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Targetability of novel immunoliposomes prepared by a new antibody conjugation technique

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Abstract

In order to develop long-circulating immunoliposomes (IL), which combine sterical stabilization with a superior targetability, we have introduced a new methodology for attaching monoclonal antibodies direcly onto the distal ends of liposome-grafted polyethylene glycol (PEG) chains. Therefore, we have synthesized a new PEG-PE derivative, which had been endgroup-functionalized with cyanuric chloride. Antibodies can simply be coupled to this membrane anchor in mild basic conditions (pH 8.8) without the need for previous antibody derivatizations. The coupling results have been determined with consideration to various liposome parameters and have been compared to several established antibody coupling procedures, where antibodies had been linked directly to the liposome surface in the presence of PEG (conventional IL). To investigate the targetability of the resulting new IL, anti E-selectin mAb have been coupled and the degree of binding selectin-containing cells has been analyzed. The terminal coupled antibodies show a 1.8-fold higher degree of in vitro cell binding compared to conventional IL, which has been attributed to the antibody position being more easy accessible at the PEG termini. Furthermore, we have illustrated the liposome surface topology and the coupled antibodies by atomic force microscopy, which for such fluid IL has been used first. These images have finely corresponded to the cell binding results, and have been discussed in terms of antibody position and flexibility at the liposome surface.

Keywords: Immunoliposomes; Drug targeting; Antibody conjugation; Selectins; Cyanuric chloride

1. Introduction

The site-specific drug delivery to cells or organs is a potentially attractive mode of therapy to increase the therapeutic effects and reduce drug toxicity. The use of liposomes as drug vehicles has

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been explored extensively. In order to obtain a liposome targeting, organ- or cell-specific homing devices have to be conjugated to the liposome surfaces. Among various opportunities, the coupling of target-specific antibodies to create immunoliposomes (IL) has appeared as most promising way in achieving a liposome targeting (Allen et al., 1994). Several methods differing in their chemical basis have been described for the attachment of antibodies to the liposome surfaces (Chua et al., 1984; Heath, 1987; Holmberg et al., 1989; Loughrey et al., 1990).

The therapeutic application of such IL is strongly restricted since liposomes of that kind were rapidly removed from circulation by cells of the reticuloendothelial system (RES) (Derksen et al., 1988). Since the early 1990s, long circulation liposomes have been designed, which were sterically stabilized by coating the liposome surface with amphipathic PEG derivatives (Blume and Cevc, 1990; Klibanov et al., 1990). The high mobility of the PEG chains and their conformational flexibility creates a sterical barrier which allows the liposomes to evade RES uptake and retain the blood circulation for a longer time, and in this way increase the possibility for targeting (Woodle and Lasic, 1992; Torchilin et al., 1994).

It could be shown that the coupling of antibodies to the liposome surface and their target binding can effectively be combined with a sterical stabilization of the IL by PEG. Nevertheless, the sterical barrier of PEG decreases the protein coupling efficiency at the liposome surface as well as the target recognition, especially in cases of higher concentrations of PEG with high molecular weights (Klibanov et al., 1991; Harasym et al., 1995). In order to overcome these problems, coupling of antibodies to the teminal ends of PEG for increase antibody accessibility has been investigated in recent studies. In analogy to the conventional antibody coupling chemistry, several workers have prepared PEG-derivatives for the terminal antibody attachment. On the basis of the thio-reaction of maleimide groups with thiol containing compounds, Maruyama et al. (1995) have introduced a PEG-PE derived lipid with a terminal maleimide group for the reaction with thiolated antibodies or Fab fragments. Allen et al. (1995) have synthesized a thiol-reactive PEG anchor (PDP-PEG-PE) for the reaction with maleimide-containing antibodies. Furthermore, Hansen et al. (1995) have used a PEG-terminal hydrazid group for the reaction with aldehyde groups from oxidized antibodies. These terminal coupled antibodies have shown increased target binding ability in most cases, compared to conventional IL. A potential drawback of these coupling procedures is the need to derivatize the antibodies to enable the attachment. A derivative with carboxy groups at the PEG terminus has also been described for protein attachment (Blume et al., 1993; Maruyama et al., 1997), which needs a two reagents activation before coupling.

In order to obtain a rapid and simple coupling procedure at the PEG terminus, we introduce a new endgroup-functionalized PEG derivative (Fig. 1) in this study. Cyanuric chloride at the PEG terminus, which has previously been used to couple proteins onto hydroxy groups of polymers for various applications, has functioned to link the antibodies via a nucleophilic substitution at a basic pH of 8.8. Antibodies or other proteins can be conjugated without previous derivatizations, which is especially economic when expensive antibodies are being used. The targetability of the IL

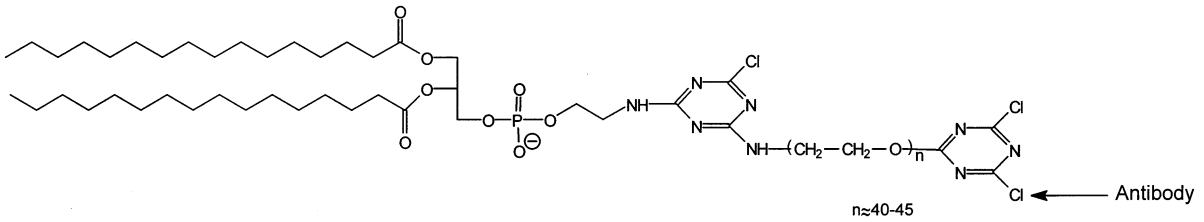


Fig. 1. Structure of the synthesized protein anchor DPPE-PEG-cyanuric chloride (cyanur-PEG-PE).

has been proved by binding E-selectin at the cell surface, which is an inflammation-related adhesion receptor at endothelial cells. These new IL have shown a significantly increased in vitro cell binding compared to conventional IL.

2. Materials and methods

2.1. Materials

Soy phosphatidylcholine (SPC) was obtained from Lucas Meyer (Hamburg, Germany). *O*-(2-Aminoethyl)polyethyleneglycol (α-amino-ω-hydroxy PEG 2000) was purchased from Fluka (Germany). 1.2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), avidin, cholesterol, 2.4.6-Trichloro-1.3.5-triazine (cyanuric chloride), *N*,*N*-diisopropylethylamine, DL-dithiothreitole (DTT), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), glutaric-anhydride, succinimidyl-4-(*p*-maleimidophenyl)butyrate (SMPB) and *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) were purchased from Sigma (Deisenhofen, Germany). Biotin-PE, PDP-PE, MPB-PE, Polyethylene glycol-PE (PEG-PE 2000) and 1.2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). The substances were used without further purification. *N*-glutaryl-PE was synthesized in our group. All salts and buffers (HEPES, MES and Borate from Sigma) were of analytical grade.

