Adoptive Immunotherapy

Methods and Protocols

Edited by

Burkhard Ludewig
Matthias W. Hoffmann

Humana Press
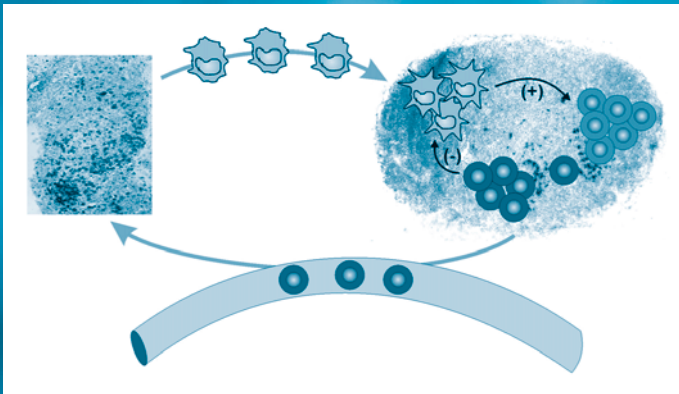
Cytotoxic Tumor Targeting
With scFv Antibody-Modified Liposomes

Cornelia Marty and Reto A. Schwendener

Summary

Specific targeting of liposome-formulated cytotoxic drugs or antigens to receptors expressed selectively on target cells represents an effective strategy for increasing the pharmacological efficacy of the delivered molecules. We have developed a feasible technique to selectively attach antibodies and fragments thereof, but also small-mol-wt ligands such as peptides, carbohydrates, or any molecules that recognize and bind target antigens or receptors to the surface of small unilamellar liposomes. Our concept is based on the site-specific functionalization of the ligands to be attached to the liposomes by thiol groups. These thiol groups can easily be introduced to antibodies or peptides by addition of cysteines, preferably at sites that do not interfere with the receptor binding domains. Optimally, the site-specific modification is introduced at the C-terminal end of the ligand, separated by an inert spacer sequence located between the thiols and the specific part of the ligand. The thiol-reactive molecules on the liposome surface are maleimides that are linked to phospholipids composing the liposome bilayer membrane. We illustrate the coupling method of a functionalized single-chain antibody fragment with binding specificity to ED-B fibronectin, an isoform of fibronectin exclusively expressed in tumor tissues, to long circulating small unilamellar poly(ethylene glycol) liposomes.

Key Words: Ligand-mediated liposome targeting; site-directed ligand modification; ligand functionalization; liposomes; immunoliposomes; thiol-maleimide coupling; cysteine modification; single-chain antibody fragment; peptides; targeted tumor therapy; vaccines.

1. Introduction

Antibody or small-molecule ligand-mediated targeting of liposome-encapsulated anticancer or antiangiogenic drugs to receptors expressed selectively on tumor and/or tumor endothelial cells represents an effective strategy for increasing the therapeutic efficacy of the liposomal drugs. Small unilamellar ligand-targeted liposomes are intended to increase the specificity of interaction with target cells and to increase the amount of drug delivered to these cells *(1,2)*. We have developed a feasible technique to attach antibodies and fragments thereof, but also small-mol-wt ligands such as peptides, carbohydrates, or any molecules that selectively recognize and bind to

target antigens or receptors, to the surface of small unilamellar liposomes (SUV) **(3)**. The random introduction of reactive groups to a protein—e.g., via amino group modification—leads to uncontrollable attachment and random orientation of the attached molecules on the liposome surfaces, causing decrease of binding efficiency. By introducing reactive groups at a distinct site on the ligand molecule, the orientation on the liposome surface can be defined. Our concept is based on the site-specific functionalization of the ligands to be attached to the liposomes by thiol groups. These thiol groups can easily be introduced onto antibodies or peptides by addition of cysteines, preferably at sites that do not interfere with the receptor binding domains. Optimally, the site-specific modification is introduced at the C-terminal end of the ligand, separated by an inert spacer sequence located between the thiols and the specific part of the ligand. The thiol-reactive molecules on the liposome surface are maleimides that are linked to phospholipids composing the liposome bilayer membrane. We illustrate the coupling method of a functionalized single-chain antibody fragment (scFv) with binding specificity to ED-B fibronectin, an isoform of fibronectin exclusively expressed in tumor tissues, to long circulating small unilamellar poly(ethylene glycol)-liposomes (PEG-liposomes).

