Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochimica et Biophysica Acta 1758 (2006) 20–28
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
http://www.elsevier.com/locate/bba

Tresylated PEG-sterols for coupling of proteins to preformed plain or PEGylated liposomes

Thomas Steenpaß, Andreas Lung, Rolf Schubert *

Institute of Pharmaceutical Sciences, Department of Pharmaceutical Technology and Biopharmacy, Hermann-Herder-Str. 9, D-79104 Freiburg, Germany

Received 27 May 2005; received in revised form 12 December 2005; accepted 21 December 2005
Available online 18 January 2006

Abstract

A simple and inexpensive method for functionalization of preformed liposomes is presented. Soy sterol–PEG_{1300} ethers are activated by tresylation at the end of the PEG chain. Coupling of bovine serum albumin as an amino group containing model ligand to the activated lipids can be performed at pH 8.4 with high efficiency. At room temperature, the mixture of sterol–PEG and sterol–PEG–protein inserts rapidly into the outer liposome monolayer with high efficiency (>100 μg protein/μmol total lipid). This method of post-functionalization is shown to be effective with fluid or rigid and plain or pre-PEGylated liposomes (EPC/Chol, 7:3; HSPC/Chol 2:1, and EPC/Chol/$MPEG_{2000}$–DSPE 2:1:0.16 molar ratios). The release of entrapped calcein upon the insertion of 7.5 mol% of the functionalized sterols is lower than 4%. Incubation of post-functionalized liposomes with serum for 20 h at 37 °C shows stable protein attachment at the liposome surface.

Keywords: Liposome; Sterical stabilization; Soy sterol-poly(ethyleneglycol); Tresylation; Protein coupling; Specific targeting

1. Introduction

Liposomes are artificial membrane vesicles which can be used as either passive or specifically targeted drug carrier systems. While the vesicle itself can be used for carrying pharmaceuticals [1] or enzymes [2], the surface of the liposome can be modified through the use of targeting motifs in order to ensure site-specific drug delivery. The grafting of hydrophilic polymers can help to avoid the rapid clearance of liposomes from the blood stream [3,4,10,11]. This is usually accomplished by the insertion of poly(ethyleneglycol) (PEG) derivatives of phosphatidylethanolamine [3,4] or PEG-cholesterol [4–9] into the vesicle surface.

Furthermore, addition of functionalized PEG-phospholipids to the lipid mixture makes the supplementary attachment of cell-targeting molecules containing amino groups such as proteins (e.g. antibodies), peptides or ligands possible. Various activating strategies have been described in the literature. While MAL-PEG-DSPE [12,13] or pyridyldithiol-PEG-PE [14,15] may be used for the attachment of thiolated homing devices, p-nitrophenyl-PEG-PE [16] or lipids functionalized with cyanuric chloride [17] are useful for the coupling of amino-group containing ligands.

A common method used in the preparation of sterically stabilized immunoliposomes is the simple mixing of activated PEG-lipids with other membrane components prior to vesicle preparation. This conventional approach for the preparation of targeted liposomes is associated not only with exposure of the functionalized lipid-PEG derivatives, but their possible chemical reaction with other membrane components or the encapsulated drugs themselves. This of course limits the usefulness of this procedure. Another disadvantage of this method, especially for smaller liposomes, is a reduction in the trapping efficiency of the material to be encapsulated due to the space

Abbreviations: BSA, bovine serum albumin; Chol, cholesterol; DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine; EPC, egg phosphatidylcholine; HBS, HEPES-buffered saline; HSPC, hydrogenated soy phosphatidylcholine; MAL-PEG–DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimido-poly(ethyleneglycol)]; $MPEG_{2000}$–DSPE, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy-poly(ethyleneglycol)-2000]; PCS, photon correlation spectroscopy; PEG, poly(ethylene glycol); sterol–PEG1300, soy sterol-poly(ethyleneglycol)-1300-ether; TEA, triethylamine; THF, tetrahydrofuran; tresylchloride, 2,2,2-trifluoroethanesulfonylchloride; TL, total lipid; TLC, thin layer chromatography

* Corresponding author. Tel.: +49 761 203 6336; fax: +49 761 203 6366.
E-mail address: rolf.schubert@pharmazie.uni-freiburg.de (R. Schubert).

0005-2736/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2005.12.010

required by the PEG chains on the inner membrane monolayer [18].

The so-called post-insertion technique was developed recently [19–22] in order to overcome these difficulties. This approach for preparing targeted liposomes makes use of the spontaneous incorporation of PEG-PE-lipids from the micellar phase into pre-formed and even drug-loaded liposomes [23–26]. This avoids the interference of activated lipids with other liposomal components such as buffer components, since the derivatization of the targeting molecule to be attached to the liposome surface takes place in a separate step. Furthermore, the surface modification of pre-formed liposomes provides a basis to specifically modify liposomal formulations currently on the market (e.g. Doxil®, Myocet®) with respect to the therapeutic needs of the individual patient [19].

A limitation of this technique is the requirement for an increase in temperature during the incorporation procedure as a result of poor transfer kinetics for PEG-PE-lipids at ambient temperature. Temperatures of 60 °C, if maintained for 1 h as suggested, may be associated with a loss in activity of coupled proteins or peptides and/or the encapsulated or membrane-incorporated drug. Furthermore, the net negative surface charge in liposomes containing PEG-PE has been shown to decrease the level of endocytosis in carcinoma cells [27].

In this study, we present the synthesis of tresylated PEG-sterols and show their suitability when used in the coupling of proteins at the distal end of the PEG-moiety. In a subsequent step, the lipid–ligand conjugates can then be effectively inserted at ambient temperature into pre-formed liposomes of various fluid or rigid membrane compositions and even into pre-PEGylated vesicles.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA) was purchased from Merck, Darmstadt, Germany. BPS-30 was a generous gift from Nikko Chemicals (Tokyo, Japan). Cholesterol (Chol) was purchased from Sigma (Deisenhofen, Germany), egg phosphatidylcholine (EPC, >98% purity) and hydrogenated soy phosphatidylcholine (HSPC, >98% purity) were a generous gift from Lipoid (Ludwigshafen, Germany). 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy-poly (ethylene glycol)-2000] ($MPEG_{2000}$–DSPE) was obtained from Genzyme Pharmaceuticals (Liestal, Switzerland), histidine–HCl from Merck (Darmstadt, Germany), triethylamine (TEA), tetrahydrofuran (THF), 2,2,2-trifluorethanesulfonylchloride (tresylchloride) from Fluka (Buchs, Switzerland), Sepharose CL-4B from Pharmacia Biotech (Uppsala, Sweden), IODO Beads from Pierce (Rockford, USA) and carrier free Sodium ^{125}Iodine from Hartmann Analytic (Braunschweig, Germany).