2.2. Monoclonal antibodies and cells

The anti-E-selectin mAb (rat-IgG) was a grateful gift from Prof. Vestweber (University Münster, Germany). Human IgG and biotinylated IgG was obtained from Sigma.

The derivatization of the antibodies into thioreactive proteines was performed as previously described by Hansen et al. (1995).

To obtain the maleimide Ab (MPB-Ab), SMPB in dimethylformamide was slowly added to the antibody solution in HEPES to gain final molar ratio of SMPB:Ab 20:1. After 90 min incubation at RT, excess SMPB was removed by gel chromatography (Sephadex G 50) eluted with buffer of pH 6.7 (25 mM Mes, 25 mM HEPES and 140 mM NaCl).

The thiolated antibody was prepared by adding SPDP in ethanol to the antibody solution in HEPES with a final molar ratio SPDP:Ab 10:1. After incubation for 30 min at RT, excess SPDP was removed by gel chromatography (Sephadex G 50) eluted with buffer of pH 4.7 (100 mM Na acetate and 100 mM NaCl). Immediately before protein coupling, the dithio group of PDP was reduced by adding DTT followed by removing excess DTT over Sephadex G 50 with buffer of pH 6.7.

2.3. Synthesis of DPPE-PEG-cyanuric chloride (cyanur-PEG-PE)

To obtain a cyanuric chloride linkage between DPPE and PEG, DPPE-cyanuric chloride was synthesized first. In order to do so, 150 mg DPPE (0.21 mmol) were dissolved in dry chloroform before adding a 3-fold molar excess cyanuric chloride (118 mg) and a 2-fold molar amount of *N*,*N*-diisopropylethylamine (58 mg). The mixture was stirred vigorously overnight at RT. The complete conversion of the DPPE (Rf 0.55) was confirmed by TLC (chloroform/methanol/water 63/33/4; RT; 50% relative humidity). The DPPE-cyanuric chloride (Rf 0.72) was isolated by preparative TLC and redissolved in dry chloroform before adding α-amino-ω-hydroxy PEG 2000 in a 0.9-fold molar ratio and a 2-fold molar excess of *N*,*N*-diisopropylethylamine. Again, this mixture was stirred for 36 h at RT. TLC of the reaction mixture showed reduced Rf-values of the product (0.63) and the nearly complete disappearance of both the free PEG derivative and DPPE-cyanuric chloride. The DPPE-PEG-OH was isolated by preparative TLC. To obtain the activation at the PEG terminus of this product, it was brought to reaction with 0.8-fold molar amount of cyanuric chloride in addition to a 2-fold molar amount of *N*,*N*-diisopropylethylamine in dry chloroform for 36 h. The final product DPPE-PEG-cyanuric chloride had a sligthly reduced Rf-value (0.57). After isolation and desiccation, the

yield of this last reaction was calculated of about 65%. The final product was stored at −20°C in a chloroform solution.

2.4. Preparation of liposomes

Liposomes were prepared by hydrating a lipid film (10 μmol) in 1 ml buffer at 60°C followed by shaking overnight. The resulting multilamellar vesicles were extruded six times (Extruder, Lipex Biomembrane, Vancouver, Canada) through a 200 nm polycarbonate membrane (Costar, Bodenheim, Germany). Vesicle size was determined by dynamic light scattering using a Malvern Autosizer II c (Malvern, UK) in mass distribution mode and estimated to be about 165 nm with a polydispersity index below 0.1. The lipid composition of vesicles was slightly modified in order to meet with the particular needs of our experiments. The basic composition was SPC/Chol 2:1 (molar ratio), whereby certain amounts of protein anchors (0.1 mol% Biotin-PE, 1 mol% PDP-PE or MPB-PE, 5 mol% *N*-glut-PE or 0.5–7.5 mol% cyanur-PEG-PE) were incorporated according to the coupling procedure. The addition of PEG-PE 2000 diminished the fraction of SPC. Likewise, for fluorescence detection, 2 mol% NBD-PE were incorporated in all vesicle preparations. These modifications in the lipid composition had no influence on the liposome size.

2.5. Antibody conjugation

For all coupling procedures, an initial phospholipid/Ab molar ratio of 1000:1 was chosen, as previously optimized by Hansen et al. (1995). The coupling yield of all reactions was quantified as a measure of the protein content with SDS/PAGE.

Liposomes containing variing amounts of cyanur-PEG-PE were prepared in 0.15 M NaCl. The indicated amounts of antibodies, which had been dissolved in borate buffer to adjust a pH of 8.8 were added to this preparation and incubated at RT for about 16 h upon shaking. Unbound antibodies were separated by passing the liposomes over a Sepharose 4B column and eluting with PBS pH 7.4.

To form a protein linkage to liposomal *N*-glut PE, 6 mg EDC were added to 10 μmol liposomes in PBS (pH 7.4) followed by an incubation period of at best 6 h at RT and by gel permeation chromatography (Sephadex G 50) to remove excess EDC. Antibodies were added and incubated overnight at room temperature. The immunoliposomes were separated from unbound antibodies by gel permeation chromatography using Sepharose 4B.

Liposomes containing biotinylated PE were prepared in HEPES (pH 7.4). This dispersion was added stepwise to a HEPES solution of avidin followed by vortexing after each step until a ratio of biotinylated DOPE/avidin of 1:1 was finally reached. After incubation for 30 min at RT, the biotinylated antibodies were added as HEPES solution before further incubating at RT for 30 min. Unbound antibodies were removed by passing a Sepharose 4B column eluted with HEPES.

Liposomes containing PDP-PE were prepared in buffer pH 6.7 (25 mM HEPES, 25 mM Mes, 140 mM Nacl). To reduce the disulfide linkage, 3.1 mg DTT were added, corresponding to a DTT final concentration of 20 mM. After 30 min of incubation at RT, liposomes were separated from excess DTT by ultra centrifugation (2 h, $200{,}000 \times g$, 20°C). The MPB-antibodies were added to the resuspended liposomes up to the indicated molar ratio PL/Ab 1000:1 to incubate for 16 h at RT before separating the unbound antibodies as described above.

MPB-PE liposomes were also prepared in buffer of pH 6.7. The freshly reduced thiolated antibody from the PDP-derivative was added to the indicated final molar ratio for incubation overnight. Again, separation of liposomes from unbound antibody was performed with a Sepharose 4B column eluted with HEPES pH 7.4.

2.6. Cell binding experiments

E-selectin-transfected Chinese Hamster Ovarial-cells (CHO-E cells) were grown in MEM-α media containing 10% fetal calf serum, 2 mM L-glutamine and 100 nM penicilline/streptomycin. Flasks seeded with 5×10^4 CHO-E cells were incubated at 37°C in 5% CO_2 for 3 or 4 days until cells were grown nearly confluent.