2. Materials

2.1. Liposome Preparation and Modification

1. Soy phosphatidylcholine (SPC) (L. Meyer GmbH, Hamburg, Germany).
2. Cholesterol Fluka (Buchs, Switzerland) (*see* **Note 1**).
3. D,L-α-tocopherol (Merck, Darmstadt, Germany).
4. 2-Dipalmitoyl-sn-glycero-3-phosphatidylethanolamine (DPPE) (Sygena, Liestal, Switzerland).
5. Methoxy-poly(ethylene glycol)-phosphatidylethanolamine (PE-PEG-OMet) (Sygena).
6. Amino-poly(ethylene glycol)-phosphatidylethanolamine (PE-PEG-NH$_2$) (Shearwater Polymers, Enschede, The Netherlands).
7. 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO) (Molecular Probes, Leiden, The Netherlands).
8. Phosphate buffer (PB): 13 m*M* KH$_2$PO$_4$, 54 m*M* NaHPO$_4$, pH 7.4.
9. Sulfosuccinimidyl *N*-(4-carboxycyclohexylmethyl)maleimide (sulfo-SMCC) (Pierce, Rockford, IL).
10. Round-bottom flasks (25–250 mL).
11. Sodium cholate.
12. Rotatory evaporator, e.g., Rotavap (Buechi AG, Flawil, Switzerland).
13. Mini-Lipoprep instrument (Harvard Apparatus Limited, Edenbridge, Kent, UK).
14. Lipex™ extruder (Lipex Biomembranes, Vancouver, Canada).
15. Nuclepore membranes of defined pore sizes: 0.4, 0.2, 0.1 µm (Whatman, Maidstone, Kent, UK).
16. Dialysis tubing, 10,000 mol wt Cut off.
17. Fluorescence spectrometer.

2.2. Antibody Coupling

1. HBSE buffer: 10 m*M* HEPES, 150 m*M* NaCl, 9.1 m*M* EDTA, pH 7.5.
2. Tributylphosphine (Fluka, Buchs, Switzerland).

3. Metrizamide (Sigma , St. Louis, MO).
4. Ultracentrifuge tubes (Beckmann).
5. Ultracentrifuge.

2.3. In Vitro Binding of scFv-Liposomes to Tumor Cells

1. Cell-culture medium (DMEM; Gibco-BRL, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% nonessential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin, and 0.1 mg/mL streptomycin (all from Gibco-BRL).
2. Phosphate buffered saline (PBS): 137 mM NaCl, 2.68 mM KCl, 8.09 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4.
3. Glass cover slips (20-mm diameter).
4. Collagen I from rat tails (Sigma) or extracted from rat tails (*see* **Note 2**).
5. Glycerin 10%.
6. Fluorescence microscope.

3. Methods

The methods described below outline (1) the preparation of liposomes by detergent dialysis and filter extrusion; (2) the liposome modification with maleimide molecules; (3) the attachment of functionalized scFv antibody fragments to liposomes via thiol-to-maleimide coupling; (4) the separation of liposomes from unbound scFv fragments; and (5) the in vitro binding of scFv-modified liposomes to cancer cells.

3.1. Liposome Preparation

In the past 30 yr a large number of methods of liposome preparation have been developed and refined. For comprehensive information, we refer to the corresponding literature. We use the two methods described below because of their ease and versatility, as well as the high quality of the liposomes they produce.

3.1.1. General Liposome Compositions

Liposomes can be composed of a large selection of phospholipids and additional lipophilic compounds, such as cholesterol, poly(ethylene glycol) lipids (PEG), and antioxidants. Depending on the intended application of the liposomes, different lipid compositions have to be selected. The "state-of-the-art" liposomes used for intravenous applications—e.g., liposomes carrying cytotoxic antitumor drugs—are those composed of lipids containing hydrophilic carbohydrates or polymers, usually a poly(ethylene glycol)-modified phopholipid. Such PEG- or "stealth" liposomes evade fast absorption in the mononuclear phagocyte system *(1)*. Liposome formulations carrying antigens intended as vaccines are administered by subcutaneous or intradermal injection and do not require PEG modification, since the targets are phagocytosing cells—macrophages and dendritic cells localized at the site of injection *(4)*. The lipid compositions used for the preparation of conventional scFv-liposomes and pegylated scFv-liposomes are given in **Table 1**.

3.1.2. Model Calculations of Numbers of Liposomes and Reactive Groups

Based on experimentally determined mean hydrodynamic diameters of the liposomes (*see* **Note 3**) and from assumptions on vesicle geometry parameters made by

Table 1
Lipid Composition in Milligrams per Milliliter of the Liposome Types A, B, C, D

Type	Liposomes	SPC [a]	Cholesterol	DPPE	DPPE-PEG-Omet	DPPE-PEG-NH$_2$
A	Conventional control liposomes	40 mg 100 mol% 52 µmol	4 mg 20 mol% 10 µmol			
B	Conventional liposomes for modification	40 mg 100 mol% 52 µmol	4 mg 20 mol% 10 µmol	2.52 mg 7 mol% 3.5 µmol		
C	Control PEG liposomes	40 mg 100 mol% 52 µmol	4 mg 20 mol% 10 µmol		9.8 mg 7 mol% 36.0 µmol	
D	PEG liposomes for modification	40 mg 100 mol% 52 µmol	4 mg 20 mol% 10 µmol			9.8 mg 7 mol% 3.5 µmol

[a] The compositions of the lipid mixtures are always calculated by taking the main lipid component (SPC, or other lipids) as 100% in moles. The additional components are given as mole percents referred to the main lipid. To lipid mixtures containing unsaturated fatty acids antioxidants such as D,L-α-tocopherol are added at 0.1–0.2 mol% (*see* **Note 3**).

