf-conjugated liposomes are stable enough to undergo nebulisation in the course of an inhalation therapy. However, PEGylation is of advantage

when it comes to interactions between the liposomes and the surfactant lining of the lungs. PEGylated liposomes are significantly more stable and retain >80% of their drug load over 48 h, which is more than sufficient time for the liposomes to be taken up by TfR-over-expressing cancer cells in the lung.

5.6 References

[1] H J Carveth, R E Kanner, Optimizing Deposition of Aerosolized Drug in the Lung: A Review, Med Gen Med. 1(3) (1999).

[2] X M Zeng, G P Martin, C Marriott, The controlled delivery of drugs to the lung, Int J Pharm. 124(2) (1995) 149--146.

[3] R W Niven, M Speer, H Schreier, Nebulization of liposomes. II. The effects of size and modeling of solute release profiles, Pharm Res. 8(2) (1991) 217--221.

[4] T R Desai, R E W Hancock, W H Finlay, A facile method of delivery of liposomes by nebulization, J Control Release. 84(1-2) (2002) 69--78.

[5] J Ally, B Martin, M Behrad Khamesee, W Roa, A Amirfazli, Magnetic targeting of aerosol particles for cancer therapy, J Magn Magn Mater. 293(1) (2005) 442--449.

[6] N V Koshkina, J C Waldrep, V Knight, Camptothecins and Lung Cancer: Improved Delivery Systems by Aerosol, Curr Cancer Drug Targets. 3(4) (2003) 251--364.

[7] R D Rao, S N Marcovic, P M Anderson, Aerosol therapy for malignancy involving the lungs, Curr Cancer Drug Targets. 3(4) (2003) 239--50.

[8] Z Wang, H T Chen, W Roa, W H Finaly, Farnesol for aerosol inhalation: nebulization and activity against human lung cancer cells, J Pharm Pharm Sci. 6(1) (2003) 95--100.

[9] N V Koshkina, E S Kleinerman, Aerosol therapy for malignancy involving the lungs, Int J Cancer. 116(3) (2005) 458--463.

[10] L W Wattenberg, T S Wiedmann, R D Estensen, Chemoprevention of cancer of the upper respiratory tract of the syrian golden hamster by aerosol administration of difluoromethylornithine and 5-fluorouracil, Cancer Res. 64(7) (2004) 2347--2349.

[11] C O' Callaghan, P W Barry, The science of the nebulised drug delivery, Thorax. 52(S2) (1997) S31--S41.

[12] W Bernhard, J Mottaghian, A Gebert, G A Rau, H von der Hardt, C F Poets, Commercial versus native surfactants, surface Activity, molecular Components, and the Effect of Calcium, Am J Respir Crit Care Med. 162(4) (2000) 1524--1533.

[13] N Ernst, S Ulrichskotter, W A Schmalix, S Gersting, C Plank, D Reinhardt, J Rosenecker, Interaction of liposomal and polycationic transfection complexes with pulmonary surfactant, J Gene Med. 1(5) (1999) 331--340.

[14] V P Torchilin, Drug targeting, Eur J Pharm Sci. 11(2) (2000) S81--S91.

[15] K Shimizu, K Tamagawa, N Takahashi, K Takayama, Y Maitani, Stability and antitumour effects of all-trans retinoic acid-loaded liposomes contained sterylglucoside mixture, Int J Pharm. 258(1-2) (2003) 45--53.

[16] P Sapra, T M. Allen, Ligand targeted liposomal anticancer drugs, Prog Lipid Res 42(5) (2003) 439--462.

[17] S Anabousi, M Laue, C M Lehr, U Bakowsky, C Ehrhardt, Assessing transferrin modification of liposomes by atomic force microscopy and transmission electron microscopy, Eur J Pharm Biopharm 60(2) (2005) 295--303.

[18] A S Derycke, P A de Witte, Transferrin-mediated targeting of hypericin embedded in sterically stabilized PEG-liposomes, Int J Oncol. 20(1) (2002) 181--187.