For binding studies, 96-well plates were seeded with nearly 75,000 cells per well and incubated at 37°C in 5% CO_2 for 2 days until cells had formed a stable monolayer at the well bottom. To reduce unspecific liposome binding, CHO-E cells were blocked with 0.5% gelatine and 0.5% albumin solution. After that, liposomes of different preparations were incubated with the cells for 90 min at 4°C (no endocytotic activity, only binding events). After this time, cells were washed three times with PBS to remove unbound liposomes. Fluorescence intensity in the several wells caused by the bound fluorescently labeled immunoliposomes (2 mol% NBD-PE) was measured by using a microplate fluorometer (Fluoroscan Ascent, Labsystem).

Alternatively, bindings studies were also evaluated using Flow cytometry.

2.7. Atomic force microscopy

Atomic force microscopy (AFM) measurements were performed on a Nanoscope™ IIIa (Digital Instruments, Santa Barbara, CA, USA) using Tapping Mode™. Commercially available cantilevers (Digital Instruments) were used. The surface images were visualized in amplitude or in height modus. The Tapping Mode™ normally gives very low contact forces and is therefore well suited for soft probes. Liposomes for the measurements were deposited on RCA-cleaned silicon wafers. The latter were measured immediately after their deposition in full hydrated state.

3. Results and discussion

3.1. Synthesis of DPPE-PEG-cyanuric chloride (cyanur-PEG-PE)

In order to find a new PEG lipid derivative, which had been expected to be able to couple proteins at the PEG terminus in a rapid, simple and safe way, we searched for a bifunctional coupling reagent, that meets all these requirements. We eventually chose cyanuric chloride. In the last decade, cyanuric chloride has been used intensively in coupling a variety of proteins or DNA to polymers, in most cases to PEG (Suzuki et al., 1984; Gombotz et al., 1991). Cyanuric chloride is a three-functional reagent with a strong reactive graduation. The first two chloride substitutions can be achieved upon reaction with nucleophiles under slight basic conditions. At further states, reactivity of the third chloride is strongly depressed.

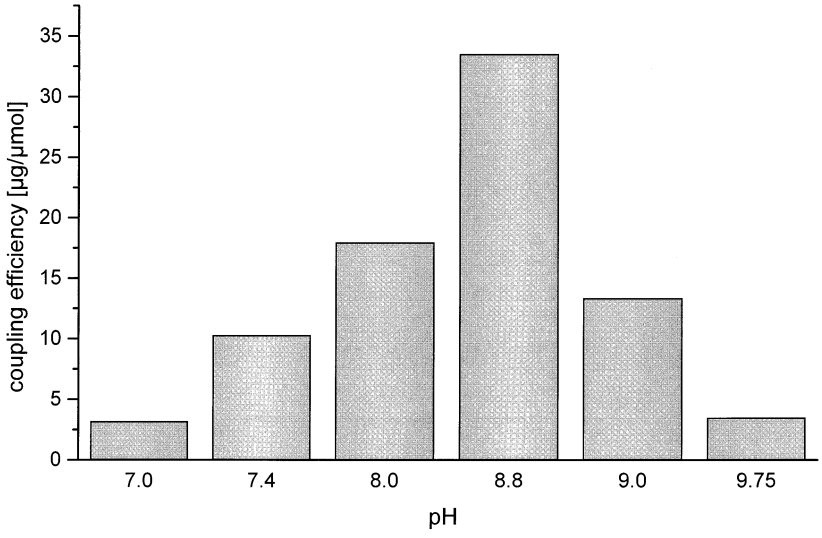


Fig. 2. Dependence of antibody coupling ability of cyanur-PEG-PE IL on pH (borate). Data represent means of three experiments. Maximum deviations from the mean value in each experiment were below 15%.

Any possible danger to organisms potentially caused by cyanuric chloride because of its reactivity has been investigated thoroughly. Although the free cyanuric chloride is regarded as a sensory respiratory irritant (Rydzynski and Jedrychowski, 1994), comprehensive cell compatibility studies have proven that cyanuric chloride does not cause accute or chronic toxicity or genotoxic or mutagenic effects (Wyszynska et al., 1994). However, cyanuric chloride can be regarded as safe since its reactivity is pH-controlled. While substitutions occur in a basic medium around pH 8.5 (which is acceptable for proteins), cyanuric chloride is nearly non-reactive under neutral physiological pH. Thus, cyanuric chloride seems to be particularly suitable to link the terminal PEG hydroxy groups with amino groups of proteins to obtain liposomal drug targeting systems.

In the first step of synthesis, cyanuric chloride was used to attach a PEG chain to a phospholipid headgroup. For this, cyanuric chloride was coupled to the amino group of DPPE. To avoid the formation of dimeric products upon attaching the PEG chain to this derivative, we used the heterobifunctional PEG derivative α-amino-ω-hydroxy-PEG 2000. Because of its higher reactivity, the amino group was selectively coupled resulting in complete conversion to a PEG-PE with terminal hydroxy group. To get a terminal-activated PEG derivative, the hydroxy groups were reacted with free cyanuric chloride. Cyanuric chloride was used in a 0.9 molar ratio to reduce dimere forming side reactions. The yield was calculated to be about 65%, which we strongly suspect to be due to sterical hindrance.

3.2. Coupling antibodies to cyanur-PEG-PE containing liposomes and comparison to established coupling procedures

To analyze at first the influence of the cyanur-PEG-PE on the liposome stability, we incorporated a scope of amounts between 0.5 and 7.5 mol% of this derivative in our SPC/Chol liposomes. It was evident that this compound was well incorporated into the liposomal membrane and does neither cause increased membrane permeability as indicated by calcein leakage studies (data not shown), nor changes in vesicle size. Dynamic light scattering investigations veryfied mean liposome diameters of about 160 nm (polydispersity index below 0.1) which are similar to