Table 2
Calculated Numbers of Liposomes As a Function
of Experimentally Measured Mean Diameters

Mean diameter (nm)	Phospholipid molecules per liposome	Liposomes per 1 mg phospholipid ($\times E^{13}$)	Trapped volume per 1 mg phospholipid (microliters)
25	4,250	18.400	0.52
50	19,930	3.930	1.60
80	54,000	1.450	2.90
100	86,070	0.911	3.80
120	125,500	0.625	4.70
200	357,500	0.219	8.20

Huang and Mason *(5)*, it is possible to approximate liposome numbers per volume and, e.g., the numbers of amino groups available on one liposome of a given composition and mean diameter. In **Table 2**, examples of calculations based on mean liposome sizes of 25 to 200 nm are listed. For example, a liposome with a mean diameter of 50 nm contains about 20,000 SPC molecules, based on the volume of one SPC molecule of 1.253 nm^3. Thus, the same given amount of lipid in mg/mL yields more than four times more liposomes with a diameter of 50 nm (1.6×10^{15}/mL) than liposomes having a diameter of 100 nm (3.6×10^{14}/mL; *see* **Table 2**).

A graphic plot of the liposome diameters (nm) vs the numbers of liposomes formed with 1 mg phospholipid/mL yields an exponential curve. After further transformations the following equation is obtained:

$$\text{Number of liposomes/mL} = a \times d^b \times c$$

where $a = 1.594 \times 10^{17}$ (per nm/mg), $b = -2.118$, c = phospholipid concentration of the liposome solution (mg/mL), and d = mean diameter of the prepared liposomes (nm).

Under the assumption that all other additional membrane-forming molecules occupy the same volume of 1.253 nm^3 in the bilayer, and that all molecules are evenly distributed in both lipid monolayers, liposomes containing, for example, 2 mol% amino groups with a mean diameter of 50 nm carry approx 400 amino groups distributed over the bilayer membrane. Hence, 200 amino groups are located on the outer monolayer surface, available for chemical modification. Consequently—as shown in **Table 3** for liposomes of 40 mg SPC/mL (*see* **Table 1**) and diameters of 50 and 100 nm, and considering losses during production—the vesicles are composed of different numbers of lipid molecules and reactive groups. A liposome containing 7 mol% amino groups and having a diameter of 50 nm carries on average 640 amino groups on its surface, whereas on a larger liposome of 100 nm, approx 2800 amino groups are available for modification.

3.1.3. Liposome Preparation by Dialysis

Small unilamellar vesicles (SUV) of 50–200 nm mean size can be prepared using the detergent dialysis method *(6)*. This method is based on the controlled removal of

Table 3
Calculation of Reactive Groups on Liposomes Prepared As Listed in Table 1

Diameter (nm)	NH$_2$-groups (mol%)	Initial concentration (NH$_2$-groups/mL)	Calculated numbers (NH$_2$-groups/lip)[a]	Yield after modification (maleimide groups/mL) [b]	Yield after modification (maleimide groups/lip) [c]
50 ± 15	2	3.1×10^{17}	200	1.6×10^{17}	120
	7	1.0×10^{18}	640	5.1×10^{17}	400
100 ± 15	2	3.1×10^{17}	850	1.6×10^{17}	510
	7	1.0×10^{18}	2800	5.1×10^{17}	1670

[a] lip, liposome.
[b] Yield calculation: [initial lipid concentration] $\times 0.95 \times 0.6 \times 0.9 = 0.513$ (based on yields of liposome preparation [95%], yield of sulfo-SMCC modification calculated from lipid-NH$_2$-group labeling with BODIPY [60%] and loss or dilution of lipid during dialysis [90%]).
[c] Corresponding to a sulfo-SMCC modification efficiency of 60 % at a 5X molar excess of sulfo-SMCC (*see* **Subheading 3.1.6.**).

detergent from mixed lipid/detergent micelles. The compositions of the lipid mixtures are calculated by taking the main bilayer-forming lipid component (SPC, or other lipids) as 100% in moles. The additional components are calculated as mole percents referred to the main lipid:

1. For liposome preparation, a given lipid mixture (SPC, 40 mg/mL; cholesterol, 4 mg/mL, 20 mol%; DPPE, 2.5 mg, 7 mol%; D,L-α-tocopherol, 0.2 mg, 1 mol%; *see* **Table 1**) is dissolved in methanol/methylene chloride (1:1 v/v) in a round-bottom flask (*see* **Notes 4** and **5**).
2. Sodium cholate (*see* **Note 6**) at a ratio of total lipids, including all lipophilic molecules, to detergent of 0.6 moles is added.
3. This mixture is evaporated at 40°C to dryness (30–60 min) using a rotatory evaporator.
4. The dry lipid/detergent film is dispersed in PB and left 30–60 min at room temperature for equilibration.
5. Dialyze the formed mixed lipid/detergent micelles against 3–5 L of PB (volume ratio = 1 to 1000) for 12–15 h at room temperature, e.g., using a Mini-Lipoprep instrument (*see* **Notes 7** and **8**).

3.1.4. Liposome Preparation by High-Pressure Filter Extrusion

SUVs can also be prepared by sequential filter extrusion of multilamellar liposome dispersions in PB through Nucleopore membranes (Sterico, Dietikon, Switzerland) of 0.4, 0.2, 0.1, and 0.05 µm pore diameter with a Lipex™ extruder (Lipex Biomembranes Inc., Vancouver, Canada) *(7)*. Using this method, lipid films without detergents are prepared as described in **Subheading 3.1.3.** and dispersed in PB, followed by sequential extrusion through Nucleopore membranes of decreasing pore sizes (*see* **Notes 9** and **10**).

3.1.5. Fluorescence Labeling of Liposomes and scFv Antibodies

The lipophilic dye DiO is used for the labeling of liposomes. DiO dissolved in methanol/methylene chloride (1:1, v/v) is added at 0.2–0.4 mg/mL (0.2–0.4 mol%) to the organic lipid mixture. To determine the initial lipid concentration, aliquots of the lipid dispersion are taken before dialysis or extrusion. These aliquots and those taken after liposome preparation are measured in a fluorescence spectrometer for the determination of the lipid concentrations after liposome preparation. Functionalized antibodies or peptides can be trace labeled with fluorescent dyes such as Alexa Fluor or BODIPY succinimidyl ester (Molecular Probes), allowing unspecific amino-group labeling with dyes of different fluorescence properties.

3.1.6. Introduction of Maleimide Groups onto Liposome Surfaces

The liposomes are modified with the bifunctional coupling reagent sulfo-SMCC to introduce maleimide groups onto their surface.

1. The liposome types B or D that contain amino groups introduced with corresponding lipids (*see* **Table 1**) in 0.5 mL PB are incubated under gentle stirring with 0.6 mg crystalline sulfo-SMCC corresponding to a fivefold molar excess relative to the reactive amino groups, for 30–60 min at 30°C.
2. Nonreacted sulfo-SMCC is removed by dialysis using a tube with 10,000 mol-wt cutoff (*see* **Notes 11–14**).
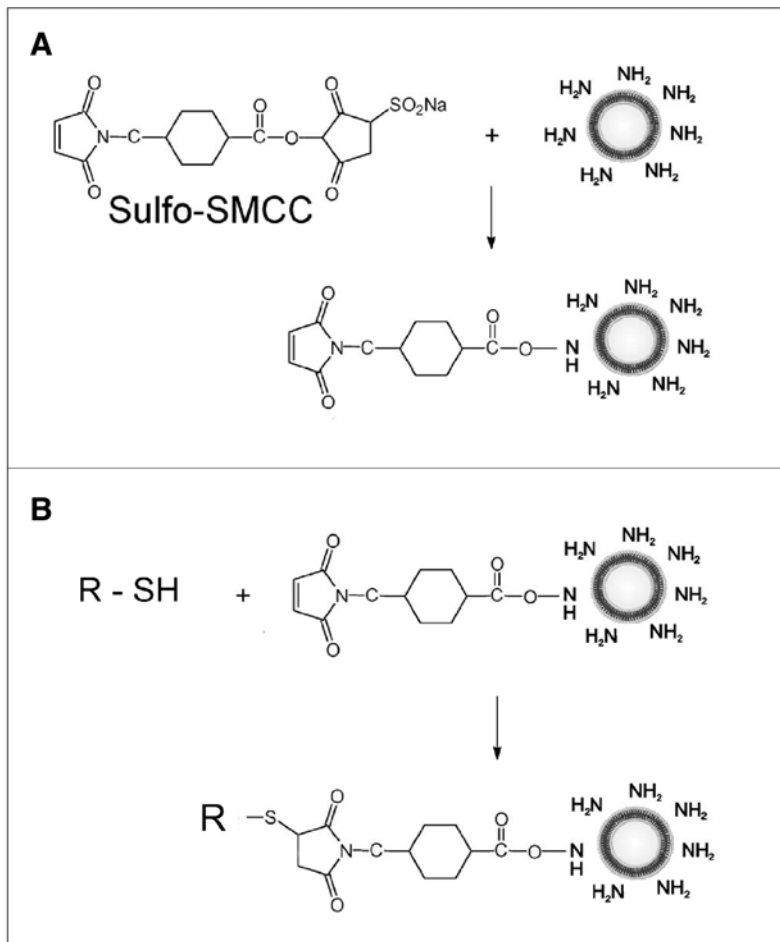
Fig. 1. **(A)** Introduction of maleimide groups to the surface of amino-group-containing conventional or PEG-liposomes. **(B)** Covalent coupling of thiol-functionalized molecules R-SH (scFv antibody fragments, peptides, and so on) to maleimide liposomes.