2.2. Purification of sterol–PEG_{1300}

Sterol–PEG_{1300} (Fig. 1a) mainly consisting of PEG ethers (mean oxyethylene number: 30, i.e., Mw of PEG, 1320) from soy sterols (sitosterol: campesterol: stigmasterol in a molar ratio of 2:1:1) was effectively separated from free PEG (2–5 wt.%) and further impurities by micellar chromatography on a Sepharose CL-4B column (3.5 × 10 cm) using water as eluent. 500 mg of BPS-30 dissolved in water (100 mg/ml) was fractionated in 2.5–5 ml samples.

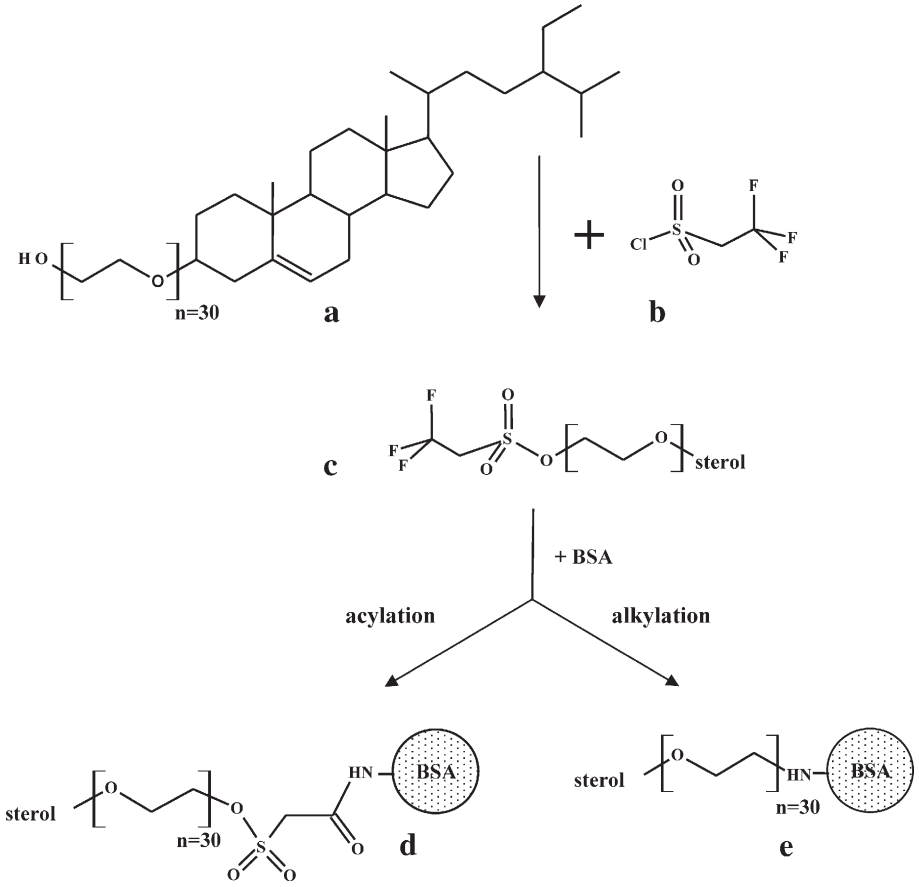

Fig. 1. Chemical activation of sterol–PEG_{1300} (a) by tresylchloride (b). The resulting sterol–PEG_{1300}–TRE (c) is coupled to BSA by acylation (d) or alkylation (e).

Each fraction was examined for free PEG by TLC on silica gel 60 plates developed with chloroform/methanol (85/15; v/v). Spots were visualized by Dragendorff's reagent *R* [Ph. Eur. 5.2]. Fractions without free PEG were pooled and freeze-dried yielding approximately 300 mg of purified sterol–PEG_{1300}.

2.3. Synthesis of tresylated sterol–PEG_{1300} (sterol–PEG_{1300}–TRE)

The tresylation of sterol–PEG_{1300}–TRE was done in principle as described by Nilson and Mosbach [28]. 500 mg (0.29 mmol) of purified sterol–PEG_{1300} and 120 µl (0.87 mmol) of triethylamine (TEA) were dissolved in 10 ml of dried THF. The solution was cooled down to 0 °C and 64 µl (0.58 mmol) of tresylchloride (Fig. 1b) in 5 ml of THF were added dropwise under nitrogen atmosphere. The reaction was allowed to continue at room temperature with constant stirring for 4 h, after which the TEA salts were removed by filtration. The filtrate was rotary evaporated under reduced pressure and redissolved in a small amount of diethylether. The solution was stored at −27 °C overnight and the white precipitate was collected by centrifugation at −10 °C. 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 an eluent. The fractions containing the product were pooled and vacuum-dried overnight to yield approximately 300 mg tresylated sterol–PEG_{1300} (sterol–PEG_{1300}–TRE, Fig. 1c). Stored under a nitrogen atmosphere at −27 °C and when monitored by ^{1}H NMR, it was stable for at least 6 months (data not shown).

Mean Mw: 1880; ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.55 (s, 1H, –C=CH–sterol), 4.52 (m, 2H, CH_2OSO_2), 4.22 (q, 2H, $^3J_{H,F}$=9.0 Hz, CH_2CF_3); 3.61 (m, oxyethylene–CH_2), 0.68–2.5 (m, sterol–CH).

2.4. Liposome preparation

Liposomes were prepared by the film method using EPC/Chol (molar ratio 7:3) or HSPC/Chol (molar ratio 2:1) representing liquid-crystalline (L_α) or gel (L_β) membrane phases at room temperature, respectively. A third kind of liposome was prepared following the membrane composition of liposomal doxorubicin (Doxil®) and consisted of HSPC/Chol/$MPEG_{2000}$–DSPE (molar ratio 2:1:0.16).

Lipids were dissolved in $CHCl_3$/MeOH (3:1 v/v) and the solvent was rotary evaporated to dryness. The film was hydrated using the appropriate volume of HBS (HEPES buffered saline, HEPES 20 mM, NaCl 130 mM, pH 7.4), resulting in the formation of multilamellar vesicles. The raw vesicle dispersion was sized down and transformed to almost unilamellar liposomes by subsequent extrusion (LiposoFast, Avestin, Ottawa, Canada) eleven times each through 200 nm and 80 nm pores of polycarbonate membranes (Nuclepore; Pleasanton, USA). Particle size was determined by photon correlation spectroscopy (Zetamaster S, Malvern, Herrenberg, Germany; auto analysis option, Malvern Software Version 1.4.1) to be within a range of 115 to 125 nm with a polydispersity index below 0.12.

