Doctoral Thesis

Immunoliposomes for the targeted inhibition of tumour growth and angiogenesis

Author(s):
Marty, Cornelia

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IMMUNOLIPOSOMES FOR THE TARGETED INHIBITION OF
TUMOUR GROWTH AND ANGIOGENESIS

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZÜRICH

for the degree of
Doctor of Natural Sciences

presented by

CORNELIA MARTY

dipl. Natw. ETH
born March 10, 1969
citizen of Zürich, Switzerland

accepted on the recommendation of
Prof. Dr. G. Folkers, examiner
Prof. Dr. R.A. Schwendener, co-examiner

Zürich, 2000
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SUMMARY

Non-specific side effects, short circulation times and development of resistance represent important drawbacks of cytotoxic drugs currently used in chemotherapy.

The aim of this study was to find a system which could avoid these disadvantages. The idea was to combine the good pharmacokinetic properties of liposomes as a drug carrier system and the specificity of antibodies or antibody fragments recognising specific tissues.

This thesis describes the construction of F(\(ab\))\(_2\)-immunoliposomes recognising the carcinoembryonic antigen (CEA) on tumour cells, of single chain Fv (scFv)-immunoliposomes directed against the ED-B domain of B-fibronectin located in the extracellular matrix of newly formed blood vessels in tumours (angiogenesis) and of arginine-glycine-asparagine acid (RGD)-immunoliposomes specific to \(\alpha\_5\beta\_3\)-integrin expressed by different tumour cells and involved in angiogenesis and metastasis formation. In addition, the immunoliposomes were loaded with N\(^4\)-octadecyl-1-\(\beta\)-D-arabinofuranosylcytosine (NOAC) and 2\(^\prime\)-deoxy-5-fluorouridylyl-N\(^4\)-octadecyl-1-\(\beta\)-D-arabinofuranosylcytosine (5 FdU-NOAC), two lipophilic derivative of the chemotherapeutic agent 1-\(\beta\)-arabinofuranosyl-cytosine (ara-C) and 5-fluorodeoxyuridine, respectively.

A vast part of this work contained the search of suitable forms of molecules able to be coupled to the liposomes. This included the fragmentation of IgG antibodies to F(\(ab\))\(_2\)-fragments, cloning of new protein constructs, protein production in bacteria and yeast, protein purification and chemical modification.

For the construction of immunoliposomes small unilamellar liposomes containing amino groups at the distal end of poly(ethylene glycol) (PEG) chains were modified with sulfosuccinimidyl 4-(\(N\)-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC). This molecule carries a succinimidyl group which can react with amino groups on liposomes and a maleimide group which reacts with thiol groups on proteins or peptides. The thiol groups were introduced into the proteins either by modification with \(N\)-succinimidyl-S-
Summary

Acetylthioacetate (SATA) (α-CEA F(ab)2 antibody fragments) or by introduction of additional cysteines to the C-terminus of the proteins (α-EDB scFv) or of the peptides (RGD). Up to 3 F(ab)2 - , 30 - 70 scFv - and 70 RGD molecules could be coupled to the surface of one liposome with a mean diameter of 50 nm.

First, the binding properties of these immunoliposomes were analysed in vitro. The specificity of the F(ab)2-immunoliposomes to CEA was shown by flow cytometry on CEA positive HCT116A cells. The binding properties of the α-ED-B scFv- and the α-αβ3 RGD-immunoliposomes were confirmed on ED-B positive Caco-2 cells and on αβ3-integrin expressing MDA-MB-231 cells by fluorescence microscopy.

Finally, the properties of the α-ED-B scFv- and the α-αβ3 RGD-immunoliposomes were tested in vivo. The biodistribution of α-ED-B scFv-immunoliposomes was analysed in nude mice bearing subcutaneous F9 tumours. The intravenous administration of 114mIndium labelled α-ED-B scFv-immunoliposomes to mice resulted in a higher accumulation in the tumour compared to unmodified liposomes within the first 2 hours. Furthermore, α-ED-B scFv-immunoliposomes loaded with 5 FdU-NOAC reduced the tumour growth by 45% in nude mice bearing subcutaneous F9 tumours. The antimetastatic properties of α-αβ3 RGD-immunoliposomes containing NOAC were examined by co-injection of liposomes and B16F10 cells into mice. The RGD-immunoliposomes could inhibit metastasis formation in the lung by 35%.

Although this examination describes the complexity of immunoliposomes, liposomes containing cytotoxic agents and specific molecules on their surface are promising vehicles for a more specific and efficient cancer therapy.
ZUSAMMENFASSUNG

Viele der heute eingesetzten Substanzen in der Krebsbekämpfung haben unerwünschten Nebenwirkungen, führen zur Resistenzentwicklung und haben wegen ihrer kurzen Verweildauer im Blut nicht den gewünschten therapeutischen Effekt.
Ein grosser Teil dieser Arbeit bestand darin, geeignete Formen von Molekülen zu finden, die an Liposomen gekoppelt werden konnten. Dies beinhaltete die Fragmentierung von IgG Antikörpern zu F(ab)₂-Fragmenten, Klonierung von neuen Proteinkonstrukten, Proteinproduktion in Bakterien und Hefe, Proteinreinigung sowie chemische Modifikationen.
Zur Herstellung von Immunoliposomen wurden mit Polyethylenlykol-Ketten modifizierte Liposomen (PEG-Liposomen) am distalen PEG-Ende mit sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexan-1-carboxylat (sulfo-
Zusammenfassung

SMCC) modifiziert. Dieses Molekül trägt einerseits eine Succinimidylgruppe, welche mit Aminogruppen auf den Liposomen eine kovalente Bindung eingehnt, andererseits eine Maleimidylgruppe, die mit Thiolgruppen auf Proteinen oder Peptiden reagieren kann. Diese Thiolgruppen wurden entweder durch Modifikation der Proteine mit N-succinimidyl-S-acetylthioacetat (\(\alpha\)-CEA F(ab)\(_2\) Fragment) oder zusätzlichen Cysteinen am C-Terminus der Proteine (\(\alpha\)-ED-B scFv) oder der Peptide (RGD) eingeführt. Bis zu 3 \(\alpha\)-CEA F(ab)\(_2\), 30 -70 \(\alpha\)-ED-B scFv- und 70 \(\alpha\)-\(\alpha_\nu\beta_3\) RGD-Moleküle konnten an ein Liposom mit einem mittleren Durchmesser von 50 nm gekoppelt werden.

Die Immunoliposomen wurde zuerst in vitro getestet. Die Spezifität der F(ab)\(_2\)-Immunoliposomen zu CEA wurde mittels Durchflusszytometrie auf CEA positive HCT116A Zellen gezeigt. Die Bindung der \(\alpha\)-ED-B scFv- und der \(\alpha\)-\(\alpha_\nu\beta_3\) RGD-Immunoliposomen konnte auf ED-B positiven Caco-2 Zellen, beziehungsweise auf \(\alpha_\nu\beta_3\)-Integrin positiven MDA-MB-231 Zellen durch Fluoreszenzmikroskopie bestätigt werden.

Schlussendlich wurden die Eigenschaften der \(\alpha\)-ED-B scFv- und der \(\alpha\)-\(\alpha_\nu\beta_3\) RGD-Immunoliposomen in vivo untersucht. Die Verteilung von \(\alpha\)-ED-B scFv-Immunoliposomen in Nacktmäusen mit subkutan implantierten F9 Tumoren wurde analysiert. Die mit \(^{114}\text{m} \text{Indium}\) radioaktiv markierten Immunoliposomen zeigten eine erhöhte Anreicherung im Tumor während den ersten 2 Stunden im Vergleich zu unmodifizierten Liposomen. Die Behandlung von Mäusen mit subkutan injizierten F9 Tumoren mit \(\alpha\)-ED-B scFv-Immunoliposomen beladen mit 5 FdU-NOAC führte zu einer Hemmung des Tumorwachstums von 45%. Die antimetastatische Wirkung von \(\alpha\)-\(\alpha_\nu\beta_3\) RGD-Immunoliposomen beladen mit NOAC wurde bei gleichzeitiger Injektion von Liposomen und B16F10 Zellen in Mäusen demonstriert. Die RGD-Immunoliposomen bewirkten eine Hemmung der Metastasenbildung in der Lunge von 35%.

Obwohl diese Arbeit die Komplexität von Immunoliposomen aufzeigt, sind Liposomen beladen mit zytotoxischen Substanzen und versehen mit spezifischen Antikörpern auf ihrer Oberfläche vielversprechende Vehikel zur gezielten und effizienten Bekämpfung von Krebs.
1 INTRODUCTION

1.1 Carcinogenesis and cancer treatment

Carcinogenesis is based on genetic changes resulting in uncontrolled cell proliferation, decrease in cell death, blocking of differentiation and metastatic spread (Figure 1.1). Cancer is caused by a combination of environmental and many different genetic factors (1, 2). One of three persons will be stricken with cancer in his life time and 50% - 60% of these persons will die from this disease.

![Figure 1.1: Development of carcinogenesis and tumour angiogenesis illustrated with the example of breast cancer: A) normal breast duct; B) hyperplasia; C) dysplasia, carcinoma in situ; D) angiogenic carcinoma in situ; E: invasive carcinoma (adapted from Ref. (3)).](image)

Nonsurgical methods of cancer treatment, chemotherapy and radiation therapy, are based on agents that kill cells. Therefore, they are associated with considerable unwanted side effects. The main problem with the current treatment methods is that in general they do not have specificity for cancer cells. For anticancer drugs, it is primarily the rapid proliferation of many cancer cells that makes them more sensitive to cancer cell killing than to their normal cellular counterparts. Improvements in chemotherapy have to be achieved by reduction of side effects and by obtaining increased efficacy.

Recent therapeutic approaches for the treatment of cancer aim to the development of novel delivery systems to increase the therapeutic indices of the agents by targeting drugs to diseased cells. One strategy uses the expression of cell surface epitopes as targets for selective delivery. Many antibodies and
antibody fragments were raised against antigens located on the surface of tumour cells. Cytotoxic molecules with tumour cell specificity can be prepared by the covalent coupling of different agents to antibodies (see 1.3) (4, 5).

A large number of cytotoxic agents have very a short half-life in the body. This property requires high doses and repetitive administrations. Different approaches like poly(ethylene glycol) modification of the drug (6, 7) or the encapsulation of the drug into long circulating molecules like liposomes were established in the past (see 1.4 and 1.5) (8, 9).

1.2 Angiogenesis

The physiology of solid tumours differs from that of normal tissues in a number of important aspects. The major variation lies between the two vasculatures. Compared with the regular, ordered vasculature of normal tissues, blood vessels in tumours are often highly abnormal and have distinguished capillaries with leaky walls and sluggish blood flow. Furthermore, correlation between microvessel density and tumour invasiveness has been reported (10). Tumour growth requires continuous formation of new blood vessels (angiogenesis) (Figure 1.1). Angiogenesis is essential for normal embryogenesis, reproduction and growth, but it also occurs in the development of many diseases like rheumatoid arthritis, ischemic peripheral vascular disease, ischemic heart disease, diabetic retinopathy, tumour growth and formation of metastasis (11, 12). The switch to the antigenic phenotype involves a change in the local equilibrium between positive angiogenic regulators (e. g. α and β fibroblast growth factors, vascular endothelial growth factor (VEGF), placental growth factor, transforming growth factor, hepatocyte growth factor, angiogenin, interleukin 8, etc.) and negative angiogenic regulators (e. g. thrombospondin-1, angiostatin, interferon-α, prolactin 16-kD fragment, metalloproteinase inhibitors, platelet factor 4, etc.) (3, 12).

Because tumour growth depends on angiogenesis the selective delivery of toxic agents to new-forming blood vessels should offer a new therapeutic benefit. Therefore, great effort to search specific angiogenic markers has been done in the past. The major presently known angiogenic markers are listed in Table 1.1. VEGF is one of the most relevant positive angiogenic factors. Not surprisingly
its receptors fetal liver kinase 1 (flk-1) and fms-like tyrosine kinase 1 (flt-1) could represent promising markers for angiogenesis (13, 14). But unfortunately an overexpression of flk-1 and flt-1 in other tissues was also described (15). Other promising angiogenic markers are the angiopoietin-receptors called Tie-1 and Tie-2 (16). They are receptor tyrosine kinases like flt-1 and flk-1 and are expressed on vascular endothelial cells. Angiostatin is a circulating inhibitor of angiogenesis generated by proteolytic cleavage of plasminogen. The mechanisms responsible for its anti-angiogenic properties are still unknown. There is some evidence that angiostatin functions as a non competitive inhibitor of plasminogen activation resulting in a reduced invasive activity (17). In addition, binding of angiostatin to the $\alpha/\beta$-subunits of ATP-synthase on the cell surface may mediate the down-regulation of endothelial cell proliferation and migration (18). Another potent inhibitor of angiogenesis is endostatin which is a carboxyl-terminal proteolytic fragment of collagen XVIII. The action mechanism of endostatin and its binding to endothelial cells is still unclear (19).

**Table 1.1: Overview of some important angiogenic markers.**

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<td>Receptors</td>
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<td></td>
<td>- Tie-2,Tie-2/Tek (receptors for angiopoietin)</td>
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<td></td>
<td>- Angiostatin receptor</td>
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<td>- Endostatin receptor</td>
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<td>- Prolactin 16 kDa fragment receptor</td>
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<td>Integrins</td>
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<td>- $\alpha_v\beta_5$</td>
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<tr>
<td>Adhesions molecules</td>
<td>- E-selectin</td>
</tr>
<tr>
<td></td>
<td>- ED-B</td>
</tr>
<tr>
<td>Proteases</td>
<td>- uPA</td>
</tr>
<tr>
<td></td>
<td>- MMP-7/MMP-9</td>
</tr>
</tbody>
</table>

Formation of new blood vessels also needs interaction with the extracellular matrix. Molecules in the extracellular matrix such as the ED-B domain of B-fibronectin (see Chapters 4 - 6, pages 61 - 114) (20), $\alpha_v\beta_3$-integrin (see Chapter 7, pages 115 126) (21) or E-selectin (22) are upregulated or even induced on stimulated angiogenic vessels. Prevention of this interaction, for example by blocking $\alpha_v\beta_3$-integrin, could represent a tool to aid inhibition of angiogenesis. The proteases involved in extracellular matrix degradation are important for
endothelial cell migration and thereby they are involved in angiogenesis. They include the urokinase-type plasminogen activator (uPA) and the metalloproteases (MMP) which are dramatically upregulated in malignant tumours (23). MMP-7 and MMP-9 may block angiogenesis by converting plasminogen into angioatin (24).

Deeper focus on the structure of the vasculature targeting blood vessels or their extracellular matrix is a complex process where many parameters play a role. Potential targets on tumour blood vessels are molecules that are expressed on the endothelial surface exposed to the blood stream (Figure 1.2A) or to the vascular basement membrane (Figure 1.2B), on the vascular basement membrane itself (Figure 1.2C), or to the extracellular matrix on the luminal (Figure 1.2D) or the abluminal side of endothelial cells (Figure 1.2E).

![Figure 1.2: Targets in the tumour vasculature: A) molecules on the endothelial surface exposed to the blood stream; B) molecules on the endothelial surface exposed to the basement membrane; C) molecules on the basement membrane; D) molecules on the extracellular matrix of the luminal side of endothelial cells; E) molecules on the extracellular matrix of abluminal side of endothelial cells.](image)

Both examples in this thesis (ED-B domain and $\alpha_v\beta_3$-integrin) of tumour vessel-specific targets are adhesion molecules that mediate binding of endothelial cells to the vascular basement membrane. $\alpha_v\beta_3$-Integrin is found on the endothelial cell surface, where it mediates binding to vitronectin, a component of the vascular basement membrane. B-fibronectin is a component of the extracellular matrix where it may act as a ligand for endothelial cell adhesion. These findings
highlight the importance of cell adhesion molecules in the formation of new blood vessels.

An antiangiogenic therapy could have several important advantages over standard anticancer treatments: (a) Different types of tumours could be treated by using the same targeted molecule; (b) Endothelial cells are closer to the blood stream in contrast to tumour cells which are located many layers distant from vasculature; (c) It targets a process that is tumour specific and therefore likely to have little systemic toxicity; (d) The genetic stability of endothelial cells (as opposed to tumour cells) should prevent the development of drug resistance on repeated administration of such agents (25).

1.3 Antibodies and antibody fragments

The use of antibodies for imaging and therapy of human tumours has been a subject of considerable interest for many years. Research has concentrated on several aspects, including the development of suitable antibodies and on the attachment of diagnostic or toxic agents to the antibodies to permit tumour imaging and therapy (26). In recent years many different monoclonal antibodies have been raised and shown to detect human tumours in vivo. However, numbers of problems remain that limit their effective clinical use. One problem is the patient’s generation of an immune response to the administered mouse monoclonal antibody resulting in a rapid clearance. One approach to this problem is the generation of human monoclonal antibodies or chimeric antibodies where mouse variable domains are fused to the human constant region at the gene level. These molecules are expected to be less immunogenic in men than the original antibodies (27). Another approach to minimise the immune response is to reduce the size of the molecule (Figure 1.3). A first generation of truncated antibodies was created by removing of the Fc part to obtain F(ab)₂ antibody fragments by enzymatic digestion (Figure 1.3B). Intact immunoglobulin (IgG) antibodies with a molecular weight of 150 kDa may not penetrate well from blood through the endothelium and to extravascular tumour tissues. It is proposed that F(ab)₂ antibody fragments (100 kDa) achieve a more effective penetration because of their smaller molecular size (28).
A new generation of small molecules recognizing antigens are minibodies, single chain antibody fragments (scFv), or peptides (Figure 1.3C-E). Minibodies are engineered antibody fragments containing the antibody variable light chain (VL), variable heavy chain (VH) and a constant region of the heavy chain of IgG (CH3). They form dimers and have a molecular weight of 80 kDa (29). ScFv are composed of an antibody variable light chain (VL) tethered to a variable heavy chain (VH) by a flexible peptide linker (30). These antibody fragments retain antigen-binding characteristics of their parental antibodies (31). ScFv can be obtained in different ways. One method of scFv engineering is accomplished by the conversion of monoclonal antibodies whose characteristics are known. The specific parts (i.e., binding site domains) of the antibody are cloned and expressed in bacteria or eukaryotic cells. Another approach of obtaining scFv is by phage display technology (32). This technique is based on the display of an scFv on the surface of the bacteriophage, which contains the corresponding gene. The smallest molecules are peptides which are composed of only a few specific amino acids (Figure 1.3E).

1.4 Liposomes

Liposomes are vesicles containing lipid bilayers surrounding aqueous compartments. Depending on the preparation and the lipid composition they have a diameter between 20 nm (SUV: small unilamellar vesicles) to 1 μm.
Introduction

(MLV: multilamellar vesicles). Research extensively investigated liposomes for more than 20 years as carriers for the improved delivery of a broad spectrum of agents including chemotherapeutic agents, imaging agents, antigens, immunomodulators, chelating compounds, lipids and genetic material (33, 34). Hydrophilic compounds can be encapsulated in the inner aqueous phase and lipophilic molecules can be incorporated into the bilayer of liposomes. A few anticancer drugs like doxorubicin, daunorubicin, muramyl tripeptide phosphatidylethanolamine, cisplatin and amphotericin B encapsulated in liposomes have successfully proceeded to clinical evaluation and human application (35).

Figure 1.4: Schematic representation of four major liposomes: A) conventional liposomes; B) sterically stabilised ("stealth") liposomes with PEG; C) conventional and stealth immunoliposomes; D) cationic and glycosylated liposomes.

The liposome system has the advantage that its structural and physicochemical characteristics can be altered. Figure 1.4 summarises the most used types of liposomes based on the formulation of the lipid composition and the modification of the liposomes. Conventional liposomes are composed of phospholipids (e.g. phosphatidylcholine (PC), phosphatidylethanolamine (PE)) and cholesterol (Figure 1.4A). Unfortunately, they have a relatively short blood
circulation time and are rapidly removed by the reticuloendothelial system (RES). The major organs of accumulation are the liver and the spleen. The liposome half-life in the circulation can be prolonged by steric stabilisation of the liposomes. This can be done by insertion of poly(ethylene glycol)-derived-lipids or ganglioside GM₁ (36, 37). Liposomes coated with poly(ethylene glycol) (PEG) are often referred as "sterically stabilised" or "stealth liposomes" (Figure 1.4B) (38). It is believed that polymer coating of liposomes increases their biological half-life due to reduced interactions with plasma proteins or cell surface receptors on macrophages (36). PEG changes the surface properties and alters the solubility of molecules to which it is attached (39). This might influence the capacity of the constructs to cross the extracellular matrix and other structures (40). Small sterically stabilised liposomes are able to extravasate into solid tumours and to sites of infection and inflammation. This observation could be explained by the high number of capillary fenestration found in newly formed blood vessels of this tissues and the endocytotic uptake of vesicles by the cells (36, 38).

The most recently developed type of liposomes are the cationic liposomes, which are used for gene transfer (Figure 1.3D). Their cationic lipid components can interact with the negatively-charged DNA and condense the DNA to a compact and neutral structure. The resulting lipid-DNA complex, rather than DNA encapsulated within liposomes, provides protection and promotes cellular internalisation and expression of condensed plasmids.

The advantages of using liposomes as drug delivery systems have been well documented including: (a) Liposomes are biodegradable and non-toxic; (b) Molecules can be entrapped into the liposomes without modification; (c) Liposomes can carry a large dose of drugs compared to the direct conjugation of the drug to antibodies; (d) Compounds are protected from enzymatic degradation or immunological reaction in the blood stream; (e) The host cells are protected from drug; and (f) Liposomes can interact with the target cells and are therefore able to promote the intracellular delivery of different molecules (34).
1.5 Immunoliposomes

Immunoliposomes carry specific antibodies or antibody fragments coupled on their surface (Figure 1.4C). The attachment of antibodies to liposomes containing anticancer drugs represents a potentially viable method to increase the specificity and efficacy of cancer therapy (41). Several theoretical advantages of this approach, as compared to the use of antibody drug conjugates, are that: (a) The drug is not chemically modified and thus, there is no loss of the cytotoxic activity; (b) It is not necessary to cleave the antibody from the drug prior to exerting its effect upon the target cell; (c) Multiple antibodies on the surface enhance the binding capacity; and (d) A very high number of cytotoxic molecules can be incorporated into a liposome. Successful attempts have been made to prolong the half-life of immunoliposomes by coating them with PEG, thus giving them a greater chance to reach their target sites before being removed by the RES. Numerous studies have shown that PEG-immunoliposomes have significantly increased target cell binding in vitro and also an improved therapeutic efficacy in vivo for the treatment of early solid tumours. However, PEG-immunoliposomes seem to lose their advantage in treating more advanced solid tumours most likely because the binding site barrier restricts penetration of PEG-immunoliposomes into the interior of the tumour. Thus, targeting of immunoliposomes to molecules in the vasculature, where the PEG-immunoliposomes could reach the target cell easier by the blood stream seems to be a more promising strategy (42).

1.6 Attachment of molecules on the surface of liposomes

It is necessary to develop methods for the stable association of antibodies on the liposome surface such that the antibodies retain their ability to recognise and bind to their target. Several techniques have been evaluated to attach proteins to conventional- and PEG-liposomes. These techniques can be subdivided into different categories, depending on whether the protein is coupled to the lipids before or after the formulation of the liposomes or protein can be bound to liposomes with covalent or non-covalent methods (43, 44). Another approach is to attach a lipophilic tag to the protein using DNA-
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engineering which enables partitioning into the lipid bilayer of the liposomes (45). Generally, the proteins can be attached directly to the surface of the liposomes or at the distal end of the PEG-chains. Using PEG-liposomes it may be advantageous to attach the antibody to the distal tip of the PEG-molecule. PEG-liposomes with antibodies linked directly to the surfaces of the liposomes showed lower binding capacity to the antigen because of the sterical hindrance of the PEG-chains (46, 47). Furthermore, the PEG-molecule can be used as a spacer between the antibodies and the liposome surfaces. Attachment of proteins to the distal end of PEG-termini minimises the interference of the polymer chains observed in antibody-antigen interactions (48).

The strategies for covalent coupling of proteins to liposomes used in the literature are summarised in Table 1.2. The chosen methods depend on the chemical features of lipids and proteins. The most common reactive groups on the head groups of natural lipids are the amino groups of phosphatidyl-ethanolamine. In contrast, the twenty amino acids commonly found in proteins possess a wide range of different side chain functions. The reactive groups on proteins that are used for modification are amino groups (lysine, N-terminus), carboxylate groups (aspartic acid, glutamic acid, C-terminus), carboxamide groups (asparagine, glutamine), thiol groups (cysteine) or hydroxyl groups (serine, threonine). In this study the modifications of amino groups and thiol groups were used and described.

In protein amino conjugation chemistry there has been a historical development in the use of different reactions. Early (pre 1970s) methods employed a range of fairly non-specific chemistries such as aryl halides and isothiocyanates. In a later state aldehyde chemistry and methods based on succinimide esters have dominated (1980). In lipid chemistry amino groups are often modified with bifunctional molecules to introduce other reactive groups (e. g. maleinimide) (Table 1.2).
Table 1.2: Overview of the coupling strategies used in literature.

<table>
<thead>
<tr>
<th>Lipid in the liposome</th>
<th>Reactive group on the liposome</th>
<th>Reactive group on the protein</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modification of PE with SPDP</td>
<td>thiol</td>
<td>thiol</td>
<td>(45, 49)</td>
</tr>
<tr>
<td>PDP-PE1</td>
<td>thiol</td>
<td>thiol</td>
<td>(50)</td>
</tr>
<tr>
<td>PDP-DPPE2</td>
<td>thiol</td>
<td>thiol</td>
<td>(46, 51)</td>
</tr>
<tr>
<td>MPB-PE3</td>
<td>maleinimide</td>
<td>thiol</td>
<td>(52, 58)</td>
</tr>
<tr>
<td>MP-PL4</td>
<td>maleinimide</td>
<td>thiol</td>
<td>(59)</td>
</tr>
<tr>
<td>MP-PE5</td>
<td>maleinimide</td>
<td>thiol</td>
<td>(59)</td>
</tr>
<tr>
<td>EMC-PL6</td>
<td>maleinimide</td>
<td>thiol</td>
<td>(59)</td>
</tr>
<tr>
<td>EMC-PE7</td>
<td>maleinimide</td>
<td>thiol</td>
<td>(59)</td>
</tr>
<tr>
<td>MPB-DPOPE8</td>
<td>maleinimide</td>
<td>thiol</td>
<td>(34, 46, 60)</td>
</tr>
<tr>
<td>PDP-PEG-DSPE9</td>
<td>PEG-thiol</td>
<td>maleinimide</td>
<td>(41)</td>
</tr>
<tr>
<td>M-PEG-DSPE10</td>
<td>PEG-maleinimide</td>
<td>thiol</td>
<td>(41, 47, 54, 61, 62)</td>
</tr>
<tr>
<td>M-PEG-PE11</td>
<td>PEG-maleinimide</td>
<td>thiol</td>
<td>(63)</td>
</tr>
<tr>
<td>Hz-PEG-DSPE12</td>
<td>PEG-hydrazide</td>
<td>oxidised protein</td>
<td>(34, 42, 46, 64, 65)</td>
</tr>
<tr>
<td>COOH-PEG-DSPE13</td>
<td>PEG-carboxy</td>
<td>amino</td>
<td>(66)</td>
</tr>
</tbody>
</table>

1PDP-PE: N-(3-(2-pyridylthio)propionatephosphatidylethanolamine;  
2PDP-DPPE: N-(3-pyridylthio)propionoylphosphatidylethanolamine;  
3MPB-PE: 4-(p-maleinimidophenylethynylphosphatidylethanolamine;  
4MP-PL: N-(3-maleinimidopropionyl-N'-palmitoyl-L-lysine methyl ester;  
5MP-PE: N-(3-maleinimidopropionyl)phosphatidylethanolamine;  
6EMC-PL: N'-6-maleinimidocaproyl-N-maleinimido-L-lysine methyl ester;  
7EMC-PE: N-(6-maleinimidocaproyl) phosphatidylethanolamine;  
8MPB-DPPE: N-(4'-maleimidophenyl)butyroyldioleoylphosphatidylethanolamine;  
9PDP-PEG-DSPE: N-(3-pyridylthio)propionylamino-poly(ethylene glycol)-distearoylphosphatidylethanolamine;  
10M-PEG-DSPE: maleimide-poly(ethylene glycol)-distearoylphosphatidylethanolamine;  
11M-PEG-PE: maleimide-poly(ethylene glycol)-phosphatidylethanolamine;  
12Hz-PEG-DSPE: hydrazide-poly(ethylene glycol)-distearoylphosphatidylethanolamine;  
13COOH-PEG-DSPE: carboxy-poly(ethylene glycol)-distearoylphosphatidylethanolamine.

