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Research Paper

Receptor-Specific Targeting with Liposomes *In Vitro* Based on Sterol-PEG$_{1300}$ Anchors

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Purpose. The challenge in developing liposomes to be used in active drug targeting is to design a method that can be used for modifying liposomal membranes that is applicable for a number of different specific ligands. In this study, the post insertion technique was used with activated sterol-PEG$_{1300}$ anchors and was evaluated with regard to its effectiveness in active targeting *in vitro*. The key advantage of these anchors is that the insertion step into the liposomal membrane takes place at room temperature and is very fast.
Materials and Methods. For *in vitro* experiments, neuroblastoma cell lines overexpressing GD2 antigen on their surface as a target structure were chosen. This allowed the use of anti-GD2 antibodies coupled to the liposomal surface for testing of specific binding. These modified liposomes were labelled with rhodamine-PE and their cellular association was analyzed by flow cytometry.
Results. It was shown that the activated sterol-PEG$_{1300}$ anchors allow specific and significant interactions of the modified liposomes with GD2 positive cells.
Conclusion. Coupling using sterol-PEG$_{1300}$ anchors is both simple and rapid. It is reproducible and applicable for all ligands bearing amino groups. This method demonstrates the advantage of a *ready-to-use* system for the modification of pre-formed liposomes with different ligands.

KEY WORDS: liposome; post-insertion technique; soy sterol-poly(ethyleneglycol); specific targeting; sterol-based post-insertion technique (SPIT).

INTRODUCTION

Various efforts have been undertaken in recent years to achieve active targeting in order to increase the effectiveness of liposome associated drugs, as well as to reduce their side-effects (1–5).

The challenge in developing a liposome formulation for active targeting is to design a coupling method which is straightforward from a technical point of view. It should also be fast, reproducible, and widely applicable to different ligands. The resulting surface-modified preparations have to meet numerous requirements such as uniform particle size, particle size stability and a ligand-density corresponding to the respective target (6–8).

Figure 1 illustrates two different methods for the modification of liposomal surfaces. The conventional method of coupling ligands to liposomal surfaces is based on the principle of integrating an active anchor into the lipid film, and thus into the liposomal bilayer, at the production stage (Fig. 1A). In a second step, the anchor reacts with the corresponding ligand via different reactive groups such as amino- (9,10) or thiol-groups (11,12). However, a severe disadvantage of this method is that this approach does not allow controlling selective integration of the active anchor into the outer bilayer only. As a consequence of this, it is possible for the still reactive anchor to interact with the encapsulated material (6,13,14).

Therefore, newly designed approaches have concentrated on a different principle. With the so-called post-insertion technique (PIT) (Fig. 1B) the active anchor reacts with the ligand first. The resulting conjugate is then selectively inserted into the outer bilayer upon incubation with plain

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ABBREVIATIONS: Ab, antibody; BB, borate buffer; BSA, bovine serum albumine; CE, coupling efficiency; Chol, cholesterol; EE, encapsulation efficiency; EPC, egg phosphatidylcholine; HBS, HEPES buffered saline; MAL-PEG$_{2500}$-DSPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[maleimido-poly(ethyleneglycol)]; NHS, *N*-hydroxysuccinimide; PBS, phosphate buffered saline; PCS, photon correlation spectroscopy; PEG, poly(ethyleneglycol); PIT, post-insertion technique; PL, phospholipid; Rh-PE, rhodamine-B-PE; SPIT, sterol-based post-insertion technique; sterol-PEG$_{1300}$, soy sterol-poly(ethyleneglycol)-1300-ether; TL, total lipid; TLC, thin layer chromatography; TRE, tresyl chloride (2,2,2-trifluoroethanesulfonylchloride); 2-IT, 2-iminothiolane.

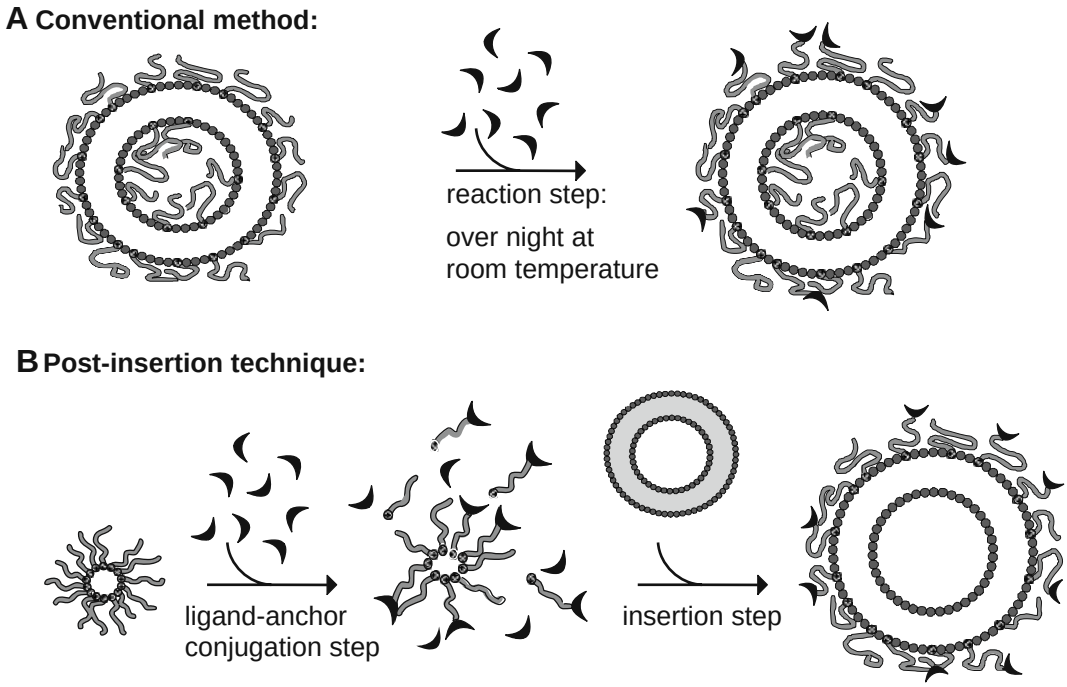


Fig. 1. Coupling methods to modify liposomal surfaces—**A** Conventional technique: The activated protein is coupled to the anchor already incorporated in the bilayer overnight. **B** Post-insertion technique: The ligand is first coupled to the anchor in the *ligand-anchor conjugation step*. In the subsequent *insertion step* the ligand-anchor conjugate is incorporated into the outer liposomal membrane.

liposomes, either at an increased temperature of 60°C for 1 h or with a long incubation time (overnight) (15–17).

The sterol-based post-insertion technique (SPIT) proposed for *in vitro* targeting in this paper is based on PIT, with the advantage that stable insertion into the liposome can be achieved at room temperature due to the different anchor structure (18). Furthermore, the whole procedure can be performed in less than one hour (ligand-anchor conjugation step: 15 min; termination of conjugation step with histidine-HCl: 10 min; insertion step: 30 min). The key difference between conventional anchors and the newly developed anchors used in SPIT is found in the lipophilic part, which is inserted into the liposomal membrane. Conventional anchors exhibit a lipid structure with two hydrocarbon chains, whereas anchors used in SPIT have a sterol framework (Fig. 2).

