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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

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Abstract

We have attempted to simplify the procedure for coupling various ligands to distal ends of liposome-grafted polyethylene glycol (PEG) chains and to make it applicable for single-step binding of a large variety of a primary amino group-containing substances, including proteins and small molecules. With this in mind, we have introduced a new amphiphilic PEG derivative, *p*-nitrophenylcarbonyl-PEG-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (pNP-PEG-DOPE), synthesized by reaction of DOPE with excess of bis(*p*-nitrophenylcarbonyl)-PEG in a chloroform/triethylamine mixture. pNP-PEG-DOPE readily incorporates into liposomes via its PE residue, and easily binds primary amino group-containing ligands via its water-exposed pNP groups, forming stable and non-toxic urethane (carbamate) bonds. The reaction between the pNP group and the ligand amino group proceeds easily and quantitatively at pH around 8.0, and remaining free pNP groups are promptly eliminated by spontaneous hydrolysis. Therefore, pNP-PEG-DOPE could serve as a very convenient tool for protein attachment to the distal ends of liposome-grafted PEG chains. To investigate the applicability of the suggested protocol for the preparation of long-circulating targeted liposomes, we have coupled several proteins, such as concanavalin A (ConA), wheat germ agglutinin (WGA), avidin, monoclonal antimyosin antibody 2G4 (mon2G4), and monoclonal antinucleosome antibody 2C5 (mon2C5) to PEG-liposomes via terminal pNP groups and studied whether the specific activity of these immobilized proteins is preserved. The method permits the binding of several dozens protein molecules per single 200 nm liposome. All bound proteins completely preserve their specific activity. Lectin-liposomes are agglutinated by the appropriate polyvalent substrates (mannan for ConA-liposomes and glycophorin for WGA-liposomes); avidin-liposomes specifically bind with biotin-agarose; antibody-liposomes demonstrate high specific binding to the substrate monolayer both in the direct binding assay and in ELISA. A comparison of the suggested method with the method of direct membrane incorporation was made. The effect of the concentration of liposome-grafted PEG on the preservation of specific protein activity in different coupling protocols was also investigated. It was also shown that pNP-PEG-DOPE-liposomes with and without attached ligands demonstrate increased stability in mouse serum.

Keywords: Liposome; Long-circulating liposome; Immunoliposome; Polyethylene glycol; Protein immobilization; *p*-Nitrophenylcarbonyl

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1. Introduction

Liposomes have long been considered promising pharmaceutical carriers in vivo [1,2] and have already found their way into a real clinical practice [3–5]. It was believed that the use of targeted liposomes, i.e. liposomes with a specific affinity for the affected organ or tissue, should increase the efficacy of the liposomal pharmaceutical agents [6]. To target liposomal pharmaceuticals to organs other than the liver and spleen, liposomes have been modified with specific targeting moieties (antibodies) [7]. However, similar to 'plain' liposomes, immunoliposomes also do not exhibit prolonged circulation and fail to accumulate sufficiently in targets with diminished blood supply and/or low antigen concentration [8,9].

On the other hand, to make liposomes capable of delivering pharmaceutical agents to targets other than the reticulo-endothelial system, attempts have been made to prolong their lifetime in the circulation. Such long-circulating liposomes were expected to accumulate in various areas with 'leaky' vasculature, such as infarcts and tumors [8–12], via the so-called enhanced permeability and retention (EPR) effect [13,14]. The problem of how to achieve long circulation was solved with the development of polymer-coated liposomes with decreased opsonization rate and sharply increased circulation times (sterically protected liposomes, 'stealth' liposomes, long-circulating liposomes) [15]. Alternatively, it was repeatedly shown that small liposomes also possess an increased longevity compared to liposomes of a larger size [16–19]. However, since the entrapment capacity of liposomes for drugs strongly diminishes with decrease of liposome size, larger polymer-coated liposomes were considered more promising pharmaceutical carriers. The usual preparation method of such liposomes includes liposome coating with poly(ethylene glycol) or PEG [20–22], though some other polymers can also be successfully used [23–27].

Further development of the concept of long-circulating liposomes involves the attempt to combine the properties of long-circulating liposomes and immunoliposomes in a single preparation [8,9]. Such an approach is especially important for efficient targeted delivery of liposomal drugs to targets with diminished blood supply and/or low antigen concentration, when increased circulation times for efficient liposome-to-target interaction are required. This approach requires the simultaneous presence of both the protecting polymer and the targeting moiety (usually a monoclonal antibody or its fragment) on the liposome surface.

It is evident that the protective polymer may create steric hindrances for target recognition with the targeting moiety if simple co-incorporation of an antibody and protecting polymer into the liposome membrane is performed [28–30], though those hindrances can be overcome by thorough selection of a ratio (relative quantity) of protective polymer and targeting moiety on the liposome surface. Using this approach, we were able to achieve effective targeting of long-circulating immunoliposomes into infarcted myocardium in vivo [8]. On the other hand, the targeting moiety can be attached above the protecting polymer layer, for example, by coupling it with the distal end of an activated liposome-grafted polymer molecule. Several protocols were developed to prepare PEG and other polymers which contain both a hydrophobic group at one end and a reactive group on the other end, and to attach antibodies to such polymers on liposomes [31–37]. Thus, maleimide-derivatized antibody was coupled to liposome surface-attached PEG containing thiol groups on its distal termini [32,33]. A comparison was made in [31,32] between liposomes with antibody and polymer co-incorporated into the membrane and liposomes with antibody attached to PEG termini, which clearly demonstrated better characteristics (binding efficacy, association with target cells) of immunoliposomes with PEG termini-attached antibodies. Similar results were obtained with partially oxidized antibodies coupled to the PEG terminus through a terminal hydrazide group [34]. Antibodies were also coupled to terminal carboxylic groups of amphiphilic PEG via water-soluble carbodiimide [35]. In [36], long-circulating immunoliposomes were prepared by antibody attachment to PEG end groups functionalized with cyanuric chloride. All mentioned protocols, though quite effective, require a separate step of antibody pre-modification or involve the use of toxic intermediates.