The other reactive groups used in this study were thiol groups. The only amino acid with a reactive thiol group is cysteine. Thiol groups have unpaired electrons which make them strong Lewis bases, giving them nucleophilic and reducing properties. The thiol groups react with haloacyl compounds, maleinimides and disulfides. The strong reactivity is the reason that cysteine is most commonly found in proteins as dimeric cystine moieties, or occasionally coupled to other groups such as sugars or porphyrins (67). By using natural thiol groups of cysteines the modifications of the thiol groups have to be carried out under reducing conditions. The most commonly used reducing agents β-mercaptoethanol or dithiothreitol are not in all cases useful because they carry thiol groups themselves and could therefore react with the thiol reactive agents. Because of their useful reactivity, thiol groups are also introduced artificially into proteins. Different heterobifunctional molecules were synthesised. Because thiol
groups are prone to atmospheric oxidation they are often introduced into the proteins in an unreactive protected form. Protected thiol derivatives include the disulfides described already, in particular those with good leaving groups such as pyridyl disulfides, and thioesters such as S-acetyl compounds. The reactive thiol groups are generated by a suitable deprotection step immediately before use. The most commonly protein thiolation reagents are those which exhibit reactivity towards amines (2-iminothiolane, N-succinimidyl S-acetylthioacetate (SATA), S-acetyl-mercaptosuccinic anhydride (SAMSA) and N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP)). The reagent SATA has the advantage that the acetyl function can be removed by treatment with hydroxylamine, whereas by using SPDP the thiol groups must be released by reduction of a disulfide (60, 68).

The coupling strategies used in this study are summarised in Figure 1.5. Briefly, liposomes containing amino groups on their surface were modified with sulfo-SMCC to introduce maleinimide groups (Figure 1.5A). The thiol groups which can react with the maleinimide groups were introduced into the protein either by modification with SATA (Figure 1.5B) or by adding additional cysteines to the C-terminus of proteins (Figure 1.5C) or peptides (Figure 1.5D).
1.7 Aim of this study

As mentioned before the major problems in treatment of cancer are: (a) The short circulation time in blood of drug molecules require repeated and high dose drug administrations; (b) The unspecificity of cytotoxic agents leading to unwanted side effects; (c) The high diversity in development of cancer; and (d) The rise of resistance against one type of treatment.

The aim of this study was to find a system which could possibly circumvent these disadvantages. The idea was to combine the use of liposomes as drug carrier system with the antibody specificity or other specific molecules (scFv, peptides) to tumour cells or tumour endothelial cells providing the following advantages: (a) The incorporation of drugs in long circulating liposomes leads to more favourable pharmacokinetic properties; (b) The attachment of tumour specific molecules and tumour endothelial cell specific molecules to transport the cytotoxic agent to the target cell; (c) The high complexity of tumour cells can be circumvented by using angiogenic markers; and (d) The combination of tumour and endothelial cell specific molecules may have a better chance to reach the aim of killing diseased cells.

The goal of this study was to construct F(ab)$_2$-immunoliposomes (Figure 1.5B) recognising the carcinoembryonic antigen (CEA) on tumour cells, scFv-immunoliposomes (Figure 1.5C) recognising the ED-B domain of B-fibronectin located in the extracellular matrix of newly formed blood vessels in the tumour and finally arginine-glycine-asparagine acid (RGD)-immunoliposomes (Figure 1.5D) recognising $\alpha_v\beta_3$-integrin expressed by different tumour cells and involved in angiogenesis and formation of metastasis. This results in three types of immunoliposomes with three different target molecules in the tumour and its environment (Figure 1.6).
**Figure 1.5:** A) Introduction of maleimide groups to the surface of liposomes by modification with sulfo-SMCC; B) Construction of F(\(ab\)\(_2\))-immunoliposomes.
**Figure 1.5:** C) Construction of scFv-immunoliposomes; D) Construction of RGD-peptide-immunoliposomes.
The presented dissertation is divided into 8 chapters. Following the introduction (Chapter 1) the preparation of different types of liposomes in Chapter 2 and the coupling methods of F(ab)\textsubscript{2} fragments to liposomes (Chapter 3) are described. Furthermore, binding and cytotoxic effects of these immunoliposomes were tested. Based on these results the $\alpha$-ED-B scFv was produced in bacteria (Chapter 4) and in yeast (Chapter 5) and coupled to liposomes (Chapter 6). In Chapter 7 the $\alpha$-$\alpha_{v}\beta_{3}$-integrin RGD peptide was attached to liposomes. Finally, the specific binding and the effects of these immunoliposomes were tested \textit{in vitro} and \textit{in vivo} (Chapters 6 and 7). In the discussion (Chapter 8) all results are summarised and an outlook of future experiments is given.
2 PREPARATION, CHARACTERISATION AND MODIFICATION OF LIPOSOMES

2.1 Abstract

Different types of liposomes were prepared using the detergent dialysis or the extruder method to obtain small unilamellar vesicles with mean diameters of 50 or 100 nm, respectively. All types of liposomes contained 100 mol% phosphatidylcholine (PC) and 20 mol% cholesterol. Methoxy-poly(ethylene glycol)-phosphatidylethanolamine (7 mol%; PE-PEG-OMet) was added for the preparation of long circulating “stealth” liposomes (PEG-liposomes). Depending on further experiments the liposomes were either radioactively labelled with $^{3}$H-cholesteryl hexadecylether ($^{3}$H-chol), $^{114}$mIndium ($^{114}$mInCl$_3$) or labelled with fluorescent dyes $N,N'$-bis(1-hexyl-heptyl)-3,4:9,10-perylenbis (dicarboximide) (BHPD) or 3,3'-diocta-decyloxacarbo-cyanine perchlorate (DiO). Lipids containing amino groups like dipalmitoyl-sn-glycero-3-phosphatidylethanolamine (DPPE) or amino-poly(ethylene glycol)-phosphatidylethanolamine (PE-PEG-NH$_2$) were added for the preparation of immunoliposomes. The liposomes containing accessible amino groups were modified with sulfosuccinimidyl 4-(N-maleimimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) to introduce maleimide groups for the reaction with thiol groups present on proteins and peptides.

2.2 Introduction

Liposomes are spherical vesicles of colloidal properties composed by lipids. They are classified into multilamellar large vesicles (MLV), small unilamellar vesicles (SUV) and large unilamellar vesicles (LUV) (for more details see 1.4, page 16). Homogeneous unilamellar vesicles ranging in size of 50 to 200 nm are most often used in medical applications.

The preparation of MLV is very simple. The liposome components are mixed in an organic solvent, which is then evaporated. A thin lipid film is formed. Hydration and agitation with buffer produces a suspension of MLV. Ohsawa et al. described another method to form MLV by freeze-drying (69). However, the
MLV are too large and too heterogeneous for bio-medical applications. Therefore, different methods like sonication, filter extrusion, homogenisation or dialysis have been established to prepare SUV. The preparation of SUV by sonication of the hydrated MLV is very easy but it can result in a heterogeneous population of liposomes (70). Another preparation method was developed by Brunner et. al (71). SUV of 30 nm diameter are formed by solubilisation of the lipid mixture with sodium cholate and by removal of the detergent by gel filtration. The detergent dialysis method is based on the same principle. Sodium cholate or other suitable detergents are added to the lipid mixture and after hydration the detergents are removed by dialysis (72, 73). The most commonly used strategy to prepare SUV is the extruder method. The liposome suspension is extruded at high pressure through a polycarbonate filter with a defined pore size (70). In this study both the extruder and the detergent dialysis methods were used.

Liposomes contain as major component phosphatidylcholine (PC), which is also the main component of most biological membranes and which can be extracted from natural sources such as egg yolks, soy beans or brain tissues or which can be prepared synthetically. Charged lipids such as phosphatidylserine (PS), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) are often added to introduce charges and to increase liposome stability. Cholesterol is ordinarily included to further improve mechanical stability of the bilayer and to decrease leakage of the contents. Poly(ethylene glycol) (PEG) attached to the polar end of a lipid leads to longer circulation in the blood. Liposomes without PEG are called conventional liposomes and liposomes containing PEG chains are defined as stealth liposomes. The circulation time is proportional to the PEG chain length (PEG5000-PE > PEG2000-PE > PEG750-PE). The half life for liposome blood clearance increased from 0.7 h, 1.7 h to 6.2 h by inclusion of PEG750-PE, PEG2000-PE and PEG5000-PE in the lipid composition, respectively. The highest possible amount of PEG to include into liposomes is 7 mol% (37).

The lipids used in this project were soy PC (SPC), cholesterol, PE, PE-PEG2000-OMet and PE-PEG2000-NH₂ (Figure 2.1).
The variation in lipid composition and modification leads to a large number of different liposomes. In this study several forms of liposomes were prepared. Generally, four different fundamental liposome types were prepared (Figure 2.2). Table 2.1 on page 36 lists the lipid compositions of these liposomes. Liposomes of type A and C contained no amino groups and were used as negative controls. Liposomes of type B and D contained 2 mol% or 7 mol%
amino groups introduced for further modifications. In some experiments the characteristics of conventional liposomes (types A and B) and stealth liposomes (types C and D) were compared.

**Figure 2.2:** Types of liposomes prepared in this study: A) control conventional liposomes; B) conventional liposomes for amino group modification; C) control stealth liposomes; E: stealth liposomes for amino group modification.

The structure of liposomes enables them to carry both lipid and water soluble components such as drugs, fluorescent or radioactive markers. The fluorescent dyes BHPD and DiO, the radioactivity marker \[^{3}H\]-chol and the cytotoxic compounds N\(^{4}\)-octadecyl-1-\(\beta\)-D-arabinofuranosylcytosine (NOAC) and 2'-deoxy-5-fluorouridylyl-N\(^{4}\)-octadecyl-1-\(\beta\)-D-arabinofuranosylcytosine (5FdU-NOAC) (Chapters 3, 6, and 7) used in this project were lipophilic and added to the organic solvent during the liposome preparation (Figure 2.3, and Table 2.2, page 38). For *in vivo* studies the liposomes were labelled with \(^{114m}\)Indium which was entrapped into the captured aqueous volume by complexion to encapsulated nitrilotriacetic acid.
Figure 2.3: Structures of the compounds incorporated into the lipid bilayer: A) BHPD; B) DiO; C) NOAC; D) 5FdU-NOAC.

Liposomes containing amino groups (types B and D) were modified with sulfo-SMCC to be able to introduce maleimide groups (Figure 2.4). This bifunctional reagent can crosslink molecules via amino groups and thiol groups, respectively, resulting in covalent thioether bonds (-S-C-) which, in contrast to disulfide bonds (-S-S-), are more stable in vivo (74).
Figure 2.4: Scheme of the modification of stealth liposomes containing amino groups with sulfo-SMCC to introduce maleimide groups.
2.3 Methods

2.3.1 Material

Soy phosphatidylcholine (SPC) was from L. Meyer (Hamburg, Germany) and cholesterol recrystallised from methanol from Fluka (Buchs, Switzerland). 2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine (DPPE) and methoxy-poly(ethylene glycol)-phosphatidylethanolamine (PE-PEG-OMet) were from Sygena (Liestal, Switzerland). Amino-poly(ethylene glycol)-phosphatidylethanolamine (PE-PEG-NH₂) was synthesised by Shearwater Polymers, Inc. (Enschede, The Netherlands). [³H]-cholesteryl-hexadecylether ([³H]-cholesterol) and [⁵-³H]-N⁴-octadecyl-1-β-D-arabinofuranosylcytosine ([⁵-³H]-NOAC) were from Amersham (Zürich, Switzerland) and ¹¹⁴⁻Indium hydrochloride (¹¹⁴⁻InCl₃) from NEN life science products (Boston, MA). N,N'-bis(1-hexylheptyl)-3,4:9,10-perylenbis(dicarboximide) (BHPD) was synthesised according to (75) and 3,3'-dioctadecyloxacarboxyanine perchlorate (DiO) and 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, succinimidyl ester (BODIPY) from Molecular Probes (Leiden, The Netherlands). N⁴-octadecyl-1-β-D-arabinofuranosylcytosine (NOAC) and 2'-deoxy-5-fluorouridylyl-N⁴-octadecyl-1-β-D-arabinofuranosylcytosine (5FdU-NOAC) were synthesised as described in (76) and (77), respectively. All buffer salts and other chemicals were from Fluka or Sigma (Buchs, Switzerland).

2.3.2 Liposome preparation by dialysis

SUV were prepared using the detergent dialysis method as described by Rubas (73). Briefly, the lipid mixture was dissolved in methanol / methylenechloride (1 : 1 v/v). Sodium cholate at a ratio of total lipids to detergent of 0.6 mols was added and the mixture evaporated to dryness. The formed lipid film was dispersed in PB (13 mM KH₂PO₄, 54 mM NaHPO₄, pH 7.4) and dialysed against 5 litres of PB overnight using a Lipoprep-GD-1 instrument (Diachema, Langnau, Switzerland). Size and stability of the liposomes were analysed as a dilution of 1 : 4 (v/v) in PB with a submicron particle sizer (Nicomp Model 370, Santa Barbara, USA). Table 2.1 on page 37 summarises the lipid compositions
of the different liposomes prepared. Depending on the experiments markers or additional lipids were added as listed in Table 2.2 on page 38.

2.3.3 Liposome preparation by extrusion
SUV were prepared by sequential filter extrusion of multilamellar liposomal preparations in PB through Nuclepore membranes (Sterico, Dietikon, Switzerland) of 0.4 µm and 0.1 µm pore diameter with a Lipex extruder (Lipex Biomembranes Inc., Vancouver, Canada). Size and stability of the liposomes were measured as described above (2.3.2).

2.3.4 Modification of liposomes with BODIPY FL®, SE
The amino-reactive dye BODIPY FL®, SE was used for determination of the modification efficiency of the amino groups on liposomes. Liposomes containing 40 mg/ml SPC and 2 mol% PE-PEG-NH₂ corresponding to 9.3 x 10¹⁶ amino groups on the surface of the liposomes (Table 2.3, page 41) in 300 µl 0.1 M sodium bicarbonate, pH 8.3 were modified with BODIPY FL®, SE (10 mg/ml in dimethylformamide (DMF)) for 1 h at RT at a molar ratio of amino groups to BODIPY of 0.5, 1 : 1, 1 : 1.5 or 1 : 5. The reaction was stopped with 30 µl hydroxylamine (1.5 M, pH 8.5) for 1 h at RT. Unreacted BODIPY was removed by loading the reaction mixture on a Biogel P6 column (1 x 15 cm; Biorad, Glatbrugg, Switzerland) equilibrated in PB. The modified liposomes were eluted with PB at 1 ml/min. Fractions of 1 ml were diluted in methanol and analysed using a fluorescence spectrofluorometer SFM 23 (Kontron, Zürich, Switzerland) with 510 nm excitation and 502 nm emission of wavelengths.

2.3.5 Modification of liposomes with sulfo-SMCC
The liposomes were modified with sulfo-SMCC to introduce maleimimide groups. Liposomes containing 40 mg/ml SPC and 2 mol% PE-PEG-NH₂ corresponding to 1.5 x 10¹⁷ amino groups on the surface of the liposomes (Table 2.3, page 40) in 500 µl PB were incubated with 0.6 mg crystalline sulfo-SMCC (7.8 x 10¹⁷ molecules; Pierce, Lausanne, Switzerland) for 30 min at 30° C. The molar ratio of amino groups to sulfo-SMCC was 1 : 5. Unreacted sulfo-SMCC was removed by loading the reaction mixture on a Biogel P6 column (1 x
5 cm) equilibrated in HBSE (10 mM Hepes, 150 mM NaCl, 9.1 mM EDTA, pH 7.5), followed by centrifugation (2 min, 100 g).

2.3.6 Labelling of liposomes with $^{114}$mIndium

Liposomes were labelled with $^{114}$mIndium as described by Proffitt (78). Briefly, liposomes containing 80 mg/ml SPC and 1 mg/ml of the ionophore A23187 for the diffusion of $^{114}$mIndium were prepared by extrusion in 1 mM nitrilotriacetic acid (NTA) in PB. Unencapsulated NTA was removed from the SUV by buffer exchange on a Sephadex G-50 column (Pharmacia) equilibrated in PB. Control unmodified liposomes were loaded with $^{114}$mInCl$_3$ for 30 min at 60° C and immunoliposomes for 2 h at RT. The initial radioactivity ranged between $7 \times 10^5$ cpm to $7 \times 10^6$ cpm per 100 µl liposomes. The reaction was stopped by adding EDTA to a final concentration of 10 mM. Free $^{114}$mIn$^{3+}$ was removed by size exclusion on a Sephadex G-50 column (1 x 90 cm) equilibrated in PB. The radioactivity was measured with a gamma counter (Cobra, Packard Instruments, Illinois, USA).
2.4 Results and Discussion

2.4.1 Liposome preparation and characterisation

Liposomes prepared by detergent dialysis had a diameter of $50 \pm 15 \text{ nm}$ and those prepared by extrusion a diameter of $100 \pm 15 \text{ nm}$. The mean diameter of liposomes is a critical parameter for their behaviour \textit{in vitro} and \textit{in vivo}. Liposomes larger than $300 \text{ nm}$ in diameter have an increased tendency to accumulate in the spleen whereas small liposomes of less than $80 \text{ nm}$ in diameter accumulate in the liver very efficiently (79). The chosen method for the preparation in this thesis depended on the composition and the volume of the liposomes. Liposomes containing the fluorescent dyes BHPD and DiO could only be prepared with the dialysis method because the dyes clog the filter membranes in the extruder. For experiments where more than two different types of liposomes were used the dialysis method was applied. Using this method all different types of liposomes could be prepared in parallel. The instrument has up to 20 separated dialysis cells each for 1 ml liposomes. If more than 1 ml liposomes were needed (e. g. \textit{in vivo} experiments) they were prepared with the extrusion method. Depending on the experiments liposomes containing 2 or 7 mol\% amino groups were prepared. Table 2.1 and Table 2.2 on pages 37 and 38 summarise the different lipid compositions used. In some experiments 100 mol\% SPC corresponded to 80 mg/ml and also to the double amount of the other components. The total number of liposomes and mean number of all lipophilic compounds were calculated from the mean diameters obtained from the laser light scattering data and from the assumptions on vesicle geometry parameters as described by Huang et al. (80). Table 2.3 and Table 2.4 on page 41 summarise these calculations. For example, a liposome with a mean diameter of 50 nm contains 20'000 SPC molecules, based on the volume of one SPC molecule of $1.253 \text{ nm}^3$. Thus, from 40 mg/ml SPC $1.6 \times 10^{15}$ liposomes with a mean diameter of 50 nm are formed (Table 2.3, page 41). Assuming the same volume of $1.253 \text{ nm}^3$ for all other membrane forming molecules, liposomes containing 2 mol\% amino groups with a mean diameter of 50 nm carry approximately 400 amino groups distributed over the bilayer of one liposome or 200 amino groups on the outer monolayer surface of one liposome.
(Table 2.4, page 41). Therefore, the size variation leads to different amounts of calculated lipid molecules and of reactive groups per liposome. The same amount lipid (mg/ml) yields more than four times more liposomes with 50 nm diameter \((1.6 \times 10^{15} /\text{ml})\) than liposomes with 100 nm diameter \((3.6 \times 10^{14} /\text{ml})\) (Table 2.3, page 41). A liposome containing 7 mol\% amino groups and having a diameter of 50 nm has 640 amino groups on its surface, whereas a liposome with a diameter of 100 nm carries 2780 amino groups (Table 2.4, page 41). The loss of lipid during the preparation was generally 5 ± 2 % as determined with radioactively labelled lipids. All prepared liposomes were stable for 3 weeks at 4°C.

**Table 2.1: Lipid composition (mg/ml) of the four liposome types (A, B, C, D) corresponding to Figure 2.2 on page 29.**

<table>
<thead>
<tr>
<th>Type</th>
<th>Name of liposomes</th>
<th>SPC (^1)</th>
<th>Cholesterol (^1)</th>
<th>DPPE (^1)</th>
<th>PE-PEGMet (^1)</th>
<th>PE-PEG-NH(_2) (^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Control</td>
<td>40 mg</td>
<td>4 mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>conventional</td>
<td>100 mol%</td>
<td>20 mol%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>liposomes</td>
<td>52 (\mu)mol</td>
<td>10 (\mu)mol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Conventional</td>
<td>40 mg</td>
<td>4 mg</td>
<td>0.7 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>liposomes 1 for</td>
<td>100 mol%</td>
<td>20 mol%</td>
<td>2 mol%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>modification</td>
<td>52 (\mu)mol</td>
<td>10 (\mu)mol</td>
<td>1 (\mu)mol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Conventional</td>
<td>40 mg</td>
<td>4 mg</td>
<td>2.5 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>liposomes 2 for</td>
<td>100 mol%</td>
<td>20 mol%</td>
<td>7 mol%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>modification</td>
<td>52 (\mu)mol</td>
<td>10 (\mu)mol</td>
<td>3.5 (\mu)mol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Control</td>
<td>40 mg</td>
<td>4 mg</td>
<td>9.8 mg</td>
<td>7 mol%</td>
<td>36 (\mu)mol</td>
</tr>
<tr>
<td></td>
<td>stealth</td>
<td>100 mol%</td>
<td>20 mol%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>liposomes</td>
<td>52 (\mu)mol</td>
<td>10 (\mu)mol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>Stealth</td>
<td>40 mg</td>
<td>4 mg</td>
<td>7.0 mg</td>
<td>2.8 mg</td>
<td></td>
</tr>
<tr>
<td></td>
<td>liposomes 1 for</td>
<td>100 mol%</td>
<td>20 mol%</td>
<td>5 mol%</td>
<td>2 mol%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>modification</td>
<td>52 (\mu)mol</td>
<td>10 (\mu)mol</td>
<td>2.6 (\mu)mol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>Stealth</td>
<td>40 mg</td>
<td>4 mg</td>
<td>9.8 mg</td>
<td></td>
<td>3.5 (\mu)mol</td>
</tr>
<tr>
<td></td>
<td>liposomes 2 for</td>
<td>100 mol%</td>
<td>20 mol%</td>
<td>7 mol%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>modification</td>
<td>52 (\mu)mol</td>
<td>10 (\mu)mol</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Depending on the experiment 80 mg/ml SPC and consequently also double amounts of the other components were used.

### 2.4.2 Labelling of liposomes

The liposomes were labelled by adding the fluorescent dyes BHPD or DiO to the lipid mixture to detect the binding of the immunoliposomes *in vitro* (Table 2.2, page 38 and Figure 2.3A-B, page 31). These fluorescent dyes are very stably incorporated into the liposome membrane. Due to their high fluorescence quantum yield, very low concentrations are sufficient to obtain highly fluorescent...
liposomes. BHPD does not interfere with other membrane components and because of its high lipophilicity no measurable leakage from the liposomes occurs (59). The lipophilic carbocyanine DiO is weakly fluorescent in water but highly fluorescent and quite photostable when incorporated into membranes. The disadvantage of using BHPD and DiO is that the liposomes cannot be prepared by using the extruder method. The dyes clog the polycarbonate membranes. BHPD with an emission maximum at 534 nm and DiO with an emission maximum at 510 nm can be used with standard fluorescein optical filters (emission maximum at 518 nm) in flow cytometry and fluorescent microscopy experiments. For measuring the yield of liposomes during the preparation and the different modification steps the liposomes were labelled with $[^3\text{H}]$-cholesterol.

Table 2.2: Overview of markers and additional lipids added to liposomes containing 40 mg/ml SPC.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fluorescence</strong></td>
<td>0.2 mg</td>
<td>0.4 mg</td>
<td>0.5 mol%</td>
<td>0.4 mol</td>
<td>0.3 μmol</td>
<td>0.4 μmol</td>
<td></td>
</tr>
<tr>
<td><strong>Radioactivity</strong></td>
<td>2.5x10⁵</td>
<td>4x10⁷</td>
<td>5 mg</td>
<td>10 mg</td>
<td>12.5 μmol</td>
<td>25 mol%</td>
<td></td>
</tr>
<tr>
<td><strong>Cytotoxicity</strong></td>
<td></td>
<td></td>
<td>10 μmol</td>
<td>20 mol%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Ionophore</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.5 mg</td>
<td>0.1 μmol</td>
</tr>
</tbody>
</table>

For in vivo studies (see Chapter 6) the liposomes were labelled with $^{114}\text{mInCl}_3$. This $\gamma$-labelling method has different advantages. The liposomes can be labelled with $^{114}\text{mInCl}_3$ after their preparation and all the modification steps. $^{114}\text{mInCl}_3$ has a half life of 50 days allowing its use over long time periods. $\gamma$-emitting isotopes have the advantage over $\beta$-emitters that probes can immediately be analysed. The $^{114}\text{mIn}^{3+}$ can diffuse passively into the liposomes. The diffusion can be increased significantly by incorporation of the cation ionophore A23187 into the bilayer of the liposomes. In the liposomes $^{114}\text{mIn}^{3+}$ ions form a complex with the chelator NTA (Figure 2.5). Different loading conditions were tested. Loading the liposomes with $^{114}\text{mInCl}_3$ at higher
temperatures and addition of more ionophore molecules gave a higher labelling efficiency. The labelling of immunoliposomes had to be carried out at RT to prevent inactivation of the protein. For in vivo studies 3 ml control liposomes and 3 ml immunoliposomes were labelled with $^{114m}$In. The final radioactivity of the control liposomes was $9.3 \times 10^6$ cpm/ml and of the immunoliposomes $1.3 \times 10^7$ cpm/ml.