Sterol-PEG has previously been used to achieve steric stabilization and a prolonged circulation time of liposomes (19–21). Due to the additional possibility of simple functionalization of pre-formed liposomes, sterol-PEG anchor molecules are of growing interest for specific targeting. Successful coupling and insertion of bovine serum albumin as a model ligand in pre-formed liposomes using tresyl-activated sterol-PEG_{1300} has already been shown by our group (18). In addition, successful targeting using a MAL-PEG-cholesterol anchor has been described in the literature (22). However, before coupling to this MAL-PEG-anchor, the ligand has to be thiolated at pH 8. The ligand-anchor conjugation step then takes 4 h at pH 6.5 and the insertion of the conjugate into pre-formed liposomes was reported to need an additional hour at 37°C.

Supplementary to our previous studies using tresyl-activation of a sterol-PEG_{1300}, activation using succinimide (NHS) is presented in this study and is compared with the previous method. Neither strategy requires pre-activation of the ligand. Furthermore, the ligand-anchor conjugation step takes place in only 15 minutes at room temperature. An additional advantage of the NHS activation is that the procedure can be performed at neutral pH.

For *in vitro* experiments an established cell model which has already been successfully used for active liposomal targeting was chosen (23–25). The model is based on neuroblastoma cell lines which strongly overexpress the disialogangliosid GD2 on the cell surface as a target structure (26). The overexpression of the tumor-associated antigen GD2 occurs primarily in neuroblastoma and is therefore specific for active targeting. This structure is targeted via anti-GD2 antibodies (Ab) coupled to the liposome surface, which can then bind to the GD2 antigen present on the cell. Interactions with different neuroblastoma cell lines (Kelly, WAC 2, SK-N-AS) were studied with respect to strong overexpression of the epitope and their potential suitability as a neuroblastoma xenograft model (27). Each cell line overexpresses the disialogangliosid GD2 at a different level and was therefore of interest for evaluating targeting differences.

In order to quantify non-specific cell adhesion, two cell lines which have been described as GD2-poor (HeLa and A431) were used (25).

MATERIALS AND METHODS

Materials

Egg phosphatidylcholine (EPC, >98% purity) was a generous gift from Lipoid (Ludwigshafen, Germany). Cholesterol (Chol), human immunoglobulin (IgG) and 2-iminothiolane hydrochloride (2-IT) were purchased from Sigma (Deisenhofen, Germany). MAL-PEG_{2500}-DSPE was

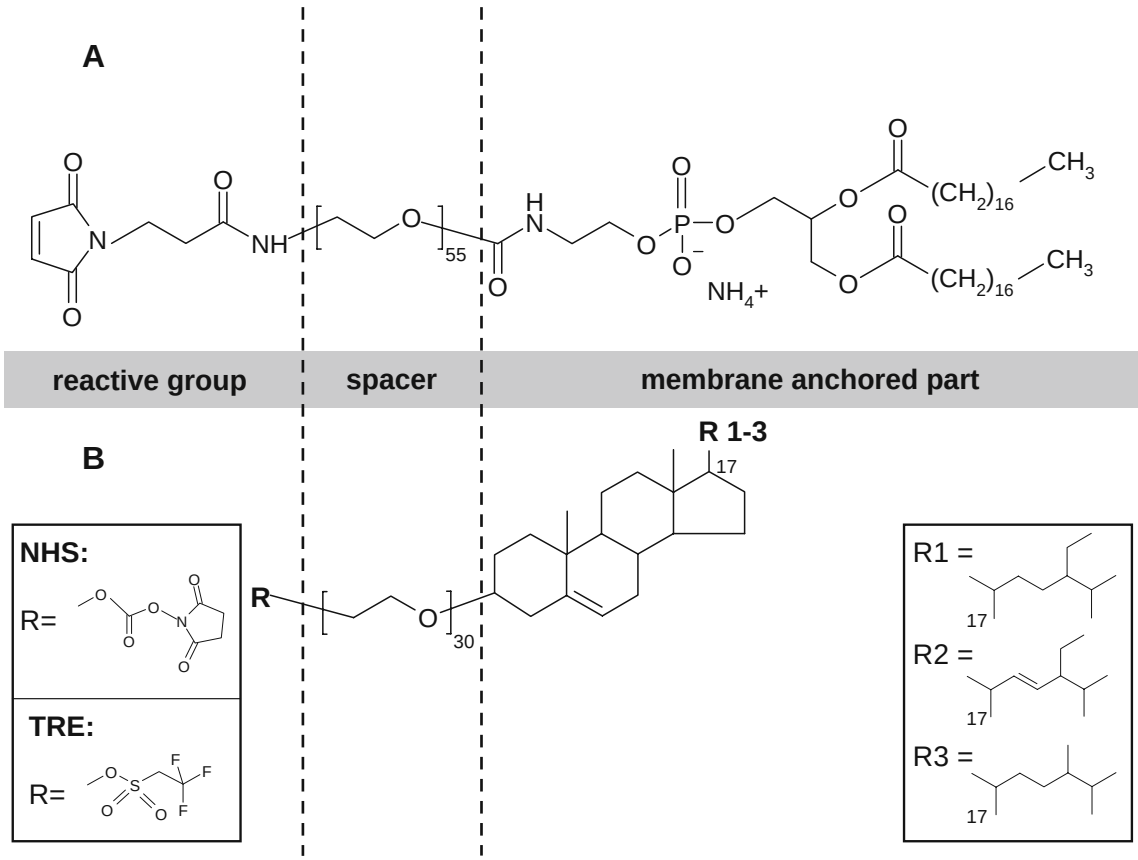


Fig. 2. Types of anchor to modify the liposomal surface—**A** MAL-PEG$_{2500}$-DSPE: a typical anchor used for the conventional method and the conventional post-insertion technique consisting of a lipid membrane anchoring part, a spacer and a reactive group. **B** Two different activated sterol-PEG$_{1300}$ anchors used for the post-functionalization technique consisting of a sterol as a membrane anchoring part, a PEG spacer and a reactive group, either TRE or NHS.

obtained from Shearwater Polymers, Inc. (Huntsville, Alabama, USA) and Rhodamine-B-PE (Rh-PE) from Molecular Probes (Karlsruhe, Germany). The anti-GD2 antibodies (human, chimeric), dissolved in PBS pH 7.4 were a generous gift of Rupert Handgretinger (University Children's Hospital Tübingen, Tübingen, Germany). Sterol-PEG$_{1300}$ was purified as described (18) from BPS-30, which was a generous gift from Nikko Chemicals (Tokyo, Japan). Histidine-HCl and silica gel 60 were obtained from Merck (Darmstadt, Germany), Sepharose CL-4B from Pharmacia Biotech (Uppsala, Sweden), carrier free Sodium ^{125}Iodine from Hartmann Analytic (Braunschweig, Germany), IODO beads, dextran desalting columns and BCA protein assay from Pierce (Rockford, USA). The Vivaspin 500 filter was purchased from Vivascience AG (Hannover, Germany). Kelly and WAC 2 cells were obtained from the Division of Pediatric Hematology and Oncology, University of Freiburg (Freiburg, Germany), SK-N-AS from the Division of Pediatric Hematology and Oncology, University Hospital of Essen (Essen, Germany), HeLa cells from the DSMZ (Heidelberg, Germany) and A431 cells from Stefan Förster (Institute for Physical Chemistry, Hamburg, Germany). RPMI 1640 medium, FCS, glutamine, PBS buffer (10 mM with or without Ca^{2+} and Mg^{2+}) and trypsin/EDTA 0.05%/0.25% were purchased from Biochrome (Berlin, Germany). FACSflow sheath fluid and 7-amino-actinomycin D (7AAD) staining solution were from BD Biosciences (Heidelberg, Germany) and the CellTiter-Glo® luminescent cell viability assay from Promega (Mannheim, Germany). All other chemicals were of analytical grade.