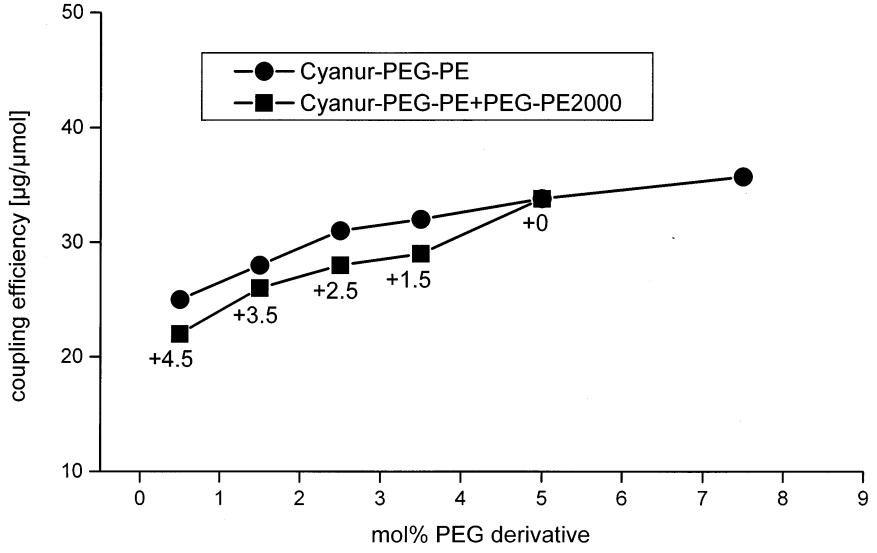


Fig. 3. Antibody coupling ability (µg protein/µmol lipid) of IL in dependence on the cyanur-PEG-PE concentration without (round symbols) and with (angular symbols) added PEG-PE 2000. Data are means of four measurements. Deviations of repeated measurements were below 10 relative%.

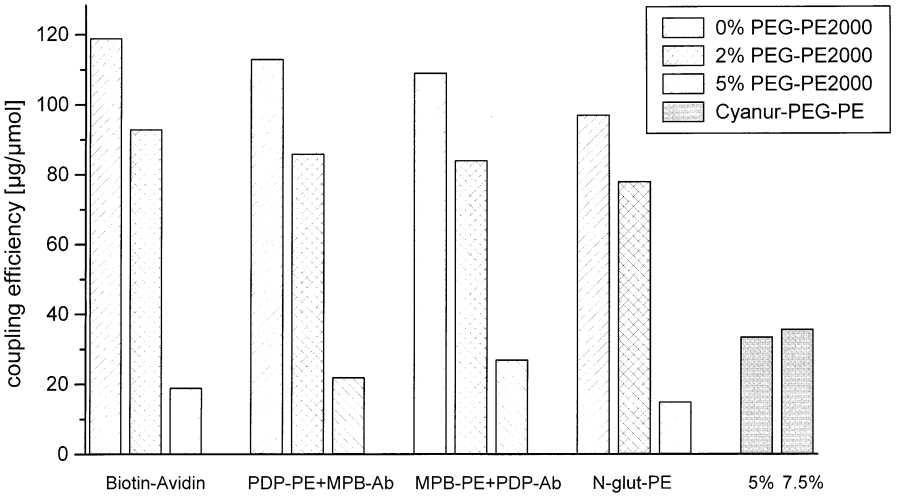


Fig. 4. Amounts of conjugated antibodies by cyanur-PEG-PE IL compared to other coupling methods in the absence or in the presence of 2 and 5 mol% PEG-PE 2000. Data are means of at least three measurements. Deviations from the mean value for repeated measurements were below 15%.

these of the plain SPC/Chol liposomes (about 165 nm). On this basis we started to explore for the optimal antibody coupling conditions.

Initially, we investigated the pH-dependence of the coupling reactions to both find a balance between coupling maximum and a protein-compatible pH and to estimate the chemical reactivity and safety under neutral-physiological conditions. The nucleophilic substitution requires basic conditions. Consequently, we performed several antibody coupling reactions in borate in a pH range between 7 and 9.75 using liposomes with 5 mol% cyanur-PEG-PE. As illustrated in Fig. 2, the coupling results are strongly pH-dependent. At a neutral pH of 7 there is nearly no antibody coupling ability. Raising the basic conditions up to a pH of 8.8 leads to a drastic increase in coupling efficiency. A pH of 8.8 seems to be the optimum for the reaction since stronger basic conditions show drastic reduced coupling results, which should be attributed to a hydrolytic degradation of the cyanuric chloride in strong basic media. Based upon these results, all coupling procedures were consequently performed at a pH of 8.8, and the danger of liposome reactivity with substrates under physiological conditions was regarded as possible to neglect.

The concentration of the reactive anchor lipid in the liposomes is an important factor affecting the antibody coupling efficiency. Thus, we analyzed the amount of conjugated antibodies with regard to the cyanur-PEG-PE concentration, ranging from 0.5 to 7.5 mol%. As illustrated in Fig. 3, the attached protein amount increases with increasing anchor–lipid concentration (round symbols). The increase is more pronounced at small cyanur-PEG-PE concentrations (0.5–2.5 mol%) whereas higher anchor concentrations do not clearly improve the antibody coupling ability.

The small amounts of cyanur-PEG-PE (0.5–2 mol%) are insufficient to form an effective sterical barrier. Further increase of the cyanur-PEG-PE concentration results in stronger sterical stabilization of the liposomes which might also affect the antibody linkage. To focus on these repulsive effects, we repeated these coupling procedures with a constant concentration of PEG derivatives within the liposomes, composed of the analog amounts of cyanur-PEG-PE and added PEG-PE 2000 to reach 5 mol%. Addition of PEG-PE 2000 does not strongly influence the protein attachment (angular symbols), as the comparison in Fig. 3 illustrates. This confirms the prominent position of the terminal cyanuric chloride at the liposome

surface which is unaffected by sterical stabilizing derivatives.

In order to compare these binding data to well established antibody coupling procedures, we chose a spectrum of four chemically different coupling methods which lead to conventional IL, with antibodies directly linked to the liposome surface. We performed the non-covalent biotin-avidin method and three covalent coupling mechanisms using PDP-PE, MPB-PE and *N*-glutaryl PE. In the non-covalent biotin-avidin method, avidin with its four binding sites functions to cross-link biotinylated antibodies and biotinylated lipids in the liposomal membrane. In literature, the two thioreactive procedures using PDP-PE or MPB-PE are the most favoured methods for the preparation of conventional IL. Their chemical basis is a thioether-linkage between a thiol group (reduced form of PDP-PE or PDP-antibody) and the corresponding maleimide group of the MPB-antibody or MPB-PE. The detailed mechanisms of these procedures have been described and reviewed by Allen et al. (1994), Hansen et al. (1995).

Upon activating the free carboxyl-group of *N*-glutaryl-PE with a water soluble carbodiimide (EDC) and *N*-hydroxy-sulfosuccinimide, *N*-glutaryl PE creates an amide bond to an amino group of the protein (Weissig et al., 1986). In our previous investigations we have found the coupling at neutral pH without sulfosuccinimide as the most effective variant of this method (Bendas et al., 1998).

Fig. 4 compares the coupling data of these methods with the cyanur-PEG-PE procedure. In addition to this, we incorporated two different concentrations of PEG-PE 2000 to investigate the influence of a sterical liposome stabilization on these procedures.