2.5. Incorporation of PEG-lipids into preformed liposomes

The incorporation of sterol–PEG_{1300} or $MPEG_{2000}$–DSPE into EPC/Chol, HSPC/Chol and HSPC/Chol/$MPEG_{2000}$–DSPE liposomes was followed by ^{1}H NMR, which is similar to the experimental setup described by Sou et al. [23].

For calibration, 200 µl of a 50 mM liposome dispersion (phospholipid content adjusted after phosphorus colorimetric assay [29]) was mixed with varying amounts of sterol–PEG_{1300} or $MPEG_{2000}$–DSPE in HBS. This resulted in an amount between 0.07 and 10 mol% PEG-lipid referring to total lipid (TL, sterol–PEG_{1300} not included). Samples were adjusted to 1.5 g with aqueous 50 mM NaCl solution, freeze-dried (Lyovac GT 2, Finn Aqua, Huerth, Germany), redissolved in $CDCl_3$, then centrifuged for the removal of precipitated NaCl to avoid peak broadening if necessary, before ^{1}H NMR spectra (Varian U-300, Varian, Palo Alto, USA) were recorded. Calibration was obtained by linear regression of mol% PEG-lipid vs. the peak area ratio (determined using MestRe-C software) of the methylene proton of PEG (3.6–3.7 ppm) to the choline methylene proton of EPC or HSPC (3.35–3.4 ppm).

For the measurement of incorporation kinetics, 500 µl containing finally 7.5 mol% of TL of sterol–PEG_{1300} or $MPEG_{2000}$–DSPE were added to 1000 µl of the liposome dispersions (50 mM) at 21±0.5 °C. After the appropriate incubation times, samples (300 µl) were taken and free PEG-lipid was separated from liposomal associated PEG-lipids by size exclusion chromatography using a Sepharose CL-4B column (12×1.5 cm) equilibrated with 50 mM NaCl. Fractions containing most of the liposomes were pooled, adjusted to 1.5 g with 50 mM NaCl and treated according to the calibrators. The amount of PEG-lipid transferred into the vesicles was calculated from the calibration data (R^2>0.99) and was expressed as the percentage of total PEG-lipid injected into the liposome dispersion.

2.6. Preparation of the protein-anchor conjugate

In order to assay the suitability of sterol–PEG_{1300}–TRE as an anchor for subsequent attachment of targeting devices to liposomes, a simple standard coupling procedure was established with BSA as a model protein.

10 µl of methanolic solutions of 0.489 µmol (915 µg) sterol–PEG_{1300}–TRE were dried in 1.5 ml reaction vials by spin evaporation (Concentrator 5301, Eppendorf, Wesseling-Berzdorf, Germany) or by high vacuum (P<0.05 mbar). The sterol–PEG_{1300}–TRE-coated vials were stored at −27 °C.

For coupling experiments varying amounts of BSA were dissolved in 100 mM borate buffer pH 8.4. They were then labelled with approximately 10 kBq of ^{125}I-labelled BSA, which was prepared using the IODO-bead method (Pierce, Rockford, USA) and purified on Sephadex-G25 (Pharmacia, Uppsala, Sweden), followed by dialysis overnight using a highly permeable 10 kDa cut-off cellulose membrane (Dianorm Munich, Germany).

After this step 150 µl of labelled BSA solution were pipetted into each of the sterol–PEG_{1300}–TRE-coated vials, bath sonicated (Sonorex RK 106 S, Bandelin, Berlin, Germany) for 30 s and then vortexed for 30 s. To determine the coupling efficiency, the derivatization of the protein (first incubation step) was allowed to continue at room temperature overnight.

2.7. Insertion of the protein-anchor conjugate into preformed liposomes

Attachment of BSA to the liposomes (second incubation step) was initiated by the addition of 300 µl of a 20 mM liposome dispersion resulting in 7.5 mol% sterol–PEG_{1300}–TRE of TL. The mixture was incubated for at least 4 h at room temperature in order to allow the incorporation to proceed.

Thereafter, a 300-µl sample was taken and free (^{125}I labelled) BSA was separated from liposomal bound BSA on Sepharose 4B-CL. The amount of BSA attached to EPC, HSPC and sterically stabilized HSPC liposomes was calculated by counting the radioactivity of each fraction, using the unfractionated liposome dispersion as a reference.

Focussing on the kinetics of the reaction of sterol–PEG_{1300}–TRE with BSA, the derivatization during the first incubation step was terminated by the addition of 100 µl of an aqueous solution of histidine–HCl (pH approximately 4.5). This solution contained an 80-fold molar excess over the moles of amino groups provided by BSA (0.5 mg/ml, borate buffer) before preformed EPC-liposomes were added for the second incubation step, which lasted overnight.

2.8. Liposome leakage during incorporation of the sterol derivatives

For quantification of liposome leakage induced by incorporation of sterol–PEG_{1300}–proteins into preformed liposomes of different membrane compositions, dried lipid films were hydrated with 50 mM calcein solution (self-quenching concentration) in HBS, pH 7.4, resulting in a 50 mM liposome raw dispersion. After three freeze/thaw cycles liposomes were sized as described above. Non-encapsulated calcein was separated from calcein liposomes on a Sepharose CL-4B column. Liposomes were adjusted to 10 mM lipid by addition of HBS. Protein–sterol–PEG_{1300}-conjugates were prepared as described in Section 2.6. Calcein loaded liposomes were added to the conjugates to yield a final amount of 7.5 mol% sterol–PEG_{1300} of the total lipid. Leakage was followed by measuring fluorescence increase at λ_{ex} 470 nm and λ_{em} 510 nm (LS-50B Perkin Elmer, Buckinghamshire, UK). When no further increase of fluorescence could be observed, 2 µl samples of the mixture were taken and the final percentage of dye released was determined. A calcein release of 100% was

obtained by lysing the liposomes with 10 μl of 10% (m/m) Triton X-100 in deionised water. Calcein release was calculated as

$$\%\ \text{calcein}_{\text{free}} = \frac{I_t - I_0}{I_\infty - I_0} \cdot 100$$

where I_t is the fluorescence intensity after incubation of liposomes for the desired period of time, I_0 the fluorescence baseline intensity and I_∞ the fluorescence after liposomes lysis. Basal release of calcein from the different liposome species was monitored for at least 6 h, and turned out to be negligible. The linearity of fluorescence intensity vs. released calcein concentration was controlled in a parallel experiment.

2.9. In vitro stability of liposome modification in human serum

The stability of membrane anchoring and covalent linkage between BSA and sterol–PEG_{1300} was investigated by incubating surface modified liposomes in human plasma (50% v/v) at 37 °C for 20 h. The amount of ^{125}I-BSA dissociated from the vesicles was determined by chromatography on Sepharose CL-4B in HBS according to Ishida et al. [19].