Synthesis of Activated Sterol-PEG$_{1300}$

Sterol-PEG$_{1300}$ was activated using two alternative kinds of reagents. Activation with tresyl chloride (TRE, trifluoroethane sulfonic acid) results in sterol-PEG$_{1300}$-TRE and was synthesized as recently described (18). The synthesis of succinimide activated sterol-PEG$_{1300}$ (sterol-PEG$_{1300}$-NHS) was performed as follows: 520 mg (0.3 mmol) of purified sterol-PEG$_{1300}$ were dissolved in 15 ml of dried dichloromethane. After adding 120 µl (0.9 mmol) triethylamine (TEA) and 154 mg (0.6 mmol) *N*,*N*′-disuccinimide carbonate (DSC) to the solution, the reaction was allowed to continue for 6 h at room temperature under a nitrogen atmosphere and with constant stirring. The organic solvent was then rotary evaporated under reduced pressure until dried, redissolved in methanol and stored overnight at −27°C. The resulting precipitate was collected by centrifugation, dissolved in methanol and dried by rotary evaporation under reduced pressure. The product was recrystallized twice overnight (−27°C), first with methanol and then with diethylether.

The pellet was redissolved in chloroform and then further purified by column chromatography (3.5×10 cm) on silica gel 60 using chloroform/methanol (85/15; *v*/*v*) as the

eluent. The fractions containing the product were pooled and vacuum-dried overnight to yield amorphous sterol-PEG_{1300}-NHS. The product was stored under a nitrogen atmosphere at −27°C.

Mean Mw: 1,875; ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.55 (s, 1H, –C=CH– sterol), 4.45 (t, 2H, $COOCH_2$), 3.75–3.78 (m, 4H, $COOCH_2CH_2$), 3.52–3.58 (m, oxyethylene-CH_2), 2.82 (s, 4H, succinimidyl), 0.68–2.5 (m, sterol-CH).

Liposome Preparation

The basic liposome composition consisted of EPC/Chol 7:3 (molar ratio). When coupling was performed by the conventional technique, 3 mol% of MAL-PEG_{2500}-DSPE was added. For analysis by flow cytometry 0.5 mol% rhodamine-PE was added as a liposomal membrane marker. Lipids were dissolved in methanol and the solvent was dried in a rotary evaporator, followed by high vacuum for 1 h. The lipid film was hydrated using the appropriate volume of HBS buffer at pH 7.4 (HEPES 20 mM, NaCl 130 mM, EDTA 5 mM), yielding a final lipid concentration of 10 or 20 mM. The resulting dispersion of multilamellar large vesicles was homogenized by subsequent extrusion, in which the dispersion was extruded 11 times through polycarbonate membranes with 200 nm pores, and 21 times through 80 nm pores (Nuclepore, Pleasanton, USA) using a hand-extrusion device (LiposoFast, Avestin, Ottawa, Canada).

Particle size was determined by photon correlation spectroscopy (PCS) (Zetamaster S, Malvern, Herrenberg, Germany; auto analysis option, Malvern Software Version 1.4.1) to be within a range of 110±5 nm with a polydipersity index below 0.13.

Radiolabelling of Antibody

Coupling efficiency was determined using radioactively labelled anti-GD2 Ab. For labelling, 3 MBq of Sodium ^{125}Iodide were incubated for 5 min with nonporous polystyrene beads for the iodination of soluble proteins (IODO beads®). At this point 400 μg of either original anti-GD2 Ab for SPIT, or sulfhydryl-activated anti-GD2 Ab for conventional coupling, were added and then incubated for 15 min under moderate agitation. Free ^{125}Iodine was removed by membrane centrifugation (Vivaspin 500, 13,000×*g*, polyethersulfone membrane, cutoff 10 kDa). The Ab solution was washed with BB (borate buffer, 100 mM, pH 8.4) or with HBS pH 7.4 (10 mM HEPES, 140 mM NaCl) for the coupling with sterol-PEG_{1300}-TRE or sterol-PEG_{1300}-NHS, respectively, until the centrifugate showed no significant radioactivity.

Coupling Procedure

For coupling to the liposomal surface hu14.18 and ch14.18 (28,29) were used as anti-GD2 Abs.

Conventional Technique

For coupling of anti-GD2 Ab to MAL-PEG_{2500}-DSPE containing liposomes, 2-iminothiolane (IT) was added to an Ab solution (BB 100 mM, 5 mM EDTA, pH 8.0) at a molar 2-IT/Ab ratio of 20:1 and the solution was lightly stirred for 1 h at room temperature. Thiolated Ab was separated from any excess of 2-IT using a dextran desalting column and HBS buffer pH 6.8 (HEPES 20 mM, NaCl 130 mM, EDTA 5 mM) for elution. Protein content was determined using the BCA protein assay.

Liposomes (EPC/Chol/MAL-PEG_{2500}-DSPE) were incubated with thiolated Ab overnight (Ab/PL molar ratio of 1:1,000). Unbound Ab was separated from immunoliposomes on Sepharose CL-4B.

Sterol-based post-insertion Technique (SPIT)

Different volumes of a methanolic stock solution of activated sterol-PEG_{1300} were dried in 1.5 ml reaction vials by spin evaporation (Concentrator 5301, Eppendorf, Wesseling-Berzdorf, Germany). The resulting activated sterol-PEG_{1300}-coated vials were stored at −27°C until further use.

A schematic representation of the coupling procedure is shown in Fig. 1B. The process consists of two steps, the first of which is the chemical preparation of the Ab-anchor conjugate, denoted as the *ligand-anchor conjugation step*. For the coupling with sterol-PEG_{1300}-TRE, the PBS (pH 7.4) of the GD2 Ab solution was first replaced by 100 mM BB (pH 8.4) via Vivaspin 500. Buffer exchange was not necessary for the reaction of sterol-PEG_{1300}-NHS with anti-GD2 Ab. Different amounts of the protein solution were pipetted into sterol-PEG_{1300}-TRE/NHS coated vials (resulting in an Ab/phospholipid (PL) molar ratio of 1/1900 for TRE and 1/700 for NHS), sonicated in a water bath (Sonorex RK 106 S, Bandelin, Berlin, Germany) for 30 s, and then vortexed for another 30 s. The derivatization was terminated at different time points by adding 100 μl of a histidine-HCl aqueous solution (250 mM), containing a several-fold molar excess of amino groups over those provided by the anti-GD2 Ab.

The second step of the standard coupling procedure is the *insertion step*. At room temperature, the resultant mixture of sterol-PEG_{1300} and sterol-PEG_{1300}-anti-GD2 Ab rapidly inserts into the outer liposome monolayer. This step was initiated by adding 300 μl of liposomes (either 10 (NHS) or 20 mM (TRE) lipid) to the reaction vials. Free Ab-conjugate was separated from the liposome associated anti-GD2 Ab on Sepharose CL-4B using HBS as an eluent.

Coupling Efficiency

The amount of anti-GD2-Ab attached to liposomes was calculated by counting the radioactivity of each fraction using 300 μl of the unfractionated liposome dispersion as a 100% value (Eq. 1).

$$\%\mathrm{CE} = \frac{\sum \mathrm{AF_{lip}}}{\sum \mathrm{AF_{tot}}} \times 100\% \qquad (1)$$

%CE relative coupling efficiency
ΣAF_{lip} radioactivity of all liposome containing fractions
ΣAF_{tot} radioactivity of all fractions

The number N_{IgG} of IgG molecules coupled to the liposomes was calculated according to Equation 2.

$$N_{\mathrm{IgG}} = \frac{\%\mathrm{CE} \times m_{\mathrm{IgG}}}{\mathrm{Mr_{IgG}}} \times N \qquad (2)$$

m_{IgG} mass of total protein (g) added for reaction
Mr molar mass of IgG1 (146,000 g × mol^{-1})
N Avogadro's number (6.022×10^{23} mol^{-1})

The total area At (nm^2) of a membrane bilayer (half the area of a corresponding monolayer) formed by the total membrane lipid in the sample was calculated as in (30).

$$A_t = N \times L \times V_t \times A \times 0.5 \quad (3)$$

L lipid concentration of the sample (mol × L^{-1})
V_t sample volume (L)
A mean area of a single lipid in the liposomal membrane, i.e. 0.53 nm^2 for a 7:3 molar mixture of EPC and Chol as calculated from reference (31).