3.1.7. Coupling of scFv Antibody Fragments to Maleimide-Modified Liposomes

For the method of cloning, protein expression in *Pichia pastoris*, and purification of functionalized scFv antibody fragments, we refer to our publication *(8)*. Briefly, an original anti-ED-B-fibronectin scFv antibody was engineered by functionalization at its C-terminal end by the introduction of a hydrophilic spacer (GGSSGGSSGS) and the terminal cysteine -Cys-Gly-Cys-Ser-Cys sequence (*see* **Note 15**). The scheme of the scFv-liposome coupling is shown in **Fig. 1**.
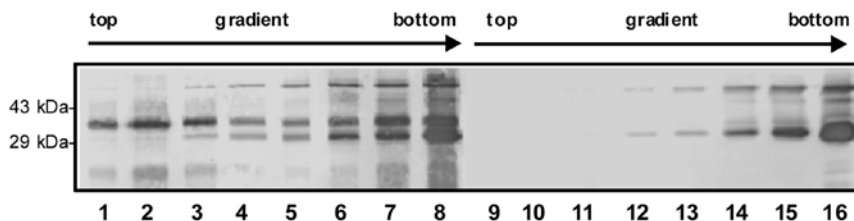
Fig. 2. Separation of scFv-liposomes from unbound scFv antibody molecules. Western blot analysis of aliquots taken from top to bottom of a metrizamide gradient: lanes 1–8, probes from an incubation of scFv-liposomes (positive reaction); lanes 9–16, probes from an incubation of scFv with unmodified control liposomes (negative control). Lanes 1–3 and 9–11 correspond to fractions containing liposomes.

Due to the presence of the introduced cysteines, the scFv antibodies form dimers in solution. Thus, coupling to the liposomes has to be effected in the presence of reducing agents (*see* **Note 16**). The purified scFv antibody dimer (0.5 mg/mL) in HBSE buffer is reduced in the presence of a 2 m*M* final concentration of tributylphosphine (TBP) for 4 h at 20–25°C (room temperature) under an argon or nitrogen atmosphere. Maleimide-modified and fluorescence-labeled liposomes (types B or D, conventional or "stealth") in 100 µL HBSE are incubated with 100 µg reduced scFv antibody in 200 µL HBSE for 10–20 h at 20–25°C (room temperature) in the presence of 2 m*M* TBP under argon or nitrogen atmosphere (*see* **Note 17**). Nonreacted maleimide groups can be blocked by addition of an excess of cysteine, followed by further incubation (e.g., 5- to 10-fold molar excess cysteine relative to the maleimide concentration, 2 h incubation). The modified liposomes are separated from nonreacted scFv on a metrizamide gradient as described in **Subheading 3.2.**

3.2. Separation of scFv-Liposomes From Nonreacted scFv on a Metrizamide Gradient

Nonreacted scFv fragments are separated from the scFv-liposomes by flotation on a discontinuous metrizamide gradient.