[19] R J Hunter, Zeta Potential in Colloid Science: Principles and Applications, Academic Press, New York (1981).

[20] V Oberle, U Bakowsky, I S Zuhorn, D Hoekstra, Lipoplex formation under equilibrium conditions reveals a three-step mechanism, Biophys. J. 79(3) (2000) 1447--1454

[21] J Senior, G Gregoriadis, Is half-life of circulating liposomes determined by changes in their permeability?, FEBS Lett. 145(1) (1982) 109--114.

[22] T R Desai, R E W. Hancock, W H Finlay, A facile method of delivery of liposomes by nebulization, J Control Release. 84(1-2) (2002) 69--78.

[23] R M Fielding, The use of inhaled liposome formulations for drug delivery to the lungs and systemic circulation, Proc West Pharmacol Soc. 32 (1989) 103--106.

[24] C Fonseca, J N Moreira, C J Ciudad, M C Pedroso de Lima and S Simões, Targeting of sterically stabilised pH-sensitive liposomes to human T-leukaemia cells, Eur J Pharm Biopharm. 59(2) (2005) 359--366.

[25] G Blume, G Cevc, Liposomes for the sustained drug release in vivo, Biochim Biophys Acta. 1029(2) (1990) 91--97.

[26] I Ahmad, T M Allen, Antibody-mediated specific binding and cytotoxicity of liposome-entrapped doxorubicin to lung cancer cells in vitro, Cancer Res. 52(17) (1992) 4817--4820.

Summary and outlook

1. Summary

The lungs are the common site of both metastasis and 1 neoplasia. The average lung cancer mortality rate is 90% making lung cancer the leading cause of cancer-related deaths in both men and women.

Current lung cancer treatments include surgery, radiation therapy and chemotherapy, but in recent years, a novel approach for cancer therapy based on Ehrlich's concept of the 'magic bullet' has been developed. Cytotoxic conjugates, composed of two components, a carrier molecule with high affinity and specificity for cancer cell-associated antigens or other tumour targets coupled to a potent cytotoxic drug, are being designed for active anti-cancer drug targeting.

Recent results have shown that the expression of transferrin receptors by human cancer cells is directly correlated with the anti-tumour effectiveness of transferrin receptor conjugates. Transferrin (Tf) is a glycoprotein involved in iron transport into cells that is internalised upon binding to its receptors on the cell surface. It is now widely accepted that the density of transferrin receptors (TfR) is a marker for cell proliferation and that tumour cells usually over-express TfR. These free TfRs are therefore ideal targets for cancer therapy, especially for malignancies that are refractory to conventional therapy, such as lung tumours.

Receptor-mediated endocytosis pathways can be exploited for specific targeting of liposomes to the cells. Coupling liposomes to a ligand, that is directed towards an over-expressed receptor in cancer cells and that normally undergoes endocytosis, is a strategy that can improve selectivity and facilitate access of liposomes to the intracellular compartment.

Goal of this PhD project was the development of a novel anti-cancer therapy by using aerosols of Tf-modified liposomes for a local, thus less problematic, treatment of lung tumours. Since not much published data was available, the first step was to estimate the expression levels of TfR on cells originating from lung cancer, as well as from healthy lung tissues. The data show that TfR expression levels in healthy alveolar epithelial cells in primary culture were significantly lower than in A549 cells, a cell line derived from an adenocarcinoma of the distal lung. TfR expression levels in the continuously growing bronchial epithelial cell lines, Calu-3 and 16HBE14o-, were again significantly higher than those observed for the alveolar cell types.

In general, all cell types showed TfR molecules located predominantly at their basolateral membranes, but cells undergoing mitotic proliferation at the time of fixation showed an additional strong signal for TfR on their apical aspect as well, due to the loss in cell polarity.