2.10. Cryo electron microscopy

Cryo electron microscopic studies of the aggregation behaviour of liposomes and sterol–PEG_{1300}–BSA were performed as described elsewhere [30].

3. Results and discussion

Using plain or sterically stabilized liposomes a novel method was developed for coupling BSA, as a model protein, to liposomes. The method consists of three simple steps. Sterol–PEG_{1300} is activated by tresylation. To the formed sterol–PEG_{1300}–TRE (Fig. 2a), protein is covalently bound at pH 8.4 resulting in sterol–PEG_{1300}–protein molecules (Fig. 2c), which then spontaneously and effectively insert into the outer membrane of preformed liposomes to yield functionalized liposomes (Fig. 2e). To better understand the various steps of this novel method, we looked at a number of aspects. These included the kinetics of the coupling reaction and insertion, the occurrence of possible membrane disturbances during the insertion step, as well as the stability of protein attachment to liposomes.

3.1. Kinetics of insertion of PEG-lipids

Insertion kinetics of PEG-sterols and PEG-phospholipids into liposome membranes was determined in order to get information about the time-scale of steric stabilization of preformed liposomes, as well as the insertion of activated lipids or protein–lipid-conjugates. The transfer of 2.7 mM sterol–PEG_{1300} or $MPEG_{2000}$–DSPE at 21±0.5 °C into the vesicle membranes (33.3 mM lipid) with different lipid compositions was followed by ^{1}H NMR after separating free from liposomally bound PEG-lipid at different time points on Sepharose CL-4B.

As shown in Fig. 3A, the addition of 2.7 mM sterol–PEG_{1300} (7.5 mol% of total lipid, i.e. 35.8 mM, including PEG lipids) to the liposome dispersion leads to almost complete incorporation of the sterol–PEG_{1300} in the case of EPC membranes. However, only approximately 40% or 30% were inserted into HSPC/Chol or sterically stabilized HSPC/Chol liposomes, respectively (Fig. 3A). The different amount of insertion of sterol–PEG_{1300} into membranes could be explained by different loading capacities for fluid and rigid membranes. As the CMC value for sterol–PEG_{1300} is 3 μM [31], the insertion of the added PEG-lipids at almost 1000-fold CMC occurs from coexisting micelles and monomers.

The insertion of the sterol anchor into the different liposome species takes place very rapidly, reaching equilibrium within 15–30 min. Since insertion of entire micelles into the membrane is improbable, the rapid kinetics point to rapid demicellization of sterol–PEG_{1300} and insertion of the monomeric PEG-lipid.

The rate of insertion of BSA-PEG lipids was estimated by incubating sterol–PEG_{1300}–BSA with EPC/Chol (7:3 mol/mol) liposomes. After 1 min of incubation followed by separation of the non-inserted BSA by column chromatography almost 80% of the BSA was attached to the membrane, which would suggest very fast and efficient membrane insertion of the albumin-linked PEG sterol.

Interestingly, 5 mol% $MPEG_{2000}$–DSPE present in the vesicles has only a minor inhibiting effect on anchor insertion, suggesting that even subsequent protein attachment to sterically stabilized liposomes is possible. Additional insertion into

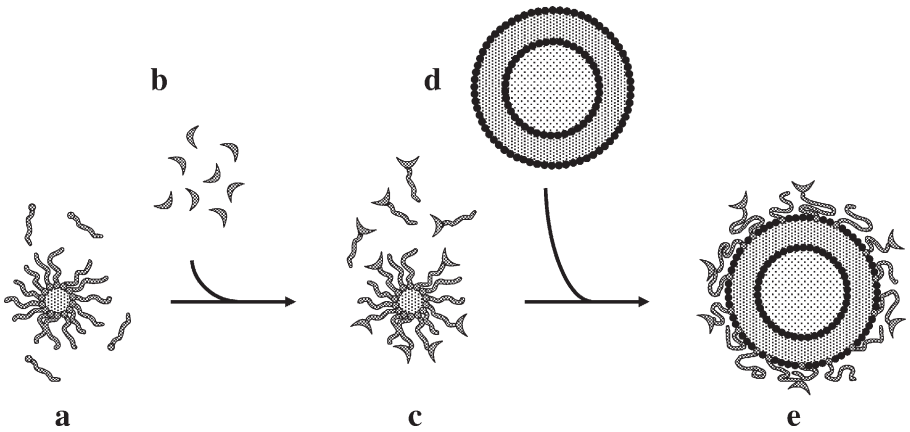

Fig. 2. Protein coupling by the tresyl method: Tresylated sterol–PEG_{1300} (sterol–PEG_{1300}–TRE), coexisting in solution as monomers and in micelles (a) is coupled to BSA (b) at pH 8.4 in order to form sterol–PEG_{1300}–BSA (c). Liposomes (d) are then added to the conjugates, which insert into the outer liposome monolayer (e) at room temperature.

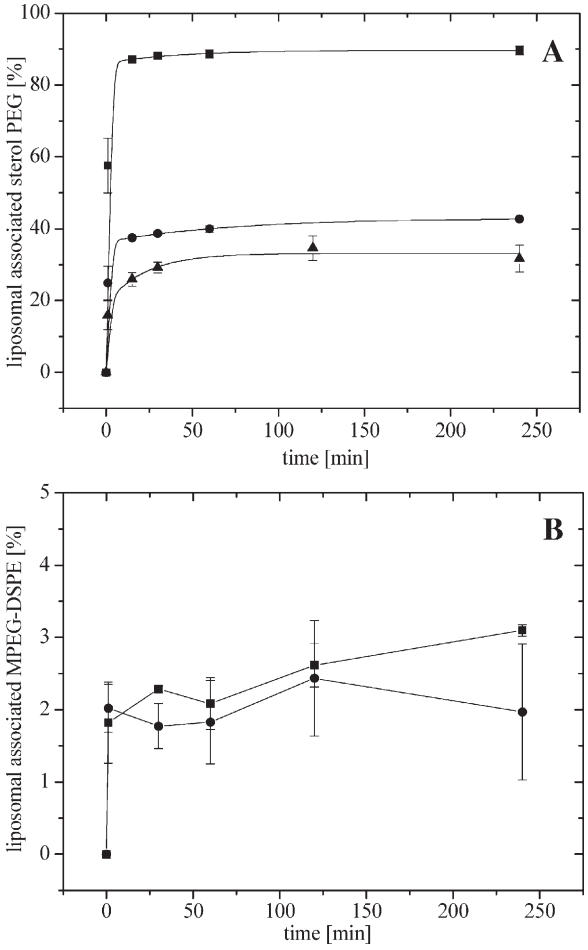

Fig. 3. Incorporation kinetics of PEGylated lipids at room temperature into liposomes composed of: ■ EPC/Chol (7/3 mol/mol); ▲ HSPC/Chol (2/1 mol/mol) or ● HSPC/Chol/$MPEG_{2000}$–DSPE (2/1/0.16 mol/mol/mol) followed by ^1H NMR. Values of membrane inserted PEG–lipids are given as the percentage of the added amount (7.5%) of total lipid. A, sterol–PEG_{1300}; B, $MPEG_{2000}$–DSPE. Mean of three experiments ± S.D.

liposomes containing $MPEG_{2000}$–DSPE has been described for micellar IgG-PEG_{2000}–DSPE as well. However, even at elevated temperature a very low degree of insertion has been reported [19,20].