![Figure 2.5: Scheme of $^{114m}$In labelling of liposomes. $^{114m}$In$^{3+}$ ions diffuse through the ionophor into the liposome. Inside the liposome the positively charged indium forms a strong complex with the negatively charged NTA.](image)

2.4.3 Modification of liposomes

Proteins containing reactive thiol groups can be attached to liposome surfaces via maleimide groups. These maleimide groups were introduced by modification of the liposomes containing amino groups on DPPE or at the distal end of PEG with sulfo-SMCC (Figure 2.4, page 32). The best conditions for the coupling of a molecule containing a succinimide group to the amino groups on the liposomes were established with the use of a fluorescent molecule containing a succinimide group (BODIPY FL®, SE). A liposome containing 2 mol% DPPE or PE-PEG-NH$_2$ and with a diameter of 50 nm carries an average of 200 amino groups on its surface. As shown in Figure 2.6 using a molar ratio of BODIPY FL, SE to amino groups of 5 : 1 a modification efficiency of 60% was obtained. This corresponds to about 120 maleimide groups on the surface of one liposome. During the whole modification procedures about 20% of the liposomes were lost, resulting in $1.4 \times 10^{17}$ maleimide groups/ml. The same calculations for all other types of liposomes are listed in Table 2.4 on page 41.
In this work the reactive maleimide groups were introduced via a modification step by coupling sulfo-SMCC either to the surface to the liposomes or at the distal end of the PEG chains. Other authors used lipids that contained maleimide groups (52) or PEG modified with a distal maleimide (46). The modification with sulfo-SMCC yields only 60% reactive groups available for further modification. The advantage of this additional step is that the modification is very easy compared to the synthesis of the maleimide containing lipids (41, 61, 65). Furthermore, at the beginning of this work no PEG-lipids containing maleimide groups were commercially available.

2.4.4 Final remarks
In the first part of this thesis work different liposomes were prepared, characterised and modified for further experiments. Using various labelling methods the preparation and the modification of the liposomes could be analysed. Tables 2.3 and 2.4 show the yields after preparation and modification of the different types of liposomes. The numbers of liposomes after each modification step was measured by labelling with $[^3]$H]-chol. The preparation of the liposomes gave a mean yield of 95%. The efficiency of the modification with sulfo-SMCC (60%) was calculated by fluorescence labelling with BODIPY and the modification resulted in a final liposome yield of 80%. The liposome
preparations described in this chapter and the numbers listed in Table 2.3 and 2.4 were used for the preparation of immunoliposomes as described in the Chapters 3, 4, 6 and 7.

**Table 2.3:** Yields of liposomes after preparation and modification.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>50 ± 15</td>
<td>1.6 x 10^{15}</td>
<td>1.2 x 10^{15}</td>
</tr>
<tr>
<td></td>
<td>100 ± 15</td>
<td>3.6 x 10^{14}</td>
<td>2.7 x 10^{14}</td>
</tr>
<tr>
<td>80</td>
<td>50 ± 15</td>
<td>3.2 x 10^{15}</td>
<td>2.4 x 10^{15}</td>
</tr>
<tr>
<td></td>
<td>100 ± 15</td>
<td>7.2 x 10^{14}</td>
<td>5.4 x 10^{14}</td>
</tr>
</tbody>
</table>

1 Volume SPC = 1.253 cm³ assuming sphere geometry, according to Ref. (80)
2 Yield calculation: [Initial] x 0.95 x 0.8 (based on yield of liposome preparation (95%) and yield of liposome modification (80%))

**Table 2.4:** Calculations of reactive groups on liposomes.

<table>
<thead>
<tr>
<th>SPC [mg/ml]</th>
<th>Diameter [nm]</th>
<th>Amino groups [mol%]</th>
<th>Initial concentration [amino groups/mol]</th>
<th>Calculated numbers of amino groups per liposome</th>
<th>Yields after sulfo-SMCC modification [maleimide groups/liposome]</th>
<th>Yields after sulfo-SMCC modification [maleimide groups/liposome]</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>50 ± 15</td>
<td>2</td>
<td>3.1 x 10^{17}</td>
<td>200</td>
<td>1.4 x 10^{17}</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1.0 x 10^{18}</td>
<td>640</td>
<td>4.5 x 10^{17}</td>
<td>390</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.1 x 10^{17}</td>
<td>850</td>
<td>1.4 x 10^{17}</td>
<td>510</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1.0 x 10^{18}</td>
<td>2780</td>
<td>4.5 x 10^{17}</td>
<td>1670</td>
<td></td>
</tr>
<tr>
<td>100 ± 15</td>
<td>2</td>
<td>3.1 x 10^{17}</td>
<td>850</td>
<td>1.4 x 10^{17}</td>
<td>510</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1.0 x 10^{18}</td>
<td>2780</td>
<td>4.5 x 10^{17}</td>
<td>1670</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>50 ± 15</td>
<td>2</td>
<td>6.2 x 10^{17}</td>
<td>200</td>
<td>2.8 x 10^{17}</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>2.0 x 10^{18}</td>
<td>640</td>
<td>9.1 x 10^{18}</td>
<td>390</td>
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<td></td>
<td>2</td>
<td>6.2 x 10^{17}</td>
<td>850</td>
<td>2.8 x 10^{17}</td>
<td>510</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>2.0 x 10^{18}</td>
<td>2780</td>
<td>9.1 x 10^{18}</td>
<td>1670</td>
<td></td>
</tr>
</tbody>
</table>

1 Yield calculation: [Initial] x 0.95 x 0.8 x 0.6 (based on yield of liposome preparation (95%), yield of liposome modification (80%) and modification efficiency (60%))
2 Corresponding to modification efficiency of 60%
Seite Leer /
Blank leaf
3 PREPARATION AND PROPERTIES OF ANTI CARCINO-EMBRYONIC ANTIGEN F(AB)2-IMMUNOLIPOSOMES

3.1 Abstract

A new type of immunoliposomes which bind to carcinoembryonic antigen (CEA) expressing tumour cells was developed. The α-CEA antibody A5B7 was digested with bromelain to the A5B7-F(ab)2 fragment and coupled with a new method to the terminal end of the poly(ethylene glycol) chains (PEG) on liposomes. Sulfo succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) modified liposomes prepared with 2 mol% of amino poly(ethylene glycol)-phosphatidylethanolamine (PE-PEG-NH2) or 2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine (DPPE) were coupled to N-succinimidyl-S-acetylthio-acetate (SATA) modified A5B7-F(ab)2 fragments. Up to 3 A5B7-F(ab)2 fragments could be attached to the surface of one liposome with a mean diameter of 50 nm. The coupling efficiency and the binding specificity of α-CEA A5B7-F(ab)2-immunoliposomes without PEG (conventional liposomes) and with PEG (stealth liposomes) were compared. The binding properties of these immunoliposomes were tested on the human colon carcinoma cell lines HCT116A (CEA positive) and HCT116B (CEA negative) by cytofluorometric measurement of liposomal BHPD fluorescence. Both types of A5B7-F(ab)2-immunoliposomes showed a specific binding to the CEA positive cell line HCT116A. Because of the tumour targeting properties of the α-CEA-A5B7-F(ab)2-immunoliposomes they appear attractive as carriers of chemotherapeutic agents, toxins and macromolecular drugs. The cytotoxicity of the immunoliposomes by loading the liposomes with N⁴-octadecyl-1-β-arabinofuranosylcytosine (NOAC) was analysed in vitro.

3.2 Introduction

Various attempts have been made to deliver anti-tumour agents selectively to tumour cells for imaging and therapy of cancer. Monoclonal antibodies have been raised against tumour-associated antigens. High tumour to blood ratios, improved tumour images and more effective therapies have been described
Antibodies and antibody fragments have been directly conjugated to drugs or to vehicles encapsulating drugs (62, 82). The pharmacological properties of antibodies can be altered by modification with PEG (83, 84) or by coupling to the surface of PEG-liposomes (63, 66). Such modifications lead to an extended plasma half-life, reduced immunogenicity, increased solubility and resistance to proteolysis (7). Antibody fragments are usually prepared by proteolytic digestion for example with pepsin, papain or bromelain. An alternative approach is to produce Fab' molecules and other fragments by DNA engineering (27) (see Chapter 4).

In this chapter the A5B7-IgG antibody (Mr: 150 kDa) recognising the carcinoembryonic antigen (CEA) was digested with bromelain to obtain the 100 kDa A5B7-F(ab)2 fragment which was linked to liposomes (Figure 3.1). The α-CEA A5B7 antibody has been used for tumour imaging and therapy of colorectal tumours, in nude mice bearing human xenografts (85), in sheep (86) and in humans (26).

A5B7-F(ab)2 fragments were coupled either to conventional or stealth liposomes containing reactive maleimide groups (see Chapter 2). Maleimide groups react with thiol groups. To introduce thiol groups to proteins, they can be modified with SATA (Figure 3.1) (59, 68). Sulfhydryl-maleimide coupling is a potential technique to attach proteins to the surface of liposomes (87).

For determination of the specific binding of these α-CEA A5B7-F(ab)2-immunoliposomes the human colon carcinoma cell lines HCT116A and HCT116B were used (88). The three tumour cell lines HCT 116, HCT116A and HCT116B differ in a number of biological properties such as tumourigenicity in nude mice, growth rate, colony formation and in cell surface glycosylation. HCT116A is the most aggressive subline, HCT116 of intermediate and HCT116B of lowest aggressiveness (89). Furthermore, the HCT116A line expresses high levels of CEA in contrast to the HCT116B cell line. CEA is a 180 to 200 kDa human glycoprotein which is expressed on a wide range of human carcinomas, including colorectal, gastric, pancreatic and non-small cell lung and breast carcinomas. It is perhaps the most widely studied human tumour marker. Serum CEA determinations are routinely used to follow potential disease progression in patients. The role of CEA in cancer is not fully understood (90).
Figure 3.1: Scheme of the fragmentation of IgG to F(ab)_2 fragments and coupling to sulfo-SMCC modified stealth liposomes.
The *in vitro* binding was tested by incubation of HCT116A and HCT116B cells with fluorescence labelled α-CEA A5B7-F(ab)₂-immunoliposomes followed by flow cytometry. Based on the successful binding assay the immunoliposomes were loaded with N⁴-octadecyl-1-β-arabinofuranosylcytosine (NOAC), a lipophilic derivative of the cytotoxic agent ara-C (59). The widely used antitumour drug ara-C has the disadvantage of being rapidly degraded into the ineffective uracil arabinoside. Furthermore, it is highly toxic due to its high activity against rapidly dividing normal cells. This has lead to numerous attempts to improve the pharmacodynamic characteristics of this antineoplastic compound. The modification of ara-C by the attachment of a octadecyl alkyl chain makes the drug lipophilic. The resulting molecule can be incorporated into liposomes, is less toxic and has longer circulating time *in vivo* (73).

The successful construction, binding to CEA and cytotoxic effects *in vitro* of A5B7-(Fab)₂-immunoliposomes are demonstrated in this chapter.
3.3 Methods

3.3.1 Material and cells
The A5B7-lgG antibody was a gift of D. King (Celltech, Slough, UK). HCT116A and HCT116B cells were obtained from J. Roth (University Hospital Zürich, Switzerland) (91). SATA was synthesised according to (92) and NOAC according to (76). All buffer salts and other chemicals were from Fluka or Sigma (Buchs, Switzerland).

3.3.2 Gel electrophoresis
Gel analysis of the proteins (SDS-PAGE) was performed as described by Laemmli (93) followed by staining with Coomassie brilliant blue or silver staining. Depending on the molecular weight of the proteins 10, 14, 16, 23 and 10-20% acrylamide was added. As standards the prestained molecular weight marker (Gibco BRL, Basel, Switzerland) and the SDS-PAGE molecular weight standard high and low range markers (Biorad, Glattbrugg, Switzerland) were used.
For Coomassie staining the gels were treated with Coomassie R250 (2.5 g/L) in 45% methanol and 10% acetic acid and destained in 45% methanol and 10% acetic acid.
For silver staining the gels were soaked in 10% ethanol for 5 min, in 1% HNO₃ for 3 min, washed in H₂O, soaked in 11.8 mM AgNO₃ for 30 min and washed with H₂O. The bands were visualised with 0.28 M NaCO₃ containing 0.02% formaldehyde and the reaction was stopped with 50 mM EDTA for 5 min.

3.3.3 Determination of protein concentration
Protein concentration was determinate as described by Bradford (94) or by Schaffner (95).
For detection with Coomassie blue, probes of 100 μl were incubated with 5 ml diluted Biorad dye reagent (Cat. Nr 550-0006; Biorad) for 5 min to 1 h. The absorption was measured at 595 nm with a UV spectrophotometer (Ultrospec 3000, Pharmacia, Dübendorf, Switzerland).
For the determination with Amidoblack, probes of 1 μl were spotted on a cellulose filter (Millipore, Volketswil, Switzerland) and air dried. The spots were cut out, stained in a solution of 0.1% Amidoblack 2B (Merck, Basel, Switzerland) in methanol : glacial acetic acid : distilled water (45:10:45, (v/v%)) and washed in destaining solution (methanol : glacial acetic acid : distilled water (90:2:8, (v/v%)). The probes were shaken in 600 μl eluant solution (25 mM NaOH, 0.05 mM EDTA in 50 vol% aqueous ethanol) for 2 h. The samples were analysed by measuring their absorption at 630 nm with a UV spectrophotometer.

3.3.4 Digestion of A5B7-lgG antibodies with bromelain
The A5B7-lgG antibody was digested as described by Pedley (7). Briefly, bromelain (2 mg, 91 nmol) was activated by incubation with 6 ml of freshly prepared 50 mM cysteine in digestion solution (0.1 M sodium acetate, 3 mM EDTA, pH 5.5) for 30 min at 37° C. The cysteine was removed by buffer exchange on two small Sephadex G-25 columns (2 x 6 cm; Pharmacia) equilibrated in digestion solution. The A5B-lgG antibody (20 mg, 133 nmol) in 2 ml digestion solution was incubated with the activated bromelain for 4 h at 37° C. The reaction mixture was adjusted to pH 6 with 492 μl NaOH (0.1 M) on ice. The digestion was analysed on a 10% non-reducing and a 10-20% reducing SDS-PAGE gel.

3.3.5 Purification of A5B7-F(ab)2 fragments
The A5B7-F(ab)2 fragments were purified immediately after fragmentation by size exclusion chromatography on a Sephacryl S-200 column (2.6 cm x 0.5 m; Pharmacia) equilibrated in 0.1 M sodium acetate, 0.1 M potassium chloride, 3 mM diethylenetriamine penta-acetic acid (DTPA), pH 6.0 and eluted in the same buffer at a flow rate of 0.3 ml/min. Fractions of 3 ml were collected, pooled and analysed on a 10% non-reducing SDS-PAGE gel.
3.3.6 Labelling of A5B7-F(ab)2 fragments with [3H]-NSP

For controlling the coupling reaction the proteins were modified with N-succinimidyl [2,3,3H] propionate ([3H]-NSP) which reacted with the amino groups. [3H]-NSP in 60 μl toluene (4 x 10^7 cpm; Amersham, Switzerland) was placed in a round bottom flask and the solvent was removed by a nitrogen flow. A5B7-F(ab)2 fragments (1 mg in 200 μl PBS (0.137 M NaCl, 2.68 mM KCl, 8.09 mM Na2HP04, 1.76 mM KH2PO4, pH 7.4)) were added and incubated for 2 h at RT. Unbound [3H]-NSP was removed on a Biogel P6 column (1 x 5 cm; Biorad) equilibrated in PBS. Fractions of 200 μl were collected and the radioactivity of 2 μl of each fraction determined by a liquid scintillation counter (Packard Instruments, Groningen, Netherlands) using Ultima Gold scintillation cocktail (Packard). The proteins were analysed on a non-reducing 10% SDS-PAGE gel and exposed to scientific imaging film (Kodak, New York, USA). The A5B7-F(ab)2 fragments could be labelled with 5 x 10^6 cpm/mg corresponding to 23.3% of initial radioactivity.

3.3.7 Modification of A5B7-F(ab)2 fragments with SATA

A5B7-F(ab)2 fragments (300 μg in 100 μl PBS) trace labelled with [3H]-A5B7-F(ab)2 fragments (3 x 10^6 cpm) were incubated with 10 μl SATA (15 mM in DMF, corresponding to 9.2 x 10^{16} molecules) at a molar ratio of protein to SATA of 1 : 100 for 1 h at RT. Unreacted SATA was removed by dialysis against 1 litre HBSE (10 mM Hepes, 150 mM NaCl, 9.1 mM EDTA, pH 7.5). The concentration of protein was analysed by measuring the radioactivity. After removing of the acyl-protecting groups by addition of hydroxylamine hydrochloride to a final concentration of 50 mM in 50 mM potassium phosphate, 25 mM EDTA, pH 7.4 and incubation for 2 h at RT under nitrogen, the number of the thiol groups was determined using the Ellman's reagent (see below 3.3.8).

For binding experiments the A5B7-F(ab)2 fragments were modified with SATA at molar ratios of protein to SATA of 1 : 100, 1 : 1000, or 1 : 10'000, respectively.
3.3.8 Determination of thiol groups
The thiol groups were determinate as described by Ellman (92). Freshly prepared Ellman's reagent (2.5 mM 5,5'-dithio-bis-2-nitrobenzoic acid in 100 μl PBS containing 2 mM EDTA) was added to the probes (max. 200 μl). The absorption at 412 nm was measured using a spectrofluorometer (Kontron, Zürich, Switzerland). Cysteine was used as a reference.

3.3.9 Coupling of SATA modified A5B7-F(ab)2 fragments to sulfo-SMCC modified liposomes
The protecting groups on the SATA modified A5B7-F(ab)2 fragments were removed (see 3.3.7) and 500 μg A5B7-F(ab)2 fragments corresponding to 4.5 x 10^{16} thiol groups in 166 μl HBSE were incubated with sulfo-SMCC modified conventional or stealth liposomes prepared with 40 mg/ml SPC and 2 mol% amino groups corresponding 1.4 x 10^{16} maleimide groups (Table 2.4, page 41) in 100 μl HBSE for 20 h at 4°C.

3.3.10 Separation of immunoliposomes and unreacted proteins on a metrizamide gradient
Uncoupled A5B7-F(ab)2 fragments were separated from the immunoliposomes by flotation on a discontinuous metrizamide gradient. The immunoliposome coupling solution (200 μl) was mixed with 100 μl metrizamide (60% in PBS (w/v); Sigma) in ultracentrifuge tubes (Beckman) and overlaid with 2 ml metrizamide (10% in PBS (w/v)) followed by PBS as a top layer. The density gradient was centrifuged (7 h, 85'000 g, 4° C) using a Ti60 rotor in a Beckman ultracentrifuge. Fractions of 400 μl were carefully removed from the bottom to the top and analysed on a 10% non-reducing SDS-PAGE gel.

3.3.11 Binding of A5B7-F(ab)2 fragments, SATA modified A5B7-F(ab)2 fragments and A5B7-F(ab)2-immunoliposomes to CEA positive tumour cells in vitro
HCT116A and HCT116B cells were maintained in RPMI 1640 supplemented with heat-inactivated 10% fetal calf serum, 5.32 mM L-glutamine, 1.5 mM...
sodium pyruvate, 14 mM NaOH, 100 U/ml penicillin, 100 µg/ml streptomycin, 6.55 mg/L L-serin, 12.5 mg/L L-asparagin, 1.25 x MEM nonessential amino acids solution (Cat. 11140-035), 0.6 x basal medium Eagle vitamin solution (Cat. 21040-035) and 0.6 x basal medium Eagle amino acids solution (Cat. 51051-019). All supplements were from Gibco BRL.

HCT116A and HCT116B cells were trypsinised and washed once with PBS. Aliquots of 10^6 cells in 100 µl PBS were incubated with 1 µg / 100 µl A5B7-F(ab)_2 fragments, SATA modified A5B7-F(ab)_2 fragments or fluorescence labelled immunoliposomes (1.8 x 10^{13} liposomes in 100 µl) for 30 min at 4° C. For incubations with the A5B7-F(ab)_2 fragments or SATA modified A5B7-F(ab)_2 fragments, the cells were washed twice with PBS and incubated with 1 µg / 100 µl FITC labelled rabbit anti-mouse antibody (Southern Biotechnology, USA) for 30 min at 4° C. Cells were washed and resuspended in 1 ml FACS buffer (PBS containing 2% fetal calf serum and 0.01% NaN₃) and analysed by flow cytometry using an Epics Elite Analyser (Coulter, Florida, USA). The binding was determined in gated detection windows which were set individually for chosen populations of viable HCT116A and HCT116B cells. 10'000 gated cells were analysed.

3.3.12 Cytotoxicity assay of α-CEA A5B7-F(ab)_2-NOAC-immunoliposomes

HCT116A cells were cultured as described in 3.3.11 in sterile 96-well plates for 48 h (50'000 cells/well). The cells were washed with PBS and incubated in the corresponding medium with the liposomes for 16 h at a final concentration of 30 - 500 µM NOAC in liposomes or mitoxantrone. The supernatants were removed and freshly diluted WST-1 solution in medium (100 µl/well; (4-[3-iodophenyl]-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disul-fonate; Boehringer) was added and incubated for 15 min at 37° C and 5% CO₂. Cell viability was determined by measuring the absorption at 450 nm using a Dynatech MR5000 plate reader (Microtec Produkte, Embrach, Switzerland).
3.4 Results

3.4.1 Fragmentation of A5B7-lgG antibodies with bromelain

IgG molecules can be digested with different types of proteases. Milenic (96) compared the effects of the enzymes papain, pepsin and bromelain on IgG digestion. Pepsin digestion did not always result in immunoreactive fragments. Using papain stable consistent products were often difficult to obtain. In contrast, the F(ab)2 fragments generated by bromelain retained 100% immunoreactivity and could be generated with consistent results from one preparation to another. Based on these results bromelain was used to digest A5B7-lgG antibodies to A5B7-F(ab)2 fragments (Figure 3.1). After 4 h of incubation at 37° C with a molar ratio of antibody to enzyme of 1 : 1.5 the A5B7-lgG antibodies were digested quantitatively. Incubation for more than 5 h resulted in degradation of the A5B7-F(ab)2 fragments.

![Image](Image.png)

Figure 3.2: Coomassie stained SDS-PAGE analysis of A5B7 antibodies and antibody fragments after purification: A) 10% acrylamide, non-reducing condition; B) 1–20% acrylamide, reducing condition: lanes 1: molecular weight marker; lanes 2: A5B7-lgG antibody; lanes 3: A5B7-F(ab)2 fragment.

After digestion the A5B7-F(ab)2 fragments had a molecular weight of 100 kDa as determined on a SDS-PAGE gel (Figure 3.2A, lane 3). By addition of the reducing agent β-mercaptoethanol the disulphide bridges were cleaved resulting in products of 27 kDa and a 24 kDa corresponding to the heavy and
light chain fragments. This is shown on a reducing 10-20% SDS-PAGE gel (Figure 3.2B, lane 3).

3.4.2 Purification of A5B7-F(ab)\(_2\) fragments
Different systems for the purification of A5B7-F(ab)\(_2\) fragments were compared. Two ion exchange columns (Hydroxylapatite, SP-Sepharose) and one size exclusion column (Sephacryl S-200) were evaluated. The best purification was obtained with the Sephacryl-200 column. The F(ab)\(_2\) fragments had to be purified immediately after digestion because of degradation. The purified A5B7-F(ab)\(_2\) fragments eluted in the first peak followed by bromelain and the Fc part of the IgG (Figure 3.3). The yield of A5B7-F(ab)\(_2\) fragments was 80%.

![Figure 3.3: Elution profile of the purification of A5B7-F(ab)\(_2\) fragments on a size exclusion Sephacryl-200 column (2.6 cm x 0.5 m) equilibrated in 0.1 M sodium acetate, 0.1 M potassium chloride, 3 mM DTPA, pH 6. The protein was eluted in the same buffer at 0.3 ml/min and collected in 3 ml fractions.](image)

3.4.3 Introduction of thiol groups to antibody fragments
One possibility to introduce thiol groups to antibodies consists in the reduction of F(ab)\(_2\) fragments to Fab fragments. A5B7-F(ab)\(_2\) fragments were reduced with β-mercaptoethanol or sodium dithionite. In both cases the protein was fully reduced, but after removing of the reducing agents the Fab molecules dimerised back to F(ab)\(_2\) fragments.
Another more general possibility to attach thiol groups on proteins consists in the modification of proteins with the bispecific coupling molecule SATA or other suitable reagents. The succinimide groups of SATA reacts with amino groups of proteins (Figure 3.1). The advantage of SATA is that thiol groups are protected by an acyl group. After removing of this protecting group the free thiol group is capable to react with sulfo-SMCC modified liposomes. The A5B7-F(ab)\textsubscript{2} fragments could be modified with an average of 15 SATA molecules per antibody fragment using a molar ratio of A5B7-F(ab)\textsubscript{2} fragments to SATA of 1 : 100 determined with Ellman's reagent. In comparison, Duncan et al. (68) obtained 4 SATA molecules per antibody using a molar ratio of 1 : 8.

### 3.4.4 Coupling of SATA modified A5B7-F(ab)\textsubscript{2} fragments to sulfo-SMCC modified liposomes

SATA modified A5B7-F(ab)\textsubscript{2} fragments were attached to sulfo-SMCC modified conventional and stealth liposomes (liposomes type B and type D, Figure 2.2, page 30 and Figure 3.1). The molar ratio of maleimide groups to thiol groups was 1 : 3 or the ratio of liposomes to A5B7-F(ab)\textsubscript{2} fragments was 1 : 25. After separation of immunoliposomes and unbound A5B7-F(ab)\textsubscript{2} fragments the amount of coupled protein was determined by loading the immunoliposomes on a SDS-PAGE gel and compared with a dilution series of A5B7-F(ab)\textsubscript{2} fragments. The concentration of the liposomes was calculated by measuring the BHPD fluorescence. The loss of liposomes ranged between 20 to 40%. The yield of protein coupled to the liposomes varied between 3 to 7%. Thus 1 - 3 A5B7-F(ab)\textsubscript{2} fragments could be coupled to the surface of one liposome containing 120 maleimide groups. Surprisingly, a higher coupling efficiency of stealth liposomes compared to the conventional liposomes was found.

### 3.4.5 Separation of liposomes and antibodies

In first pilot experiments the separation of proteins from liposomes was tested by loading liposomes and [\textsuperscript{3}H]-labelled A5B7-F(ab)\textsubscript{2} fragments on different size exclusion columns (Sephadex G-25, G-75, G-100, G-200 and Biogel P6). It was expected that the liposomes eluted in the excluded volume, followed by the unreacted proteins. In all experiments high amounts of radioactivity were
Anti CEA immunoliposomes

measured in the liposome fractions. Meaning that the liposomes and the proteins could not be well separated. Therefore, immunoliposomes were separated from unbound A5B7-F(ab)₂ fragments on a discontinuous metrizamide gradient. The floating immunoliposomes, which were visible as a pink fluorescent band, were carefully removed from the underlying metrizamide phases. Collected fractions from the top to the bottom of the gradient were analysed on a 10% non-reducing SDS-PAGE gel (Figure 3.4). During separation and removal of the metrizamide by dialysis the liposomes were diluted by a factor of 2. The immunoliposomes were stable for at least 2 weeks at 4°C.

![Figure 3.4](image)

**Figure 3.4:** Separation of liposomes and A5B7-F(ab)₂ fragments. Coomassie stained SDS-PAGE analysis of aliquots taken from top to bottom of the metrizamide gradient: *lanes 1 – 5:* probes from an incubation of liposomes prepared with PE-PEG-NH₂ (positive reaction); *lanes 6 – 10:* probes from an incubation of liposomes prepared without PE-PEG-NH₂ (negative control). *Lanes 1 and 6* correspond to the liposome fractions.