The bilayer area A_v (nm^2) of a single vesicle when calculated in the middle of the membrane, is

$$A_V = 4\pi \times \left(R - \frac{D}{2}\right)^2 \quad (4)$$

R mean hydrodynamic radius of the vesicle (32)
D thickness of the membrane, approx. 5 nm

The number of vesicles N_V in the sample, assuming they are essentially unilamellar and homogeneous in size, is then

$$N_V = \frac{A_t}{A_V} \quad (5)$$

The mean number of protein molecules coupled to a single vesicle N_{PV} is then

$$N_{PV} = \frac{N_{IgG}}{N_V} \quad (6)$$

Cell Experiments

Cell Culture and Cell Experiments

All cell lines were cultured in RPMI 1640 medium supplemented with 10% FCS and 2% glutamine. Cells were seeded into 24-well culture plates 24 h prior to the experiment at a density of 7.5×10^4 cells per well, and were maintained at 37°C in a humidified incubator with 5% CO_2 atmosphere.

The culture medium was renewed 1 h prior to incubation and the cells were then incubated with different liposomal preparations (75 nmol lipid of each preparation in 0.5 ml RPMI medium) for 2 h at either 37°C or 4°C.

Flow Cytometry

After incubation the cells were prepared for flow cytometry. First, liposomes and medium were removed. The cells were then washed with 1 ml PBS and harvested by trypsinization for 2–4 min, depending on the cell line. All supernatants were collected and centrifuged (5 min at 1,200×*g*), washed and resuspended in 200 µl PBS containing Ca^{2+}/Mg^{2+} either with or without 7AAD staining solution (1%). These samples were stored on ice in the dark until used.

Flow cytometry analysis was performed using a four-color FACS Calibur® (Becton-Dickinson, Heidelberg, Germany) in combination with Lysis II software for analysis.

In order to determine the percentage of cell-associated fluorescence of Rh-PE and 7AAD, 10,000 cells were analyzed from each sample.

Viability Assay

In order to determine cell viability the CellTiter-Glo® luminescent cell viability assay was performed.

Cells were prepared and incubated as described in the section *flow cytometry*. After incubation the 24-well plates and their contents were allowed to equilibrate to room temperature for 15 min. The samples were stored in the dark during the whole procedure, which is described below. A volume of 500 µl of the CellTiter-Glo® reagent was added to each well, followed by mixing on an orbital shaker for 2 min. Afterwards, the plates were again incubated at room temperature for an additional 10 min. Three 200 µl samples from each well were transferred into a luminometer-compatible multiwell plate and sample luminescence was recorded (MicroLumat Plus LB 96V luminometer, Win Glow software Version 1.24 from EG&G Berthold GmbH, Bad Wildbach, Germany.).

RESULTS

Properties of GD2 Modified Liposomal Preparations

Table I reveals the coupling efficiency achieved with different mol% of sterol-PEG$_{1300}$-TRE (TRE) and sterol-PEG$_{1300}$-NHS (NHS), the Ab densities of the liposomes used in the cell experiments, and the increase in particle size after coupling of the Ab.

The size of the modified liposomes was measured before as well as after coupling of the Ab. The initial size (110± 5 nm) showed an increase of from 4 nm (TRE 1 mol%, ligand-anchor conjugation time 10 min) up to 52 nm (TRE 7.5 mol%). The polydispersity index for all modified formulations was smaller than 0.13.

Focussing on the coupling of the Ab with sterol-PEG$_{1300}$-TRE, it can be seen that the increase in particle size correlates with rising anchor concentration (data with anti-GD2 Ab hu 14.18), as well as with extended reaction time (data with anti-GD2 Ab ch 14.18). For the sterol-PEG$_{1300}$-TRE preparations, Ab densities on the liposomal surface were raised from 12 to 41 µg/µmol phospholipid (PL), corresponding to increasing anchor concentrations.

Coupling with 5 mol% of sterol-PEG-NHS led to Ab densities of 62 µg/µmol PL.

In order to compare SPIT with the conventional technique, the Ab was also coupled via a MAL-PEG-DSPE anchor. A concentration of 3 mol% of MAL-PEG-DSPE resulted in an Ab density of 38 µg/µmol PL.

Optimization of SPIT Using Sterol-PEG$_{1300}$-NHS *In Vitro*

The cellular association of modified liposomes labelled with Rh-PE was analyzed by flow cytometry.

A first set of experiments was performed with the anti-GD2 Ab hu 14.18.

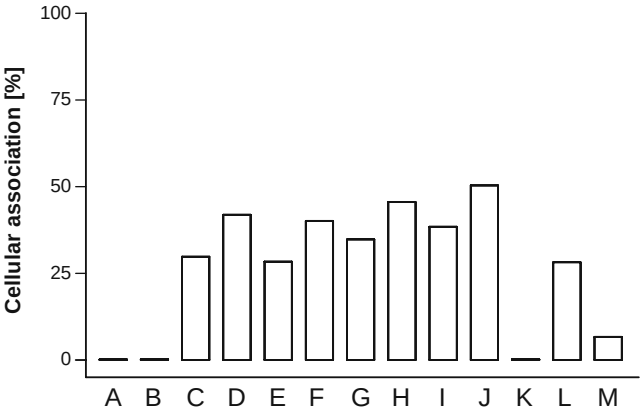
For optimization of the coupling protocol and the *in vitro* assay, the influence of anchor concentration, incubation time

Table I. Coupling Efficiency of Anti-GD2 Ab and Particle Size Increase due to the Coupling Procedure (Initial Size 110 nm±5)

Sample (mol% anchor)	Antibody type	Coupling efficiency (%)	Coupled antibody (μg/μmol PL)	Coupled antibodies per liposome	Increase in size (nm)
NHS 5 mol %	ch	28±5	61±3.0	37	30±4
	hu	n.d.	n.d.	n.d.	46±7
TRE 2 mol %	hu	27±2	20±0.4	12	29±3
TRE 3.7 mol %	hu	37±4	28±1.1	17	34±5
TRE 7.5 mol %	hu	53±1	41±0.2	24	52±8
TRE 1 mol % ligand-anchor conjugation time (10 min)	ch	16±3	12±0.4	7	4±2
TRE 1 mol % ligand-anchor conjugation time (20 min)	ch	21±2	15±0.3	9	7±1
TRE 1 mol % ligand-anchor conjugation time (30 min)	ch	24±4	17±0.7	11	11±3
MAL-PEG-DSPE 3 mol %	hu	25±1	38±0.4	23	12±3

The two types of antibody (*hu* human; *ch* chimeric) were coupled with varying mol% of the different anchors (n=3)

with cells, and amount of TL/well were examined in a preliminary experiment with SK-N-AS cells (Fig. 3). As shown in the diagram, different levels of cellular association were found for the various liposomal preparations. Control experiments were performed with both cells which were incubated without liposomes (Fig. 3, A) and cells incubated with plain EPC/Chol liposomes (Fig. 3, B).