1. The liposome–scFv reaction solution (200 µL) is mixed with 100 µL metrizamide (60% in HBSE [w/v]) in ultracentrifuge tubes (5 mL, nitrocellulose) and overlaid with 2 mL metrizamide (10% in HBSE [w/v]) followed by HBSE as a top layer.
2. The density gradient is centrifuged for 7 h at 85,000*g* and 4°C using a swing-out rotor in an ultracentrifuge.
3. Fractions of 400 µL are carefully removed from the bottom to the top of the tubes by cautious insertion of a capillary tube or Pasteur pipet and analyzed for protein content on a non-reducing 14% SDS-PAGE gel followed by Western blot analysis (*see* **Note 18**).
   As shown in **Fig. 2**, fractions containing scFv-coupled liposomes (lanes 1–3) give a 33 kDa band on a Western blot, corresponding to the mol wt of the scFv antibody monomer plus the attached PEG-lipid. Nonreacted scFv monomers and dimers remain at the bottom of the metrizamide gradient (lanes 5–8 and 14–16). Incubation of unmodified
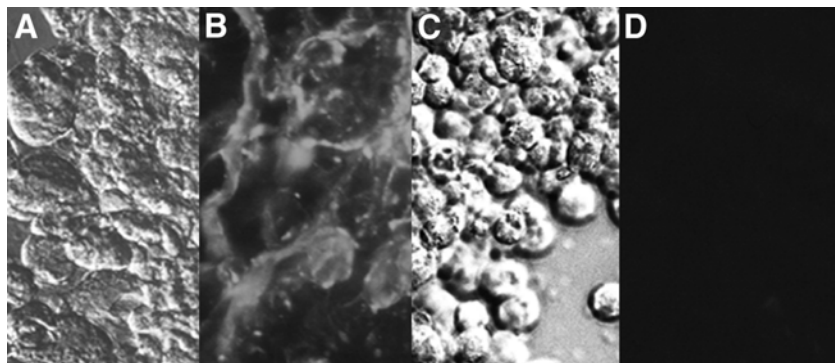
Fig. 3. Binding of α-ED-B scFv-PEG-immunoliposomes to human colon carcinoma cells Caco-2 (E-DB fibronectin positive) and Co-115 (E-DB fibronectin negative) cultured on collagen-I-coated cover slips. Phase contrast image **(A)** and immunofluorescence **(B)** showing binding of DiO-labeled α-ED-B scFv-PEG-immunoliposomes to ED-B-positive Caco-2 cells. On the ED-B-negative Co-115 cells shown in phase contrast **(C)** and by immunofluorescence **(D)**, binding was not detectable. Unmodified DiO-labeled control liposomes did not bind to both cell types (not shown).

   control liposomes with the scFv antibody (lanes 9–16) results in no protein detection in the liposome fractions (lanes 9–11).
4. To remove the metrizamide, the pooled liposome fractions are dialyzed against PB using a 10,000-mol-wt cut-off dialysis tube (volume ratio = 1 to 1000).

3.3. Binding of scFv-Liposomes to Tumor Cells In Vitro

An example of how the binding properties of the prepared scFv-liposomes can be evaluated in vitro is given below (*see* **Fig. 3**).

1. Human colon carcinoma cells Caco-2 (ED-B fibronectin positive) and Co-115 (ED-B fibronectin negative) are cultured in complete DMEM medium. Glass cover slips (15–20 mm diameter) are placed into 12-well tissue-culture plates and coated with 100 μL rat-tail collagen I (10 mg/mL) and incubated for 30 min at 37°C.
2. Caco-2 and Co-115 cells (3×10^5 cells/well) are plated on the cover slips and cultured for 72 h in a humidified 5% CO_2 atmosphere at 37°C.
3. Then the washed cells (PBS) are incubated with 100 μL DiO-labeled α-ED-B scFv-liposomes corresponding to 9–10×10^{13} liposomes or 12.5–31.5 μg scFv, respectively, in PBS for 30 min at 4°C.
4. After another washing step, the cover slips are removed, treated with 10% glycerine, placed on a microscope slide, and analyzed on a fluorescence microscope (Leica DLMB). As negative controls, the cells are incubated with unmodified fluorescence-labeled liposomes (liposome types A, C; *see* **Table 1** and **Note 19**).

4. Notes

1. Cholesterol (e.g., from Fluka, purum quality, >95%) should be recrystallized from methanol. Cholesterol of lower quality should be avoided, since liposome membrane stability can be reduced.

2. Rat-tail collagen can be extracted from fresh rat tails according to the method developed by Ellsdale *(9)*.

3. Mean hydrodynamic diameters of vesicles (liposomes, nanospheres, nanobeads) can be determined with dynamic laser light-scattering instruments, e.g., the NICOMP 380 particle sizer (Particle Sizing Systems, Santa Barbara, CA).

4. For steric reasons, the maximal amount of total PEG-lipids that can be incorporated into a lipid bilayer is 7 mol%. Within this amount, the contents of PE-PEG-Omet and PE-PEG-NH$_2$ can be varied—e.g., 5 mol% PE-PEG-Omet and 2 mol% PE-PEG-NH$_2$, depending on the required degree of amino group modification.