3.4.6 Binding of A5B7-F(ab)₂ fragments, SATA modified A5B7-F(ab)₂ fragments and A5B7-F(ab)₂-immunoliposomes *in vitro* to HCT116 cells

The binding was determined by flow cytometry on CEA-positive HCT116A and on CEA-negative HCT116B cells. As shown in Figure 3.5 the increase of the molar ratio of SATA for the modification of A5B7-F(ab)₂ fragments resulted in a very low decrease of their binding capacity to CEA-positive HCT116A cells.
Figure 3.5: Binding properties of SATA-modified A5B7-F(ab)_2-fragments to CEA on HCT116A cells analysed by flow cytometry using a FITC labelled second antibody.

Figure 3.6A shows the specific binding of the unmodified A5B7-F(ab)_2 fragments to CEA and Figure 3.6B the binding of the A5B7-F(ab)_2-PEG-immunoliposomes to the CEA-positive HCT116A cells but not to CEA-negative HCT116B cells. Unmodified control liposomes showed a very low unspecific binding after an extensive washing procedure (Figure 3.6C).

SATA-modified A5B7-F(ab)_2 fragments were coupled to liposomes containing 0, 0.5, 1 and 2 mol% PE-PEG-NH₂ and their binding properties on the HCT116A cells were compared (data not shown). All three liposome preparations could be modified with A5B7-F(ab)_2 fragments but as expected at different amounts. For the specific binding to HCT116A cells at least 1 mol% of PE-PEG-NH₂ were needed. Incubation times ranging from 30 min to 4 h had no significant influence on the binding properties (data not shown) and incubation of the cells with increasing amounts of liposomes (1.8 x 10^{13} to 3.6 x 10^{14}) in a constant volume of 250 µl resulted in a linear increase of binding (data not shown).
Figure 3.6: Cytofluorometric determination of binding to HCT116 cells: A) A5B7-F(ab)₂ fragments on HCT116A (CEA positive); B) A5B7-F(ab)₂-PEG-immunoliposomes on HCT116A (CEA positive) and on HCT116B (CEA negative); C) unmodified control PEG-liposomes, A5B7-F(ab)₂-PEG-immunoliposomes and conventional A5B7-F(ab)₂-immunoliposomes on HCT116A (CEA positive). The binding of F(ab)₂ fragments and immunoliposomes was detected with a FITC labelled second antibody and by incorporation of the fluorescent dye BHPD in the liposomes, respectively. BHPD fluorescence is identical to FITC.
Furthermore, the binding properties of A5B7-F(ab)2-PEG-immunoliposomes to conventional A5B7-F(ab)2-immunoliposomes were compared. As shown in Figure 3.6C, immunoliposomes without PEG have better binding properties in this in vitro model.

3.4.7 Effect of liposomes containing NOAC on cell growth in vitro

![Graph showing cell viability of HCT116A cells after 16 h incubation with different treatments.]

**Figure 3.7:** Cell viability of HCT116A cells after 16 h incubation with: mitoxantrone (△); A5B7-F(ab)2-NOAC-immunoliposomes (■); A5B7-F(ab)2-NOAC-PEG-immunoliposomes (▼); NOAC-liposomes (●); empty liposomes (○). Cytotoxicity was measured by incubation with WST-1 dye followed by fluorometric determination.

In order to test if the immunoliposomes were able to bind and to deliver cytotoxic agents to the tumour cells, they were loaded with NOAC and added to HCT116A cells. As comparison, mitoxantrone was used as positive cytotoxic control drug. Figure 3.7 shows the cytotoxicity of different NOAC-immunoliposomes compared to unmodified liposomes loaded with NOAC and empty liposomes. A5B7-F(ab)2-immunoliposomes had the best effect followed by A5B7-F(ab)2-PEG-immunoliposomes and unmodified liposomes. Cell viability was reduced by 50% by treatment of the cells with A5B7-F(ab)2-NOAC-
immunoliposomes (■) compared to the unmodified PEG-NOAC-liposomes (▼) at a low concentration of 25 - 50 μM. These results correspond to the binding experiments obtained by flow cytometry. Conventional A5B7-F(ab)2-immunoliposomes bound better to the CEA positive cells than A5B7-F(ab)2-PEG-immunoliposomes (Figure 3.6C).
3.5 Discussion

The successful digestion of A5B7-IgG to A5B7-F(ab)2 fragments and their attachment to conventional and stealth liposomes is described. Working with immunoglobulin fragments is preferable for several reasons. Removal of the Fc segment can minimise the host response to the xenogeneic antibody, reduce the non-specific in vivo binding of the antibody via Fc receptors found on a number of normal cells and reduce the uptake of the antibody into the RES (49). The removal of the Fc part by bromelain digestion of A5B7 IgG resulted in no decrease of binding activity to CEA measured by flow cytometry (data not shown).

Other molecules like PEG (7) or enzymes (97) were attached to the A5B7-IgG antibody. One possibility to couple these molecules to antibodies is by the introduction of a sulfhydryl group to the antibodies with SATA. SATA has a number of advantages compared to other bispecific coupling molecules (see 1.6, pages 19-22). Firstly, the electrostatic charge of the protein is less disturbed. Secondly, only one form of derivative can be formed (68). The most important advantage of using SATA is that no reducing agent like β-mercaptoethanol or dithiothreitol are required for the activation of the thiol groups. Remaining traces of the reducing agent could lower the efficiency of the coupling reaction. In addition, other types of modifications could lead to precipitation of the protein (59). The preparation of these α-CEA A5B7-F(ab)2-immunoliposomes included many experimental steps, namely the fragmentation of IgG to F(ab)2-fragments, purification and modification of F(ab)2-fragments with SATA, removing of the protecting group, modification of liposomes with sulfo-SMCC, the coupling reaction and separation of immunoliposomes from unbound proteins. On the protein side the fragmentation and the modification could be eliminated by construction of F(ab)2- fragments or scFv and by introducing additional cysteines by DNA engineering (see Chapter 4).

Finally, only 1 to 3 A5B7-F(ab)2 fragments containing 15 thiol groups per fragment could be coupled to one liposome with a mean diameter of 50 nm containing 120 maleimide groups on its surface. This corresponds to a rather low coupling efficiency of 2.5% of the initial protein. The coupling efficiency was
higher by attaching the A5B7-F(ab)_2 fragment to PEG-liposomes (± 3 F(ab)_2 fragments / liposome) than by the modification of the conventional liposomes (± 1 F(ab)_2 fragment / liposome). In future experiments more than 2 mol% amino-PEG should be tested. The incorporation of PEG into the liposomes becomes saturated at 7 mol% (37). Therefore, the number of fragments that can be attached to liposomes containing 7 mol% amino-PEG could be increased theoretically by a factor of 3 - 4. There are controversial reports in the literature reporting that more antibodies per liposome increase binding efficiency. Conventional A5B7-F(ab)_2-immunoliposomes had better binding properties to CEA compared to A5B7-F(ab)_2-PEG-immunoliposomes (Figure 3.6C). The number of immunoliposomes added to the cells was normalised based on the liposome fluorescence and not on the amount of bound protein. Thus, three times more protein was added to the cells in the PEG-immunoliposome probes compared to conventional immunoliposomes. It can be assumed that the freedom of movement of the PEG chains leads to a reduction of binding of the immunoliposomes to the antigen.

The cell viability test showed that the binding properties of the A5B7-F(ab)_2-immunoliposomes enhanced the effect of NOAC encapsulated in liposomes on CEA positive HCT116A cells in vitro (Figure 3.7). The concentration of NOAC-liposomes added was calculated based on [³H]-NOAC in the liposomes. Three times more A5B7-F(ab)_2 fragments on the NOAC-PEG-immunoliposomes were added to the cells compared to the conventional NOAC-immunoliposomes. The conventional A5B7-F(ab)_2-NOAC-immunoliposomes showed a better effect compared to the A5B7-F(ab)_2-NOAC-PEG-immunoliposomes in the cytotoxic assay (Figure 3.7). The reason for this observation could be the lower binding affinity or that the PEG-chain could hinder NOAC to be released into the cell. Nevertheless, the advantage of stealth liposomes according to the circulation time in vivo will probably overcome these in vitro effects. Therefore, the A5B7-F(ab)_2-immunoliposomes should be tested in vivo. Furthermore, other cytotoxic compounds than NOAC have to be tested (e.g. liposomal mitoxantrone). The biodistribution and cytotoxic effects could be analysed in the LS174T colon xenograft model in nude mice. The human colon adenocarcinoma cell line LS174T is a well described model for the detection and binding to the CEA (98).
In contrast to stealth F(\(ab\)_2)-A5B7-immunoliposomes, the biodistribution of A5B7-IgG, A5B7-F(\(ab\)_2) and A5B7-Fab' fragments (99) and their PEG-modified constructs (7) as well as conventional A5B7-F(\(ab\)_2)-immunoliposomes (49) were described. PEG modification of the intact A5B7-IgG had little effect on biodistribution. In contrast, PEG-modification of A5B7-F(\(ab\)_2) and A5B7-Fab' significantly prolonged plasma half-life and increased accumulation in the tumour. Conjugation of PEG to the A5B7-F(\(ab\)_2) fragments doubled the circulation time compared to the unmodified A5B7-F(\(ab\)_2) fragments.
4 SINGLE CHAIN FV ANTIBODY FRAGMENT BINDING TO THE ED-B DOMAIN OF THE B-ISOFORM OF FIBRONECTIN PRODUCED IN E. COLI

4.1 Abstract
Based on the positive results obtained with the α-CEA A5B7-F(ab)_2-immunoliposomes, the construction of immunoliposomes for targeting tumour blood vessels was investigated. A single chain Fv antibody fragment (scFv) directed against the ED-B domain of the B-fibronectin isoform which was found to be present only in newly formed blood vessels during tumour angiogenesis was used as the specific molecule. This α-ED-B scFv was produced in E. coli. The yield of the purified protein was 10-15 mg/litre culture medium. For the coupling to the liposomes the α-ED-B scFv was modified with N-succinimidyl-S-acetylthioacetate (SATA) to introduce thiol groups to the protein. Up to 4 SATA molecules per protein could be introduced. Unfortunately, this modification inhibited the binding activity of the α-ED-B scFv on ED-B coated ELISA plates by 100%. Therefore, an effort was made to introduce a thiol group into the protein by adding one cysteine near the C-terminus using DNA engineering. The new α-ED-B scFv, called α-ED-B scFv-CM1, was produced in E. coli with a yield of 3 mg/litre culture medium. However, the coupling of the α-ED-B scFv-CM1 to the liposomes was not successful, assuming that one cysteine was not enough or that it was not accessible for coupling. Thus, in the following experiments other α-ED-B scFv constructs containing more than one cysteine were engineered (see Chapter 5).

4.2 Introduction
Single chain Fv antibody fragments (scFv) represent potential molecules for the targeted delivery of drugs, toxins or radionuclides and for coupling to liposomes for diagnostic and therapeutic applications. A scFv molecule is an engineered antibody derivative that includes heavy- and light-chain variable regions joined by a peptide linker (Figure 1.3D, page 16). ScFv are potentially more effective
than unmodified IgG antibodies. The reduced size of 27-30 kDa permits them to penetrate tissues and solid tumours more readily (30).

Carnemolla et al. (20) isolated and characterised a scFv binding to the human ED-B domain of the B-fibronectin (B-Fn) isoform using phage display technology. The successful use of phage antibodies and bacterially expressed antibody fragments as immunological reagents has been demonstrated and they are expected to largely replace the hybridoma-based monoclonal antibody technology (100). Repertoires of scFv derived from human V-gene segments were displayed on phages and selected using immobilised recombinant human ED-B domains. The scFv secreted into bacterial cultures were characterised by their binding to Fn isoforms. Fn is an extracellular adhesion molecule which mediates interactions between cells and extracellular matrix components. Fn is a dimeric glycoprotein composed of two subunits joined by disulfide bonds. The protein is involved in different biological processes, such as establishment and maintenance of morphology, cell migration, haemostasis and thrombosis, wound healing and oncogenic transformation (101). Fn polymorphism originates from alternative splicing patterns of the primary transcript of a single gene in three regions (IICS, ED-A, ED-B) (101, 102). The B-Fn isoform with the ED-B oncofetal domain is present in the stroma of foetal and neoplastic tissues and in adult and neoplastic blood vessels during angiogenesis but it is not detectable in mature vessels (103, 104). Angiogenesis is defined as the formation of new capillary blood vessels. This process is essential for normal embryogenesis, reproduction and growth, but it also occurs in the development of many diseases like rheumatoid arthritis, ischemic peripheral vascular disease, ischemic heart disease, diabetic retinopathy, tumour growth and tumour metastasis (see 1.2, page 12) (12, 105). Therefore, the ED-B isoform of Fn represents a promising marker for angiogenesis in growing solid tumours. The targeting properties of a radiolabelled human high-affinity antibody fragment (L19) directed against the ED-B domain of Fn was recently demonstrated by Tarli et al. (106). This affinity-maturated scFv selectively accumulated in vessels of growing tumours of human and murine origin.

The aim of this chapter was to find a method to attach the α-ED-B scFv to the surface of liposomes. Two constructs of α-ED-B scFv were produced in E. coli
for coupling to the surface of liposomes. First the clone CGS-1 producing α-ED-B scFv was selected and subcloned into the Sfi/NotI sites of the pDN268 expression vector (107) which encodes the sequence for the flag-tag (DYKDDDDK) for detection and a histidine tag for purification at the C-terminus of the scFv. The sequence of the insert of the resulting plasmid pDN351 and the corresponding amino acid sequences is shown in Figure 4.1.

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Cysteine introduced for coupling

**Figure 4.1:** Scheme of the scFv construct containing different C-termini produced in E. coli. VL: variable light chain; VH: variable heavy chain.

The modification of this αED-B scFv with the bifunctional molecule SATA could not be used for coupling the protein on the surface of liposomes because the SATA modification inhibited the binding of the scFv to ED-B on ELISA plates. Therefore, another α-ED-B scFv containing a cysteine near the C-terminus was constructed by DNA-engineering. Cysteine engineering is a highly successful approach widely applicable to chemical modification (107, 108). Figure 4.1 shows the new scFv construct called CM1.
4.3 Methods

4.3.1 Material
The plasmids pQE-12/ED-B, pDN28 and pDN351 were a gift of D. Neri (ETH Zürich, Switzerland). All buffer salts and other chemicals were from Fluka or Sigma (Buchs, Switzerland).

4.3.2 Gel electrophoresis and Western blot analysis
Gel analysis of the proteins (SDS-PAGE) was performed as described by Laemmli (93) followed by staining with Coomassie brilliant blue and silver staining according to 3.3.2, page 45 or immunoblotting.
For Western blot analysis the proteins were transferred onto a Immobilon-P transfer membrane (Millipore, Volketswil, Switzerland) using a semi-dry system (1 h, 0.8 mA/cm²) with transfer buffer (48 mM Tris base, 39 mM glycine, 0.0375% SDS, 20% methanol). The membranes were soaked for 1 h in TBST (10 mM Tris pH 8, 150 mM NaCl, 0.05% Tween 30) containing 5% milk. An incubation for 1 h with a 1 : 2000 (v/v%) dilution of α-flag M2 antibody (Integra Biosciences, Wallisellen, Switzerland) or α-myc 9E10 antibody (ATCC, Rockville, United States) in TBST containing 1% milk followed. Then the membranes were washed three times for 10 min with TBST and incubated for 1 h with a 1 : 7000 (v/v%) dilution of an alkaline phosphatase-labelled goat α-mouse IgG (Promega, Zürich, Switzerland) in TBST containing 1% milk. After three washings with TBST the proteins were detected with a ProtoBlot II AP system (Promega).

4.3.3 Production and purification of ED-B and coupling to sepharose
Production and purification of ED-B was performed as described by Carnemolla (20). Briefly, E. coli TG1 bacteria transformed with the pQE-12 vector containing the sequence for ED-B were grown at 37°C in 2 x TY (1.6% tryptone, 1% yeast extract, 85 mM NaCl) medium containing 100 μg/ml ampicillin (Sigma), 25 μg/ml kanamycin (Sigma) and 0.1% glucose. When the cell suspension reached an A600nm of 0.8, isopropylthiogalactoside (IPTG, Appligene, Basel, Switzerland) was added to a final concentration of 1 mM and growth continued overnight at
30 °C. After centrifugation (30 min, 4'000 g, 4° C), the pellet was resuspended in 60 ml sonication buffer (PBS (0.137 M NaCl, 2.68 mM KCl, 8.09 mM Na₂HPO₄, 1.76 mM KH₂PO₄) containing 20 mM imidazole and 250 mM NaCl) and sonicated three times for 1 min on ice using a cell disrupter (Kontron, Zürich, Switzerland). The soluble protein was separated from insoluble compounds by centrifugation (20 min, 17'000 g, 4° C). The supernatant with the protein was incubated with 4 ml Ni-NTA resin (Qiagen, Basel, Switzerland) equilibrated in sonication buffer for 30 min on ice and loaded onto a column (1.5 x 10 cm) followed by washing with sonication buffer. ED-B was eluted with PBS containing 200 mM imidazole and 250 mM NaCl. The fractions were analysed on a 23% non-reducing SDS-PAGE gel and the protein was stored at -20° C at a concentration of 1 mg/ml.

Purified ED-B was linked to CNBr-activated Sepharose 4B resin (Pharmacia, Dübendorf, Switzerland) by incubation of 4 g gel with 30 ml ED-B (0.6 mg/ml) in coupling buffer (0.1 M NaHCO₃, 0.5 M NaCl, pH 8.6) for 1 h. The reaction was periodically controlled by measurement of the A₂₈₀nm of the supernatant and analysis on a non-reducing 23% SDS-PAGE gel. After incubation with the ED-B protein, the resin was washed with three cycles at different pH. Each cycle consisted of a wash with 5 gel volumes of 0.1 M sodium acetate, 0.5 M NaCl, pH 4 followed by 0.1 M Tris and 0.5 M NaCl, pH 8.

4.3.4 Production and purification of α-ED-B scFv in E. coli

Competent E. coli TG1 (100 μl) bacteria and 2 μl plasmid pDN351 (1 ng/μl) were incubated for 1 h on ice and 2 min at 42° C. After adding 1 ml 2 x TY the mixture was incubated for 1 h at 37° C. The bacteria were spread on an agar plate containing 100 μg/ml ampicillin and incubated at 37° C overnight. One clone was picked and grown at 37° C in 2 litres 2 x TY medium containing 100 μg/ml ampicillin and 0.1% glucose. When the cell suspension reached an A₅₀₀nm of 1, IPTG was added to the final concentration of 1 mM and growth continued overnight at 30° C. After centrifugation (30 min, 4'000 g, 4° C) the supernatant containing the α-ED-B scFv was loaded onto the ED-B modified CNBr activated Sepharose 4B column (2 x 6 cm) (see 4.3.3). The column was washed with 300 ml PBS containing 1 mM EDTA followed by 300 ml PBS containing 1 mM EDTA.
and 500 mM NaCl. The α-ED-B scFv was eluted with 100 mM triethylamine in PBS. Fractions of 3 ml were analysed on a 18% non-reducing SDS-PAGE gel. The scFv was diluted to a concentration of 1 mg/ml and stored at −20°C.

4.3.5 Modification of α-ED-B scFv with Sata

ScFv (3.7 x 10¹⁶ molecules; 300 μg in 500 μl PBS were incubated with 1-36 μl Sata (9.2 x 10¹⁵ - 4.4 x 10¹⁷ molecules; 15 mM in DMF) for 1 h at RT. Unreacted Sata was removed by dialysis against 1 litre PBS. The reactions were analysed by determination of thiol groups according to 3.3.8, page 50.

4.3.6 Binding of scFv to ED-B by ELISA

ELISA plates (96 wells) were coated overnight at 4° C with 1 μg ED-B in 100 μl PBS) per well. After each of the following steps the wells were washed three times with PBS. To prevent unspecific binding the probes were blocked with 200 μl PBS containing 2% milk (PBSM) for 2 h at RT. The ED-B was incubated with 200 μl scFv or Sata-modified scFv (6 ng – 60 μg) in PBSM for 2 h at RT followed by 1 μg biotinylated-M2 (Integra Biosciences) or 1 μg biotinylated-9E10 antibody (ATCC, Rockville, United States) in 100 μl PBSM for 2 h at RT. For the colour reaction the probes were incubated with 100 μl streptavidin-POD conjugate (Boehringer Mannheim, Germany) diluted 1 : 1000 (v/v%) in PBSM for 1 h at RT and followed by 100 μl BM blue POD substrate (Boehringer) for 10 min. The reaction was stopped with 50 μl H₂SO₄ (1 M). The probes were analysed by measurement of the absorption at 450 nm using a Dynatech MR5000 plate reader (Microtec Produkte, Embrach, Switzerland).

4.3.7 Construction, production and purification of α-ED-B scFv-CM1 in E. coli

The plasmids pDN351 and pDN28 were digested with NcoI/NotI. The insert of pDN351 and the vector of pDN28 were purified on agarose gels with the QIAquick gel extraction kit (Qiagen) and then ligated. The ligation was performed in 5 μl ligase buffer (Boehringer) containing 150 ng insert, 600 ng vector and 1 U of T4 DNA ligase (Boehringer) for 12 h at 16° C. The resulting
vector pCM1 was transformed into freshly prepared competent *E. coli* TG1 bacteria. Transformed colonies were selected on agar plates containing 100 µg/ml ampicillin. Twelve clones were picked and analysed for protein production. Production and purification of the α-ED-B scFv-CM1 were performed as described in 4.3.4, page 67. The thiol groups were determined with the Ellman's reagent according to 3.3.8, page 50.

**4.3.8 Coupling of α-ED-B scFv-CM1 to sulfo-SMCC modified liposomes**

Conventional sulfo-SMCC modified liposomes prepared with 40 mg/ml SPC and 2 mol% DPPE corresponding to 7.5 x 10^{15} maleimide groups (Table 2.4, page 41) in 50 µl HBSE (10 mM Hepes, 150 mM NaCl, 9.1 mM EDTA, pH 7.5) were incubated with 500 µg α-ED-B scFv-CM1 corresponding to 8.3 x 10^{16} thiol groups in 400 µl HBSE for 20 h at 4°C. The immunoliposomes and the unreacted α-ED-B scFv-CM1 were separated on a metrizamide gradient according to 3.3.10, page 50. The fractions of the metrizamide gradient were analysed on a SDS-PAGE gel followed by Western-blot analysis as described in 4.3.2, page 66.
4.4 Results

4.4.1 Production and purification of ED-B and α-ED-B scFv

The ED-B protein was purified on a Ni-NTA column which is a one-step method for rapid protein purification. The system is based on the affinity of Ni-NTA resin for proteins that contain six consecutive histidine residues at either their N- or C-terminus. The protein was eluted with imidazole and the yield was 40 mg/litre culture medium (Figure 4.2A). Figure 4.2C lane 2 shows the purified protein with a molecular weight of 10 kDa.

![Graph A](image1.png)

Absorbance at 280 nm vs. Fraction numbers

![Graph B](image2.png)

Absorbance at 280 nm vs. Fraction numbers

![Graph C](image3.png)

The $\alpha$-ED-B scFv was purified on ED-B coupled to a Sepharose column and eluted with triethylamine (Figure 4.2B). The yield after elution was 15 mg/litre culture medium. Figure 4.5 lane 3 shows the purified protein with the molecular weight of 30 kDa. The solubility of the $\alpha$-ED-B scFv caused problems. The protein aggregated at a concentration higher than 1 mg/ml.

4.4.2 Modification of $\alpha$-ED-B scFv with SATA and binding to ED-B

$\alpha$-ED-B scFv were modified with SATA for the introduction of thiol groups similar to the modification of $\alpha$-CEA A5B7-F(ab)² fragments as described in Chapter 3 at a molar ratio of amino groups to SATA ranging from 1 : 0.25 to 1 : 12 corresponding to a molar ratio of protein to SATA of 1 : 4 to 1 : 48. After removing the protecting groups the thiol groups of SATA linked to the $\alpha$-ED-B scFv were determined with the Ellman’s reagent according 3.3.8, page 48. One $\alpha$-ED-B scFv contains 7 lysine residues which can react with SATA. Using a 12-fold excess of SATA, 60% of the amino groups could be modified, corresponding to 4.2 SATA molecules/scFv (Figure 4.3).

![Amino group modification graph](image)

**Figure 4.3:** SATA-modification efficiency of $\alpha$-ED-B scFv by increasing the molar ratio of amino groups on proteins to SATA molecules after incubation for 1 h at RT. 100% amino groups correspond to 7 lysines/scFv.

The binding properties of $\alpha$-ED-B scFv and of SATA modified $\alpha$-ED-B scFv were tested on ED-B coated ELISA plates. In contrast to the $\alpha$-CEA A5B7-
F(ab)2-fragment, the SATA modification of α-ED-B scFv prevents the scFv from binding to ED-B (Figure 4.4). A modification of 13% amino groups corresponding to one SATA molecule per scFv was already enough to inhibit the binding of the SATA modified α-ED-B scFv to ED-B (Figure 4.4 (●)). Therefore another strategy had to be found to introduce thiol groups to the α-ED-B scFv.

![Graph showing binding of different SATA-modified scFv to ED-B coated ELISA plates](image)

**Figure 4.4**: Binding of different SATA-modified scFv to ED-B coated ELISA plates. Modification of α-ED-B scFv with SATA at a molar ratio of amino groups to SATA of 1:0 (■); 1:0.25 (▲); 1:0.5 (▼); 1:0.75 (●); 1:1 (▲); 1:2 (●) and 1:12 (▲). The binding was detected by using a biotinylated second antibody M2 recognising the flag-tag on the scFv followed by streptavidin coupled to a colour reaction system.

### 4.4.3 Construction, production and characterisation of α-ED-B scFv-CM1

Based on the negative results obtained with the modification of α-ED-B scFv with SATA, a thiol group was introduced into the α-ED-B scFv by addition of one cysteine in vicinity of the C-terminus of the protein. To construct the new plasmid called pCM1 the sequence for the α-ED-B scFv (CGS-1) was cut out of vector pDN351 and was cloned into the vector pDN28 (108). This vector contained the sequence for the following amino acids at the C-terminus of the
Anti ED-B scFv protein: GGSSGSGSC-EQK-LISRRDLN (Figure 4.1, page 65). The position of the cysteine near to the C-terminus was confirmed by sequence analysis of the plasmid pCM1. The determination of the thiol groups on the protein with the Ellman's reagent resulted in 0.83 thiol groups per scFv. The α-ED-B scFv-CM1 had the same binding activity on ED-B coated ELISA plates as the α-ED-B scFv without the cysteine (data not shown). The yield of the purified α-ED-B scFv-CM1 was only 3 mg/litre culture medium. A part of the α-ED-B scFv-CM1 formed dimers of 60 kDa due to the cysteines as shown on a non-reducing SDS-PAGE gel (Figure 4.2C lane 4, page 70).