Considering the PEG-free preparations, it is evident that all the used conventional methods bind the antibodies much more effective than the cyanur-PEG-PE liposomes. Most of these results are in accordance to data from literature (Hansen et al., 1995). As has already been described in literature, these conventional coupling procedures are strongly be influenced by the addition of

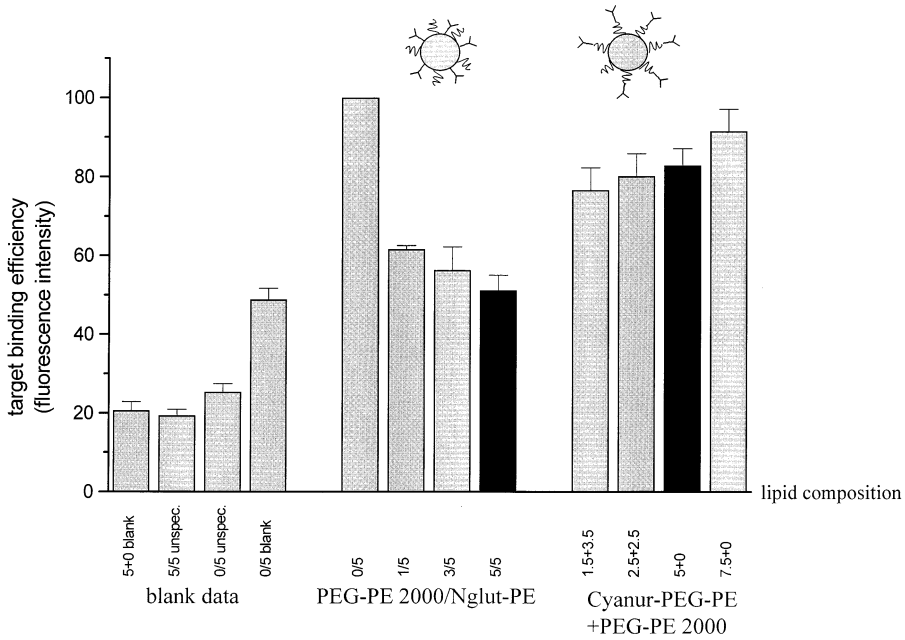


Fig. 5. Comparison of in vitro target cell binding of cyanur-PEG-PE IL and conventional IL (*N*-glut-PE). Data are given in relative fluorescence intensities, taken the PEG-free conventional IL (specific and unspecific liposome-cell interactions) as standard of 100. Data are means of five measurements ± S.D.

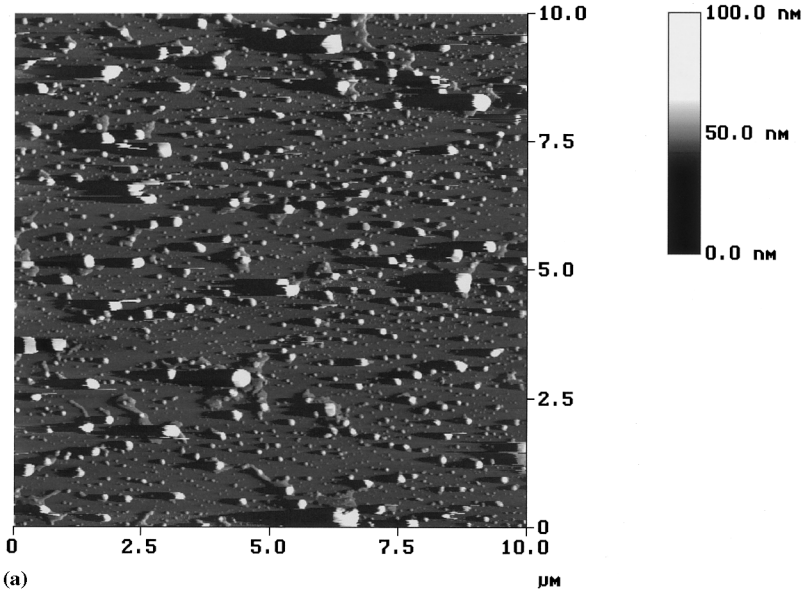


Fig. 6. (a) AFM image in height modus of a cyanur-PEG-PE IL dispersion. (b) AFM image and surface scan in amplitude modus along the body of one cyanur-PEG-PE IL. The scan indicates no detectable surface structures in the dimension of antibodies. (c) AFM scan of surface structures at the liposomal border zone (on top of Fig. 6b). The trimeric structure represents the dimension and the typical appearance of one IgG molecule.

PEG-PE. Whereas 2 mol% PEG-PE 2000 cause only a slight shift in the coupling results, further increase in the PEG concentration leads to a drastically reduced antibody coupling efficiency, which should be attributed to a sterical hindrance of the reactivity at the liposome surface by PEG. This has rendered the effective application of these conventional IL, since a concentration of about 5 mol% PEG PE 2000 has been proved to be essential for sterical stabilization and prolonged circulation. At these PEG concentrations, the cyanur-PEG-PE liposomes are more effective in conjugating proteins.