Column	Anchor [mol%]	Incubation time [h]	TL / well [nmol]	Initial lipid conc. [mM]
A	0	2	0	0
B	0	2	75	10
C	0.14	1	40	10
D	0.14	1	75	10
E	0.14	2	40	10
F	0.14	2	75	10
G	5	1	40	10
H	5	1	75	10
I	5	2	40	10
J	**5**	**2**	**75**	**10**
K*	5	2	75	10
L	5	2	75	50
M**	5	2	75	50

* Ligand-anchor incubation time extended to 12 hours
** Preparation L after 7 days storage

Fig. 3. Cellular association of modified liposomes with SK-N-AS. Anti-GD2 Ab (hu 14.18) was coupled to liposomes via sterol-PEG_{1300}-NHS. Preliminary experiments with SK-N-AS cells indicated the influences in cell experiments by varying the anchor concentration, incubation time on cells, amount of total lipid (TL) and the initial lipid concentration.

The increased ratios of anchor to lipid (5 mol% *vs.* 0.14 mol%) within the liposomal formulations and the longer incubation time (2 h *vs.* 1 h) led to a higher association.

Furthermore, an increase in the amount of lipid per cell was correlated with an increase in cellular interaction. Incubation with 75 nmol lipid exhibited a consistently higher association than with 40 nmol/well.

Lengthening the time for the first *ligand-anchor conjugation step* between anchor and Ab from 15 min to 12 h resulted in a total loss of affinity (Fig. 3, K), indicating a loss in Ab activity. When coupling was performed using 50 mM liposomes (Fig. 3, L) the preparations also interacted significantly but the effect was less pronounced. The 50 mM preparations increased in size by about 150 nm over a period of 7 days (data not shown), whereas the 10 mM preparations only increased by about 10 nm. An additional drawback to the increase in size was a decrease in the cellular association of preparation L (Fig. 3) after 7 days storage (Fig. 3, M).

In summary, the highest cellular association obtained in this preliminary experiment was achieved using 75 nmol lipid/well with an incubation time of 2 hours at 37°C (Fig. 3, J).

Optimization of SPIT Using Sterol-PEG_{1300}-TRE *In Vitro*

In Fig. 4 different preparations with either sterol-PEG_{1300} -TRE or sterol-PEG_{1300} –NHS anchor are compared according to their binding to Kelly, WAC 2 and SK-N-AS cells. It was shown that these cell lines exhibit GD2 in different densities on their cell surface (data not shown). The optimized sterol-PEG_{1300}-NHS anti-GD2 preparation showed the most specific interaction compared to plain liposomes with each of these GD2 positive cell lines (Fig. 4, D; Kelly 65±5% *vs.* 0.03±0,02, p=0,001; WAC 2 82±3% *vs.* 0.07±0,08, p=0.0002; SK-N-AS 39±1% *vs.* 0,2±0.05%, p=0,004). Their interaction with plain liposomes as well as with liposomal preparations carrying a non-specific homing device was also examined in order to exclude any non-specific interaction (Fig. 4, B). Therefore, bovine serum albumin (data not shown) and IgG (Fig. 4, C and E) were coupled to liposomes via the sterol-PEG_{1300}-NHS or PEG_{1300}-TRE anchor. Neither of these couplings resulted in a preparation which interacted with the GD2 positive cell lines.

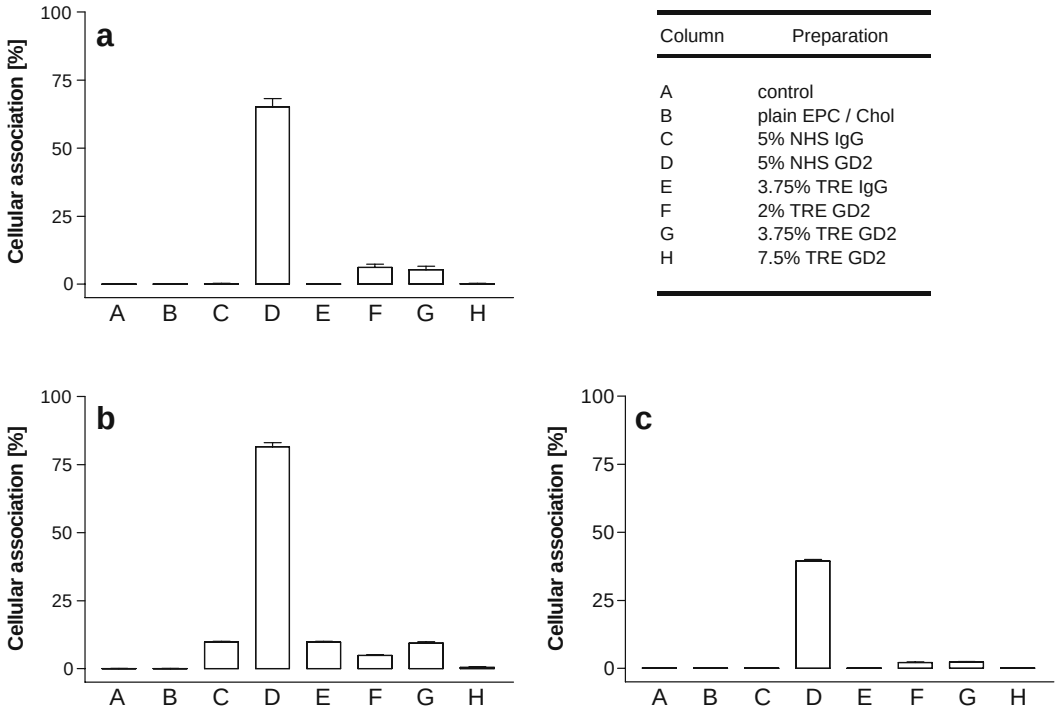


Fig. 4. Cellular interaction of immunoliposomes with different GD2-positive cell lines (**a** Kelly, **b** WAC 2, **c** SK-N-AS) as analyzed by flow cytometry. For the GD2 specific preparations hu14.18 anti-GD2 Ab was used (n=3).

The sterol-PEG$_{1300}$-TRE preparations carrying anti-GD2 Ab also show a specific interaction which is dependant on anchor concentration (Fig. 4, F–H). As can be seen in Fig. 4b, the interaction with WAC 2 cells when using the sterol-PEG$_{1300}$-TRE anchor preparations were optimal when applying 3.75% anchor (Fig. 4 b, G; 5±0,5%, p=0,002). For Kelly cells and SK-N-AS cells it can be assumed that the use of 7.5% anchor leads to a loss of specific interaction (Fig. 4a and c, H). However, the sterol-PEG$_{1300}$-NHS preparation was clearly superior to the sterol-PEG$_{1300}$-TRE preparation. Nevertheless, there was a correlation between protein coupling with fewer anchors and an increase in interaction with all three cell lines. Upon applying a concentration of 7.5% sterol-PEG$_{1300}$-TRE anchor (H), the affinity disappears. The Kelly cells appeared to be the most suitable for use in further optimization experiments because they showed high significance and no non-specific binding. Additionally, it is a cell line which is stable and easy to handle, i.e. it displays a homogeneous population when examined by flow cytometry.