In our study, the amount of $MPEG_{2000}$–DSPE transferred to preformed vesicles at room temperature was found to be very small (Fig. 3B), independent of membrane fluidity. After 4 h of incubation, less than 3% were co-eluted with the vesicles for EPC/Chol and 2% for HSPC/Chol liposomes of the $MPEG_{2000}$–DSPE. This resulted in a total PEG–lipid ratio of less than 0.2 mol% of TL in the outer membrane monolayer. The different degrees of insertion for both components is not caused by a difference in initial concentrations of monomers coexisting with the micelles because CMC values are around 5 μM in each case. Sterical hindrance of the two flexible fatty acid moieties may contribute to the lower amount of insertion of $MPEG_{2000}$–DSPE as compared to that seen with sterol–PEG_{1300}. The sterol–PEG_{1300} molecule contains a rigid, single portion, which can overcome the water–membrane interface more easily. However, considering the rapid insertion of approximately 2% of added $MPEG_{2000}$–DSPE in Fig. 6, it is more probable that the activation energy of demicellization is much higher and/or its kinetics are much slower than that of sterol–PEG_{1300}.

3.2. Leakage during incorporation

The accumulation of the sterol anchor, which is initially most likely restricted to the outer leaflet of the liposomes, can induce membrane tensions due to differing lipid amounts in the two monolayers [32]. This effect may cause significant loss of encapsulated material by provoking membrane reorganization. As shown in Fig. 4, the release of calcein after mixing with sterol–PEG_{1300}–BSA (7.5 mol% of TL) is at maximum 5% from EPC liposomes with high membrane fluidity and in the order of 1–3% for plain or sterically stabilized rigid HSPC liposomes. Maximum release was reached 30 min after addition of the protein–sterol conjugate and then remained essentially constant.

The release rate of calcein from liposomes exposed to sterol–PEG_{1300}–BSA amounts to up to 7.5 mol% of TL, which is convenient for protein attachment, is comparable to the release rate found for doxorubicin [19] or carboxyfluorescein [24] when using the post insertion technique.

3.3. Protein attachment to preformed liposomes

The activation of hydroxyl groups by tresylation was introduced by Nilsson and Mosbach [28] and has become a widely used method for PEGylation of proteins [33–35] and other amine-containing materials [36,37]. The coupling of tresylated PEG was formerly believed to take place by alkylation of amino groups [28], resulting in a secondary amide bond to the protein to be coupled (Fig. 1e). Later studies suggested an alternative route of PEGylation of the protein forming a sulfoacetamide linkage [38,39] to the proteins to be modified (Fig. 1d). While the secondary amide is stable in vitro and in vivo, sulfonyl linkages may be subject to slow hydrolytic cleavage. Furthermore, the formation of the stable secondary amine

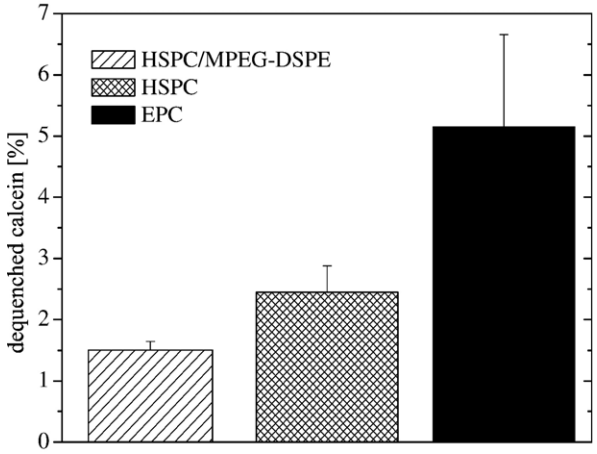

Fig. 4. Calcein release from liposomes 6 h after mixing with sterol–PEG_{1300}–BSA (7.5 mol% of TL) at room temperature.

preserves the positive charge of the protein amino group and therefore may not decrease the biological function to the same extent as the alternative sulfoacetamide bond [40]. As carbamate, p-nitrophenylcarbonyl [16] or amide bonds, resulting from other amine-reactive coupling procedures also eliminate the positive charge, the coupling of proteins to liposomes may result in more effective ligand receptor interaction when tresyl-activated lipids are used for their attachment. Since pH values slightly above 8 are most favourable for achieving high rates of secondary amide bonds [39], the coupling of protein to sterol–PEG_{1300}–TRE was performed in borate buffered solution at pH 8.4.

As shown in Fig. 5, more than 50% of the radioactive labelled protein is coupled to the activated sterol–PEG_{1300} within 1 h. After 12 h the reaction is almost completed.

3.4. Coupling efficiency

The absolute amount of sterol–PEG_{1300}–BSA, which is attachable to different liposome types as a function of protein concentration during the first incubation step is shown in Fig. 6. Protein:lipid ratio (mol/mol) of the final liposome dispersions during coupling experiments reached from 1:2700 (1 mg/ml BSA during first incubation step) to 1:540 (5 mg/ml), which is similar to the conditions used for antibody coupling (1:1000) as described in [17,41].

Although the relative coupling efficiency decreases (Fig. 6) with increasing concentrations of the protein to be coupled, the subsequent grafting of proteins via tresylated sterol–PEG_{1300} easily allows protein densities necessary for targeting purposes [42]. Because an antibody density above 75 μg/μmol of TL was reported to accelerate the clearance of sterically stabilized immunoliposomes [15], and keeping in mind that the molar mass of antibodies is more than 2-fold higher than that of BSA, one could calculate that the protein/lipid ratio of 1:2000 should supply sufficient coupling efficiency in the case of antibody coupling.