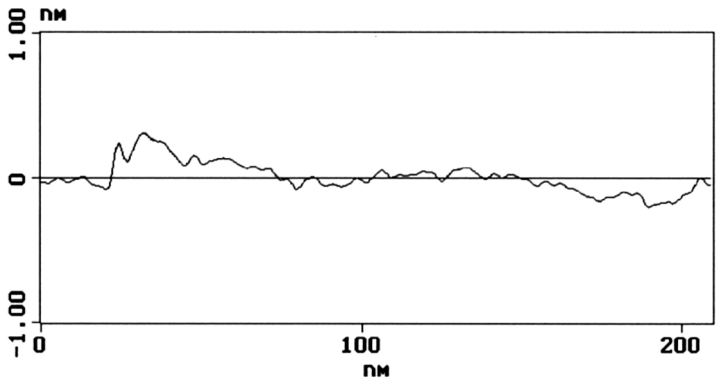
3.3. In vitro cell binding studies

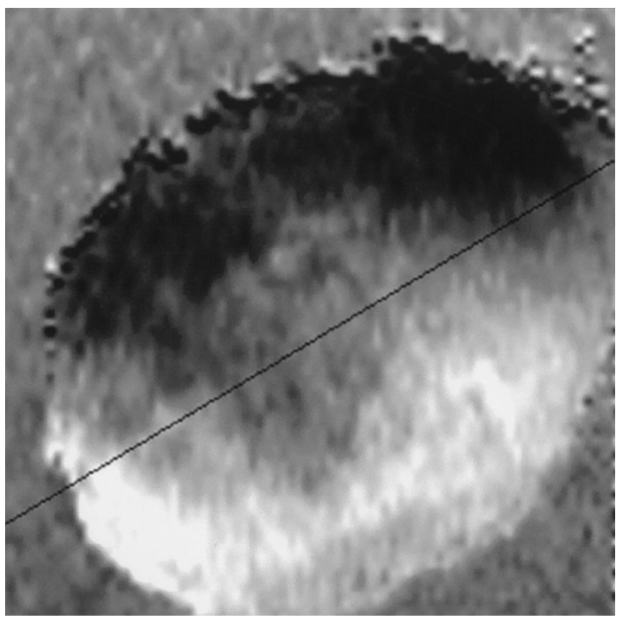
In order to investigate the influence of the antibody coupling results on the targetability of the liposomes, we performed several in vitro cell binding studies. To evaluate the cell binding ability of the cyanur-PEG-PE IL, we compared them with the behavior of conventional IL. Being compatible to our experiences and previous results with this technique (Bendas et al., 1997, 1998), we chose *N*-glut-PE IL for comparison. E-selectin, which is an inflammation-related surface receptor on endothelial cells, was chosen as target molecule. Normally, E-selectins were expressed strictly time and spatially related in response to inflammatory stimuli to mediate a trafficking of leukocytes to sites of inflammation (for review: McEver, 1997). This has offered a very promising new principle for targeting drug delivery systems to inflammatory sites (Spragg et al., 1997; Bendas et al., 1998). For the present study we chose Chinese Hamster Ovarial (CHO)-cells, which stably express a certain amount of E-selectins. Therefore, anti-E-selectin IgG were conjugated to the liposomes, and the binding of the fluorescently labeled IL to the cells has either been analyzed in a well plate assay or by flow cytometry.

We started by analyzing the cell binding ability of the conventional *N*-glut PE IL. The PEG-free population (0/5) shows a strong tendency to bind the CHO-E cells, as illustrated in Fig. 5. This value represents specific (antibody-selectin bonds) and unspecific liposome–cell bindings and was regarded as standard with fluorescence intensity

of 100. To determine the fraction of unspecific liposome–cell interactions of this value, we stepwise increased the PEG-PE 2000 concentration (1/5–5/5). Furthermore, we analyzed various blank liposomes, either with a plain surface or coupled with unspecific IgG. As illustrated in Fig. 5, the PEG-free blank liposomes (0/5) without protein show a binding of about 50 relative fluorescence units or 25 units with coupled unspecific IgG (0/5 unspec.), which should approximately be regarded as the degree of unspecific liposome binding. PEG-containing blank liposomes (5/5) or cyanur-PEG-PE blank liposomes (5 + 0) show lower cell interactions.

Increasing the PEG concentration in the *N*-glut-PE liposomes leads to reduced binding amounts, whereas the most excessive decrease is achieved at 1 mol% PEG-PE. The latter facts maintain that this PEG-PE concentration is nearly sufficient to depress most of the unspecific interactions under the in vitro conditions of our experiments. Applying higher PEG-PE concentra-





(b)

Fig. 6. (*Continued*)

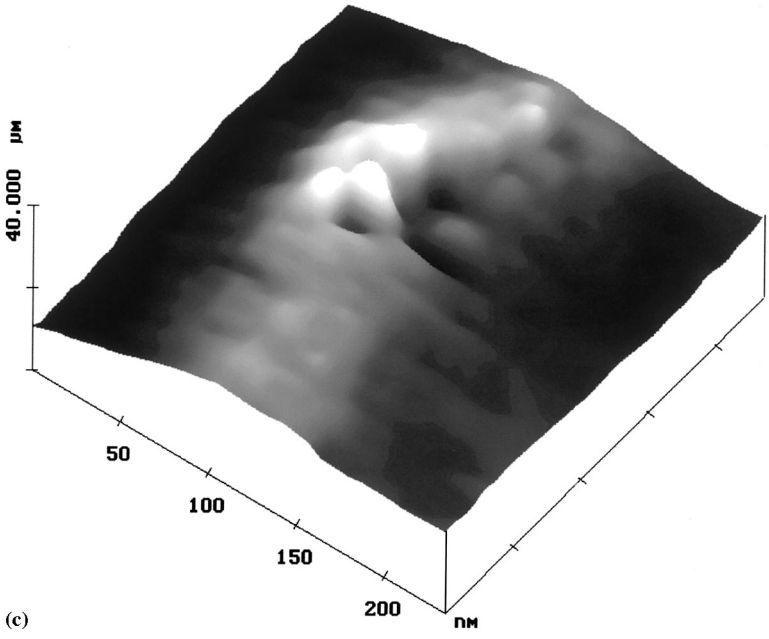


Fig. 6. (*Continued*)

tion, which were necessary for in vivo applications, further reduce the cell binding ability, which should presumably be caused by either a reduced antibody accessibility or by the reduced antibody coupling results (Section 3.2.).

The terminal coupled IL, which themselves mediate a sterical stabilization, were analyzed in accordance to the cyanur-PEG-PE concentration, with added PEG-PE 2000 to reach 5 mol% PEG derivatives. Fig. 5 clearly indicates in detail that all cyanur-PEG-PE liposomes are more effective in cell binding than the conventional IL, despite of their partly less effective antibody conjugation results shown in Fig. 4. The increase in cyanur-PEG-PE concentration leads to higher cell binding results, whereby the most convincing result can be obtained using 7.5 mol% cyanur-PEG-PE, which is about 1.8-fold more effective that the cell binding by conventional IL. This clearly indicates the advantage of terminal coupled antibodies for targeting by combining a reduced unspecific cell binding with improved antibody accessibility. Under in vivo conditions, where higher amounts of PEG-PE would probably be necessary to achieve prolonged circulation, we expect the differences between conventional IL on one hand, and the cyanur-PEG-PE IL on the other to be more pronounced.

3.4. Atomic force microscopic investigations

AFM is a high resolution method with the ability to illustrate the surface topology and characteristics of biological or model membranes on solid supports. The application of this technique to the field of IL with the aim of illustrating antibodies at the membrane surface has never been described. We tried to illustrate the liposome surfaces with this technique. As we used two different IL population in this study (terminal coupled versus conventional IL) which obviously differ in their antibody accessibility and flexibility, we tried to correlate their surface appearance with their targetability.