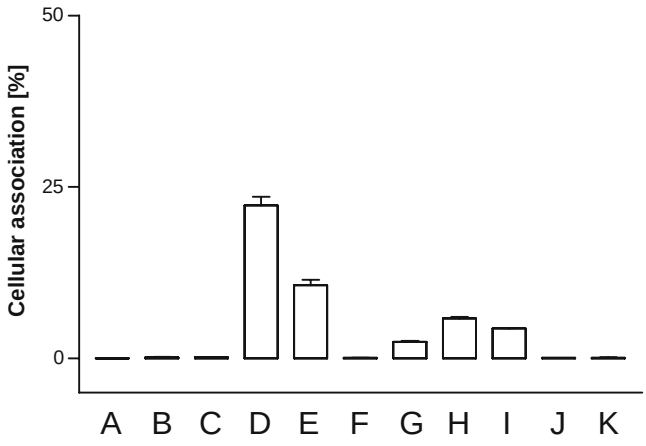
As human anti-GD2-Ab was limited further experiments were performed with chimeric Ab (Fig. 5). Again, preparations coupled via the sterol-PEG$_{1300}$-NHS anchor proved to be more efficient than those coupled via sterol-PEG$_{1300}$-TRE, yet both preparations showed significantly higher binding compared to the plain liposomes (Fig. 5, B) as well as to the controls with non-specific target structures (Fig. 5, C and F). However, it should be mentioned that this effect clearly declined upon change of Ab (hu ~75%/ch ~25%). Nevertheless, the model allows for comparative optimization of the various parameters, as shown for sterol-PEG$_{1300}$-TRE. The latter showed that coupling of 2 mol% anchor with chimeric Ab, in contrast to humanized Ab, does not result in any measurable cell interaction (data not shown). Therefore, the anchor concentration was further reduced. Upon using 1 mol% sterol-PEG$_{1300}$-TRE anchor (Fig. 5, G–I) significant binding in comparison to plain liposomes (0.07±0.08%) was again detected (G 2.5±0.2, p=0.001; H 5.8±0.4, p=0.001; I 4.4±0.1, p<0.001). Furthermore, the *ligand-anchor conjugation step* between the anchor and the Ab was also varied, with an optimum observed at 20 min (Fig. 5, H). As with the sterol-PEG$_{1300}$-NHS preparation, the coupling with the sterol-PEG$_{1300}$-TRE anchor was also performed in PBS, which is the medium the Ab was stored in. As seen in Fig. 5, J and K a buffer change cannot be avoided when applying the sterol-PEG$_{1300}$-TRE anchor. However, no effect independent of the reaction time could be detected (10 min up to 12 h).

Each of the preparations was also tested with cell lines which were GD2 poor (A431, HeLa) (25). As expected, neither specifically targeted preparations nor controls displayed any kind of interaction with these cell lines (data not shown, activity less than 1%).

Comparison to Conventional Coupling Using MAL-PEG-DSPE

For a final comparison between the anchors used in this paper and conventional anchors, the optimized sterol-PEG$_{1300}$-NHS preparation used in the above experiments as well as a Mal-PEG preparation were incubated with Kelly cells. Figure 6 shows that both anchors interacted significantly and displayed an equally efficient binding.

Each of the preparations used in the above experiments were tested with respect to their viability (CellTiter-Glo® test) and toxicity (7AAD in flow cytometry) in order to exclude a negative impact of the preparations on cellular metabolism. None of the samples, including the free Ab, showed any unfavorable effects on cell viability (100±10%) or on toxicity (data not shown).



Column	Preparation
A	control
B	plain EPC / Chol
C	5 % NHS IgG
D	5 % NHS GD2
E	5 % NHS GD2, 1:1
F	3 .75% TRE IgG
G	1% TRE GD2 reaction time 10 min
H	1 % TRE GD2 reaction time 20 min
I	1 % TRE GD2 reaction time 30 min
J	1% TRE GD2 reaction time 20 min (PBS)
K	1 % TRE GD2 reaction time 12 h (PBS)

Fig. 5. Cellular interaction of anti-GD2 (ch 14.18) immunoliposomes with Kelly cells (n=3).

Furthermore, it has been shown in independent experiments that the reproducibility of liposomal protein attachment with SPIT was very high (SD±5%).

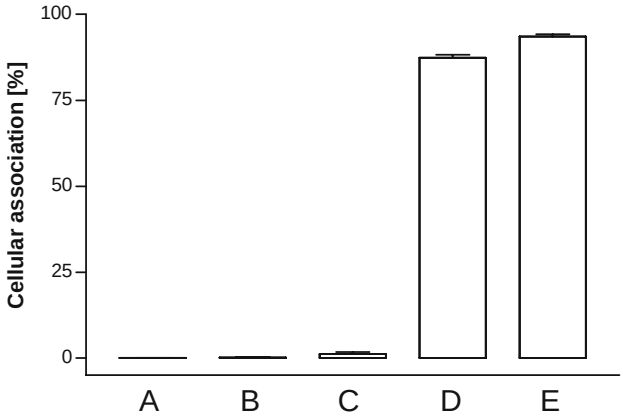
DISCUSSION

The aim of this paper was to show that active targeting can be performed with sterol-anchor post-functionalized liposomes. SPIT is a recently published procedure (18) which allows convenient modification of the liposomal surface. Similar to PIT but in contrast to the conventional method, a key feature of SPIT is the coupling of the ligand and the anchor independent of liposome production. In this way, both the loading of the liposome with the encapsulated material as well as the active targeting can be optimized independently of each other. The advantage of SPIT compared to PIT lies in the fact that the insertion of the ligand-anchor conjugate in the liposomal membrane is achieved at room temperature over a short period of time, thus avoiding chemical or physical inactivation of the liposomal drug or homing device.

Summarizing the results in this paper, it was shown that active targeting with sterol-PEG_{1300} anchors is possible *in vitro*. Significant cellular association was achieved with humanized as well as with chimeric 14.18 anti-GD2 Ab coupled via SPIT. The interaction with the target proved to be highly specific since coupled non-specific Ab did not display any affinity to GD2 positive cell lines, while specific Ab within the GD2 poor cell lines did not show any interaction. The sterol-PEG_{1300}-NHS anti-GD2-liposomes showed the strongest cellular binding to each of the GD2 positive cell lines. When employing chimeric antibodies these values declined to a third of the values achieved for the humanized antibodies (Fig. 4 *vs.* Fig. 5). A similar effect was observed for the coupling with the sterol-PEG_{1300}-TRE anchor. It should be noted that these differences in total affinity values comparing hu14.18 and ch14.18 Ab are due to different purities of batches. This loss in effectiveness cannot be attributed to a change in the coupling between anchor and Ab because there are only marginal differences in the amino acid sequence of the two anti-GD2 antibodies. Both anchors react with the same structures within the Ab: the amines (Fig. 2) (32–34). The independence of coupling efficiency and amino acid sequences for primary antibodies has also been reported in another study (35).

The reduced interaction of tresyl modified liposomes with cells was not of primary concern to us since we focused on presenting an alternative, simple coupling method for *in vitro* targeting, as well as on evaluating the suitability of the different activated sterol-PEG_{1300} anchors. As has been described in the literature, the optimal density of Ab per liposome must be redetermined for each new ligand (6). The aim is to find an optimal balance between the required ligand density, which is necessary for the cellular interaction, and the suppression of undesirable side-effects which include the crosslinking of liposomes during the *insertion step* and a lack of reproducibility in the *ligand-anchor conjugation step* (Fig. 1).