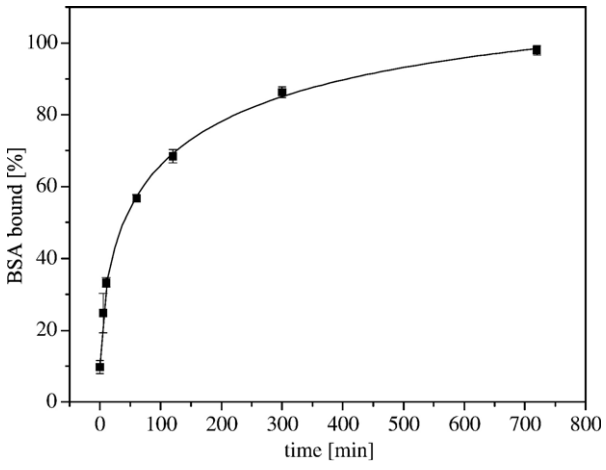

Fig. 5. Binding efficiency of BSA to liposomes depending on the reaction time between BSA (0.5 mg/ml) and 7.5 mol% (of TL) of sterol–PEG_{1300}–TRE. The coupling reaction of protein and sterol–PEG_{1300}–TRE was terminated at different times by addition of a molar excess of amino-groups, before the BSA-conjugate was mixed with vesicles and incubated for 2 h. Mean of three experiments ± S.D.

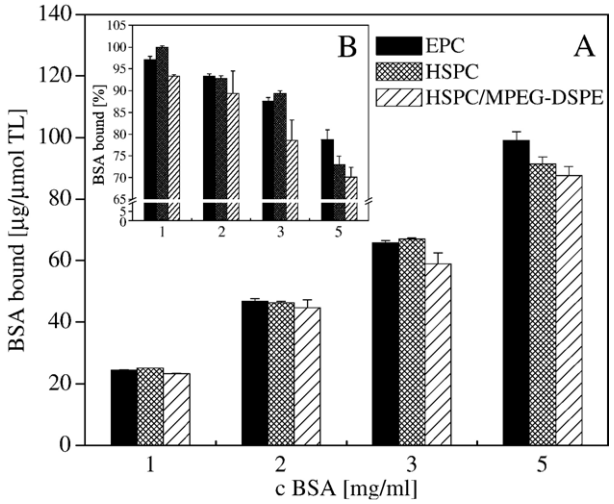

Fig. 6. Absolute (A) amount of BSA (μg protein/total lipid) and (B) relative amount (% of total BSA) coupled to different liposome preparations (20 mmol lipid/l) depending on the protein concentration during the first incubation step, after addition of sterol–PEG_{1300}–BSA to pre-formed liposomes.

Since the quantity of sterol–PEG_{1300}–TRE was kept constant over all coupling experiments, the ratio of protein supplemented amino groups to activated lipid becomes more and more unfavourable as protein concentration increases.

For the highest protein concentration used during coupling experiments a 43-fold molar excess of activated lipid results in 0.62 sterol–PEG_{1300}–TRE anchors per lysine of BSA. Therefore, it can be supposed that every BSA molecule bears several sterol-anchors and is multiply linked to the liposome.

In this context, it may be understandable that the differences in the level of protein grafting of the varying liposome types is less pronounced than may be assumed from the amount of sterol–PEG_{1300} incorporated (see Fig. 3A). The number of sterol anchors accepted by the sterically stabilized vesicles seems to be sufficient for a stable protein–liposome linkage.

3.5. Increase of particle size

Upon incubation of liposomes with BSA bearing several sterol anchors, the linkage of liposomes and the resulting increase in particle size is unavoidable. This problem of liposome cross-linking is rarely discussed in the literature, even though it has a high probability of occurring with each coupling technique. Table 1 illustrates that size increase is dependent on protein amount and liposome composition as a function of time. As expected, increasing protein amounts favours the formation of liposome aggregates. In some cases particle diameters are two-fold larger, which would suggest the covalent linkage of several liposomes via the protein molecules bearing several PEG–sterol moieties. The structure of such aggregates is shown in a representative cryo-EM picture (Fig. 7). Besides some isolated vesicles, most of the liposomes are tightly connected. At an aggregation number of more than four, the aggregates become

Table 1
Increase of particle size (hydrodynamic diameter in nm) during the incorporation of sterol–PEG_{1300}–proteins into different liposome types

BSA [mg/ml]	EPC/Chol			HSPC/Chol			HSPC/Chol/MPEG		
	0	1	5	0	1	5	0	1	5
z-Av									
Initial	116.2±1.38	124.3±1.45	119.6±2.13	112.6±1.67	113.5±1.14	111.9±1.02	118.7±1.13	119.4±1.12	118.6±1.45
4 h	–	170.1±3.46	374.3±3.21	–	121.8±1.73	228.1±2.51	–	130.3±5.82	172.6±2.7
12 h	118.2±1.56	209.9±10.61	816.2±18.4	115.3±2.31	120.5±1.91	335.0±11.72	119.2±1.13	136.0±3.11	226.6±3.6
PI									
Initial	0.1±0.01	0.11±0.03	0.09±0.04	0.06±0.02	0.05±0.04	0.06±0.02	0.09±0.01	0.06±0.02	0.1±0.01
4 h	–	0.12±0.02	0.28±0.01	–	0.04±0.02	0.2±0.1	–	0.12±0.01	0.26±0.03
12 h	0.098±0.02	0.2±0.01	0.56±0.09	0.076±0.02	0.06±0.04	0.31±0.02	0.083±0.01	0.11±0.01	0.38±0.02

three-dimensional (see upper half in the middle or bottom left) and also show flattened areas formed by cross-linked bilayers. Cryo studies of control preparations of plain liposomes with or without the insertion of non-activated sterol–PEG showed that these liposomes were essentially non-aggregated (not shown).

Particle size can be limited by the rapid usage of the functionalized liposomes. Alternatively, modification of the liposome surface using 7.5% sterol–PEG_{1300} during vesicle preparation or by subsequent insertion into plain liposomes leads to a stabilization of particle size during the second incubation step (data not presented). After 12 h of incubation of the sterol–PEG_{1300} modified liposomes (EPC/Chol 20 mM, z-Average: 120.4) with the sterol–PEG_{1300}–protein conjugate (c BSA: 1 mg/ml) a particle size of approximately 160 nm was reached and this did not significantly change over a period of 48 h.

3.6. Stability of protein attachment

Investigating the stability of the protein linkage in the presence of serum, BSA coupled liposomes were rechromatographed after incubation for 20 h at 37 °C in 50% (v/v) human serum. Dissociated ^{125}I-labelled protein was not detectable in any of the preparations, suggesting a covalent attachment which is stable with regards to enzymatic stress, as well as a hydrophobic anchoring in the membrane which is resistant to interference by serum.