4.4.4 Coupling of α-ED-B scFv-CM1 to sulfo-SMCC liposomes

The coupling of the α-ED-B scFv-CM1 to liposomes according to the method used with α-CEA A5B7-F(ab)2 fragments described in Chapter 3 was not successful. One explanation could be the low number of free thiol groups available for coupling. Only 0.83 thiol groups per scFv molecule seemed not to be sufficient for successful coupling. In comparison, 15 thiol groups could be attached to the A5B7-F(ab)2 fragments by SATA modification without loss of antibody binding properties. In 100 μl liposomes prepared with 2 mol% DPPE or PE-PEG-NH₂ an average of 1.4 × 10¹⁶ maleimide groups were available for the attachment of the protein (Table 2.4, page 41). In the case of the α-ED-B scFv-CM1 only 8.3 × 10¹⁵ thiol groups in 500 μg protein were accessible compared to the A5B7-F(ab)2 fragment where 4.5 × 10¹⁶ thiol groups were present in 500 μg protein, corresponding to 50 times more thiol groups.
4.5 Discussion

Scheme 4.5 gives an overview of the attempts made to introduce thiol groups into the different α-ED-B scFv constructs for the coupling to liposomes. In the first experiments the α-ED-B scFv was modified with SATA according to the modification of α-CEA A5B7-F(ab)2 fragment. Unfortunately, a modification of 13% amino groups which only corresponds to one SATA molecule/scFv was enough to inhibit the binding of α-ED-B scFv to ED-B on ELISA plates (Figure 4.4, page 72). A scFv has a molecular weight of 30 kDa compared to a F(ab)2-fragment with 100 kDa. The probability that a SATA molecule is coupled to the active binding site is greater on a small molecule than on a large one. The α-ED-B scFv (CGS-1) has 7 lysine residues which could potentially react with SATA and which are located in the FR2-, FR4- and CDR2-region of the VL chain and in the FR2-, 2 in FR3- and CDR2- region of the VH chain. Thus, modification of scFv with SATA could not be used to introduce thiol groups on the protein without significant loss of binding activity.

Another method to attach a protein via thiol groups to liposomes consists of the thiol groups of the cysteines present in the protein or by adding cysteines by DNA engineering. These methods overcome the biochemical modification step which could change the binding properties of the protein. The α-ED-B scFv (CGS-1) has 4 cysteine residues in the FR1 and FR3 region of the VH and VL chains. Assuming that these cysteines are necessary for the three-dimensional structure of the protein, one additional cysteine was introduced near the C-terminus. However, the new construct α-ED-B scFv-CM1 could not be coupled to the liposomes. In the vector pDN28 the sequence for the cysteine was not at the very C-terminal end of the protein. Behind the spacer sequence containing the cysteine the sequence for the myc tag (LISRRDLN) followed (Figure 4.1, page 65). This myc-tag sequence was probably responsible for hindering the liposomes to react with the thiol groups.

Another possibility to couple a protein to the surface of liposomes consists in the usage of carboxyl groups on the protein. Carboxyl groups of proteins and amino groups on the surface of liposomes can be converted into amides.
catalysed by a water-soluble carbodiimide by a two step reaction (109). Unfortunately, this kind of coupling was not successful either.

![Diagram](image)

**Figure 4.5: A) B) Overview of the experiments for the modification of the α-ED-B scFv for coupling to liposomes and the results obtained in this chapter. C) Further strategies to introduce thiol groups to the α-ED-B scFv (see Chapter 5).**

One cysteine was not enough to obtain a successful coupling reaction to the liposomes (Scheme 4.5B). Based on these negative results other constructs of the scFv should be produced (Scheme 4.5C). The new α-ED-B scFv should carry more than one cysteine at the C-terminus of the protein. A second negative result was the low amount of scFv-CM1 produced in *E. coli*. The yield of 3 mg/litre culture medium was not enough for liposome coupling experiments. Therefore, another protein expression system like *P. pastoris* had to be established (see Chapter 5).
5 PRODUCTION OF FUNCTIONALISED \( \alpha \)-ED-B SINGLE CHAIN FV ANTIBODY FRAGMENTS IN PICHIA PASTORIS

5.1 Abstract

The Pichia pastoris expression system was used to produce different constructs of scFv that bind to the ED-B domain of B-fibronectin. Four functionalised \( \alpha \)-ED-B scFv (called \( \alpha \)-ED-B scFv-CM2, \( \alpha \)-ED-B scFv-CM3, \( \alpha \)-ED-B scFv-CM4, \( \alpha \)-ED-B scFv-CM5) were constructed for attachment to liposomes. The C-termini of the new \( \alpha \)-ED-B scFv contain 1-3 cysteine residues that are separated from the active protein by a hydrophilic linker (GGSSGGSSGS) and are accessible for coupling to liposomes. Plasmid expression, culture conditions and purification were optimised in 1 litre cultures. The \( \alpha \)-ED-B scFv were purified by ammonium sulphate precipitation followed by anion exchange chromatography. The yields were 5-20 mg/litre culture medium. The large-scale production of the \( \alpha \)-ED-B scFv-CM3 in a 3.7 litre fermenter gave a yield of 60 mg. The reactivity of the cysteines was demonstrated by labelling with the thiol-reactive fluorescent dye ABD-F. All four \( \alpha \)-ED-B scFv bound specifically to ED-B modified Sepharose and binding was further confirmed by immunofluorescence on cell cultures using ED-B positive human Caco-2 tumour cells. This results indicate that the P. pastoris expression system is useful for the large-scale production of cysteine-functionalised \( \alpha \)-ED-B scFv.

5.2 Introduction

The methylotrophic yeast P. pastoris has been shown to be suitable for high yield expression of various proteins by secretion into the culture supernatant or by intracellular localisation (110, 111). Protein expression in P. pastoris is based on the use of the alcohol oxidase gene 1, AOX1. Transcription of the gene is regulated by the AOX1 promoter when the AOX1 coding sequence is replaced by a gene of interest. The activity of this promoter is strictly regulated by the concentration of the carbon source in the culture medium. Full transcriptional activity is achieved by addition of methanol. The protein yield can be enhanced
significantly by manipulation of the factors that influence gene expression and product stability (112).

The sequence for the α-ED-B scFv (CGS-1) corresponding to the insert of pDN351 (Figure 5.1) was cloned into the pPICZαA yeast vector containing a sequence for secretion, for zeocin resistance and for flag-tag detection. Based on the problems described in Chapter 4 (Scheme 4.5, page 73), the four α-ED-B scFv called α-ED-B scFv-CM2, α-ED-B scFv-CM3, α-ED-B scFv-CM4, α-ED-B scFv-CM5 were constructed. As shown in Figure 5.1, they were different in the number of cysteines and spacer length between the cysteines and the α-ED-B sequence.

**Figure 5.1:** Scheme of all scFv constructs made in this study containing different C-termini produced in E. coli (DN351, CM1) and in P. pastoris (CM2, CM3, CM4, CM5). VL: variable light chain; VH: variable heavy chain; hydrophilic amino acids (GGSSGGSSGS); C: cysteines introduced for the functionalisation.

The α-ED-B scFv were functionalised for the linkage to the surface of liposomes that can function as carriers of different molecules like antiangiogenic and cytostatic drugs.

The specific binding of these α-ED-B scFv were demonstrated on an ED-B modified Sepharose column and on cell culture. Some cancer cell lines are able
to synthesise and deposit the two splicing variations ED-B and ED-A fibronectin by culturing the cells on collagen (113). The human colorectal carcinoma cell line Caco-2 expresses ED-B and was used as the positive target for the $\alpha$-ED-B scFv. As a negative control the human colorectal carcinoma cell line Co-115 expressing ED-A was used.

The construction of the four $\alpha$-ED-B scFv, their overexpression in *P. pastoris* and their purification and characterisation are described in this chapter.
5.3 Methods

5.3.1 Material
The Caco-2 cell line was obtained from H. Wunderli-Allenspach (ETH, Zürich, Switzerland) and the Co-115 cell line was obtained from B. Sordat (Swiss Institute for Cancer Research, Lausanne, Switzerland). The pPICZαA vector, the X-33 yeast strain, the 5‘AOX1 and 3′AOX1 primers and the antibiotic zeocin were obtained from Invitrogen BV (Leek, the Netherlands). All yeast culture media were from Difco (Detroit, USA). The oligonucleotides were synthesised by Microsynth (Balgach, Switzerland). All buffer salts and other chemicals were from Fluka or Sigma (Buchs, Switzerland).

5.3.2 Construction of the yeast vectors (pCM2-pCM5)
The sequence of the α-ED-B scFv (CGS-1) was modified by PCR using pDN351 as a template (114). The forward primer (5’-CCG GAA TTC GAG GTG CAG CTG GTG GAG 3’) included an EcoRI site for cloning into the P. pastoris expression vector pPICZαA. Four different reverse primers were used for the four scFv:
pCM2: 5’-GCA TCT AGA TCA TCA TGA ACA CCC ACA TGA CCC CTT GTC ATC GTC GTC CTT GTA GTC-3’;
pCM3: 5’-GCA TCT AGA TCA ACA TGAACA CCC ACA TGA CCC TGA TGA CCC CCC TGATGA CCC CCC CTT GTCATC GTC CTT GTA-3’;
pCM4: 5’-GCA TCT AGA TCA TCA ACAACA ACA TGA CCC TGA TGA CCC CCC TGATGA CCC CCC CTT GTCATC GTC GTC CTT GTA GTC-3’;
pCM5: 5’-GCA TCT AGA TCA TCAACA TGA CCC TGA TGA CCC CCC TGA TGA CCC CCC CTT GTC ATC GTC GTC CTT GTA GTC-3’.
They contained the sequence for the flag-tag, for the hydrophilic spacer amino acids (GGSSGGSSGS) and for the cysteines, a stop codon and an Xba site for cloning into the Xba site of the P. pastoris expression vector. The PCR reactions were performed with the ExpandTM High Fidelity PCR System (Boehringer Mannheim, Germany) in 50 µl containing 120 ng of the DNA template, 25 pmol of each primer, 100 µM dNTP (Boehringer) and 1.75 U Polymerase. Twenty five cycles were carried out in a DNA thermocycler (Perkin
Elmer Certus) each consisting of 1 min at 94°C, 1 min at 55°C and 75 s at 72°C. The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Basel, Switzerland), digested with EcoRI and Xba, purified by electrophoresis on agarose gels and isolated with the QIAquick gel extraction kit (Qiagen). The four inserts were ligated into the pPICZαA vector which had been digested with EcoRI and Xba, treated with alkaline phosphatase and purified by phenol extraction. The ligations were performed in 30 μl ligase buffer (Boehringer) containing 100 ng vector, 300 ng insert and 1 U of T4 DNA ligase (Boehringer) for 12 h at 16°C.

5.3.3 Transformation of the pPICZαA vectors into E. coli
Competent E. coli XL1blue bacteria were freshly prepared using PEG-6000. A 1 ml overnight culture of XL1blue was grown in 100 ml low salt LB (1% tryptone, 5% yeast extract, 171 mM NaCl) to an A600nm of 0.3. The bacteria were centrifuged (15 min, 600 g) and resuspended in 1 ml TSS (low salt LB containing 10% PEG 6000, 50 mM MgCl2, 5% DMSO). The four different vectors (pCM2, pCM3, pCM4, pCM5) were transformed into competent bacteria and incubated with the ligation mixture for 30 min on ice, 2 min at 42°C and again 2 min on ice. After addition of 1 ml low salt LB medium the bacteria were incubated for 1 h at 37°C. Transformed colonies were selected on low salt LB plates containing 25 μg/ml zeocin. Six zeocin resistant clones of each construct were inoculated with 2 ml low salt LB containing 25 μg/ml zeocin and grown overnight at 37°C. The DNA was isolated using the following protocol. The bacteria were centrifuged (1 min, 11'000 g) and resuspended in 350 μl STET buffer (0.1 M NaCl, 10 mM Tris HCl pH 8, 1 mM EDTA, 8% Triton X-100). After addition of 35 μl freshly prepared lysozyme solution (10 mg/ml in 10 mM Tris, pH 8) the tubes were mixed and boiled for 45 s. The lysed bacteria were centrifuged (10 min, 11'000 g) and the pellets removed. To the supernatant 30 μl sodium acetate (3 M, pH 5.2) and 400 μl isopropanol were added and kept for 5 min at RT. The precipitated DNA was centrifuged (15 min, 11'000 g), the supernatant removed and after drying on air the pellet was resuspended in 50 μl TE buffer (10 mM Tris, 1 mM EDTA, pH 8). The plasmids containing an insert
were identified by restriction digest analysis using BstEII and SacI. Clones containing the required insert were identified by DNA sequencing using the 5’AOX1 and the 3’AOX1 primers. The four plasmids (pCM2, pCM3, pCM4, pCM5) containing the correct insert were amplified using a plasmid maxiprep kit (Qiagen).

5.3.4 Transformation of the pPICZαA vectors into P. Pastoris

The four plasmids linearised with SacI were transformed into the P. pastoris strain X-33 by electroporation. The X-33 yeast cells were grown overnight at 30°C in 5 ml yeast extract peptone dextrose medium (YPD; 1% yeast extract, 2% peptone, 2% dextrose). Fresh YPD medium (500 ml) was inoculated with the cultures and grown overnight to reach an A_{600nm} of 1. The cells were centrifuged (5 min, 1500 g) and the pellets resuspended in 500 ml sterile ice cold water, followed by sequential centrifugation steps with resuspension of the pellet in 250 ml sterile ice cold water, 2 ml sterile ice cold sorbitol (1 M) and 1 ml sterile ice cold sorbitol (1 M), respectively. The competent cells were electroporated by pulse discharge (1500 V, 25 μF, 200 Ω; Genepulser, Biorad) in the presence of 7 μg of each linearised plasmid. After adding 1 ml sorbitol (1 M) the cultures were incubated for 1 h at 30°C without shaking. The electroporation mixtures were plated on YPDS (YPD with 1 M sorbitol) plates containing 100 μg/ml zeocin. After 72 h incubation at 30°C 20 clones of each construct were picked and spotted on minimal methanol medium (MM) agar plates (1.34% yeast nitrogen base, 4 x 10^{-5}% biotin, 0.5% methanol), on minimal dextrose medium (MD) agar plates (1.34% yeast nitrogen base, 4 x 10^{-5}% biotin, 2% dextrose) and on YPDS plates containing different amounts of zeocin (2000, 1000, 500 and 100 μg/ml). After growing for 3 days at 30°C transformants that were growing on MD medium and on medium containing 2000 μg/ml zeocin but only very slowly on MM medium were retained for further characterisation.

5.3.5 Screening for high expression clones

Five positive clones of each construct were used to inoculate 10 ml BMGY medium (1% yeast extract, 2% peptone, 1% glycerol, 100 mM potassium
phosphate (pH 6), 1.34% yeast nitrogen base with ammonium sulphate and without amino acids) in 50 ml tubes overnight at 30° C. These cultures were used to inoculate 100 ml BMGY medium in 250 ml shaker flasks which were grown for 48 h. The cells were centrifuged (10 min, 2'000 g) and the pellets resuspended in 100 ml BMMY medium (BMGY containing 0.5% methanol instead of glycerol) and incubated in 250 ml shaker flasks for 72 h. To induce expression, the cultures were supplemented every 24 h with methanol to a final concentration of 0.5% (v/v). Culture supernatants were sampled at different time points to monitor the α-ED-B scFv production by Western blot analysis as described in 5.3.9. The best clones of each construct were stored in 25% glycerol at -70° C.

5.3.6 Scaling-up of expression

One single clone of each of constructs was picked and grown overnight in 10 ml BMGY. This overnight culture was used to inoculate 500 ml BMGY in a 1 litre shaker flask for 48 h at 30° C and 250 rpm until the culture reached an A600nm of 15. To induce the α-ED-B scFv production the cells were centrifuged (10 min, 2'800 g) and the pellets resuspended in 500 ml BMM (BMMY without yeast extract) containing 0.001% structol (Schill + Seilacher, Hamburg, Germany) as an antifoam agent. The cells were shaken in a 1 litre baffled flask for 72 h at 30° C and 250 rpm. For α-ED-B scFv production, cultures were supplemented every 24 h with methanol to a final concentration of 0.5% (v/v). Culture supernatants were sampled to monitor the α-ED-B scFv yield by Western blot analysis as described in 5.3.9.

5.3.7 Production α-ED-B scFv-CM3 by fermentation

One single clone of pCM3/X-33 was picked and grown overnight in 5 ml BMGY. This overnight culture was used to inoculate 250 ml BMGY in a 1 litre shaker flask for 48 h at 30° C and 250 rpm until the culture reached an A600nm of 15. This inoculum was transferred into a 3.7 litre fermenter (Bioengineering, Wald, Switzerland) containing 1.3 litres synthetic medium (580 ml 10 x basal salt containing in one litre: 52 ml phosphoric acid, 1.8 g CaSO4x2H2O, 28.6 g
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K$_2$SO$_4$, 6.5 g KOH, 7 g MgSO$_4\times7$H$_2$O) plus 7.8 ml trace salt solution (containing in one litre: 6 g CuSO$_4\times5$H$_2$O, 0.08 g NaI, 3 g MgSO$_4\times$H$_2$O, 0.2 g MoNa$_2$O$_4\times2$H$_2$O, 0.02g H$_3$BO$_3$, 0.5 g CoCl$_2$, 20 g ZnCl$_2$, 65 g FeSO$_4\times7$H$_2$O)) plus 70 g glycerol, 0.2 g biotin and 5 ml sulfuric acid (25%). After 12 h a glycerol feed (50% v/w) containing 12 ml/litre trace salts was started at 10 ml/h for the duration of 12 h. The temperature was kept at 30° C and dissolved oxygen maintained above air saturation. A pH of 5.5 was regulated with 20% ammonium hydroxide. The protein production was induced by addition of methanol after 30 h inoculation and reaching an A$_{600\text{nm}}$ of 100. The methanol concentration was controlled at 0.5% (v/v) final concentration and the production stopped after 24 h.

5.3.8 Purification of $\alpha$-ED-B scFv produced in P. pastoris

The yeast cells were removed by centrifugation (10 min, 2'800 g, 4° C) and the cell free medium further clarified by 0.2 μm filtration. The proteins were either precipitated or the supernatant was directly loaded on an anion exchange column. The proteins were precipitated by addition of ammonium sulphate at 80% saturation for 1 h on ice under slow stirring. Precipitated proteins were pelleted by centrifugation (20 min, 14'000 g, 4° C) and resuspended in PB (67 mM phosphate buffer, pH 7.4). Ammonium sulphate was removed by dialysis against PB overnight at 4° C and the remaining protein solution loaded on a DEAE-Sephacel anion exchange column (2 x 10 cm, Pharmacia, Dübendorf, Switzerland) equilibrated with PB. After washing the column with PB the protein was eluted with a step gradient of 0 – 250 mM NaCl in PB with steps of 50 mM NaCl at 4° C. Fractions of 5 ml were collected and analysed on 14% SDS-PAGE gels. The amount of protein was determined according to 3.3.3, page 47. The four $\alpha$-ED-B scFv were concentrated to 0.5 mg/ml in PB and stored at -20° C.

5.3.9 Gel electrophoresis, Western and lectin blot analysis

Gel analysis of the proteins (SDS-PAGE) was performed as described by Laemmli (93) followed by staining with Coomassie brilliant blue or silver staining according to 3.3.2, page 45 or immunoblotting according to 4.3.2, page 66.
For lectin blot analysis the membranes were soaked for 40 min with in TBST 10 mM (Tris pH 8, 150 mM NaCl, 0.05% Tween 30) containing 1% milk (TBSTM) followed by incubation in TBSTM containing a 1 : 1000 (v/v %) dilution of digoxigenin-conjugated lectin ConA (Boehringer). Membranes were washed three times for 10 min with TBST and incubated for 1 h with a 1 : 5000 (v/v %) dilution of an alkaline phosphatase-conjugated polyclonal sheep α-digoxigenin Fab' fragment (Boehringer) in TBSTM. After three washings with TBST and wash buffer (100 mM MgCl₂, 100 mM Tris, 100 mM NaCl) the enzyme activity was visualised by using the nitroblue tetrazolium/X-phosphate substrate system (Sigma).

5.3.10 Cysteine labelling of the α-ED-B scFv
Stock solutions of 40 mM tributylphosphine (TBP, Fluka) and 40 mM 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F, Molecular Probes, Eugene, Oregon) in DMSO were prepared immediately before use. The labelling of proteins (0.5 mg/ml) was performed for 15 min at 30° C and with 2 mM TBF and 4 mM ABD-F final concentration in PB (115). The labelled proteins were loaded on 16% SDS-PAGE gels followed by photographic examination using a transilluminator UV (312 nm) light source and Coomassie blue staining.

5.3.11 Binding of scFv to ED-B on Sepharose
ED-B was produced, purified and coupled to the CNBr activated Sepharose according to 4.3.3, page 64. After loading the α-ED-B scFv (0.1 mg) onto the ED-B Sepharose the column was washed with PBS (0.137 M NaCl, 2.68 mM KCl, 8.09 mM Na₂HPO₄, 1.76 mM KH₂PO₄), and the α-ED-B scFv eluted with 100 mM triethylamine in PBS.

5.3.12 Binding of scFv to ED-B positive tumour cells in vitro
Caco-2 cells were maintained in DMEM medium (Gibco BRL, Basel, Switzerland) supplemented with heat-inactivated 10% fetal bovine serum, 1% L-glutamine, 1% nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptavidin. Co-115 cells were maintained in supplemented DMEM without nonessential amino acids and pyruvate. Cover
slips (20 mm diameter) were placed into 12 well plates, coated with 100 µl rat tail collagen I (10 mg/ml) isolated from rat tail as described by Eldsdale (116) and incubated for 30 min at 37° C. Caco-2 and Co-115 cells (3 x 10^5 cells/well) were plated and cultured for 48 h in a humidified 5% CO₂ atmosphere at 37° C. Washed cells were incubated with α-ED-B scFv (5 µg/well) in 100 µl PBS containing 3% bovine serum albumin (BSA) for 30 min at 4° C. Likewise, after two washings with PBS the cells were incubated with α-flag M2 antibody (1 µg/well, Integra Biosciences, Wallisellen, Switzerland) followed by a FITC labelled rabbit α-mouse IgG (1 µg/well, Southern Biotechnology, USA). After another washing step the cover slips were removed, treated with 10% glycerine, placed on a microscope slide and analysed on a fluorescence microscope (Leica DLMB). As negative controls the cells were incubated with the M2 antibody followed by the FITC labelled rabbit α-mouse IgG or the FITC labelled rabbit α-mouse IgG alone.
5.4 Results

5.4.1 Construction and transformation of the vectors
The sequence of the α-ED-B scFv (CGS-1) was modified by PCR by introduction of an EcoRI site at the 5'-end. At the 3'-end the sequences for a flag-tag, a Xba site and the four different C-termini were introduced, respectively (Figure 5.1, page 75). The 0.7 kb inserts were ligated into the multiple cloning site region downstream of the P. pastoris AOX1 promoter and of the α-factor signal sequence of the pPICZαA vector using the EcoRI/Xba restriction site. The resulting plasmids (pCM2, pCM3, pCM4, pCM5) were transformed into the E. coli XL1blue strain. The pPICZαA plasmids encode resistance to zeocin for selection of E. coli and P. pastoris. Three hundred transformants were recovered on low salt LB plates containing zeocin. The 3'- and 5'- nucleotide sequences of one positive clone of each construct were confirmed by DNA sequencing using 3'- and 5'-AOX1 sequencing primers. Vectors linearised with Saci will integrate by gene insertion into the host's 5'-AOX1 region. Thus, the linearised plasmids were introduced into the X-33 strain by electroporation. The recombinants were selected on YPDS plates containing zeocin. Several hundred colonies were obtained. Five clones of each construct were selected for high zeocin (2 mg/ml) resistance on YPDS plates and for slow growth on MM plates. Transformants with the integration of the vector in the 5'-AOX1 region yield Mut⁺ (methanol utilisation positive) strains. Mut⁺ refers to the ability of wild type strains to metabolise methanol as the sole carbon source.

5.4.2 Expression, production and purification of α-ED-B scFv (CM2-CM5)
Expression of the four α-ED-B scFv was assessed in a small culture volume experiment. The protein production of totally 20 clones of each construct was tested by analysis of the supernatants by Western blotting. All tested clones were able to secrete the α-ED-B scFv into the supernatant but at different amounts. The clones with the highest yields of each construct were chosen for scaled-up expression. A small overnight culture was used to inoculate one litre BMGY medium. The cells were grown for 48 h. When they had reached an A₆₀₀nm of 15 the medium was changed to BMM for induction of protein
production. The large-scale protein production was made in medium without yeast extract (BMM) for easier purification. To prevent protein degradation the concentration of methanol was kept at 0.5%. The use of baffled flasks gave a higher yield compared to conventional bottles. The pH value was critical and optimised for the production of the α-E-DB scFv. At pH values of >6 proteases were active, whereas at pH values below 5 protein degradation by hydrolysis was observed. Based on these observations the production was started at pH 5.5 and, if necessary the pH adjusted frequently. The duration of the protein production was also critical. As shown in Figure 5.2 the protein (α-ED-B scFv-CM3) was detected after 24 h in the supernatant in its monomeric as well as in its dimeric form. The highest yield of protein was obtained after 48 h. Protein degradation was observed after longer production periods (Figure 5.2, lane 4).

![Figure 5.2: Western blot analysis of the supernatant during α-ED-B scFv-CM3 production. Samples were taken at different time points after methanol induction: lane 1: after 0 h; lane 2: after 24 h; lane 3: after 48 h; lane 4: after 72 h. The same result was obtained with the other α-ED-B scFv constructs.](image)

The isolation of the α-E-DB scFv by ammonium sulphate precipitation was detrimental for the proteins. Consequently, purification on an anion exchange column with elution by a stepwise NaCl gradient was chosen (Figure 5.3A). The eluted fractions were analysed on a 14% SDS-PAGE gel (Figure 5.3B). The majority of the purified protein eluted with 150 mM NaCl with a yield of 15 - 20 mg/litre culture medium. For further scaling-up the α-ED-B scFv-CM3 was produced in a 3.7 litre fermenter. Based on the results of the production in the baffled flasks the production in the fermenter was stopped after 24 h. The yield of the production by fermentation was 60 mg.
Figure 5.3: Purification of the \( \alpha \)-ED-B scFv-CM3. A) Elution profile of \( \alpha \)-ED-B scFv on a DEAE-Sephacel column. The protein was eluted with a step gradient of NaCl in PB, detected at 280 nm and collected in 3 ml fractions. B) Coomassie stained SDS-PAGE analysis after purification: lane 1: supernatant before purification; lane 2: flow through; lane 3: fractions eluted with 50 mM NaCl (peak 3 in Figure A); lane 4: fractions eluted with 100 mM NaCl (peak 4 in Figure A); lane 5: fractions eluted with 150 mM NaCl (peak 5 in Figure A); lane 6: fractions eluted with 200 mM (peak 6 in Figure A); lane 7: fractions eluted with 250 mM NaCl (peak 7 in Figure A); lane 8: fractions eluted with 1 M NaCl (peak 8 in Figure A).

5.4.3 Characterisation of \( \alpha \)-ED-B scFv (CM2-CM5)

In contrast to the unmodified scFv without cysteines produced in \( E. coli \) (see Chapter 4) the functionalised \( \alpha \)-ED-B scFv containing cysteines produced in \( P. pastoris \) formed dimers. These \( \alpha \)-ED-B scFv could be reduced to monomers with TBP (Figure 5.4A, lanes 1 - 4). This indicated that the C-terminal cysteines were accessible for chemical modification. To confirm this observation the
cysteine groups were modified with the fluorescent thiol reactive dye ABD-F (115). As shown in Figure 5.4B only the reduced α-ED-B scFv reacted with ABD-F (lanes 1 - 4) and were detectable under UV-light. The α-ED-B scFv were not glycosylated which was confirmed by treatment of a Western blot membrane with lectin for the detection of N-glycosylation (data not shown). The monomers and dimers of the α-ED-B scFv were separated on a Superdex 75HR 10/30 column (Pharmacia) at a flow rate of 0.5 ml/min using a Pharmacia Aecta system. The collected fractions were analysed on SDS-PAGE gels (data not shown).