After validating the applicability of TfR as a target molecule for our strategy, in an attempt to produce Tf-modified liposomes, three different linker lipids (DSPE-PEG_{2000}-maleimide, *N*-glutaryl-PE and DSPE-PEG_{2000}-COOH) were used. Many protocols have been published on the conjugation of proteins to surfaces of liposomes, but (visual) data on the quality and quantity of the claimed modifications are scarce. We successfully used atomic force microscopy (AFM) and transmission electron microscopy (TEM) as novel tools to visualise the actual conjugation of transferrin to the liposomal carrier. In addition, to quantify the conjugation efficiency, BCA-assays were performed and the phospholipid concentration was measured spectrophotometrically according to Stewart's method. The obtained date were then compared to the AFM and TEM results. AFM and TEM were able to detect Tf at the liposomal surface on the molecular level in a fast and reproducible manner. Both microscopic techniques can deliver semi-quantitative visual information on the actual functionalisation of nano-scale drug carriers with protein or antibody molecules. While the use of AFM does not require any fixation or preparation of the sample prior to the measurement, the advantage of TEM is the possibility to reach a higher level of specificity when using antibodies raised against the protein under investigation.

The third step of the project was to test if the developed Tf-liposomes were actually able to delivery the payload to cancer cells at a higher rate than to their healthy counterparts. Again, *in vitro* cell culture techniques of several pulmonary epithelial cell types (both healthy and cancerous) were employed to give us some answers. Binding/uptake studies as well as cytotoxicity assays were carried out using the most promising candidates from our first study. Furthermore, we tested sterically-stabilised liposomes to assess the repulsive effect of the PEG-chains on the Tf-mediated uptake. We found that our Tf-liposomes can efficiently deliver the model drug, doxorubicin (DOX), to tumour cells and bypass the drug efflux mechanisms characteristic to multidrug resistance. The time-dependent uptake of liposomes into the cells showed a significantly higher uptake for the Tf-modified liposomes. When performed at 4°C or in the presence of excess free Tf, the experiments resulted in significantly lower fluorescence signals. The results of the cytotoxicity tests showed that primary cells were less affected by DOX encapsulated in Tf-liposomes than all cancerous cell lines used in this study. PEGylation generally decreased the binding/uptake, but only at a neglectable level.

In the last part of my work, the liposomal system were tested for their suitability for aerosolisation. Plain and PEGylated Tf-liposomes were aerosolised by two different nebuliser

types (i.e., air-jet and ultrasound) and their morphology was assessed by AFM and the integrity of the liposomal membranes by measuring the loss of encapsulated calcein. In additional studies, the stability of the liposomes in buffer solution was compared to the stability in an artificial lung surfactant (Alveofact®). The results lead to the conclusion that PEGylated and plain Tf-conjugated liposomes were both stable enough to undergo nebulisation, however, PEGylation was of advantage when it came to interaction between liposomes and the surfactant lining of the lungs. PEGylated liposomes were significantly more stable and retained >80% of their drug load over 48 h in artificial lung surfactant, which is more than sufficient time for the liposomes to be taken up by transferrin receptor over-expressing tumours in the lung.

In general conclusion, this work could proof the feasibility to produce transferrin-conjugated liposomes, which can successfully deliver high amounts of anti-cancer drugs to pneumocytes of cancerous origin while having a low level of cytotoxicity in healthy cells. Furthermore, the developed liposomes are stable enough to undergo aerosolisation, a necessary prerequisite of oral inhalation. The liposomes also endure prolonged times in lung surfactant solutions without being compromised.

2. Zusammenfassung

Bösartige Erkrankungen der Lunge fordern weltweit weiterhin die meisten Todesopfer, sowohl bei Männern wie auch Frauen. Ursächlich hierfür ist, dass sich in der Lunge neben Primärtumoren (d.h. Tumoren, die vom Lungengewebe ausgehen) auch überproportional viele Metastasen im engen Kapillarnetz der Lunge festsetzen und somit für Sekundärtumoren verantwortlich sind. Therapieoptionen bei Lungenkrebs sind chirurgische Eingriffe, Bestrahlung und seit einiger Zeit auch Chemotherapie. Die verschiedenen Therapieformen können auch kombiniert angewendet werden, jedoch ist die Prognose bei Lungenkrebs generell eher schlecht, da die Krankheit in der Regel erst in einem späten Stadium diagnostiziert wird.