As illustrated in Fig. 6a, the cyanur-PEG-PE liposomes appear as a very homogenous dispersion with a mean liposome size of about 150 nm, which excellently corresponds to the dynamic light scattering results, indicating mean liposome diameters of about 160 nm. Investigating a cya-

nur-PEG-PE IL in detail (Fig. 6b) we can see that it is shaped ideally round, but obviously without coupled antibodies along the scanned surface. IgG molecules that are in the dimension of about 10–15 nm, should unquestionably be visible in the surface topology scan. Still, only in the edge area on top of the vesicle, homogenous surface structures could be detected at the membrane border, those can be regarded as attached proteins. We were able to focus on those surface structures in the border area, demonstrated in Fig. 6c. The magnification of the membrane border area in this image illustrates three round structures with diameters of about 8–10 nm. This should be one complete IgG molecule, because it is in excellent agreement with recent AFM images of IgG, which appear as trimeric structures of this dimension (Fritz et al., 1997). Consequently, we can optically prove that IgGs were coupled to the cyanur-PEG-PE IL, which confirms our coupling results (Section 3.2.). We conclude, that we can perfectly illustrate coupled IgGs in the border zone of the cyanur-PEG-PE IL, but not at the entire liposome body. To gain confirmation whether this is a technical problem of AFM or is rather caused by the characteristics of the cyanur-PEG-PE IL, we analyzed the behavior of the *N*-glut-PE IL.

The strong magnification of one *N*-glut-PE IL in Fig. 7a gives us good reason to believe that there is a homogenous distribution of surface structures. The analysis in the height modus reveals a surface coverage by structures of about 7–8 nm in size. This can be explained by a relatively dense coupling of antibodies around the vesicle. *N*-glut PE liposomes are more effective in antibody coupling than the cyanur-PEG-PE liposomes. Still, this can not solely explain the differences in the AFM images. We tried to find the reason in changes in the surface/protein flexibility, investigating why proteins on the cyanur-PEG-PE liposomes could not be scanned. Antibodies on these liposomes are attached via a PEG 2000 anchor, which is about 4 nm in length grafted from the surface. This should not strongly influence the size of the proteins, but their flexibility to some extent, because the PEG chain is certainly coiled randomly, causing high protein mobility. Contrary, antibody immobilization directly on the liposome surface at the *N*-glut PE vesicles would lead to restricted protein mobility, because several

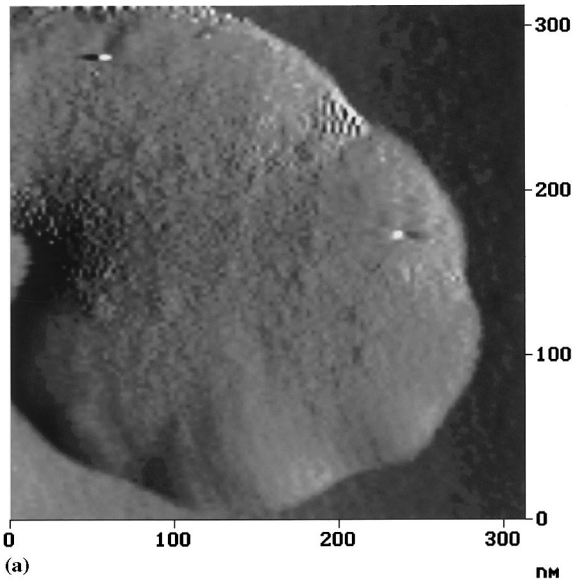
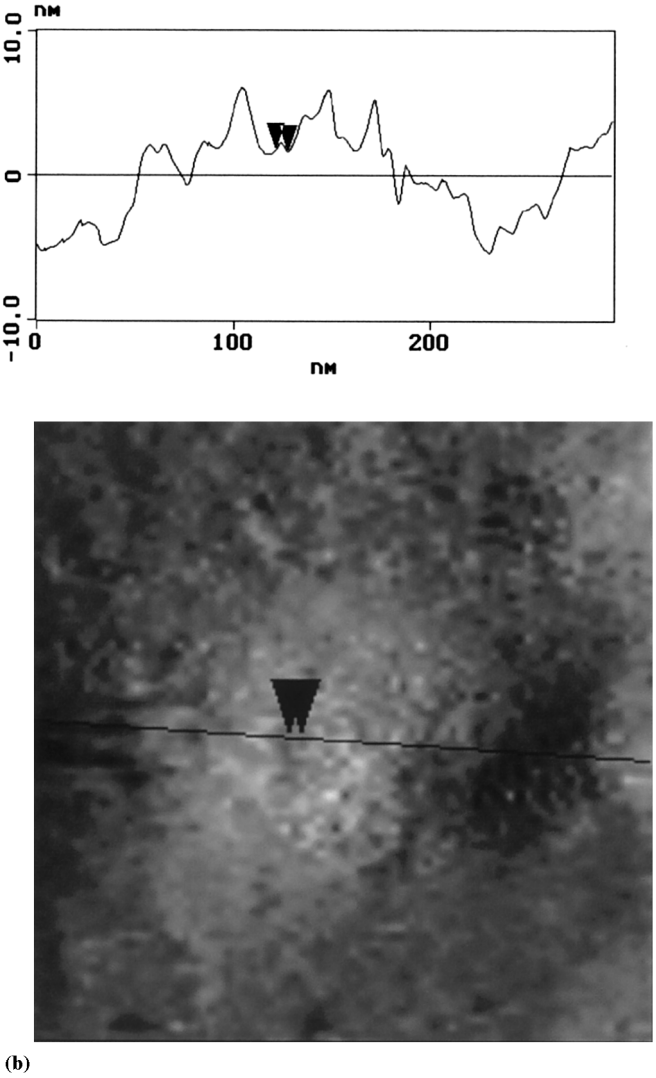


Fig. 7. (a) Surface structure of a *N*-glut PE IL with coupled IgGs scaned by AFM with amplitude modus. (b) Determination of the dimension of the surface structures at a *N*-glut-PE IL. Structures are in the dimension of 5–10 nm and homogenously distributed along the surface, as indicated by the scan line.



(b)

Fig. 7. (*Continued*)

anchor molecule bind one antibody. Because AFM images result from a direct, force-induced scan along the membrane surface, the highly flexible proteins at the cyanur-PEG-PE liposomes can not exactly be imaged as proteins on *N*-glut-PE IL. The latter affair indirectly proves differences in the protein attachment in both kinds of liposomes, which we suspect be the reason for their differences in their targetability. These explanations are in excellent agreement with our in vitro cell binding experiments and prove the advantage of terminal antibody coupling for IL.

4. Conclusions

In the present study we introduced a new liposomal membrane anchor for the covalent attachment of proteins to liposomes. On the basis of the reactivity of cyanuric chloride, antibodies can

simply be coupled to the distal ends of liposomal grafted PEG-chains without previous derivatizations. The antibody coupling conditions were optimized and were compared to other, well established coupling procedures. The resulting IL show sterical stabilization combined with superior targetability. Compared to conventional IL, these terminal coupled IL bind E-selectins as their molecular target structures under the used in vitro conditions much more effective, which was attributed to a better accessibility and flexibility of the antibodies at the liposome surface. AFM images of these liposomes confirm this fact and display a higher protein flexibility of the terminal coupled antibodies.