**Figure 5.4:** Labelling of the thiol groups of the four α-ED-B scFv with ABD-F: A) coomassie stained SDS-PAGE analysis after purification; B) fluorescence of the ABD-labelled α-ED-B scFv photographed using UV light (312 nm). Reduced: lane 1: scFv-CM2; lane 2: scFv-CM3; lane 3: scFv-CM4; lane 4: scFv-CM5; lane 5: marker; non reduced: lane 6: scFv-CM2; lane 7: scFv-CM3; lane 8: scFv-CM4; lane 9: scFv-CM5.

### 5.4.4 Binding of scFv (CM2 - CM5) to ED-B

The binding properties of the four scFv produced in *P. pastoris* were tested on an ED-B modified Sepharose column and in cell cultures on ED-B positive tumour cells. Figure 5.5 shows the binding properties of α-ED-B scFv-CM3 and
of an irrelevant, randomly picked scFv to immobilised ED-B on a Sepharose resin. The irrelevant scFv eluted in the void volume of the column, whereas the \( \alpha \)-ED-B scFv-CM3 was only released from ED-B column after addition of triethylamine. The other \( \alpha \)-ED-B scFv (scFv-CM2, scFv-CM4 and scFv-CM5) had comparable binding properties to ED-B Sepharose (data not shown).

**Figure 5.5:** Binding of the \( \alpha \)-ED-B scFv-CM3 versus an irrelevant scFv to ED-B modified Sepharose. The scFv was eluted with 100 mM triethylamine. Elution of \( \alpha \)-ED-B scFv-CM3 (——) and of the irrelevant scFv (---).

**Figure 5.6:** Specific binding of \( \alpha \)-ED-B scFv-CM3 to cells cultured for 48 h on collagen I coated cover slips: A) C) Phase contrast images of the sections shown in B and D; B) \( \alpha \)-ED-B scFv-CM3 on ED-B positive Caco-2 cells; D) \( \alpha \)-ED-B scFv-CM3 on ED-B negative Co-115 cells. Detection of specific binding was demonstrated by immunofluorescence staining with a \( \alpha \)-flag-tag antibody M2 followed by a FITC-labelled antibody.
Caco-2 tumour cells express ED-B (113) by culturing on collagen which allowed to demonstrate the specific binding of the scFv *in vitro*. As shown in Figure 5.6 by immunofluorescence, it could be demonstrated that the scFv-CM3 binds to ED-B positive Caco-2 cells but not to the ED-B negative Co-115 control cells. The other α-ED-B scFv (scFv-CM2, scFv-CM4 and scFv-CM5) had comparable binding properties to ED-B positive cells (data not shown).
5.5 Discussion

Four functionalised α-ED-B scFv with or without hydrophilic spacers and different numbers of cysteines located at their C-termini (Figure 5.1, page 78) were produced and characterised. High yield production and purification of the α-ED-B scFv in the P. pastoris yeast expression system was established and the specific binding of the scFv to the ED-B domain of fibronectin was demonstrated. The binding properties could not be demonstrated by flow cytometry, because the ED-B is located in the extracellular matrix and not on a cell surface. Therefore, a method was established to demonstrate the binding on cells cultured on collagen.

The ease of the expression and purification of functional scFv in P. pastoris point out the potential of this system for producing large quantities of protein needed for therapeutic purposes. The production and the purification has to be established and optimised for each individual protein. Recently, other proteins have been expressed in P. pastoris at large but variable yields (111, 117, 118). Thus, protein expression levels depend on copy numbers and the sites of integration as well as on fermentation parameters. The production of large amounts of proteins of high purity and stability becomes an important issue for the development of therapeutic recombinant proteins. Proteins like endostatin (119, 120), IL-17 (121, 122) and insulin (123) have been produced in the P. pastoris system.

As shown in Chapter 4 (Figure 4.4, page 72) the modification of proteins with bispecific coupling molecules like SATA which interact with amino groups on the protein could potentially reduce the binding activity of the antibody fragments. C-terminal cysteine thiols offer a large variability for site-specific modifications of the scFv molecules having the advantage of not interfering with antigen binding domains. The thiol groups on the α-ED-B scFv were accessible for small molecules like the fluorescence dye ABD-F. In further experiments the coupling to liposomes with a mean diameter of 50 nm has to be established (see next chapter). The location of the thiol groups at the C-terminus should be more accessible than the thiol groups inside the protein. All four α-ED-B scFv formed dimers. The disulfide bridges have to be cleaved for the attachment to
maleimide-modified liposomes. TBF represents a good reducing agent which did not decrease the binding activity of the α-ED-B scFv up to a final concentration of 4 mM (data not shown) and which had not to be removed for the coupling reaction because it carries no thiol groups itself, compared to β-mercaptoethanol which could react with the liposomes.

ScFv production in *P. pastoris* gave a higher yield compared to the production in *E. coli* (see Chapter 4). The cysteines in α-ED-B scFv-CM2-CM5 were located at the C-terminus and were more available for the attachment to liposomes. In the scFv-CM1 (Figure 5.1, page 78) the myc tag was located at the C-terminus which was a hindrance for the attachment to the liposomes. The α-ED-B scFv (CM2, CM3, CM4, CM5) will be used for the preparation of immunoliposomes that serve as carriers for therapeutic or diagnostic molecules (47, 50).

Generally, the C-terminal cysteine modification of scFv can serve as an excellent tool for the attachment of active compounds.
6 CONSTRUCTION, BINDING AND CYTOTOXICITY STUDIES OF α-ED-B SCFV-IMMUNOLIPOSOMES IN VITRO AND IN VIVO

6.1 Abstract

All four α-ED-B scFv constructs containing terminal cysteines called α-ED-B scFv-CM2, α-ED-B scFv-CM3, α-ED-B scFv-CM4, α-ED-B scFv-CM5 produced in P. pastoris were coupled to the distal end of PEG chains on sulfo-SMCC modified liposomes prepared with 2 mol% PE-PEG-NH₂. Different numbers of scFv molecules per liposome could be attached. The coupling efficiency correlated with the number of cysteines at the C-terminus of the protein and the spacer between the protein and the cysteines. As expected, the α-ED-B scFv-CM3 containing three cysteines and a spacer between the cysteines and the protein showed the best coupling efficiency with 70 α-ED-B scFv molecules on one liposome with a diameter of 50 nm and up to 220 molecules on a liposome prepared with 7 mol% of PE-PEG-NH₂ and with a diameter of 100 nm. With the other scFv 28 - 30 molecules could be attached to one liposome with a diameter of 50 nm.

Target cell binding of these α-ED-B scFv-PEG-immunoliposomes was studied in vitro and in vivo. All four types of scFv-PEG-immunoliposomes showed specific binding to the ED-B positive cell line Caco-2 compared to ED-B negative Co-115 cells in vitro as analysed by incubation of the cells with fluorescence labelled α-ED-B scFv-PEG-immunoliposomes and examination by fluorescence microscopy. Pharmacokinetics and tissue distribution of ¹¹⁴mIndium labelled α-ED-B scFv-CM3-PEG-immunoliposomes and unmodified control PEG-liposomes were compared in nude mice bearing subcutaneous F9 tumours. The α-ED-B scFv-PEG-immunoliposomes showed a two fold higher accumulation in the tumour 2 h after injection compared to the control liposomes. Compared to unmodified PEG-liposomes, the α-ED-B scFv-PEG-immunoliposomes were rapidly taken up by the liver and the spleen. This resulted in a high tumour to blood ratio. Treatment of F9 tumour bearing mice
with \(\alpha\)-ED-B scFv-PEG-immunoliposomes loaded with the new cytotoxic agent 2'-deoxy-5-fluorouridyl-N^4-octadecyl-1-\(\beta\)-D-arabinofuranosylcytosine (5FdU-NOAC) reduced tumour growth by 45% compared to untreated mice after five daily injections of 600 \(\mu\)g 5FdU-NOAC encapsulated in scFv-PEG-immunoliposomes.

### 6.2 Introduction

The group of Prof. Neri raised different clones of scFv binding to the ED-B domain using a phage display library (for more details see 4.1, page 65). The four new \(\alpha\)-ED-B scFv constructs produced in this project (scFv-CM2, scFv-CM3, scFv-CM4 and scFv-CM5) contain the \(\alpha\)-ED-B scFv sequence of the clone CGS-1 which has a binding affinity to ED-B of 53 nM (20). At the beginning of this study, only this clone CGS-1 was available. During the last three years new affinity matured clones with significantly higher binding affinities were produced (Table 6.1). All scFv clones listed in Table 6.1 were also constructed as dimers which showed better binding capacity compared to the monomers.

**Table 6.1: Overview of the different clones of scFv binding to ED-B.**

<table>
<thead>
<tr>
<th>Name of the construct</th>
<th>Dissociation constant [nM]</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC-1 (monoclonal antibody)</td>
<td>-</td>
<td>(124)</td>
</tr>
<tr>
<td>CGS-1</td>
<td>54</td>
<td>(20)</td>
</tr>
<tr>
<td>CGS-2</td>
<td>1.1</td>
<td>(20)</td>
</tr>
<tr>
<td>E1</td>
<td>41</td>
<td>(125)</td>
</tr>
<tr>
<td>L19</td>
<td>0.054</td>
<td>(106)</td>
</tr>
</tbody>
</table>

Attachment of proteins to the distal end of the maleimide modified PEG chains on liposomes requires the presence of thiol groups on the proteins (Figure 6.1). Chapter 5 described the production of four different constructs of \(\alpha\)-ED-B scFv containing additional cysteines in yeast (Figure 5.1, page 78). In this Chapter the coupling efficiencies of these \(\alpha\)-ED-B scFv to liposomes are compared. The successful modification of a scFv containing one additional cysteine near the C-terminus with the \(^{99}\text{m}\)technetium isotope was described (126). The coupling of scFv molecules to liposomes with a diameter of 50 nm or 100 nm is expected to
be more difficult than the attachment of small molecules as isotopes or cytotoxic drugs.

\[ N'\text{LVl} \quad SH \quad C' \quad + \quad \text{Maleimide} \quad \text{modified amino-PEG-liposomes} \]

\[ N'\text{LVl} \quad SH \quad C' \quad + \quad \text{Maleimide} \quad \text{modified amino-PEG-liposomes} \]

**Figure 6.1:** Scheme of the coupling reaction of \( \alpha \)-ED-B scFv to maleimide modified amino-PEG-liposomes.

After the successful construction of the four \( \alpha \)-ED-B scFv-PEG-immunoliposomes their binding properties were demonstrated in cell cultures using ED-B positive (Caco-2) and ED-B negative (Co-115) cells as described with the scFv alone in Chapter 5.

In the second part of this Chapter the biodistribution of \( {^{114m}}\text{In} \) labelled \( \alpha \)-ED-B scFv-PEG-immunoliposomes and of unmodified PEG-liposomes were compared. The liposomes were injected into nude mice bearing subcutaneous the aggressive and highly vascularised murine F9 teratocarcinoma tumour cells (20). The therapeutic effect of \( \alpha \)-ED-B scFv-PEG-immunoliposomes versus unmodified PEG-liposomes was demonstrated by loading the liposomes with 5FdU-NOAC, a new amphiphilic heterodinucleoside (76).

The successful construction, binding capacity *in vitro*, biodistribution in tumour bearing mice and the cytotoxic properties *in vivo* of \( \alpha \)-ED-B scFv-PEG-immunoliposomes are described in this chapter.
6.3 Methods

6.3.1 Materials, cells and animals

5FdU-NOAC was synthesised according to (77). The Caco-2 cell line was obtained from H. Wunderli-Allenspach (ETH, Zürich, Switzerland) and the Co-115 cell line was obtained from B. Sordat (Swiss Institute for Cancer Research, Lausanne, Switzerland). The murine teratocarcinoma F9 cell line was obtained from D. Neri (ETH, Zürich, Switzerland). Nude mice ((CD-1<R>)-nu/nu) were purchased from Charles River Wiga (Sulzfeld, Germany). All buffer salts and other chemicals were from Fluka or Sigma (Buchs, Switzerland).

6.3.2 Coupling of α-ED-B scFv-CM2, α-ED-B scFv-CM3, α-ED-B scFv-CM4, α-ED-B and scFv-CM5 to sulfo-SMCC modified liposomes for in vitro studies

The purified α-ED-B scFv (0.5 mg/ml) in HBSE (10 mM Hepes, 150 mM NaCl, 9.1 mM EDTA, pH 7.5) were reduced with a 2 mM final concentration of tributylphosphine (TBF) for 4 h at 4°C under argon. Sulfo-SMCC modified and DiO fluorescence labelled conventional or stealth liposomes prepared with 40 mg/ml SPC and 2 mol% amino groups corresponding to 1.4 x 10^{16} maleimimide groups (Table 2.4, page 41) in 100 μl HBSE were incubated with 100 μg reduced α-ED-B scFv corresponding to 2 - 6 x 10^{15} thiol groups (depending on the number of cysteines in the scFv) in 200 μl HBSE for 20 h at 4°C under argon. Modified liposomes and unreacted scFv were separated on a metrizamide gradient as described in 3.3.10, page 50. The fractions of the gradient were analysed from the bottom to the top on a non-reducing 14% SDS-PAGE gel followed by Western blot analysis as described in 4.3.2, page 66.

6.3.3 Binding of scFv-immunoliposomes to ED-B positive tumour cells in vitro

Caco-2 cells and Co-115 cells were cultured as described in 5.3.12, page 82. Cover slips (20 mm diameter) were placed into 12 well plates, coated with 100 μl rat tail collagen I (10 mg/ml) isolated from rat tail as described by Eldsdale (116) and incubated for 30 min at 37°C. Caco-2 and Co115 cells (3 x 10^5
cells/well) were plated and cultured for 48 h in a humidified 5% CO₂ atmosphere at 37° C. Washed cells were incubated with 100 μl DiO labelled α-ED-B scFv-immunoliposomes corresponding to 9 × 10^{13} liposomes or 12.5 – 31.5 μg scFv, (depending on the construct) respectively, in PBS (0.137 M NaCl, 2.68 mM KCl, 8.09 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.4) for 30 min at 4° C. After another washing step the cover slips were removed, treated with 10% glycerine and placed on a microscope slide and analysed on a fluorescence microscope (Leica DLMB). As negative controls the cells were incubated with unmodified fluorescence labelled liposomes.

6.3.4 Preparation of scFv-PEG-immunoliposomes for in vivo studies

α-ED-B scFv-CM-3 produced by fermentation (0.2 mg/ml) in HBSE was reduced with a 2 mM final concentration of TBF for 4 h at 4° C under argon. Sulfo-SMCC modified PEG-liposomes prepared with 80 mg/ml SPC and 7 mol% PE-PEG-NH₂ corresponding to 1.8 × 10^{19} maleimid groups (Table 2.4, page 41) in 2 ml HBSE and 7.5 mg α-ED-B scFv-CM3 corresponding to 4.5 × 10^{17} thiol groups or 1.5 × 10^{17} protein molecules were incubated for 20 h at 4° C under argon. Liposomes used for therapeutic studies contained 10 mg/ml 5FdU-NOAC and were trace labelled with [³H]-NOAC (Table 2.2, page 38). Modified liposomes and unreacted scFv were separated on a metrizamide gradient as described in 3.3.10, page 50. For biodistribution experiments control liposomes and immunoliposomes were labelled with ¹¹⁴mInCl₃ as described in 2.3.6, page 35.

6.3.5 Biodistribution of ¹¹⁴mIn labelled scFv-PEG-immunoliposomes in tumour bearing mice

The murine F9 teratocarcinoma cells were maintained in DMEM medium (Gibco BRL, Basel, Switzerland) supplemented with heat-inactivated 10% fetal bovine serum, 2% L-glutamine, 100 U/ml penicillin and 100 μg/ml streptavidin. The cells were harvested and resuspended in PBS. Nude female mice (CD-1< R> nu/nu) were injected s.c. with 10⁷ cells in 50 μl on both sides of the back. After 5 to 10 days, when the tumours reached diameters of 0.5 – 1.5 cm, ¹¹⁴mIn labelled
liposomes containing 51.5 mg/ml SPC and 375 µg scFv in 200 µl PBS were injected *i.v.*. At 5 min, 1, 2, 6 and 24 h after injection the animals were anaesthetised with ether and immediately sacrificed for the removal of blood, heart, lung, liver, spleen, kidney and tumour. Three animals were used for each time point. The organs were weighted and the radioactivity was measured using a gamma counter (Cobra, Packard Instruments, Illinois, USA). Blood correction factors were applied to all organ samples (127).

6.3.6 Cytotoxicity assay on F9 tumour cells
F9 cells were cultured as described in 6.3.5 in sterile 96-well plates for 48 h (30'000 cells/well). The cells were washed with PBS and incubated for 24 h in the corresponding medium with the cytotoxic drugs mitoxantrone, doxorubicin, NOAC, 5FdU-NOAC or 5FdU-5FdC\textsuperscript{18} with a final concentration of 0.1 to 1.5 µM. The supernatants were removed and 100 µl/well freshly diluted WST-1 solution in medium ((4-[3-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate; Boehringer Mannheim, Germany) was added and incubated for 15 min at 37° C and 5% CO\textsubscript{2}. Cell viability was determined by measuring the absorption at 450 nm using a Dynatech MR5000 plate reader (Microtec Produkte, Embrach, Switzerland).

6.3.7 Therapeutic studies
F9 cells were cultured as described in 6.3.5 and 10\textsuperscript{7} cells per animal were injected s.c. on the abdominal side of nude female mice (CD-1\textsubscript{<R>}-nu/nu). The mice were divided in three groups of five animals and intravenous treatment was started when the tumour size reached a diameter of 6 - 8 mm. The mice were treated with 200 µl scFv-5FdU-NOAC-PEG-immunoliposomes or 5FdU-NOAC-PEG-liposomes containing 11 mg SPC 24 h for five consecutive days. Each injection dose contained 600 µg 5FdU-NOAC or corresponding amount of protein. Mice were checked for tumour growth every day and the tumour volumes were estimated by the following equation \(V = \pi ab^2/6\) where \(a\) is the largest tumour diameter and \(b\) is the perpendicular diameter. The tumour volume/weight values were converted into percent change from baseline by the equation \(V_t \times 100/V_0\) where \(V_t\) is the tumour volume measured at time \(t\) and \(V_0\)
baseline volume. Eight days after start of treatment all mice were sacrificed and
tumours were dissected and weighed.
6.4 Results

6.4.1 Coupling of \(\alpha\)-ED-B scFv to sulfo-SMCC modified liposomes

The new \(\alpha\)-ED-B scFv constructs (scFv-CM2, scFv-CM3, scFv-CM4 and scFv-CM5) formed in the absence of reducing agents dimers due to the cysteines. Therefore, the disulfide bridges had to be cleaved for the coupling reaction of the scFv to maleimide modified liposomes. The reduction can be achieved by using reducing agents containing thiol groups themselves (e.g. \(\beta\)-mercaptoethanol or dithiothreitol) or with agents without thiol groups (e.g. TBF or dithionite). The advantage of working with an agent without thiol groups is that the reducing agent has not to be removed before the coupling reaction takes place. Agents with thiol groups could react with the maleimide groups on the liposomes. Dithionite showed to be a very aggressive agent which inactivated the binding properties of the scFv to ED-B as tested by ELISA (data not shown). In contrast to dithionite, the reduction of the dimers of the \(\alpha\)-ED-B scFv with a 2 mM final concentration of TBF for 4 h at 4° C produced active and stable monomers (55).

In a chemical reaction the molar ratios of active groups are important. Labelling of proteins with small molecules or modification of liposomes with proteins should be carried out with a molar excess of the smaller molecules. The major problem of liposome modification with \(\alpha\)-ED-B scFv was the limited amount of available protein. For the preparation of the immunoliposomes the molar ratio of maleimide groups on the liposomes to the thiol groups on the proteins was 2 : 1 (\textit{in vitro} studies) or 45 : 1 (\textit{in vivo} studies).

Unreacted scFv was separated from the immunoliposomes on a metrizamide gradient. Collected fractions from the gradient were analysed on a 14% non-reducing SDS-PAGE gel followed by the Western blot analysis (Figure 6.2). Incubation of control liposomes without sulfo-SMCC with the scFv (Figure 6.2 lanes 9 - 16) resulted in no protein detection in the liposome fractions (lanes 9 - 11) and with monomers and dimers of the scFv molecules remaining at the bottom of the metrizamide gradient (lanes 14 - 16). Fractions containing liposomes with sulfo-SMCC gave a 33 kDa band on a Western blot corresponding to the molecular weight of the scFv monomer plus the attached
PEG-lipid (Figure 6.2, lanes 1 - 3). Although the scFv was kept at 4° C during the preparation of the immunoliposomes some degradation of the protein occurred as detected by an additional band smaller than 18 kDa (Figure, 6.2 lane 2). The yield of liposomes was 82% corresponding to $9 \times 10^{13}$ liposomes in 100 µl.

**Figure 6.2:** Separation of liposomes and unbound α-ED-B scFv. Western blot analysis of aliquots taken from top to bottom of the metrizamide gradient: **lanes 1 – 8:** probes from an incubation of liposomes prepared with PE-PEG-NH$_2$ (positive reaction); **lanes 9 – 16:** probes from an incubation of liposomes prepared without PE-PEG-NH$_2$ (negative control). Lanes 1 - 3 and 9 - 11 correspond to the liposome fractions.

All four α-ED-B scFv could be successfully attached to the surface of conventional and PEG-liposomes but at different amounts. Figure 6.3 shows the four α-ED-B scFv-PEG-immunoliposomes after metrizamide separation analysed on a Western blot. As expected the α-ED-B scFvCM-3 showed the best coupling efficiency (Figure 6.3, lane 3). The α-ED-B scFvCM-3 carries three additional cysteines at the C-terminus that separated from the protein with a hydrophilic spacer (Figure 5.1, page 75).

**Figure 6.3:** Comparison of the coupling efficiency of the four α-ED-B scFv constructs to liposomes. Western blot analysis of aliquots taken from the liposome fractions on the metrizamide gradient: **lane 1:** negative control (incubation of scFv-CM3 with liposomes without PE-PEG-NH$_2$); **lane 2:** scFv-CM2-; **lane 3:** scFv-CM-3-; **lane 4:** scFv-CM-4-; **lane 5:** scFv-CM5-immunoliposomes.
Sulfo-SMCC modified liposomes with a diameter of 50 nm and prepared with 2 mol % DPPE or PE-PEG-NH₂ carry 120 maleimide groups on the surface of one liposome (Table 2.4, page 41). The numbers of protein molecules linked to one liposome were calculated by determination of the amount of lipid based on DiO fluorescence and by determination of protein concentration on the SDS-PAGE gels. The calculated average numbers of protein molecules per liposome are summarised in Table 6.2.

**Table 6.2:** Calculation of scFv modification of PEG-liposomes with a diameter of 50 nm containing 120 maleimide groups.

<table>
<thead>
<tr>
<th>Construct</th>
<th>ScFv [µg/100 µl]</th>
<th>Yield of scFv [%]</th>
<th>ScFv/liposome</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-ED-B scFv CM-2</td>
<td>13.5</td>
<td>13.5</td>
<td>30</td>
</tr>
<tr>
<td>α-ED-B scFv CM-3</td>
<td>31.5</td>
<td>31.5</td>
<td>70</td>
</tr>
<tr>
<td>α-ED-B scFv CM-4</td>
<td>13.5</td>
<td>13.5</td>
<td>30</td>
</tr>
<tr>
<td>α-ED-B scFv CM-5</td>
<td>12.5</td>
<td>12.5</td>
<td>28</td>
</tr>
</tbody>
</table>

In addition, the coupling efficiency of α-ED-B scFv-CM3 to conventional and PEG-liposomes were compared. As already seen by attaching α-CEA A5B7-F(\(ab\)₂) fragments to liposomes (3.4.4, page 54) more protein molecules could be coupled to stealth than to conventional liposomes (Figure 6.4).

**Figure 6.4:** Comparison of the coupling efficiency of α-ED-B scFv-CM3 to conventional and PEG-liposomes. Western blot analysis of aliquots taken from the liposome fractions on the metrizamide gradient: lane 1: incubation of scFv-CM3 with conventional liposomes without amino groups (negative control); lane 2: incubation of scFv-CM3 with DPPE-liposomes; lane 3: incubation of scFv-CM3 with PEG-liposomes without amino groups (negative control); lane 4: incubation of scFv-CM3 with liposomes with PEG-amino groups.

6.4.2 Binding of scFv-immunoliposomes to ED-B positive cells in vitro

Caco-2 tumour cells cultured on collagen I express ED-B (113) which allowed to demonstrate the specific binding of the α-ED-B scFv-immunoliposomes in vitro.
The cells were incubated with DiO labelled α-ED-B scFv-immunoliposomes and binding analysed by fluorescence microscopy.

![Image](image.png)

**Figure 6.5**: Binding of α-ED-B scFv-PEG-immunoliposomes to cells cultured for 48 h on collagen I coated cover slips: **A)** **C)** **E)** phase contrast images of the sections shown in **B), D** and **F); **B)** α-ED-B scFv-PEG-immunoliposomes on ED-B positive Caco-2 cells; **D)** unmodified PEG-liposomes on ED-B positive Caco-2 cells; **F)** α-ED-B scFv-PEG-immunoliposomes on ED-B negative Co-115 cells. Detection of specific binding was demonstrated by labelling the liposomes with the lipophilic fluorescent dye DiO.

As shown in Figure 6.5 the fluorescence labelled α-ED-B scFv-CM3-PEG-immunoliposomes bound strongly to ED-B positive Caco-2 cells (Figure 6.5B) but not to ED-B negative Co-115 control cells (Figure 6.5F). Equal results were obtained with immunoliposomes prepared with the other constructs (scFv-CM2, scFv-CM4 and scFv-CM5) (data not shown).

### 6.4.3 Preparation and biodistribution of $^{111}$In labelled α-ED-B scFv-PEG-immunoliposomes in tumour bearing mice

ScFv-PEG-immunoliposomes and PEG-liposomes for *in vivo* studies were prepared using 80 mg/ml SPC and corresponding higher amounts of the other compounds and 7 mol% PE-PEG-NH$_2$ (Table 2.1, page 37). This resulted in 1670 maleimide groups per liposome (Table 2.4, page 41) and in higher molar ratio of maleimide to thiol groups (45 : 1). The protein coupling efficiency using less protein compared to the maleimide groups was nearly 100%, corresponding to 220 scFv linked to one liposome with a diameter of 100 nm.
For detection of the liposomes they were labelled with $^{114m}$Indium (2.3.6, page 35). The recovery of liposomes after scFv modification and $^{114m}$In labelling was 64%, corresponding to 51.2 mg/ml SPC.

Biodistribution of these liposomes in nude mice bearing F9 teratocarcinoma tumours was evaluated. This tumour model was used because F9 tumours express ED-B in vivo, are highly vascularized and do not invade the organs. F9 cells were injected s.c. and when the tumour reached a size of 0.5 – 1.5 cm diameter $^{114m}$In-labelled α-ED-B scFv-PEG-immunoliposomes and unmodified PEG-liposomes were injected i.v.. Tumours larger than 400 mg become necrotic. Each mouse was injected with 375 μg scFv on 3.4 x 10$^{13}$ liposomes (28.34 μg scFv / μmol SPC) containing 2 x 10$^{6}$ cpm $^{114m}$In in 200 μl PBS.