Seit Paul Ehrlich das Prinzip der selektiven Toxizität postulierte („magic bullet“) versuchen Forscher mit immer neuen Mitteln Arzneistoffe gezielt einzusetzen. So gesehen geht das moderne *drug targeting* auf eine 150 Jahre alte Idee zurück. Im konkreten Fall der Krebstherapie wird versucht zytotoxische Arzneistoffe möglichst nur im Tumorgewebe anzureichern, um somit eine effektive Therapie mit reduzierten systemischen Nebenwirkungen zu kombinieren. Hierzu werden die Arzneistoffe oder Arzneistoffträger mit Molekülen konjugiert, die eine stark erhöhte Affinität zu Strukturen (z.B. Antigenen) haben, die vornehmlich auf Tumorzellen exprimiert werden. Transferrin-Rezeptoren (TfR, CD71) gehören zu dieser Gruppe von Antigenen. Transferrin (Tf) ist ein biogenes Glykoprotein, welches die Körperzellen mit Eisen versorgt. Zwei Gründe sind für das Interesse an der Tf/TfR-Interaktion als Arzneistoff-Target verantwortlich: 1. wird Tf nachdem es am TfR gebunden hat internalisiert und 2. ist der TfR auf den meisten entarteten Zellen überexprimiert und wird teilweise sogar als Marker für die Aggressivität der Erkrankung herangezogen.

Ziel dieser Arbeit war es eine neuartige Strategie im Kampf gegen den Lungenkrebs zu entwickeln. Dazu sollten *drug targeting* mittels Tf/TfR und Lokaltherapie der Lunge mittels Inhalation von Aerosolen kombiniert werden.

Um die Validität der Hypothese zu überprüfen musste das zuerst einmal ermittelt werden, ob Krebszellen des Lungenepithels ebenfalls erhöhte TfR Werte haben. Da es in der Literatur hierzu nur lückenhafte Hinweise gab, wurde die TfR Dichte verschiedener Lungenepitheltypen (normal und entartet) mittels indirekter Immunofluoreszenz (FACS) nachgewiesen. Die Ergebnisse der Studie ergaben, dass die Beobachtungen bei anderen Tumoren auch für Lungenkrebs zutreffen. Die auf ein Adenokarzinom der distalen Lunge

zurückgehende Zelllinie A549 wies eine signifikant höhere TfR Anzahl auf als vergleichbare Alveolarepithelzellen in Primärkultur. Weiterhin stellten wir fest, dass beide untersuchten Zelllinien des Bronchialepithels (Calu-3 und 16HBE14o-) wiederum signifikant höhere Werte lieferten als die Alveolarzelltypen.

Nach diesen Ergebnissen konnte die eigentliche Arbeit – die Entwicklung eines Arzneistoffträgers – aufgenommen werden. In der Literatur sind mehrere verschiedene Mechanismen beschrieben worden um Tf an Liposomen zu koppeln. Jedoch finden sich fast keine Hinweise wie der Erfolg der Kopplungsreaktion überprüft werden kann. In der Regel begnügt man sich hier mit einem Protein-Assay (z.B. BCA). Eine zusätzliche optische Darstellung der Kopplungseffizienz wurde bisher nicht beschrieben. Deshalb wurden in von uns 3 beschriebene Kopplungsmethoden für Tf ausprobiert und die Ergebnisse verglichen. Dies geschah mittels BCA-Protein-Assay und Bestimmung der Phospholipide nach Steward. Zusätzlich wurde die Proben in einem Rasterkraftmikroskop (oder AFM) und einem Transmissionselektronenmikroskop untersucht. Die Ergebnisse konnten nicht nur signifikante Unterschiede zwischen der Effizienz der verschiedenen Kopplungsverfahren aufzeigen, wir konnten auch weiterhin AFM und TEM als schnelle und zuverlässige Verfahren zur Darstellung von Protein-*drug carrier* Konjugation etablieren. Während AFM den Vorteil bietet, dass die zu untersuchende Probe nicht behandelt oder fixiert werden muss, kann beim TEM die die Empfindlichkeit des Verfahrens durch Verwendung von Antikörpern gegen das Zielprotein noch weiter erhöht werden.