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References

Allen, T.M., Agrawal, A.K., Ahmad, I., Hansen, C.B., Zalipsky, S., 1994. Antibody-mediated targeting of long-circulating (Stealth®) liposomes. J. Liposome Res. 4, 1–25.

Allen, T.M., Brandeis, E., Hansen, C.B., Kao, G.Y., Zalipsky, S., 1995. A new strategy for attachment of antibodies to sterically stabilized liposomes resulting in efficient targeting to cancer cells. Biochim. Biophys. Acta 1237, 99–108.

Bendas, G., Vogel, J., Bakowsky, U., Krause, A., Müller, J., Rothe, U., 1997. A liposome-based model system for the simulation of lectin-induced cell adhesion. Biochim. Biophys. Acta 1325, 297–308.

Bendas, G., Krause, A., Schmidt, R., Vogel, J., Rothe, U., 1998. Selectins as new targets for immunoliposome-mediated drug delivery—A potential way of anti-inflammatory therapy. Pharm. Acta Helv. 73, 19–26.

Blume, G., Cevc, G., 1990. Liposomes for the sustained drug release in vivo. Biochim. Biophys. Acta 1029, 91–97.

Blume, G., Cevc, G., Crommelin, M.D.J.A., Bakker-Woudenberg, I.A.J.M., Kluft, C., Storm, G., 1993. Specific targeting with poly (ethyleneglycol)-modified liposomes: coupling of homing devices to the ends of the polymeric chains combines effective target binding with long circulation times. Biochim. Biophys. Acta 1149, 180–184.

Chua, M.M., Fan, S.T., Karush, F., 1984. Attachment of immunoglobulins to liposomal membranes via protein carbohydrate. Biochim. Biophys. Acta 800, 291–300.

Derksen, J.T.P., Marselt, H.W.M., Scherphof, G.L., 1988. Uptake and processing of immunoglobulin-coated liposomes by subpopulations of rat liver macrophages. Biochim. Biophys. Acta 971, 978–984.

Fritz, J., Anselmetti, D., Jarchow, J., Fernandez-Busquets, X., 1997. Probing single biomolecules with atomic force microscopy. J. Struct. Biol. 119, 165–171.

Gombotz, W.R., Wang, G.H., Horbett, T.A., Hoffman, A.S., 1991. Protein adsorption to poly(ethylene oxide) surfaces. J. Biomed. Mater. Res. 25, 1547–1562.

Hansen, C.B., Kao, G.Y., Moase, E.H., Zalipsky, S., Allen, T.M., 1995. Attachment of antibodies to sterically stabilized liposomes: evaluation, comparision and optimization of coupling procedures. Biochim. Biophys. Acta 1239, 133–144.

Harasym, T.O., Tardi, P., Longman, S.A., Ansell, S.M., Bally, M.B., Cullis, P.R., Choi, S.L., 1995. Poly(ethylene glycol)-modified phospholipids prevent aggregation during covalent conjugation of proteins to liposomes. Bioconjugate Chem. 6, 187–194.

Heath, T.D., 1987. Covalent attachment of proteins to liposomes. Methods Enzymol. 149, 111–118.

Holmberg, E., Maruyama, K., Litzinger, D.C., Wright, S., Davis, M., Kabalka, G.W., Kennel, S.J., Huang, L., 1989. Highly efficient immunoliposomes prepared with a method which is compatible with various lipid compositions. Biochem. Biophys. Res. Commun. 165, 1272–1278.

Klibanov, A.L., Maruyama, K., Torchilin, V.P., Huang, L., 1990. Amphipatic polyethyleneglycols effectively prolong the circulation time of liposomes. FEBS Lett. 268, 235–237.

Klibanov, A.L., Maruyama, K., Beckerleg, A.M., Torchilin, V.P., Huang, L., 1991. Activity of amphipathic poly (ethyleneglycol) 5000 to prolong the circulation time of liposomes depends on the liposome size and is unfavorable for immunoliposome binding to target. Biochim. Biophys. Acta 1062, 142–148.

Loughrey, H.C., Choi, L.S., Cullis, P.R., Bally, M.B., 1990. Optimized procedure for the coupling of proteins to liposomes. J. Immunol. Methods 132, 25–35.

Maruyama, K., Takizawa, T., Yuda, T., Kennel, S.J., Huang, L., Iwatsuru, M., 1995. Targetability of novel immunoliposomes modified with amphipathic poly (ethyleneglycol)s conjugated at their distal terminals to monoclonal antibodies. Biochim. Biophys. Acta 1234, 74–80.

Maruyama, K., Takizawa, T., Takahashi, N., Tagawa, T., Nagaike, K., Iwatsura, M., 1997. Targeting efficiency of PEG-immunoliposome-conjugated antibodies at PEG terminals. Adv. Drug Deliv. Rev. 24, 235–242.

McEver, R.P., 1997. Selectin-carbohydrate interactions during inflammation and metastasis. Glycocojugate J. 14, 585–591.

Rydzynski, K., Jedrychowski, R., 1994. Sensory irritating properties of cyanuric chloride as revealed with plethysmographic method. Int. J. Occup. Med. Environ. Health. 7, 149–154.

Spragg, D.D., Alford, D.R., Greferath, R., Larsen, C.E., Lee, K.D., Gurtner, G.C., Cybulsky, M.I., Tosi, P.F., Nicolau, C., Gimbrone, M.A., 1997. Immunotargeting of liposomes to activated vascular endothelial cells: A strategy for site-selective delivery in the cardiovascular system. Proc. Natl. Acad. Sci. U.S.A. 94, 8795–8800.

Suzuki, T., Kanbara, N., Tomono, T., Hayashi, N., Shinohara, I., 1984. Physicochemical and biological properties of poly(ethylene glycol)-coupled immunoglobulin G. Biochim. Biophys. Acta 788, 248–255.

Torchilin, V.P., Omelyanenko, V.G., Papisov, M.I., Bogdanov, A.B., Trubetskoy, V.S., Herron, J.N., Gentry, C.A., 1994. Poly(ethylene glycol) on the liposome surface: on the mechanism of polymer-coated liposome longevity. Biochim. Biophys. Acta 1195, 11–20.

Weissig, V., Lasch, J., Klibanov, A., Torchilin, V.P., 1986. A new hydrophobic anchor for the attachment of proteins to liposomal membranes. FEBS Lett. 202, 86–90.

Woodle, M.C., Lasic, D.D., 1992. Sterically stabilized liposomes. Biochim. Biophys. Acta 1113, 171–199.

Wyszynska, K., Przybojewska, B., Spiechowicz, E., Chwialkowska-Liro, W., Dziubaltowska, E., Rydzynski, K., 1994. Cyanuric chloride has no genotoxic and mutagenic properties in bacteria and bone marrow cells. Int. J. Occup. Med. Environ. Health. 7, 281–289.