The distribution of the liposomes was analysed by removal of tumour, heart, lung, liver, spleen, kidney and blood at given time points, followed by measuring the radioactivity. Table 6.3 and Table 6.4 summarise the biodistribution of the liposomes at different time points. The unmodified PEG-liposomes showed the known typical distribution pattern. They circulated for a long time period in the blood and accumulated with time in the liver (Table 6.3). Two hours after injection 50% of injected dose of the unmodified liposomes were still found in the blood, whereas only a small fraction accumulated in the tumour (1.9% of injected dose per gram tumour, Table 6.4). The blood clearance of the α-ED-B scFv-immunoliposomes was faster as compared to the unmodified PEG-liposomes and they accumulated in liver (57% of injected dose after 6 h) and at a high dose also in the spleen (10% of injected dose after 24 h) (Table 6.3) (128).
Table 6.3: Organ distribution of unmodified PEG-liposomes and α-ED-B scFv-PEG-immunoliposomes. Three mice bearing s. c. F9 tumours per group were injected with $^{114m}$In labelled liposomes and killed after different time points. Results are expressed as % of injected radioactivity. Lung and heart showed insignificant accumulation of the liposomes (data not shown).

<table>
<thead>
<tr>
<th>Time [min]</th>
<th>Blood [%]</th>
<th>Liver [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PEG-liposomes</td>
<td>scFv-PEG-liposomes</td>
</tr>
<tr>
<td>5</td>
<td>86.4 ± 2.3</td>
<td>83.3 ± 14.8</td>
</tr>
<tr>
<td>60</td>
<td>57.7 ± 4.3</td>
<td>24.2 ± 5.6</td>
</tr>
<tr>
<td>120</td>
<td>50.1 ± 2.2</td>
<td>25.9 ± 3.6</td>
</tr>
<tr>
<td>360</td>
<td>34.6 ± 9.7</td>
<td>13.8 ± 3.0</td>
</tr>
<tr>
<td>1440</td>
<td>10.1 ± 2.1</td>
<td>2.4 ± 0.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time [min]</th>
<th>Kidney [%]</th>
<th>Spleen [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PEG-liposomes</td>
<td>scFv-PEG-liposomes</td>
</tr>
<tr>
<td>5</td>
<td>1.6 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>60</td>
<td>1.1 ± 0.3</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>120</td>
<td>1.1 ± 0.1</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>360</td>
<td>1.5 ± 1.0</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>1440</td>
<td>2.5 ± 0.3</td>
<td>1.8 ± 0.2</td>
</tr>
</tbody>
</table>

Table 6.4: Comparison of the distribution in blood and tumour of PEG-liposomes and α-ED-B scFv-PEG-immunoliposomes. Three mice bearing s. c. F9 tumours per group were injected with $^{114m}$In labelled liposomes and killed after different time points. Results are expressed as % injected dose of radioactivity per millilitre blood or per gram tumour.

<table>
<thead>
<tr>
<th>Time [min]</th>
<th>Blood [%ID/ml]</th>
<th>Tumour [%ID/g]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PEG-liposomes</td>
<td>scFv-PEG-liposomes</td>
</tr>
<tr>
<td>5</td>
<td>45.1 ± 7.5</td>
<td>41.0 ± 9.6</td>
</tr>
<tr>
<td>60</td>
<td>31.9 ± 2.2</td>
<td>24.7 ± 2.3</td>
</tr>
<tr>
<td>120</td>
<td>31.2 ± 1.8</td>
<td>12.3 ± 3.6</td>
</tr>
<tr>
<td>360</td>
<td>19.2 ± 5.3</td>
<td>6.4 ± 0.8</td>
</tr>
<tr>
<td>1440</td>
<td>5.3 ± 1.4</td>
<td>1.2 ± 0.3</td>
</tr>
</tbody>
</table>

The rapid accumulation of the scFv-PEG-immunoliposomes in the spleen and the liver could be explained by a more pronounced interaction between the scFv and the plasma proteins than between the PEG and the same proteins. This fast clearance of α-ED-B scFv-PEG-immunoliposomes in blood results in a higher tumour to blood ratio of the scFv-PEG-immunoliposomes compared to
the unmodified PEG-liposomes (Figure 6.6). Nevertheless, the circulation time of the scFv-PEG-immunoliposomes compared to a cytotoxic agent like doxorubicine which is cleared in blood after 5 min is still remarkable.

![Graph](image)

**Figure 6.6:** Comparison of tumour to blood ratio of unmodified PEG-liposomes (■) and α-ED-B scFv-PEG-immunoliposomes (▲) at different time points. Three mice per group bearing s. c. F9 tumours were injected with $^{114m}$In labelled liposomes and killed after different time points. Results are expressed as the ratio of % injected dose of radioactivity per gram tissue or ml blood.

Figure 6.7 compares the accumulation of PEG-liposomes and scFv-PEG-immunoliposomes in the tumour over the time period of 24 h. The scFv-PEG-immunoliposomes were detected in the tumour already after 1 h and remained there at least for 24 h at high concentrations. The unmodified PEG-liposomes reached the level of scFv-PEG-immunoliposomes after 6 h. In this first 6 h the accumulation is probably due to the specific binding of the scFv to ED-B. After 6 h the longer circulation time in blood of the PEG-liposomes leads to an increase of unspecific liposome accumulation in the tumour.
Figure 6.7: Comparison of tumour accumulation of unmodified PEG-liposomes and α-ED-B scFv-PEG-immunoliposomes at different time points. Three mice per group bearing s. c. F9 tumours were injected with $^{114m}$In labelled liposomes and killed after different time points. Results are expressed as %injected dose of radioactivity per gram tissue after the indicated time points.

The circulation time in blood of the scFv-PEG-immunoliposomes could be enhanced by pre-treatment of the mice with unmodified conventional liposomes (SPC liposomes) 24 h before injection of the $^{114m}$In labelled liposomes. Figure 6.8 compares the distribution of $^{114m}$In labelled liposomes with and without this pre-treatment in the blood and the tumour at time points 1 h and 6 h after injection. The accumulation of the scFv-PEG-immunoliposomes in the tumour could be slightly enhanced by pre-treatment (Figure 6.8A and 6.8B). In contrast, after 6 h the levels of PEG-liposomes in the tumour were equal with and without pre-treatment (Figure 6.8B). This could be explained by the fact that the concentration of scFv-PEG-liposomes in the blood after 6 h is three fold higher after pre-treatment compared with the result obtained without pre-treatment (Figure 6.8D), whereas the amount of PEG-liposomes in the blood is only enhanced by a factor of 1.5.
A) 1 h after injection

B) 6 h after injection

C) 1 h after injection

D) 6 h after injection

Figure 6.8: Accumulation of liposomes by pre-treatment with empty conventional liposomes: A) in the tumour after 1 h; B) in the tumour after 6 h; C) accumulation in blood after 1 h; D) in the blood after 6 h. Three mice per group bearing s. c. F9 tumours were injected with $^{114}$In labelled liposomes and killed after different time points. Results are expressed as the ratio of % injected dose of radioactivity per gram tissue or ml blood.

6.4.4 Cytotoxicity on F9 tumour cells

Based on the accumulation of the $^{114}$In labelled scFv-PEG-immunoliposomes in F9 tumours a therapy study was carried out. Before the therapy experiments was started a cytotoxic compound killing F9 tumours cells had to be found in vitro. F9 cells were treated with the different cytotoxic agents mitoxantrone, doxorubicin, NOAC, 5FdU-NOAC, 5FdU-5FdC-18 which could be incorporated into liposomes. As shown in Figure 6.9 the most effective compound was mitoxantrone followed by doxorubicin and 5FdU-NOAC. Because loading of liposomes with mitoxantrone or doxorubicin needs another step after the liposome preparation, the new cytotoxic agent 5FdU-NOAC was used for in vivo
Anti ED-B immunoliposomes

studies. 5FdU-NOAC is lipophilic and can directly be added to the lipid mixture during the liposome preparation.

![Cell viability graph](image)

Figure 6.9: Cell viability of F9 cells after 24 h incubation with: mitoxantrone (●); doxorubicin (△); NOAC (▼); 5FdU-NOAC (●); 5FdU-5FdC-18 (○). Cytotoxicity was measured by incubation with WST-1 dye followed by fluorometric determination.

6.4.5 Treatment of mice bearing F9 tumours with scFv-PEG-immunoliposomes containing 5FdU-NOAC

Stimulated by the superior cell growth inhibition and the accumulation of the liposomes in the tumour, the activity of liposomes containing 5FdU-NOAC was tested in vivo. Mice bearing one subcutaneous F9 tumour on the abdomen with a diameter of 7 – 8 mm were treated for five days every 24 h with two types of liposomes (scFv-5FdU-NOAC-PEG-liposomes, 5FdU-NOAC-PEG-liposomes). The administration of these liposomes was not toxic for the animals. The body weight remained constant during the experiment. Each treatment contained 600 µg 5FdU-NOAC. As shown in Figure 6.10 and Table 6.4 the α-ED-B scFv-5FdU-NOAC-PEG-liposomes showed a remarkable reduction of tumour growth. After 5 days of treatment the tumour growth could be inhibited by 45% compared to the untreated control mice. At day 8 after start of treatment the mice were killed and the tumours were excised and weighted. The tumour
weights of mice treated with scFv-5FdU-NOAC-PEG-liposomes were reduced by 45%, compared to control tumours. Although 5FdU-NOAC-PEG-liposomes accumulated in the tumour (Figure 6.7) they showed nearly no effect on tumour growth. Untreated mice showed progressive tumour growth (Figure 6.10).

**Figure 6.10:** In vivo effects of liposome treatment on F9 tumour growth inoculated on the abdominal side of nude mice. Tumour bearing mice were treated five times with scFv-5FdU-NOAC-PEG-liposomes or 5FdU-NOAC-PEG-liposomes every 24 h. Each treatment contained 600 µg 5FdU-NOAC (0.75 µmol) or equal amounts of scFv. Values represent the mean of five treated mice. The tumour volumes were calculated by the following equation $V = \pi ab^2/6$, where $a$ is the largest tumour diameter and $b$ is the perpendicular diameter. The tumour volume/weight values were converted in percent change from baseline by the equation $V_t \times 100/V_0$, where $V_t$ is volume measured at time $t$ and $V_0$ the baseline volume.

The better cytotoxic effect of the scFv-5FdU-NOAC-PEG-liposomes compared to the unmodified 5FdU-NOAC-PEG-liposomes could be explained by the higher accumulation over a longer time period of the scFv-5FdU-NOAC-PEG-
liposomes in the tumour. In addition, the scFv binding to the extracellular matrix could have an antiangiogenic effect.

Table 6.4: Tumour weights from mice killed after 8 days. The results are expressed as tumour weights and the ratios of tumour weight of treated mice to untreated mice. Values represent the mean of five treated mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>Tumour weight [g]</th>
<th>T/C [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.86 ± 0.66</td>
<td>-</td>
</tr>
<tr>
<td>5FdU-NOAC-PEG-liposomes</td>
<td>1.65 ± 0.27</td>
<td>88.7</td>
</tr>
<tr>
<td>ScFv-5FdU-NOAC-PEG-liposomes</td>
<td>1.01 ± 0.15</td>
<td>54.3</td>
</tr>
</tbody>
</table>
6.5 Discussion

After the unsuccessful attempt of coupling the original α-ED-B scFv (DN351) via thiol groups introduced either by modification with SATA or by addition of one cysteine near the C-terminus (see Chapter 4), four new constructs of α-ED-B scFv containing more cysteines and produced in P. pastoris (see Chapter 5) could be attached to the surface of liposomes. As shown in Figure 6.3, page 100 the location of the thiol groups on the protein was critical. The α-ED-B scFv-CM3 containing more than one cysteine and a spacer between the cysteines and the protein showed the best coupling efficiency.

The binding properties of the α-ED-B scFv-immunoliposomes could not be tested by flow cytometry, because the ED-B is expressed in the extracellular matrix and not on the cell surface. Therefore, the results could directly not be compared with those obtained with the α-CEA A5B7-F(ab)2-immunoliposomes as described in Chapter 3. A quantitative assay to compare the binding of the four immunoliposomes could not be developed. Different attempts were made to use ED-B coated ELISA plates to analyse the binding of the immunoliposomes. Unfortunately, various technical problems were encountered which made it impossible to detect the binding of the liposomes on ELISA plates. It was only possible to detect the scFv on the liposomes using the M2 antibody, which recognises the flag-tag on the scFv. Another approach would be to measure the binding of the immunoliposomes using the Biacore technique. Finally, Caco-2 cells grown on collagen were successfully used to demonstrate the binding properties of the four α-ED-B scFv-immunoliposome preparations in vitro. With fluorescence microscopy all four α-ED-B scFv-immunoliposomes showed comparable binding properties to ED-B positive cells. Because the α-ED-B scFv-CM3 showed the best coupling efficiency it was produced in P. pastoris using a fermenter (Chapter 5) for the use in the in vivo studies.

Based on the positive results obtained in vitro, the biodistribution and the cytotoxicity of scFv-PEG-immunoliposomes loaded with 5FdU-NOAC were tested in vivo using nude mice bearing subcutaneously growing F9 tumours. The use of a syngeneic model leads to a better evaluation of the target specificity of
a molecule than with models in which a human marker is present in a human tumour xenografted in mice but is otherwise absent in other murine tissues.

Neri et al. demonstrated the accumulation of α-ED-B scFv CGS-1 in SCID mice bearing F9 tumours (114). To be able to image the targeting they labelled the α-ED-B scFv with the infrared fluorophore CY7 and used the photodetection method described by Folli (129). This methodology allows a kinetic evaluation of tumour targeting and of scFv clearance in the same living animal at different time points. The authors described an accumulation of 2% of the injected dose per gram of tumour over a time period of 2 days. However, an exact quantification of the accumulation of scFv in the tumour and in other organs is not possible using this method.

Viti et al. (125) and Tarli et al. (106) reported biodistribution experiments with the new affinity maturated α-ED-B scFv L19. They performed the same in vivo experiments as described in this work by labelling the scFv with 125I followed by analysis of the accumulation of the scFv in the tumour and in organs. They injected 1 μg protein per mouse. The dimer and the monomer of L19 showed an accumulation of 20% and 5% of injected dose per gram tumour 4 h after injection, respectively. The scFv did not accumulate in the liver and it was rapidly eliminated from blood through the kidney with a biphasic profile (106). This scFv is a promising diagnostic tool to detect newly formed blood vessels where a short half-life of the radionucleide is required to minimise the exposure of the patients to radiation. For therapeutic applications a longer circulation time in the blood would be a great advantage as obtained with liposomes (Tables 6.3 and 6.4, page 107). Circulation time of liposomes in the blood is influenced by liposome size, the presence of a protein on the liposome surface and the introduction of PEG. Liposomes larger than 300 nm in diameter accumulate in the spleen and liposomes smaller than 80 nm accumulate in the liver (130).

The highest accumulation of immunoliposomes containing α-ED-B-scFv CGS-1 was 8% of injected dose per gram tumour (Table 6.4, page 107). Reminding that the scFv CGS-1 has a 1000 fold higher dissociation constant than the scFv L19 (Table 6.1, page 96) the results obtained with the scFv-CGS-1-PEG-immunoliposomes are remarkable. The observation that B-fibronectin might be present only on the abluminal site of the endothelial cells could be the
explanation for the rather low accumulation in the tumour of the \( \alpha \)-ED-B scFv-immunoliposomes \textit{in vivo}. Risau et. al. described the presence of fibronectin exclusively on the abluminal side of the endothelial cells in chicken embryos (131).

The capillary permeability of the endothelial barrier in newly vascularised tumours is significantly greater than that of normal organs. Thus, the liposomes could extravasate through the leaky endothelium by passive transport. It has been shown that the size limitation of such liposomes is of practical significance. Under physiological conditions, only liposomes with small diameters and with prolonged circulation times have a chance to encounter and penetrate through a leaky vessel in the tumour. A method to improve the extravasation of target specific liposomes could consist in the RES blocking by pre-treatment of the mice with conventional liposomes. The immunoliposomes would remain in the circulation for longer time and thus have a higher probability of diffusing through the capillary of newly formed vessels.

Other authors described already the different biodistribution properties of liposomes with or without proteins on their surface (64, 128). Goren et al. (64) showed a lower uptake of the immunoliposomes in the tumour compared to control liposomes. At the present time only a few research groups described the possible targeting of angiogenesis \textit{in vivo} (132). Reports in antiangiogenetic therapeutic strategies focus more on the search of inhibitory molecules (133).

The cytotoxic effect detected with the scFv-5FdU-NOAC-PEG-liposomes are a very promising basis to investigate further experiments. Other therapeutic schedules or other cytotoxic agents should be tested. The cytotoxicity of 5FdU-NOAC was tested \textit{in vitro} only on tumour cells. Therefore, it still unknown weather the liposomes had an antiangiogenic effect resulting in the reduction of growing of blood vessels. Further experiments addressing this question should be performed. One approach could be done by detection of blood vessel density in the tumour using immunohistochemistry. Another feasible approach would be the construction of immunoliposomes containing the \( \alpha \)-ED-B scFv L19.

In conclusion, keeping in mind that the ED-B domain of fibronectin is conserved between mouse and man scFv-PEG-immunoliposomes containing
cytotoxic drugs (e.g. 5FdU-NOAC) are a promising approach for the targeting of the tumour neovascularature in humans.
7  PREPARATION OF RGD-IMMUNOLIPOSOMES, BINDING TO 
\( \alpha_v\beta_3 \)-INTEGRINS \textit{IN VITRO} AND ANTIMETASTATIC ANALYSIS 
WITH RGD-IMMUNOLIPOSOMES

\subsection*{7.1 Abstract}
The tripeptide arginine-glycine-asparagine acid (RGD) was coupled to the 
terminal end of PEG chains on sulfo-SMCC modified liposomes. One cysteine 
residue was added at the C-terminus of the tripeptide for the attachment to the 
surface of liposomes using the maleimide coupling method. Up to 70 peptides 
could be coupled to one liposome prepared with 7 mol\% PE-PEG-NH\textsubscript{2} and with  
a mean diameter of 50 nm. The RGD peptide recognises the \( \alpha_v\beta_3 \)-integrin which 
is expressed on different tumour cell lines and plays a key role in the adhesion  
of tumour cells and in angiogenesis. The specific binding of fluorescence 
labelled RGD-immunoliposomes to \( \alpha_v\beta_3 \)-integrin was shown \textit{in vitro} by 
fluorescence microscopy on integrin expressing tumour cells. The human breast 
cancer cell lines MDA-MB-231 (\( \alpha_v\beta_3 \)-integrin positive) and MCF-7 (\( \alpha_v\beta_3 \)-integrin  
negative) were used as targets. RGD-peptides have been found to decrease  
metastatic colonisation. The reduction of metastasis was shown by co-injection 
of B16F10 melanoma cells and RGD-immunoliposomes into C57BL/6 mice. The  
metastasis suppression efficacy was increased by 35\% by loading the RGD- 
immunoliposomes with N\textsuperscript{4}-octadecyl-1-\( \beta \)-D-arabinofuranosylcytosine (NOAC).

\subsection*{7.2 Introduction}
The \( \alpha_v\beta_3 \)-integrin is of particular interest in oncology because it has been 
implicated in tumour progression and acquisition of invasiveness (134). 
Integrins function as transmembrane rivets, link the cell to the extracellular 
matrix and are involved in signalling. They are \( \alpha/\beta \) heterodimers expressed on a  
wide variety of cells. There are 8 known \( \beta \) subunits and 14 known \( \alpha \) subunits  
which associate to give at least 20 transmembrane glycoproteins with different  
ligand specificities. The ligands for several integrins are adhesive proteins  
(135). The \( \alpha_v\beta_3 \)-integrin is expressed on endothelial cells, osteoclasts,
melanoma cells and other cell types. Though originally isolated as a receptor of vitronectin, \(\alpha_5\beta_3\)-integrin recognises a broad range of extracellular matrix protein ligands: fibronectin, fibrinogen, thrombospondin, von Willebrand factor and osteopontin which all contain the classical integrin-recognition motif arginine-glycine-asparagine acid (RGD). The specificity of \(\alpha_5\beta_3\)-integrin contrasts sharply with the selectivity of the \(\alpha_5\beta_1\)-integrin, which binds exclusively to fibronectin and is apparently capable of recognising the RGD sequence only within the context of the fibronectin molecule (136).

Tumour vasculature undergoes continuous angiogenesis and expresses molecular markers that characterise these vessels (for more details see 1.2, page 12). The markers in angiogenic endothelium are particular receptors including \(\alpha_5\beta_3\)-integrin (137) (Table 1.1, page 13). Preventing the \(\alpha_5\beta_3\)-integrin from binding to their ligands causes apoptosis of endothelial cells of newly formed blood vessels (21). The application of a monoclonal antibody LM 609 directed to \(\alpha_5\beta_3\)-integrin resulted in the suppression of angiogenesis (138, 139). This finding indicates that \(\alpha_5\beta_3\)-integrin plays a critical role in angiogenesis and could therefore be used as a angiogenic marker and RGD-immunoliposomes recognising \(\alpha_5\beta_3\)-integrin could have the potential to inhibit tumour angiogenesis.

Several studies have shown that RGD-peptides can inhibit metastasis (140). Metastasis is established by a complex cascade of activities including metastatic tumour cell detachment and extravasation from the local tumour, adhesion and interaction of the cancer cells with endothelial cells and subendothelial basement membranes (141). These processes depend on regulated cellular adhesion. Many of these functions appear to be modulated by integrins. Suppression of the interaction of the extracellular matrix proteins and the cell surface leads to a reduction of metastasis. Co-injection of RGD-peptides with tumour cells reduce the metastatic process of B16BL6 cells (140). The B16BL6 or the B16F10 cell lines are sublines of the highly lung-metastatic murine B16 melanoma cell line. They accumulate immediately after injection in the lung and have a very low release (1 %/min) into the circulation (142). RGD-peptides can prevent the attachment of the melanoma cells in the lung by
binding to $\alpha_\beta$-integrins. Since most peptides have a short circulation half-life a high dose of RGD-peptide is required to obtain acceptable antimetastatic effects. RGD-peptides attached to PEG-liposomes circulate for much longer times and at high concentration in the blood which could lead to a more effective inhibition of tumour cell accumulation and formation of metastasis.

![Figure 7.1: Structure of the RGD-peptide (M, 575.61) used to prepare RGD-immunoliposomes.](image)

In this chapter a RGD-peptide containing an additional cysteine at the C-terminus (Figure 7.1) was attached to the terminal end of maleimide modified PEG on liposomes. The coupling strategy is shown in Figure 7.2 and was the same according to the attachment of $\alpha$-ED-B scFv to liposomes in Chapter 6. 

*In vitro* targeting of RGD-PEG-immunoliposomes to the human adenocarcinoma breast cancer cell line MDA-MB-231 was investigated. These highly tumourigenic cells express relatively high levels of $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, and $\alpha_4\beta_3$-integrins compared to the MCF-7 breast cancer cell line (143, 144). The MDA-MB-231 and MCF-7 cells were cultured on collagen and incubated with the fluorescence labelled RGD-PEG-immunoliposomes and specific binding was demonstrated by fluorescence microscopy.
In the second part of this chapter the inhibition of tumour metastasis formation was analysed by co-injection of B16F10 tumour cells and RGD-PEG-liposomes containing NOAC. It is expected that this lipophilic derivative of the chemotherapeutic agent ara-C could enhance the therapeutic effect by killing the arrested tumour cells in the lung. Another study has shown that immunoliposomes containing the lipophilic prodrug 3',5'-O-dipalmitoyl-5-fluoro-2'-deoxyuridine (dpFUdR) binding to the lung endothelial anticoagulant protein thrombomodulin could inhibit experimental lung metastasis in mice (145).

The construction, binding properties in vitro and antimetastatic effects of RGD-PEG-immunoliposomes in vivo are described in this chapter.
7.3 Methods

7.3.1 Material, cells and animals
The RGD-peptide was synthesised by the Naturwissenschaftliches und Medizinisches Institut (University of Tübingen, Germany). The cell lines MDA-MB-231 and MCF-7 were purchased from the American Type Culture Collection (ATCC; Rockville, USA). The cell line PC-3 was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The high metastatic melanoma cell line B16F10 was obtained from Hoffmann-La Roche (Basel, Switzerland). NOAC was synthesised according to (76). C57BL6 female mice were purchased from rrc (Füllinsdorf, Switzerland). All buffer salts and other chemicals were from Fluka or Sigma (Buchs, Switzerland).

7.3.2 Modification of RGD-peptides with BODIPY FL®, SE
RGD peptides (3 mg, 3 x 10^18 amino groups) in 900 μl 0.1 M sodium bicarbonate, pH 8.3 were modified with 234 μl BODIPY FL®, SE (10 mg/ml in DMF) for 1 h at RT at a molar ratio of amino groups to BODIPY of 1 : 1.2. The reaction was stopped by addition 90 μl hydroxylamine (1.5 M, pH 8.5) for 1 h at RT.

7.3.3 Coupling of RGD-peptides to sulfo-SMCC modified liposomes
For the calculation of the coupling efficiency the reaction mixture (122 μl) containing 10^17 fluorescence labelled RGD-peptide molecules and unreacted and inactivated BODIPY FL® was incubated with 50 μl non-labelled and sulfo-SMCC modified PEG-liposomes. They were prepared with 80 mg/ml SPC and 7 mol% PE-PEG-NH₂ corresponding to 4.5 x 10^17 maleimide groups (Table 2.4, page 40) and incubated with a final concentration of 2 mM TBF for 48 h at 4° C under argon. Unreacted BODIPY FL® and the uncoupled peptides were removed by dialysis during 12 h against 1 litre PBS (0.137 M NaCl, 2.68 mM KCl, 8.09 mM Na2HPO₄, 1.76 mM KH₂PO₄, pH 7.4). The probes were analysed using a fluorescence spectrofluorometer SFM 23 (Kontron, Zürich, Switzerland) with an excitation wavelength of 510 nm and an emission of 502 nm.
For in vitro binding studies 750 μg unlabelled RGD-peptides corresponding to 7.5 x 10^{17} thiol groups was incubated with 200 μl DiO labelled and sulfo-SMCC-modified PEG-liposomes corresponding to 1.8 x 10^{18} maleimide groups (Table 2.4, page 41) as described before.

7.3.4 Determination of αβ3-integrin expression on tumour cells by flow cytometry

MDA-MB-231, MCF-7 and PC-3 cells were maintained in RPMI 1640 (Gibco BRL, Basel, Switzerland) supplemented with heat-inactivated 10% fetal calf serum, 2 mM L-glutamine 100 U/ml penicillin and 100 μg/ml streptomycin under a humidified atmosphere of 5% CO₂ at 37°C. The cells were trypsinised and washed once with PBS. Aliquots of 100 μl containing 10^6 cells were incubated with 1 μg anti-αβ3 antibody LM 609 (Chemicon International; Temecula, Canada) in 100 μl PBS for 30 min at 4°C. The cells were washed twice with PBS and incubated with 1 μg FITC labelled rabbit anti-mouse antibody (Southern Biotechnology, USA) in 100 μl PBS for 30 min at 4°C. Cells were washed and resuspended in 1 ml FACS buffer (PBS containing 2% fetal calf serum and 0.01% NaN₃) and analysed by flow cytometry using an Epics Elite Analyser (Coulter, Florida, USA).