Als nächstes wurde untersucht, ob die Tf-Kopplung tatsächlich zu einer erhöhten Aufnahme von Liposomen in die Zellen führt, und wie zytotoxisch die Liposomen generell sind. Dazu wurden Bindungs- und Aufnahmeversuche sowie Zytotoxizitäts-Assays in verschiedenen Lungenepithelzelltypen *in vitro* durchgeführt. Zusätzlich wurde der Einfluss von PEG-Stabilisierung auf die Effektivität der Liposomen untersucht. Wiederum waren die Ergebnisse sehr viel versprechend: Die Tf.Kopplung resultierte in einer Vervielfachung der Aufnahme von Liposomen in die Krebszellen. Diese Aufnahme war beeinflussbar durch niedrige Temperaturen (4°C) und auch durch größere Mengen freies Tf im Versuchsmedium. Die einzelnen Werte für die Internalisierung korrelierten sehr gut mit den zuvor gemessenen TrF Dichten der einzelnen Zelltypen. PEGylierte Liposomen banden zwar etwas schlechter als „nackte“, aber immer noch signifikant besser als Liposomen ohne Tf-Modifizierung. Bei den Zytotoxizitäts-Assays sah es ähnlich aus: die Tf-konjugierten Liposomen resultierten in hohen

Absterberaten bei den Tumorzellen, während die Primärzellen deutlich weniger beeinflusst wurden.

Im letzen Teil der Arbeit sollten schließlich ermittelt werden, ob die Liposomen überhaupt in der Lage sind ihre Ladung an den Wirkort zu bringen, d.h. ob sie den Scheerstress bei der Vernebelung überstehen und ob die Membranintegrität durch Lungensurfactant nachteilig beeinflusst wird. Einfache und PEGylierte Liposomen wurden in zwei verschiedenen Systemen (Druckluftdüse und Ultraschall) vernebelt und die Retention von Calcein wurde bestimmt. Zusätzlich wurde die Morphologie mittels AFM untersucht. Die liposomalen Formulierungen wurden weiterhin mit künstlichen Surfactant (Alveolfact®) inkubiert. Die Studien ergaben, dass der Einfluss von PEGylierung auf die Stabilität beim Vernebeln nicht allzu groß ist, jedoch war die Latenz von verkapselten Calcein in den Surfactant Versuchen deutlich höher.

In der Zusammenfassung aller Versuche kann man schließen, dass die Aerosoltherapie mit Transferrin-modifizierte Liposomen ein sehr interessanter und Erfolg versprechender Ansatz in der Behandlung des Lungenkrebses ist. Die von uns entwickelten liposomalen Formulierungen sind in der Lage das Zielorgan zu erreichen und dort ihre Ladung selektiv an Krebszellen/Metastasen abzugeben. Diese Lokaltherapie sollte es ermöglichen die Tumoren gezielt zu bekämpfen, ohne den Körper schädigenden Konzentrationen des Zytostatikums auszusetzen.

3. Outlook

In this dissertation, different problems were solved and the following conclusions could be drawn:

- Like other cancer types, tumours of the lung exhibit elevated levels of transferrin receptor at their surfaces. These TfR might have the potential to be exploited for targeted drug delivery.

- Conjugation by covalent linkage of transferrin to liposomes can be achieved by a variety of different methodologies. The actually coupled Tf can be visualised using AFM and TEM techniques.

- It is feasible to produce transferrin-conjugated liposomes, which can successfully deliver high payloads of anti-cancer drugs to pneumocytes of cancerous origin while showing a low level of cytotoxicity in healthy cells.

- The developed liposomes are stable enough to undergo aerosolisation, a necessary prerequisite of oral inhalation. The liposomes also endure prolonged times in lung surfactant solutions without being compromised.