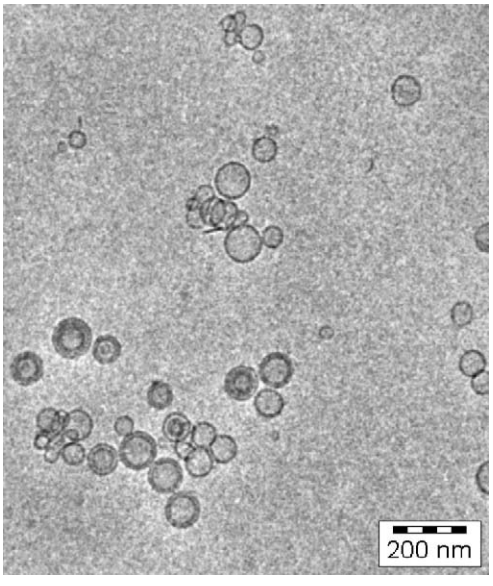

Fig. 7. Cryo electron microscopic picture of single liposomes and aggregates formed after incubating sterol–PEG_{1300}–BSA (5 mg/ml BSA) with EPC/cholesterol liposomes for 4 h. Flat connection areas and spaces between membranes in larger aggregates (middle and bottom left) indicate the covalent linkage of liposomes by sterol–PEG–BSA.

4. Conclusion

The novel and simple method presented here is suitable to functionalize plain or even PEGylated liposomes. In the standard protocol used to couple proteins such as BSA as a model ligand, in a first step sterol–PEG_{1300} is activated by tresylchloride at 0 °C for 4 h to give sterol–PEG_{1300}–TRE, which can be stored until use. In a second step, sterol–PEG_{1300}–TRE reacts quantitatively with proteins in an aqueous solution at pH 8.4 within 2–4 h, forming sterol–PEG_{1300}–protein conjugates, which in a third step spontaneously insert into preformed liposomes at room temperature. The amount of membrane leakage during the grafting process is negligible. This method can be used for the functionalization of plain fluid or rigid membranes and also of PEGylated liposomes. In contrast to conventional coupling procedures, the derivatization of the protein takes place in the absence of membrane lipids and encapsulated material, which avoids interference by the activated lipids with these compounds. An additional advantage of the post-functionalization method is the modification of liposomes at room temperature, which avoids denaturation of encapsulated or attached biomacromolecules.

In preliminary experiments in mice, post-functionalized liposomes show a half-life in the circulation of approximately 4 h. The shorter circulation time as compared to that seen with $MPEG_{2000}$–DSPE is probably due to the shorter length of the PEG-1300 moiety, rather than to instability of the anchoring of the lipid in the membrane. The strength of membrane anchoring of sterol–PEG seems to be of minor importance as seen with the results of incubating the post-functionalized liposomes with serum. However, further studies in animals using double-labelled protein-bearing liposomes can clarify if other destabilization or cleaving reactions occur under in vivo conditions.

The prolonged blood circulation compared to conventional liposomes should be sufficient, when targeting cells such as granulocytes and monocytes, which are both resident in tissues as well as circulating the blood. Therefore, with the presented

method specific targeting of liposome encapsulated protein like glucose oxidase [2] to phagocytes of blood and tissue should be possible. Furthermore, the described method may allow functionalization of polymerized liposomes [43], thus avoiding possible interference between the techniques for coupling and polymerization.

Acknowledgements

We gratefully acknowledge the excellent technical assistance of Elli Saalfelder and Sabine Barnert. We also thank Volker Brecht for the ^{1}H-NMR measurements, Markus Gantert for additional liposome preparations and Andreas Neub for the cryo-EM studies. Financial support of this work was provided by the Deutsche Forschungsgemeinschaft (Schu 800/4-1) and the Sonderforschungsbereich 428.