7.3.5 Binding of RGD-immunoliposomes to αβ3-positive cells in vitro

The binding properties of RGD-immunoliposomes was analysed as described in 6.3.3, page 95. Cover slips (20 mm diameter) were placed into 12 well plates, coated with 100 μl rat tail collagen I (10 mg/ml) isolated from rat tail as described by Eldsdale (116) and incubated for 30 min at 37°C. MDA-MB-231 and MCF-7 cells (10^5 cells/well) were plated and cultured for 48 h in a humidified 5% CO₂ atmosphere at 37°C. The washed cells were incubated with 100 μl PBS containing 1% BSA for 30 min at 4°C followed by 100 μl DiO labelled α-αβ3 RGD-immunoliposomes corresponding to 1.4 x 10^{13} liposomes and 5.6 x 10^{14} peptide molecules for 30 min at 4°C. After another washing step the cover slips were removed, treated with 10% glycerine, placed on a microscope slide and analysed on a fluorescence microscope (Leica DLMB).
7.3.6 Preparation of RGD-PEG-immunoliposomes for in vivo studies

Three types of PEG-liposomes containing NOAC and the RGD-peptide, NOAC alone or RGD-peptide alone were prepared for the treatment of mice with B16F10 melanoma cells. Briefly, 2 ml sulfo-SMCC modified liposomes prepared with 80 mg/ml SPC, 10 mg/ml NOAC (trace labelled with $[^3]$H-NOAC) and 7 mol% PE-PEG-NH$_2$ corresponding to $1.8 \times 10^{19}$ maleinimide groups (Table 2.4, page 41) were incubated with 16 mg peptide corresponding to $1.7 \times 10^{19}$ thiol groups in HBSE (10 mM Hepes, 150 mM NaCl, 9.1 mM EDTA, pH 7.5) at a final concentration of 2 mM TBF for 48 h and at 4°C under argon. Unreacted RGD-peptide was removed by dialysis. The concentration of NOAC in the liposomes was determined by measuring the radioactivity of $[^3]$H-NOAC.

7.3.7 Effects of immunoliposomes on metastasis formation of B16F10 cells in the lung

The metastatic murine B16F10 melanoma cells were cultured in DMEM medium (Gibco) supplemented with heat-inactivated 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells ($10^5$ cells/mouse) were co-injected with 100 μl liposomes (containing RGD-peptide and NOAC, NOAC alone and RGD-peptide alone; 400 μg NOAC per injection) into female C57BL6 mice (5 animals per group) via the tail vein. Fourteen days after injection the lungs were excised, photographed, and fixed in 3% formalin. The number of metastatic colonies on the lung surface was counted directly after removal.
7.4 Results

7.4.1 Modification of RGD peptides with BODIPY FL®, SE and coupling to sulfo-SMCC modified PEG-liposomes

The amino-reactive fluorescent dye BODIPY FL®, SE was used for the labelling of the RGD peptides. Unreacted RGD peptides and the fluorescent dye could not be separated because of their equal size. The reaction mixture containing the RGD peptide and BODIPY was used to attach the RGD peptides to the sulfo-SMCC modified liposomes prepared with 7 mol% PE-PEG-NH₂. The coupling efficiency using liposomes prepared with 2 mol% PE-PEG-NH₂ was very low. After the coupling reaction unreacted BODIPY and unreacted RGD-peptides were removed together by dialysis. The number of peptides linked to one liposome was calculated by measuring the BODIPY fluorescence. The molar ratio of peptides to maleinimide groups on the liposomes was 1 : 1. Up to 70 peptides per liposome with a diameter of 50 nm containing 390 maleinimide groups could be detected (Table 2.4, page 41). Assuming that not every peptide molecule was labelled with BODIPY more than 70 peptides were attached to one liposome.

7.4.2 Expression of αvβ3-integrin on tumour cells

![Figure 7.3: Cytofluorometric determination of αvβ3-integrin expression on PC-3, MDA-MB-231, MCF-7 cells. The expression was detected using the αvαvβ3-integrin antibody LM609, followed by a FITC labelled second antibody.](image)

To demonstrate specific binding of RGD-immunoliposomes αvβ3-integrin expressing cells were needed. Therefore, different cell lines (PC-3, MDA-MB-
Anti αvβ3 immunoliposomes

231, MCF-7) were screened for αvβ3-integrin expression by flow cytometry using the anti αvβ3-integrin antibody LM609. The MDA-MB-231 cell line express the highest amount of αvβ3-integrins and MCF-7 cells were used as a negative control (Figure 7.3).

7.4.3 Binding of RGD-immunoliposomes to MDA-MB-231 cells in vitro

The in vitro binding of the RGD-immunoliposomes to αvβ3-integrin was tested according to the methods described in Chapter 6. MDA-MB-231 and MCF-7 cells were cultured on collagen and incubated with DiO labelled RGD-immunoliposomes. Figure 7.4 shows the specific binding of immunoliposomes only to the αvβ3-integrin positive MDA-MB-231 cells (Figure 7.4B) but not to the αvβ3-integrin negative MCF-7 cells (Figure 7.4F). Unspecific binding of unmodified liposomes to MDA-MB-231 cells could be prevented by pre-incubation of the cells with PBS containing 1% BSA.

Figure 7.4: Binding of RGD-PEG-immunoliposomes to cells cultured for 72 h on collagen I coated cover slips: A) C) E) phase contrast images of the sections shown in B, D and F; B) RGD-PEG-immunoliposomes on αvβ3-positive MDA-MB 231 cells; D) unmodified PEG-liposomes on αvβ3-positive MDA-MB 231 cells; F) RGD-PEG-immunoliposomes on αvβ3-negative MCF-7 cells. Fluorescence was detected by labelling the liposomes with the fluorescent dye DiO.
7.4.4 Inhibition of experimental metastasis formation by RGD-PEG-immunoliposomes

The inhibition properties of RGD-PEG-immunoliposomes containing NOAC were tested by co-injection of $10^5$ metastatic murine B16F10 melanoma cells and different types of PEG-liposome preparations (liposomes containing RGD-peptide and NOAC, NOAC alone or RGD-peptide alone) i.v.. Fourteen days after injection the lungs were removed, weighed and the number of metastases counted. Reduction of 35% in the occurrence of lung metastases was observed by injection of cells and 100 µl RGD-immunoliposomes of a diameter of 100 nm containing 400 µg NOAC and 2.6 µg RGD-peptides. Liposomes loaded with NOAC alone had no effect (Figure 7.5A). The number of metastases correlated with the weight of the lungs.

![Figure 7.5: Effects of RGD-NOAC-immunoliposomes on experimental lung metastatic B16F10 cells. Five mice per group were injected i.v. with: cells alone, RGD-NOAC-immunoliposomes and cells, NOAC-liposomes and cells or RGD-immunoliposomes and cells. Fourteen days after injection the mice were sacrificed and lung colonies were counted. The number of lung metastases in the control animals was taken as 100%.](image-url)
7.5 Discussion

Based on the successful construction of α-ED-B scFv-immunoliposomes the promising RGD peptide recognising tumour cells and tumour endothelial cells was attached to liposomes. One cysteine at the C-terminus of the peptide was enough to couple the peptide to liposomes. Working with such a small molecule has its advantages and disadvantages. The separation of peptide could be easily done by dialysis. No metrizamide gradient had to be used. However, it is not easy to label such a small molecule, because the peptide and the BODIPY dye could not be separated because of their equal size. Therefore, the exact calculation of attached peptide molecules per liposome was not possible.

The nature of the vitronectin receptor is of particular interest in oncology because it has been implicated in tumour progression and acquisition of invasiveness. In melanoma, for example, the expression of αvβ3-integrin correlates with tumour invasiveness (146). In several immunohistochemical studies the expression of integrins in breast cancer compared to normal breast tissue was examined. The αvβ3- and the α1β3-integrins are overexpressed in some breast cancer cell lines (144). This leads to the assumption that integrins are possible markers for human breast cancer. Furthermore, there is some evidence that antagonists of αvβ3-integrin may offer an effective target for the treatment of tumour angiogenesis (139). RGD-liposomes containing cytotoxic agents may become a new and promising system for specific tumour killing in combination with antiangiogenic effects (132).

RGD-peptides can inhibit metastasis formation but not growth of primary tumours (147). Fujii et al reported a 50% reduction of metastasis formation by co-injection of $5 \times 10^4$ B16BL6 cells and 1'000 µg GRGDS peptide into mice (140). Clearance of small peptides in the circulation is known to be quite fast, usually occurring within several minutes. Liposomes may also provide cooperative binding to metastatic tumour cells due to a large number of RGD molecules present on a single liposome surface. In this study a 35% reduction could be demonstrated by injection 100 µl RGD-liposomes filled with 400 µg NOAC and modified with only 2.6 µg RGD-peptides.
The used RGD liposome preparation reported in the literature give very controversial results. Therefore, other RGD-constructs will have to be tested. Pasqualanini et al. (132) described a better effect of circular RGD-peptides. The circle was formed by disulphide bridges of four additional cysteines. This kind of peptide needs another strategy to couple the peptide to the liposomes.
8 GENERAL DISCUSSION

8.1 Construction of immunoliposomes

The attachment of antibodies or antibody fragments to the outer surface of liposomes is a difficult task. Introduction of active groups on proteins is often complex and involving intermediates and reactants which make the use of immunoliposomes difficult in living systems. Several methods for coupling proteins to liposomes have been described (Table 1.2, page 21). Some of the most efficient coupling chemistries compromise the conjugation of thiolated proteins to liposomes containing either thiol or maleimide groups (46). The use of PEG as protector and stabilisator against rapid elimination of immunoliposomes could result in steric hindrance for the antibody-antigen interaction, especially when the antibodies are coupled directly to the surface of the liposomes. To solve this problem, the protein should be attached to the distal end of the PEG chain.

In this thesis the successful coupling of F(ab)\(_2\) fragments recognising the carcinoembryonic antigen (CEA) (Chapter 3), scFv recognising the ED-B domain of B-fibronectin (Chapter 6) and RGD-peptides binding to \(\alpha_v\beta_3\)-integrin (Chapter 7) are described. All molecules were attached via the thiol-maleimide reaction to the liposome surfaces. The \(\alpha\)-CEA F(ab)\(_2\) fragments were modified with SATA to introduce reactive thiol groups. With this technique a high number of reactive groups can be introduced into the protein. The thiol groups are randomly localised on the surface of the protein. As disadvantage, the modification of antibodies or antibody fragments with a reagent like SATA or SPDP could lead to an inactivation of the binding properties as shown by modification of the \(\alpha\)-ED-B scFv with SATA (Chapter 4) and also reported by Uyama (49). Another disadvantage of introducing active groups randomly over the protein consists in the orientation of the proteins on liposomes. It is possible that proteins are attached to the liposome surface with the active site directed towards the liposome surface, leading to a loss of the binding properties to antigens. By introducing reactive groups at a defined site on the protein structure their orientation can be fixed and it is therefore well defined. Using
protein engineering, reactive thiol groups were introduced at the C-terminus of α-ED-B scFv or of α-αβ3 RGD-peptides by adding cysteines. In contrast to the α-CEA A5B7-F(ab)2 fragments, the scFv and RGD-peptides attached had all the same orientation on the liposome surface. All three molecules could be coupled successfully to the distal end of the PEG chains on the liposome surfaces. The coupling efficiency correlated with the size of the molecules. The F(ab)2 fragments with a molecular weight of 100'000 Da showed the lowest coupling efficiency with an average of 3 molecules bound per liposome. Depending on the number of additional cysteines introduced at the C-terminus of scFv (Mₐ: 30'000 Da) 28 to 70 scFv molecules could be attached to one liposome. The exact determination of the number of RGD-peptides (Mₐ: 575 Da) per liposome was not possible. Approximately, 70 or more peptide molecules could be coupled to the surface of one liposome.

8.2 Binding of immunoliposomes in vitro

The specific binding of all three immunoliposomes (α-CEA A5B7-F(ab)2-, α-ED-B scFv- and α-αβ3 RGD-) was demonstrated in vitro. Three different models had to be established. Unfortunately, no system was found allowing to test two or even all three immunoliposomes in a single system together. CEA is a widely studied human tumour marker and it is expressed on the surface of different tumour cells. Therefore, specific binding of the α-CEA A5B7-F(ab)2-immunoliposomes could be easily demonstrated by flow cytometry using CEA positive and CEA negative cells (Figure 3.6, page 57). The establishment of an in vitro system to test the properties of α-ED-B scFv-immunoliposomes was more difficult. The ED-B domain of the B-fibronectin isoform is not expressed on the surface of a cell, rather it is located in the extracellular matrix. Different approaches with the antigen alone were evaluated. The antigen ED-B could be produced in E.coli and was available at high amounts (Chapter 4). The attempts to test the binding of scFv-immunoliposomes on ED-B coated ELISA plates or on ED-B modified microbeads followed by flow cytometry was not successful. Because of these technical problems another system was chosen. Pujuguet et al. (113) described the expression of ED-B and ED-A, another
splicing variation of fibronectin, by the cell lines Caco-2 and Co-115, respectively. Furthermore, it was shown that cells cultured on collagen or Matrigel were able to form an extracellular matrix in vitro (148). All these observations put together resulted in a successful in vitro system which was useful for the characterisation of the binding properties of scFv-immunoliposomes to ED-B (Figure 6.5, page 105). The $\alpha_v\beta_3$-integrin is also a component of the extracellular matrix and it is expressed on the surface of different tumour cells (149). Van der Pluijm et al. (143) described $\alpha_v\beta_3$-integrin expression on the surface of the breast cancer cell line MDA-MB-231. This observation could be verified successfully in this thesis by flow cytometry with the use of the LM609 antibody recognising $\alpha_v\beta_3$-integrin. Nevertheless, it was not possible to show a specific binding of the fluorescence labelled RGD-immunoliposomes on MDA-MB-231 cells by flow cytometry. However, the binding of the RGD-immunoliposomes to $\alpha_v\beta_3$-integrin could be demonstrated in vitro using the same technical approach as with the $\alpha$-ED-B scFv-immunoliposomes (Figure 7.4, page 127).

### 8.3 Properties of immunoliposomes in vivo

This study and work from a number of other laboratories demonstrated that antibodies can successfully be used to increase the specific binding of liposomes to tumour cells in vitro. In contrast, the attempts to show target specificity with immunoliposomes in vivo have proven to be far more difficult than in vitro studies. Systemic treatments resulted in weak or no cytotoxic effects (56), except for pegylated liposomes (53, 150). The targeted liposomes were not able to gain access to tumour tissue or those liposomes which bound to the tumour could not enter the cell and/or release their drug. Therefore, it is very important to test the properties of drugs encapsulated in liposomes in vivo. In this work it was shown that the binding capacity of scFv-immunoliposomes to ED-B was specific on cell cultures (Figure 6.5, page 105), and in vivo an increased accumulation in the tumour was observed compared to unmodified liposomes up to 2 h after injection (Figure 6.7, page 109). Other authors found no significant differences between unmodified liposomes and immunoliposomes.
in vivo (64). This situation may be due to a number of factors. One of the most
commonly recognised limitations in the use of immunoliposomes in vivo has
been their rapid clearance from circulation into the RES as compared to
unmodified liposomes (49) as also reported in this thesis (Table 6.4, page 104).
This problem is due to the immune response against the proteins present on the
surface of the liposomes. Thus, the number and the type of protein molecules
linked to the liposomes is critical. Immunoliposomes with large proteins (e.g.
IgG), are rapidly taken up from the RES. Furthermore, a lower number of
proteins linked to lipids leads to a longer circulation time (64). Another
explanation for the rapid clearance of immunoliposomes is in part their tendency
to aggregate. Several possible explanations for the spontaneous aggregation of
the liposomes can be considered. Firstly, when the proteins contain more than
one reactive group (e.g. thiol group) cross-linking of the liposomes could occur.
Secondly, repeated injections of immunoliposomes could result in less efficient
binding to the target. One potential mechanism of reduced target binding is that
an immune response induced by repeated injection might prevent
immunoliposomes from accumulation in the target (145). In addition, the
quantity of conjugated protein molecules influences the binding and endocytosis
of immunoliposomes. Park et al. (54) described a plateau for binding and for
internalisation into the target cells at 40 and 10 Fab' fragments per liposome,
respectively. This ratio has to be evaluated for each individual system.
A very important question regarding the feasibility of liposomes as drug carriers
in vivo is whether they are able to cross the anatomical barriers, such as the
endothelial capillary wall, which separates tumour cells from the blood stream.
Movement of molecules through the vasculature is governed by the vascular
morphology (i.e. the number, length, diameter and geometrical arrangement of
various blood vessels) and the blood flow rate. Liposomes first need to
extravasate to reach tumour tissue and only then they can bind to tumour cell
receptors. Targeting with liposomes will only be successful if they are able to
extravasate from the circulation. Drug loaded immunoliposomes had no effect
upon established tumours that were growing outside of the vascular bed (53). In
this context, the anatomy of the microcirculation can be expected to be of
crucial importance. Blood capillaries are classified into three different groups;
continuous, fenestrated and sinusoidal capillaries. In contrast to the continuous and fenestrated capillaries the sinusoid capillaries possess relatively large gaps that allows small particle such as SUV to penetrate through the endothelium. Another factor, which might be favourable for the access of liposomes to tumours is the already mentioned increased permeability of tumour vasculature compared to normal tissue (151). In fact, it seems likely that the ability of some liposome formulations to accumulate in tumours, at least in part, is due to increased microvascular permeability. Therefore, also liposomes without target specificity can accumulate at high percentages in the tumour (e.g. the PEG-liposomes in Figure 6.6, page 108).

The preparation of immunoliposomes with the target specificity to antigens located on the surface of tumour cells has often been described (49, 56, 64). The antigens on the tumour cells are located beyond the endothelial cell barrier. Molecules recognising tumour vessels (e.g. α-ED-B scFv-PEG-immunoliposomes) could easier reach their target without having to extravasate or to be transported across the vessel wall. Markers on newly formed blood vessels should be readily accessible to specific binders injected intravenously (132). Drugs for targeting angiogenesis markers are suitable for the therapy of several different tumour types, because new-forming blood vessels are a general feature of tumour progression. But the vasculature represents only a small fraction of the total tumour mass. Therefore, combination therapies with anti-tumour and anti-angiogenic molecules should to be develop.

8.4 Future experiments

In this thesis small, rigid, neutral and sterically stabilised PEG-immunoliposomes were prepared, characterised and tested for their binding and their cytotoxic activity in vitro and in vivo. As emphasised, the factors involved in the design of immunoliposomes are complex. The conditions have to be established individually for each system. A lot of problems have to be solved before immunoliposomes can be used in clinical trials. The binding and the cytotoxic activity of A5B7-F(ab)_2- immunoliposomes loaded with NOAC were shown on cell cultures (Figure 3.6, page 57 and Figure 3.7, page 58). In a next step the binding properties of free A5B7-F(ab)_2-fragments
and A5B7-F(ab)2-immunoliposomes should be compared in *in vivo* experiments, e.g. in the L174T tumour model.

The specific binding of free $\alpha$-ED-B scFv (Figure 5.6, page 91) and $\alpha$-ED-B scFv-immunoliposomes to ED-B (Figure 6.5, page 102) was demonstrated *in vitro*. So far only the biodistribution of $\alpha$-ED-B scFv-immunoliposomes and unmodified liposomes in nude mice bearing F9 tumour were analysed. Although it is well known that free scFv have a much shorter circulation time in blood than liposomes the biodistribution of free $\alpha$-ED-B scFv and $\alpha$-ED-B scFv-immunoliposomes should be compared.

The positive effect of $\alpha$-ED-B scFv-5FdU-NOAC-PEG-liposomes on tumour growth in mice open a wide field of future experiments. Other time schedules of injection and other cytotoxic agents should be tested. Another feasible approach would be the construction of immunoliposomes with the affinity matured $\alpha$-ED-B scFv L19. In addition, the internalisation of liposomes into the tumour or the vasculature has to be studied in further experiments.

Tumour angiogenesis and other angiogenic models (chronic airways and physiological angiogenesis in mouse ovary) showed binding and internalisation of cationic and unmodified liposomes by angiogenic endothelial cells but not by normal blood vessels. In contrast no evidence of uptake of anionic, neutral or sterically stabilised liposomes by blood vessels was found (152). Therefore, further experiments should be done with a combination of immunoliposomes and cationic liposomes.

The preliminary experiments by co-injection B16F10 melanoma cells metastatic to the lung and RGD-NOAC-immunoliposomes showed promising results to inhibit the formation of metastasis (Figure 7.5, page 128). In further experiments the effects of RGD alone and RGD-liposomes have to be compared. Experiments with spontaneous lung metastasis (formed by s. c. injection of the cells) need repeated treatment (140). In addition, further experiments should be performed by repeated treatments with these immunoliposomes.
8.5 Final remarks

Several methods have been developed for the preparation of immunoliposomes capable of targeting to specific cells (Table 1.2, page 21). When considering the preparation of immunoliposomes as a targeted drug delivery system several factors should be taken into account:

(a) Conjugation of antibodies to liposomes requires the use of at least milligram quantities of purified material.

(b) The coupling methods should be simple and rapid, producing a stable and non-toxic bond.

(c) An exact quantity of antibodies must be attached to the surface of the liposomes in a reproducible way.

(d) The integrity of the liposomes should be preserved during the whole coupling process.

(e) The liposome-antibody complex must be sufficiently stable on storage and after administration in vivo.

(f) The homing capacity of the antibodies should be preserved after coupling to the liposomes.

(g) Drugs need to be loaded efficiently into the liposomes, once at the target site, released at rates which will result in improved therapeutic effects over that of free drugs.
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### 9 ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>[(^3)H]-Chol</td>
<td>[^{3}H]-cholesteryl hexadecylether</td>
</tr>
<tr>
<td>[(^3)H]-NSP</td>
<td>[^{3}H]-N-succinimidyl[2,3,(^3)H] propionate</td>
</tr>
<tr>
<td>[5-(^3)H]-NOAC</td>
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</tr>
<tr>
<td></td>
<td>arabinofuranslytosine</td>
</tr>
<tr>
<td>ABD-F</td>
<td>4-(aminosulfonyl)-7-fluoro-2,1,3-benoxadiol</td>
</tr>
<tr>
<td>AOX1</td>
<td>Alcohol oxidase gene</td>
</tr>
<tr>
<td>Ara C</td>
<td>1-(\beta)-arabinofuranslytosine</td>
</tr>
<tr>
<td>BHPD</td>
<td>(N,N)-bis(1-hexylheptyl)-3,4,9,10-perylenbis(dicarboximide)</td>
</tr>
<tr>
<td>BMGY</td>
<td>Buffered glycerol-complex medium</td>
</tr>
<tr>
<td>BMM</td>
<td>Buffered methanol-complex medium without yeast</td>
</tr>
<tr>
<td>BMMY</td>
<td>Buffered methanol-complex medium</td>
</tr>
<tr>
<td>BODIPY FL(^\circ), SE</td>
<td>4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-daza-S-indacene-3-</td>
</tr>
<tr>
<td></td>
<td>propionic acid, succinimidy ester</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CEA</td>
<td>Carcinoembryonic antigen</td>
</tr>
<tr>
<td>DiO</td>
<td>3,3'-dioctadecylloxacarbocyanine perchlorate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified medium</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DPPE</td>
<td>2-Dipalmitoyl-sn-glycero-3-phosphatidylethanolamine</td>
</tr>
<tr>
<td>DTPA</td>
<td>Diethylenetriamine penta acetic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetate</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immuno-stimulated assay</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein-5-isothiocyanate</td>
</tr>
<tr>
<td>Fn</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropylthiogalactoside</td>
</tr>
<tr>
<td>Iv</td>
<td>Intravenously</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani medium</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>MD</td>
<td>Minimal dextrose</td>
</tr>
<tr>
<td>MM</td>
<td>Minimal methanol</td>
</tr>
<tr>
<td>MLV</td>
<td>Multilamellar large vesicles</td>
</tr>
<tr>
<td>NOAC</td>
<td>N4-octadecyl-1-ß-D-arabinofuranosylcytosine</td>
</tr>
<tr>
<td>NTP</td>
<td>Ribonucleoside triphosphate</td>
</tr>
<tr>
<td>P. pastoris</td>
<td>Pichia pastoris</td>
</tr>
<tr>
<td>PB</td>
<td>Phosphate buffer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PBSM</td>
<td>PBS containing 2 % milk</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PE-PEG-NH₂</td>
<td>amino-poly(ethylene glycol)-phosphatidylethanolamine</td>
</tr>
<tr>
<td>PE-PEG-OMet</td>
<td>methoxy-poly(ethylene glycol)-phosphatidylethanolamine</td>
</tr>
<tr>
<td>RES</td>
<td>Reticuloendothelial system</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-glycine-asparagine acid</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SATA</td>
<td>N-succinimidyl-S-acetylthioacetate</td>
</tr>
<tr>
<td>Sc</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>ScFv</td>
<td>single chain Fv antibody fragment</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SPC</td>
<td>soy phosphatidylcholine</td>
</tr>
<tr>
<td>SPDP</td>
<td>N-succinimidyl-3-(2-pyridyldithio)propionate</td>
</tr>
<tr>
<td>sulfo-SMCC</td>
<td>sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate</td>
</tr>
<tr>
<td>SUV</td>
<td>Small unilamellar vesicles</td>
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<tr>
<td>TBP</td>
<td>Tributylphosphine</td>
</tr>
<tr>
<td>TY</td>
<td>Trypton yeast extract</td>
</tr>
<tr>
<td>V_H</td>
<td>Variable heavy chain</td>
</tr>
<tr>
<td>V_L</td>
<td>Variable light chain</td>
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<tr>
<td>YPD</td>
<td>Yeast extract peptone dextrose</td>
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<tr>
<td>YPDS</td>
<td>Yeast extract peptone dextrose sorbitol</td>
</tr>
</tbody>
</table>
10 REFERENCE LIST


step direct labelling of recombinant antibodies with technetium-99m. 


11 APPENDIX

11.1 List of publications

11.1.1 Poster presentations
Marty C., Klemenz R., Neri D., Schwendener R. A.: Immunoliposomes for the targeted inhibition of angiogenesis and tumour growth; 30th annual meeting of USGEB/USSBE; March 1998; Lausanne (Switzerland).

11.1.2 Oral presentations
Immunoliposomen zur gezielten Hemmung von Angiogenese und Tumorwachstum; Doktorandentag des Departements Pharmazie; April 1998; ETH Zürich (Switzerland).
11.2 *Curriculum vitae*

1976 – 1982  
Primary School, Zürich

1988  
Graduation diploma (Matura, Typus B)

1989 – 1995  
Studies in Biochemistry at the Swiss Federal Institute of Technology, Zürich

1995  
Diploma student at the Department of Biochemistry, Swiss Federal Institute of Technology Zürich, under the guidance of Prof. Dr. J. Brunner

1995  
Diploma in Biochemistry (dipl. Natw. ETH)

1996 – 2000  
Ph. D. student at the Division of Cancer Research, Department of Pathology, University Hospital Zürich and Swiss Federal Institute of Technology Zürich, under the guidance of Prof. Dr. G. Folkers and Prof. Dr. R.A. Schwendener

2000  
Examination to obtain the degree of Doctoral Sciences, Swiss Federal Institute of Technology, ETH Zürich, Switzerland