Before bringing this exciting new therapeutic strategy to the clinic, as a next step, animal xenograft models of lung cancer should be used to obtain important *in vivo* data.

Appendices

List of abbreviations

AFM	Atomic force microscopy
BCA	Bicinchoninic acid assay
BUD	Budesonide
CF	5,6-Carboxyfluorescein
Chol	Cholesterol
COPD	Chronic obstructive pulmonary disease
CLSM	Confocal laser scanning microscopy
DPPC	1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine
DPPG	Dipalmitoyl phosphatidyl glycerol
DSPC	1,2-Distearoyl-*sn*-glycero-3-phosphocholine
DSPE-PEG_{2000}-COOH	1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol) 2000]
DSPE-PEG_{2000}-MAL	1,2-Distearoyl-*sn*-glycero-3-phosphocholine-(polyethylene glycol) 2000-maleimide
DOX	Doxorubicin
DPI	Dry powder inhaler
EDC	N-(3-dimethylaminopropyl)-N-ethylcarbodiimide Hydrochloride
EMEM	Eagle's minimum essential medium
FACS	Fluorescence-activated cell sorting
HSPC	Hydrogenated soy phosphatidyl choline
IP	Intraperitoneal
IV	Intravenous
LUVs	Large unilamellar vesicles
LTL	Ligand-targeted liposomes
LED	Liposome-encapsulated doxorubicin
MDR	Multi drug resistance
MLVs	Multilamellar vesicles
N-glutaryl-PE	1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-glutaryl
NSCLC	Non small cell lung carcinoma
PC	Phosphatidylcholine
PL	Phospholipid
PEG	Polyethylene glycol
PG	Phosphatidyl glycerol
pMDI	Pressurised-metered-dose inhalers
hAEpC II	Primary human alveolar type I-like epithelial cells
hAEpC I	Primary human alveolar type II epithelial cells
PGE	Prostaglandin
RES	Reticuloendothelial system
SAGM	Small airways growth medium
SCLC	Small cell lung carcinoma
SMLVs	Small multilamellar vesicles
SUVs	Small unilamellar vesicles
S-liposomes	Sterically-stabilised liposomes

S-NHS	Sulpho-N-hydroxysuccinimide
TEM	Transmission electron microscopy
Tf	Transferrin
TfR	Transferrin receptor (CD71)
TB	Tuberculosis
UVs	Unilamellar vesicles

Curriculum Vitae

Samah Anabousi

Date of birth	Oct. 1st, 1973
Place of birth	Kuwait
Nationality	Jordanian
e-mail	samahanab@yahoo.com

In 1991, I studied Pharmacy at Jordan University of Science and Technology (JUST), Irbid, Jordan and graduated in 1996. From September 1998 to June 2000, I joined the faculty of pharmacy at Jordan University, Amman, Jordan for the M.Sc. and received my degree with GPA 3.81/4 rating excellent. Between October 2002 and June 2006, I took part in research for my PhD study at Department of Biopharmaceutics and Pharmaceutical Technology, Saarland University, Saarbrücken, Germany, under supervision of professor Claus-Michael Lehr. During the PhD study, I have been funded by a PhD scholarship from DAAD.

My academic experience involves working at the pharmacy college in Al-Zaytoonah University, Amman, Jordan as a teaching and research assistant from March 1996 to August 2000. From September 2000 until April 2002, I was appointed there as lecturer. I am a (co-author) of 5 articles in peer-reviewed journals, 1 book chapter, and 10 abstracts.

Scientific publications in refereed journals

1**.** S Anabousi, A Shervington, M Al-Ghazawi (2000) The influence of different media and hormones on the lethal concentration of gentamycin and ampicillin in tea plant tissue culture**,** *Acta Technol Leg Medic,* **59**, XI, N 3,1-6.

2**.** S Anabousi, M Laue, CM Lehr, U Bakowsky, C Ehrhardt (2005) Assessing transferrin modification of liposomes by atomic force microscopy and transmission electron microscopy. *Eur J Pharm Biopharm*, **60**(2):295-303.