5. Other lipid compositions with synthetic lipids or hydrogenated SPC (HSPC) are often used, especially for liposome formulations intended for parenteral applications (long circulating or "stealth" liposomes). Several analytical methods to follow loss of lipids during the preparation and modification steps are available. Radioactively labeled lipids (^{3}H-DPPC, ^{14}C-DPPC) or cholesterol (^{3}H-cholesterol), or ^{3}H-cholesteryl hexadecyl ether (NEN Life Science Products, Boston, MA), or lipophilic fluorescent dyes (e.g., lipophilic BODIPY derivatives, Molecular Probes) are added at appropriate amounts to the initial lipid mixtures.

6. The preparation of liposomes from mixed detergent/lipid micelles can also be done with other detergents, such as *n*-alkyl-glucosides ($n = 6 – 9$), octyl-thioglucoside, or *N*-octanoyl-*N*-methylglucamin (MEGA-8, Fluka). Interestingly, the choice of detergent influences the size of the resulting liposomes. Thus, liposomes prepared from *n*-octyl-glucoside/lipid micelles have an average size of 180 nm, whereas those made with *n*-hexyl-glucoside are 60 nm in diameter *(10)*.

7. When synthetic lipids are used, the dialysis has to be performed above the corresponding transition temperature T$_c$ of the lipid. Hence, when for example dipalmitoylphosphatidyl choline (DPPC) is used as main liposome-forming lipid, a temperature above its T$_c$ of 41°C has to be chosen. Additional membrane-forming components (cholesterol and so on) depress the T$_c$ by several degrees.

8. The use of common dialysis tubes for the preparation of liposomes is not recommended because formation of concentration gradients in the tubes leads to uncontrolled detergent removal, resulting in nonhomogeneous liposome preparations. Controlled detergent removal—e.g., using a Mini-Lipoprep instrument or, for the production of large volumes of liposomes, a capillary dialyzer method *(11)*—is very important to obtain homogeneous and reproducible small unilamellar liposomes of defined mean diameters.

9. The major differences between detergent dialysis and filter extrusion are the following: Dialysis is a very gentle method, recommended when labile and unstable molecules are incorporated into liposomes. Furthermore, we made the observation that lipophilic dyes (e.g., Texas-red-DPPE) are strongly absorbed by Nucleopore membranes using the extrusion method, whereas in detergent dialysis, such dyes are quantitatively incorporated into the liposomes.

10. The detachment of the lipid mixtures from the glass walls of the round-bottom flasks can be accelerated by addition of small glass beads (2–3 mm diameter) and vigorous shaking. The encapsulation efficiency of hydrophilic molecules into the trapped volume of the liposomes is significantly increased by using the freeze-thaw method, as described by Mayer *(12)*. Using synthetic lipids, temperatures above the corresponding T$_c$ also have to be applied to the extrusion process.

11. The concentration of maleimide groups linked to the liposome surface can be determined by using either a fluorescent thio-reagent or, e.g., radiolabeled cysteine or methionine.

Alternatively, the maleimide groups are saturated with an excess of cysteine followed by determination of unbound cysteine with the Ellman's reagent *(13)*.

12. A large selection of bifunctional coupling reagents for amino-group modification is available from Pierce, Rockford, IL.

13. As an alternative to the coupling of bifunctional maleimide reagents, such as sulfo-SMCC, to amino groups on the liposomes, the maleimide-PEG-lipid molecule distearoyl-PEG-maleimide (DSPC-PEG-Mal) can be purchased from Avanti Lipid Products, Alabaster, AL.

14. Another alternative to the specific coupling method described here is the so-called post-insertion method *(1)*. Briefly, thiol-modified ligands are coupled to DSPC-PEG-Mal micelles in buffered aqueous solutions. After removing nonreacted ligand molecules by dialysis, the modified micelles are incubated with preformed liposomes, whereby the DSPC-PEG-maleimide-ligand molecules are efficiently transferred to the outer lipid layer of the liposomes.

15. The number of cysteines introduced into the ligand molecule can vary from one to three or more. Site-specific thiol modification with a single C-terminal cysteine—e.g., as used for the attachment of peptides to liposomes—is also effective *(9)*. Ideally, the coupling reaction of a thiolated ligand with maleimide-modified liposomes should be performed at a molar excess of ligand. However, the limited availability of large quantities of recombinant proteins or the high costs of synthetic peptides do not permit working with ideal reaction conditions.

16. The reduction of dimers can be achieved by using reducing agents containing thiol groups themselves, such as β-mercaptoethanol or dithiothreitol. However, the thiol groups of the reducing agent compete directly with those of the scFv antibodies for attachment to maleimide groups on the liposomes. Therefore they have to be removed after reduction and before liposome coupling. This problem can be circumvented by using the trialkyl-phosphine reagents tributylphosphine (TBP) or tris(2-carboxy-ethyl)phosphine (TCEP) for reduction *(14)*. Thus, we use TBP at a 2 m*M* final concentration for reduction of the scFv-dimers, and we recommend maintaining the reducing conditions during the coupling reaction to the liposomes.