The excess of activated sterol-PEG-$_{1300}$ anchors, which are necessary for successful coupling, results in multiply



Column	Preparation
A	control
B	plain EPC / Chol
C	5 % NHS IgG
D	5 % NHS GD2
E	3% MAL GD2

Fig. 6. Cellular interaction of anti-GD2 (hu14.18) immunoliposomes with Kelly cells prepared either by SPIT (NHS 5%) or by the conventional method (MAL-PEG-DSPE 3%) (n=3).

activated antibodies (18). On the one hand this may lead to a loss of specific target interaction caused by steric hindrance by the PEG chain. On the other hand, the addition of plain liposomes at the *insertion step* results in aggregation of the liposomes. Furthermore, the multiple activation of one antibody will lead to a modified insertion property of the ligand–anchor conjugate. Taken together, these three facts explain the results which are shown in Fig. 3, K and Fig. 4, H, where extended incubations times (Fig. 3, K) and increased anchor concentrations (Fig. 4, H) resulted in a loss of receptor specific interactions. Crosslinking is reflected by an increase in the size of the modified liposomes - measured via PCS (Table I) - as a function of an increase in anchor concentration. If one compares the most efficient sterol-PEG$_{1300}$-NHS preparation with the most efficient sterol-PEG$_{1300}$-TRE preparation, it is evident that the optimal Ab density per liposome for the performed cell adhesion depends on the ligand as well as on the respective anchor type (Table I: NHS 5, 61 μg/μmol; TRE 1, 20 min, 15 μg/μmol). An explanation for this phenomenon is to be found in the difference in reactivity of the two anchors. The ε-amino groups of the amino acid lysine are the main points for conjugation in our system (36). Their pK_a value of approximately 9.3 results in a strong dependence on the reaction conditions, especially with regard to the pH-value (37). At higher pH-values more amino groups become suitable for the coupling reaction, i.e. the ε-amino groups are deprotonized. For coupling with sterol-PEG$_{1300}$-TRE the pH value is 8.4 compared to 7.4 for coupling with sterol-PEG$_{1300}$-NHS. The loss of specific Ab activity may be due to the required coupling conditions.

A marginal change in anchor concentration or *ligand-anchor conjugation time* can lead to a total loss in affinity (see Figs. 3 and 5). However, the sterol-PEG$_{1300}$-NHS anchor was less vulnerable to such changes. A molar ratio of anchor to Ab of 1:1 still exhibited 50% activity in comparison to the optimal preparation (Fig. 5, E). A total loss in activity of the optimized sterol-PEG$_{1300}$-NHS preparation *in vitro* was not reached before the *ligand-anchor conjugation time* was extended to 12 h (Fig. 3, K).

As outlined in the results with humanized Ab, the newly developed sterol-PEG$_{1300}$-NHS is as efficient as the conventionally used Mal-PEG-DSPE anchor *in vitro* (Fig. 6), and is superior in terms of broader applicability.

CONCLUSION

The newly designed activated anchors (sterol-PEG$_{1300}$-NHS and sterol-PEG$_{1300}$-TRE) allow a specific and significant interaction of liposomal anti-GD2 Ab with their cellular receptor. Sterol-PEG$_{1300}$-NHS exhibits coupling features which are better controlled. This is in turn reflected by the higher cellular affinity seen with these preparations *in vitro*.

In conclusion, the SPIT performed with functionalized sterol-PEG$_{1300}$ anchors is a *ready to use system* for active targeting. The developed system is simple, rapid (shorter than 1 h), reproducible, non-toxic and applicable for all ligands bearing amino groups.

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REFERENCES

1. J. M. Metselaar, E. Mastrobattista, and G. Storm. Liposomes for intravenous drug targeting: design and applications. *Mini Rev. Med. Chem.* **2**:319–329 (2002) doi:10.2174/1389557023405873.
2. R. J. Debs, T. D. Heath, and D. Papahadjopoulos. Targeting of anti-Thy 1.1 monoclonal antibody conjugated liposomes in Thy 1.1 mice after intravenous administration. *Biochim. Biophys. Acta*. **901**:183–190 (1987) doi:10.1016/0005-2736(87)90114-3.
3. I. Ahmad, M. Longenecker, J. Samuel, and T. M. Allen. Antibody-targeted delivery of doxorubicin entrapped in sterically stabilized liposomes can eradicate lung cancer in mice. *Cancer Res.* **53**:1484–1488 (1993).
4. Y. L. Tseng, R. L. Hong, M. H. Tao, and F. H. Chang. Sterically stabilized anti-idiotype immunoliposomes improve the therapeutic efficacy of doxorubicin in a murine B-cell lymphoma model. *Int. J. Cancer.* **80**:723–730 (1999) doi:10.1002/(SICI)1097-0215(19990301)80:5<723::AID-IJC16>3.0.CO;2-L.
5. J. W. Park, K. Hong, D. B. Kirpotin, G. Colbern, R. Shalaby, J. Baselga, Y. Shao, U. B. Nielsen, J. D. Marks, D. Moore, D. Papahadjopoulos, and C. C. Benz. Anti-HER2 immunoliposomes: enhanced efficacy attributable to targeted delivery. *Clin. Cancer Res.* **8**:1172–1181 (2002).
6. C. B. Hansen, G. Y. Kao, E. H. Moase, S. Zalipsky, and T. M. Allen. Attachment of antibodies to sterically stabilized lip-