3. S Anabousi, E Kleemann, U Bakowsky, T Kissel, T Schmehl, T Gessler, W Seeger, CM Lehr, C Ehrhardt (2006) Effect of PEGylation on the stability of liposomes during nebulisation and in lung surfactant, *J Nanosci Nanotechnol*, **6**(8):1-7

4. S Anabousi, U Bakowsky, M Schneider, H Huwer, CM Lehr, Carsten Ehrhardt, In vitro assessment of transferrin-conjugated liposomes as drug delivery systems for the inhalation therapy of lung cancer, *submitted to Eur J Pharm Sci*

Book chapters and invited review articles

1. M Bur, S Anabousi, CM Lehr, Transport von Arzneistoffmolkülen über die zelluläre Luft-Blut-Schranke, In G Scheuch (ed.) "*Aerosole in der Inhalationstherapie IX*", dustriTBmed, München, pp 38-51.

2. G Sharma, S Anabousi, C Ehrhardt, MNV Ravi Kumar**,** Liposomes as targeted drug delivery systems for breast cancer, *accepted for publication in J Drug Target*

Presentations and abstracts contributed to international congresses

*** S Anabousi, A Shervington, Transformation of plant cells with DNA encoding an antibiotic resistance marker. *8th International Congress of Biochemistry and Molecular Biology*, 16.-20.07.2002, United Kingdom, (2002)

* S Anabousi, U Bakowsky, U Schaefer, CM Lehr, C Ehrhardt, Design of DSPE-PEG-maleimide-modified liposomes for specific conjugation with transferrin. *3rd International Galenos Intensive Course and Workshop on Liposome Technology and Applications in Therapeutics*, 01.-11.09.2003, Patras, Greece, *Proc 3rd Int Course Liposome Technol*, 163 (2003).

* S Anabousi, U Bakowsky, UF Schaefer, CM Lehr, C Ehrhardt, Preparation and characterisation of DSPE-PEG-maleimide-modified liposomes for conjugation with receptor ligands for cell specific targeting. *International Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology 2004*, Nuremberg, Germany, 15.-18.03.2004, *Proc Int Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology*, 787-8 (2004).

* S Anabousi, U Bakowsky, M Laue, CM Lehr, C Ehrhardt, Atomic force microscopy and transmission electron microscopy are novel tools used for verification of transferrin

modification of liposomes. *CRS German Chapter Annual Meeting*, 02.04.2004, Heidelberg, Germany, *Proc CRS German Chapter Annual Meeting*, 16 (2004).

* S Anabousi, U Bakowsky, M Laue, CM Lehr, C Ehrhardt, AFM as a novel tool to verify the presence of transferrin on liposomal surfaces. *Annual meeting of the AAPS*, Baltimore, MD, 07.-11.11.2004, *AAPS J* T3263 (2004).

* S Anabousi, CM Lehr, C Ehrhardt, Binding and uptake of transferring-modified liposomes in lung epithelial cells. *CRS German Chapter Annual Meeting*, 15.03.2005, Marburg, Germany, *Proc CRS German Chapter Annual Meeting*, 17 (2005).

* S Anabousi, CM Lehr, C Ehrhardt, Design of transferrin-modified liposomes: binding and uptake in lung cancer cells. *32nd Annual Meeting of the Controlled Release Society*, Miami Beach, FL, 18.-22.06.2005, *Proc Intern Symp Control Rel Bioact Mat* **32**(1):290 (2005).

* S Anabousi, CM Lehr, C Ehrhardt, Transferrin-modified liposomes for treatment of lung cancer-binding and -uptake studies using human lung epithelial cells. *17th Mountain/Sea Liposome Workshop*. 04.-08.04.2005, Oberjoch, Germany (2005).

* S Anabousi, E Kleemann, U Bakowsky, CM Lehr, C Ehrhardt, Transferrin-modified liposomes for inhalation therapy of lung cancer. *33rd Annual Meeting of the Controlled Release Society*, Vienna, Austria, 22.-26.07.2006.
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