17. It is recommended to use screw-cap glass vials with small magnetic stirring bars for the coupling reactions. Incubation time can vary from 2 to 24 h, depending on the properties (size, solubility) of the molecule to be attached to the liposomes.

18. Instead of separating the modified liposomes from nonreacted ligand molecules by metrizamide centrifugation, unbound scFv can be removed by dialysis using high-mol-wt cut-off dialysis tubes. Lately, metrizamide has been difficult to obtain. Thus, it can be replaced by Ficoll PM70 (Amersham Biosciences, Uppsala, Sweden) at the same concentrations as metrizamide. We recommend the Spectra/Por DispoDialyzers, which offer a wide selection of mol wt cut-offs ranging from 100 to 300,000 Daltons (Spectrum Laboratories).

19. Depending on the properties of the cells expressing target receptors for ligand-modified liposomes, flow cytometry can be used for the detection of liposome-cell binding.

Acknowledgments

References

1. Sapra, P. and Allen, T. M. (2003) Ligand-targeted liposomal anticancer drugs. *Progr. Lipid Res.* **42,** 439–462.

2. Allen, T. M. (2002) Ligand-targeted therpeutics in anticancer therapy. *Nat. Rev. Cancer* **2,** 750–762.
3. Marty, C., Ballmer-Hofer, K., Neri, D., Klemenz, R., Schott, H., and Schwendener, R. A. (2002) Cytotoxic targeting of F9 teratocarcinoma tumours with anti-ED-B fibronectin scFv antibody modified liposomes. *Br. J. Cancer* **87,** 106–112.
4. Ludewig, B., Barchiesi, F., Pericin, M., Zinkernagel, R. M., Hengartner, H., and Schwendener, R. A. (2000) In vivo antigen loading and activation of dendritic cells via a liposomal peptide vaccine mediated protective antiviral and antitumor immunity. *Vaccine* **19,** 23–32.
5. Huang, C. and Mason, J. T. (1978) Geometric packing constraints in egg phosphatidylcholine vesicles. *Proc. Natl. Acad. Sci. USA* **75,** 308–310.
6. Rubas, W., Supersaxo, A. Weder, H. G., et al. (1986) Treatment of murine L1210 leukemia and melanoma B16 with lipophilic cytosine arabinoside prodrugs incorporated into unilamellar liposomes. *Int J. Cancer* **37,** 149–154.
7. Mayer, L. D., Hope, M. J., and Cullis, P. R. (1986) Vesicles of variable sizes produced by a rapid extrusion procedure. *Biochim. Biophys. Acta* **858,** 161–168.
8. Marty, C., Scheidegger, P., Ballmer-Hofer, K., Klemenz, R., and Schwendener, R. A. (2001) Production of functionalized single-chain Fv antibody fragments binding to the ED-B domain of the B-isoform of fibronectin in *Pichia pastoris. Protein Expr. Purif.* **21,** 156–164.
9. Ellsdale, T. and Bard, J. (1972) Collagen substrata for studies on cell behaviour. *J. Cell. Biol.* **54,** 626–637.
10. Schwendener, R. A., Asanger, M., and Weder, H. G. (1981) The preparation of large bilayer liposomes: controlled removal of n-alkyl-glucoside detergents from lipid/detergent micelles. *Biochem. Biophys. Res. Commun.* **100,** 1055–1062.
11. Schwendener, R. A. (1986) The preparation of large volumes of homogeneous, sterile liposomes containing various lipophilic cytostatic drugs by the use of a capillary dialyzer. *Cancer Drug Delivery* **3,** 123–129.
12. Mayer, L. D., Hope, M. J., Cullis, P. R., and Janoff, A. S. (1985) Solute distributions and trapping efficiencies observed in freeze-thawed multilamellar vesicles. *Biochim. Biophys. Acta* **817,** 193–196.
13. Eyer, P., Worek, F., Kiderlen, D., et al. (2003) Molar absorption coefficients for the reduced Ellman reagent: reassessment. *Anal. Biochem.* **312,** 224–227.
14. Console, S., Marty, C., Garcia-Echeverria, C., Schwendener, R. A., and Ballmer-Hofer, K. (2003) Antennapedia and HIV TAT "protein transduction domains" promote endocytosis of high Mr cargo upon binding to cell surface glycosaminoglycans. *J. Biol. Chem.* **278(37),** 35,109–35,114.
15. Burmeister Getz, E., Xiao, M., Chakrabarty, T., Cooke, R., and Selvin, P. R. (1999) A comparison between the sulfhydryl reductants tris(2-carboxyethyl)phosphine and dithiothreitol for use in protein biochemistry. *Anal. Biochem.* **273,** 73–80.