osomes: evaluation, comparison and optimization of coupling procedures. *Biochim. Biophys. Acta*. **1239**:133–144 (1995) doi:10.1016/0005-2736(95)00138-S.
7. H. C. Loughrey, K. F. Wong, L. S. Choi, P. R. Cullis, and M. B. Bally. Protein-liposome conjugates with defined size distributions. *Biochim. Biophys. Acta*. **1028**:73–81 (1990) doi:10.1016/0005-2736(90)90267-R.
8. T. O. Harasym, P. Tardi, S. A. Longman, S. M. Ansell, M. B. Bally, P. R. Cullis, and L. S. Choi. Poly(ethylense glycol)-modified phospholipids prevent aggregation during covalent conjugation of proteins to liposomes. *Bioconjug. Chem.* **6**:187–194 (1995) doi:10.1021/bc00032a006.
9. G. Bendas, A. Krause, U. Bakowsky, J. Vogel, and U. Rothe. Targetability of novel immunoliposomes prepared by a new antibody conjugation technique. *Int. J. Pharm.* **181**:79–93 (1999) doi:10.1016/S0378-5173(99)00002-2.
10. V. P. Torchilin, T. S. Levchenko, A. N. Lukyanov, B. A. Khaw, A. L. Klibanov, R. Rammohan, G. P. Samokhin, and K. R. Whiteman. p-Nitrophenylcarbonyl-PEG-PE-liposomes: fast and simple attachment of specific ligands, including monoclonal antibodies, to distal ends of PEG chains via p-nitrophenylcarbonyl groups. *Biochim. Biophys. Acta*. **1511**:397–411 (2001) doi:10.1016/S0005-2728(01)00165-7.
11. T. M. Allen, E. Brandeis, C. B. Hansen, G. Y. Kao, and S. Zalipsky. A new strategy for attachment of antibodies to sterically stabilized liposomes resulting in efficient targeting to cancer cells. *Biochim. Biophys. Acta*. **1237**:99–108 (1995) doi:10.1016/0005-2736(95)00085-H.
12. D. Kirpotin, J. W. Park, K. Hong, S. Zalipsky, W. L. Li, P. Carter, C. C. Benz, and D. Papahadjopoulos. Sterically stabilized anti-HER2 immunoliposomes: design and targeting to human breast cancer cells *in vitro*. *Biochemistry*. **36**:66–75 (1997) doi:10.1021/bi962148u.
13. A. R. Nicholas, M. J. Scott, N. I. Kennedy, and M. N. Jones. Effect of grafted polyethylene glycol (PEG) on the size, encapsulation efficiency and permeability of vesicles. *Biochim. Biophys. Acta*. **1463**:167–178 (2000) doi:10.1016/S0005-2736(99)00192-3.
14. J. N. Moreira, T. Ishida, R. Gaspar, and T. M. Allen. Use of the post-insertion technique to insert peptide ligands into pre-formed stealth liposomes with retention of binding activity and cytotoxicity. *Pharm. Res*. **19**:265–269 (2002) doi:10.1023/A:1014434732752.
15. D. L. Iden, and T. M. Allen. *In vitro* and *in vivo* comparison of immunoliposomes made by conventional coupling techniques with those made by a new post-insertion approach. *Biochim. Biophys. Acta*. **1513**:207–216 (2001) doi:10.1016/S0005-2736(01)00357-1.
16. T. Ishida, D. L. Iden, and T. M. Allen. A combinatorial approach to producing sterically stabilized (Stealth) immunoliposomal drugs. *FEBS Lett.* **460**:129–133 (1999) doi:10.1016/S0014-5793(99)01320-4.
17. J. Takasaki, and S. M. Ansell. Micelles as intermediates in the preparation of protein-liposome conjugates. *Bioconjugate Chem.* **17**:438–450 (2006) doi:10.1021/bc050051r.
18. T. Steenpaß, A. Lung, and R. Schubert. Tresylated PEG-sterols for coupling of proteins to preformed plain or PEGylated liposomes. *Biochim. Biophys. Acta*. **1758**:20–28 (2006) doi:10.1016/j.bbamem.2005.12.010.
19. A. J. Bradley, D. V. Devine, S. M. Ansell, J. Janzen, and D. E. Brooks. Inhibition of liposome-induced complement activation by incorporated poly(ethylene glycol)-lipids. *Arch. Biochem. Biophys.* **357**:185–194 (1998) doi:10.1006/abbi.1998.0798.
20. T. Yuda, K. Maruyama, and M. Iwatsuru. Prolongation of liposome circulation time by various derivatives of polyethyleneglycols. *Biol. Pharm. Bull.* **19**:1347–1351 (1996).
21. A. Sachse, J. U. Leike, T. Schneider, S. E. Wagner, G. L. Rossling, W. Krause, and M. Brandl. Biodistribution and computed tomography blood-pool imaging properties of polyethylene glycol-coated iopromide-carrying liposomes. *Invest. Radiol.* **32**:44–50 (1997) doi:10.1097/00004424-199701000-00007.
22. X. Pan, G. Wu, W. Yang, R. F. Barth, W. Tjarks, and R. J. Lee. Synthesis of cetuximab-immunoliposomes via a cholesterol-based membrane anchor for targeting of EGFR. *Bioconjug. Chemi.* **18**:101–108 (2007) doi:10.1021/bc060174r.
23. G. Pagnan, P. G. Montaldo, F. Pastorino, L. Raffaghello, M. Kirchmeier, T. M. Allen, and M. Ponzoni. GD2-mediated melanoma cell targeting and cytotoxicity of liposome-entrapped fenretinide. *Int. J. Cancer.* **81**:268–274 (1999) doi:10.1002/(SICI)1097-0215(19990412)81:2<268::AID-IJC17>3.0.CO;2-1.
24. S. Ohta, S. Igarashi, A. Honda, S. Sato, and N. Hanai. Cytotoxicity of adriamycin-containing immunoliposomes targeted with anti-ganglioside monoclonal antibodies. *Anticancer Res.* **13**:331–336 (1993).
25. F. Pastorino, C. Brignole, D. Marimpietri, G. Pagnan, A. Morando, D. Ribatti, S. C. Semple, C. Gambini, T. M. Allen, and M. Ponzoni. Targeted liposomal c-myc antisense oligodeoxynucleotides induce apoptosis and inhibit tumor growth and metastases in human melanoma models. *Clin. Cancer Res.* **9**:4595–4605 (2003).
26. K. Mujoo, D. A. Cheresh, H. M. Yang, and R. A. Reisfeld. Disialoganglioside GD2 on human neuroblastoma cells: target antigen for monoclonal antibody-mediated cytolysis and suppression of tumor growth. *Cancer Res.* **47**:1098–1104 (1987).
27. E. Jouanneau, L. Alberti, M. Nejjari, I. Treilleux, I. Vilgrain, A. Duc, V. Combaret, M. Favrot, P. Leboulch, and T. Bachelot. Lack of antitumor activity of recombinant endostatin in a human neuroblastoma xenograft model. *J. Neurooncol.* **51**:11–18 (2001) doi:10.1023/A:1006420200626.
28. M. M. Uttenreuther-Fischer, C. S. Huang, and A. L. Yu. Pharmacokinetics of human-mouse chimeric anti-GD2 mAb ch14.18 in a phase I trial in neuroblastoma patients. *Cancer Immunol. Immunother.* **41**:331–338 (1995) doi:10.1007/BF01526552.
29. L. S. Metelitsa, S. D. Gillies, M. Super, H. Shimada, C. P. Reynolds, and R. C. Seeger. Antidisialoganglioside/granulocyte macrophage-colony-stimulating factor fusion protein facilitates neutrophil antibody-dependent cellular cytotoxicity and depends on FcgammaRII (CD32) and Mac-1 (CD11b/CD18) for enhanced effector cell adhesion and azurophil granule exocytosis. *Blood*. **99**:4166–4173 (2002) doi:10.1182/blood.V99.11.4166.
30. R. Schubert, W. Hartwig, K.-H. Schmidt, and H. J. Roth. Loading of preformed liposomes with high entrapping efficiency by detergent-induced formation of transient membrane holes. *Chem. Phys. Lipids*. **58**:121–129 (1991) doi:10.1016/0009-3084(91)90118-U.
31. K. Tajima, and N. L. Gershfeld. Equilibrium studies of lecithin-cholesterol interactions. II. Phase relations in surface films: analysis of the "condensing" effect of cholesterol. *Biophys. J.* **22**:489–500 (1978).
32. I. D. Bianco, J. J. Daniele, C. Delgado, D. Fisher, G. E. Francis, and G. D. Fidelio. Coupling reaction and properties of poly (ethylene glycol)-linked phospholipases A2. *Biosci. Biotechnol. Biochem.* **66**:722–729 (2002) doi:10.1271/bbb.66.722.
33. C. Delgado, J. N. Patel, G. E. Francis, and D. Fisher. Coupling of poly(ethylene glycol) to albumin under very mild conditions by activation with tresyl chloride: characterization of the conjugate by partitioning in aqueous two-phase systems. *Biotechnol. Appl. Biochem.* **12**:119–128 (1990).
34. M. J. Roberts, M. D. Bentley, and J. M. Harris. Chemistry for peptide and protein PEGylation. *Adv. Drug Deliv. Rev.* **54**:459–476 (2002) doi:10.1016/S0169-409X(02)00022-4.
35. S. Mirzadeh, M. W. Brechbiel, R. W. Atcher, and O. A. Gansow. Radiometal labeling of immunoproteins: covalent linkage of 2-(4-isothiocyanatobenzyl)diethylenetriaminepentaacetic acid ligands to immunoglobulin. *Bioconj. Chem.* **1**:59–65 (1990) doi:10.1021/bc00001a007.
36. P. Tardi, M. B. Bally, and T. O. Harasym. Clearance properties of liposomes involving conjugated proteins for targeting. *Adv. Drug Deliv. Rev.* **32**:99–118 (1998) doi:10.1016/S0169-409X(97)00134-8.
37. T. M. Rana, and C. F. Meares. N-terminal modification of immunoglobulin polypeptide chains tagged with isothiocyanato chelates. *Bioconj. Chem.* **1**:357–362 (1990) doi:10.1021/bc00005a010.