References

[1] P. Sapra, T.M. Allen, Ligand-targeted liposomal anticancer drugs, Prog. Lipid Res. 42 (2003) 439–462.
[2] C.E. Gerber, G. Bruchelt, U.B. Falk, A. Kimpfler, O. Hauschild, S. Kuci, T. Bachi, D. Niethammer, R. Schubert, Reconstitution of bactericidal activity in chronic granulomatous disease cells by glucose-oxidase-containing liposomes, Blood 98 (2001) 3097–3105.
[3] G. Blume, G. Cevc, Liposomes for the sustained drug release in vivo, Biochim. Biophys. Acta 1029 (1990) 91–97.
[4] T.M. Allen, C. Hansen, F. Martin, C. Redemann, A. Yau-Young, Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo, Biochim. Biophys. Acta 1066 (1991) 29–36.
[5] S. Beugin, K. Edwards, G. Karlsson, M. Ollivon, S. Lesieur, New sterically stabilized vesicles based on nonionic surfactant, cholesterol, and poly(ethylene glycol)-cholesterol conjugates, Biophys. J. 74 (1998) 3198–3210.
[6] C. Carrion, J.C. Domingo, M.A. de Madariaga, Preparation of long-circulating immunoliposomes using PEG-cholesterol conjugates: effect of the spacer arm between PEG and cholesterol on liposomal characteristics, Chem. Phys. Lipids 113 (2001) 97–110.
[7] H. Ishiwata, A. Vertut-Doi, T. Hirose, K. Miyajima, Physical-chemistry characteristics and biodistribution of poly(ethylene glycol)-coated liposomes using poly(oxyethylene) cholesteryl ether, Chem. Pharm. Bull. (Tokyo) 43 (1995) 1005–1011.
[8] H. Ishiwata, S.B. Sato, A. Vertut-Doi, Y. Hamashima, K. Miyajima, Cholesterol derivative of poly(ethylene glycol) inhibits clathrin-independent, but not clathrin-dependent endocytosis, Biochim. Biophys. Acta 1359 (1997) 123–135.
[9] M. Fleiner, P. Benzinger, T. Fichert, U. Massing, Studies on protein–liposome coupling using novel thiol-reactive coupling lipids: influence of spacer length and polarity, Bioconjug. Chem. 12 (2001) 470–475.
[10] K. Maruyama, T. Yuda, A. Okamoto, C. Ishikura, S. Kojima, M. Iwatsuru, Effect of molecular weight in amphipathic polyethyleneglycol on prolonging the circulation time of large unilamellar liposomes, Chem. Pharm. Bull. (Tokyo) 39 (1991) 1620–1622.
[11] M.C. Woodle, D.D. Lasic, Sterically stabilized liposomes, Biochim. Biophys. Acta 1113 (1992) 171–199.
[12] S. Shahinian, J.R. Silvius, A novel strategy affords high-yield coupling of antibody Fab' fragments to liposomes, Biochim. Biophys. Acta 1239 (1995) 157–167.
[13] D. Kirpotin, J.W. Park, K. Hong, S. Zalipsky, W.L. Li, P. Carter, C.C. Benz, D. Papahadjopoulos, Sterically stabilized anti-HER2 immunoliposomes: design and targeting to human breast cancer cells in vitro, Biochemistry 36 (1997) 66–75.
[14] S. Zalipsky, N. Mullah, J.A. Harding, J. Gittelman, L. Guo, S.A. DeFrees, Poly(ethylene glycol)-grafted liposomes with oligopeptide or oligosaccharide ligands appended to the termini of the polymer chains, Bioconjug. Chem. 8 (1997) 111–118.
[15] T.M. Allen, E. Brandeis, C.B. Hansen, G.Y. Kao, S. Zalipsky, A new strategy for attachment of antibodies to sterically stabilized liposomes resulting in efficient targeting to cancer cells, Biochim. Biophys. Acta 1237 (1995) 99–108.
[16] V.P. Torchilin, T.S. Levchenko, A.N. Lukyanov, B.A. Khaw, A.L. Klibanov, R. Rammohan, G.P. Samokhin, 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 (2001) 397–411.
[17] G. Bendas, A. Krause, U. Bakowsky, J. Vogel, U. Rothe, Targetability of novel immunoliposomes prepared by a new antibody conjugation technique, Int. J. Pharm. 181 (1999) 79–93.
[18] A.R. Nicholas, M.J. Scott, N.I. Kennedy, M.N. Jones, Effect of grafted polyethylene glycol (PEG) on the size, encapsulation efficiency and permeability of vesicles, Biochim. Biophys. Acta 1463 (2000) 167–178.
[19] T. Ishida, D.L. Iden, T.M. Allen, A combinatorial approach to producing sterically stabilized (Stealth) immunoliposomal drugs, FEBS Lett. 460 (1999) 129–133.
[20] D.L. Iden, 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 (2001) 207–216.
[21] J.N. Moreira, T. Ishida, R. Gaspar, 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 (2002) 265–269.
[22] V.D. Awasthi, D. Garcia, R. Klipper, W.T. Phillips, B.A. Goins, Kinetics of liposome-encapsulated hemoglobin after 25% hypovolemic exchange transfusion, Int. J. Pharm. 283 (2004) 53–62.
[23] K. Sou, T. Endo, S. Takeoka, E. Tsuchida, Poly(ethylene glycol)-modification of the phospholipid vesicles by using the spontaneous incorporation of poly(ethylene glycol)-lipid into the vesicles, Bioconjug. Chem. 11 (2000) 372–379.
[24] P.S. Uster, T.M. Allen, B.E. Daniel, C.J. Mendez, M.S. Newman, G.Z. Zhu, Insertion of poly(ethylene glycol) derivatized phospholipid into pre-formed liposomes results in prolonged in vivo circulation time, FEBS Lett. 386 (1996) 243–246.
[25] J.M. Saul, A. Annapragada, J.V. Natarajan, R.V. Bellamkonda, Controlled targeting of liposomal doxorubicin via the folate receptor in vitro, J. Control. Release 92 (2003) 49–67.
[26] V.D. Awasthi, D. Garcia, R. Klipper, B.A. Goins, W.T. Phillips, Neutral and anionic liposome-encapsulated hemoglobin: effect of post-inserted poly (ethylene glycol)-distearoylphosphatidylethanolamine on distribution and circulation kinetics, J. Pharmacol. Exp. Ther. 309 (2004) 241–248.
[27] C.R. Miller, B. Bondurant, S.D. McLean, K.A. McGovern, D.F. O'Brien, Liposome–cell interactions in vitro: effect of liposome surface charge on the binding and endocytosis of conventional and sterically stabilized liposomes, Biochemistry 37 (1998) 12875–12883.
[28] K. Nilsson, K. Mosbach, Immobilization of ligands with organic sulfonyl chlorides, Methods Enzymol. 104 (1984) 56–69.
[29] G.R. Bartlett, Phosphorus assay in column chromatography, J. Biol. Chem. 234 (1959) 466–468.
[30] N. Kaiser, A. Kimpfler, U. Massing, A.M. Burger, H.H. Fiebig, M. Brandl, R. Schubert, 5-Fluorouracil (5-FU) in vesicular phospholipid gels (VPG) for anticancer treatment: entrapment and release properties, Int. J. Pharm. 256 (2003) 131–132.
[31] B.M. Folmer, M. Svensson, K. Holmberg, W. Brown, The physicochemical behavior of phytosterol ethoxylates, J. Colloid Interface Sci. 213 (1999) 112–120.
[32] P.F. Devaux, Reconstitution of flippase activity into liposomes, Cell. Mol. Biol. Lett. 7 (2002) 227–229.
[33] C. Delgado, J.N. Patel, G.E. Francis, 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 (1990) 119–128.
[34] J. Rostin, A.L. Smeds, E. Akerblom, B-Domain deleted recombinant coagulation factor VIII modified with monomethoxy polyethylene glycol, Bioconjug. Chem. 11 (2000) 387–396.
[35] I.D. Bianco, J.J. Daniele, C. Delgado, D. Fisher, G.E. Francis, G.D. Fidelio, Coupling reaction and properties of poly(ethylene glycol)-linked phospholipases A2, Biosci. Biotechnol. Biochem. 66 (2002) 722–729.
[36] Y. Yamamoto, M.V. Sefton, Reaction of poly(acrylamide-co-vinylamine) with tresyl-PEG in the presence of PC12 cells, Biomaterials 24 (2003) 435–442.
[37] Y. Yang, H.A. Chase, Immobilization of alpha-amylase on poly(vinyl alcohol)-coated perfluoropolymer supports for use in enzyme reactors, Biotechnol. Appl. Biochem. 28 (Pt. 2) (1998) 145–154.
[38] J.F. King, M.S. Gill, Alkyl 2,2,2-Trifluoroethanesulfonates (Tresylates): elimination—Addition vs. bimolecular nucleophilic substitution in reactions with nucleophiles in aqueous media(1), J. Org. Chem. 61 (1996) 7250–7255.
[39] J.J. Sperinde, B.D. Martens, L.G. Griffith, Tresyl-mediated synthesis: kinetics of competing coupling and hydrolysis reactions as a function of pH, temperature, and steric factors, Bioconjug. Chem. 10 (1999) 213–220.
[40] S. Zalipsky, Functionalized poly(ethylene glycol) for preparation of biologically relevant conjugates, Bioconjug. Chem. 6 (1995) 150–165.
[41] C.B. Hansen, G.Y. Kao, E.H. Moase, S. Zalipsky, T.M. Allen, Attachment of antibodies to sterically stabilized liposomes: evaluation, comparison and optimization of coupling procedures, Biochim. Biophys. Acta 1239 (1995) 133–144.
[42] V.P. Torchilin, Liposomes as targetable drug carriers, Crit. Rev. Ther. Drug Carr. Syst. 2 (1985) 65–115.
[43] O. Stauch, T. Uhlmann, M. Frohlich, R. Thomann, M. El-Badry, Y.K. Kim, R. Schubert, Mimicking a cytoskeleton by coupling poly(N-isopropylacrylamide) to the inner leaflet of liposomal membranes: effects of photopolymerization on vesicle shape and polymer architecture, Biomacromolecules 3 (2002) 324–332.