To further simplify the coupling procedure and to make it applicable for single-step binding of a large variety of amino group-containing ligands (including antibodies, proteins and small molecules) to the dis-

tal end of liposome-attached polymeric chains without the use of any potentially toxic compounds, we have introduced a new amphiphilic PEG derivative, *p*-nitrophenylcarbonyl-PEG-PE (pNP-PEG-PE) [38]. pNP-PEG-PE readily incorporates into liposomes via its phospholipid residue, and easily binds any amino group-containing compound via its water-exposed pNP group forming a stable and non-toxic urethane (carbamate) bond [39,40]. The use of a similar carbamate linker for the attachment of amino acids via their N-termini to (hydroxymethyl)polystyrene was also described in [41]. The reaction between the pNP group and the ligand amino group proceeds easily and quantitatively at pH around 8.0, while excessive free pNP groups are easily eliminated by spontaneous hydrolysis. The current study has been undertaken to further investigate the efficacy of protein binding to pNP-PEG-PE-containing liposomes, as well as specific activity preservation of liposome-attached proteins.

2. Experimental

2.1. Materials

Egg phosphatidylcholine (PC), cholesterol (Chol), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), polyoxyethylene(MW 2000)-DOPE (PEG-DOPE), stearylamine, and *N*-glutaryl phosphatidylethanolamine (NGPE) were from Avanti Polar Lipids. Concanavalin A (ConA), wheat germ agglutinin (WGA), avidin, biotin-agarose, glycophorin, α-methyl mannoside, L-fucose, diaminobutane, *p*-nitrophenol, triethylamine (TEA), octyl glycoside (OG), diethylenetriaminepentaacetic acid anhydride (DTPA), mannan from *Saccharomyces cerevisiae*, and polyoxyethylene(MW 3500)-bis(*p*-nitrophenyl carbonate) (PEG-$(pNP)_2$) were products of Sigma. 1-Ethyl-3-(3′-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysulfosuccinimide (NHS) were from Pierce. $^{111}InCl_3$ was obtained from NEN. All solvents and components of buffer solutions were analytical grade products.

Cell lines producing cardiac myosin-specific monoclonal antibody 2G4 (mon2G4) [42,43] and nucleosome-specific monoclonal antibody 2C5 (mon2C5) [44,45] (both belonging to the immunoglobulin (IgG) class) are available in our laboratory. Monoclonal antibodies were purified from corresponding murine ascites according to the standard methods of ammonium sulfate precipitation, DEAE-cellulose anion exchange chromatography, and chromatofocusing over a pH gradient of 7.0–5.0. The purity of antibody preparations was characterized by gel electrophoresis and HPLC. Since mon2C5 is very sensitive to various chemical modifications, this antibody was protected from inactivation by reversible complexation with dextran sulfate (Samokhin et al., to be published elsewhere) for all attachment and labeling procedures.

2.2. Methods

2.2.1. Synthesis of pNP-PEG-DOPE

24 mg (32.2 μmole) of DOPE was dissolved in chloroform to obtain a 50 mg/ml solution. The solution was supplemented with 80 μl (approx. 2-fold molar excess over PEG-$(pNP)_2$) of TEA. 1 g (approx. 10-fold molar excess over DOPE) of PEG-$(pNP)_2$ dissolved in 5 ml of chloroform was added to the mixture and the sample was incubated overnight at room temperature with stirring under argon. Then, the organic solvents were removed using a rotary evaporator. The pNP-PEG-DOPE micelles were formed in 0.01 M HCl, 0.15 M NaCl using water bath sonication. The micelles were separated from the unbound PEG and released pNP on a CL-4B column using 0.01 M HCl, 0.15 M NaCl as an eluent. Pooled fractions containing pNP-PEG-DOPE were freeze-dried, and pNP-PEG-DOPE was extracted with chloroform. The latter procedure was repeated twice to ensure the complete removal of NaCl from the preparation. pNP-PEG-DOPE was stored as chloroform solution at −80°C.

2.2.2. Liposome preparation

Liposomes were prepared by detergent (OG) removal from a mixture of egg phosphatidylcholine and cholesterol (7:3 molar ratio). The starting lipid mixture in chloroform was argon-dried, vacuumed, solubilized with OG in HEPES-buffered saline (HBS), pH 7.4 (final total lipid concentration may vary from 5 to 20 mg/ml), and dialyzed overnight against HBS at 4°C. Liposomes obtained were sized by repeated passing through the polycarbonate filters

with gradually decreasing pore size, 0.6, 0.4 and 0.2 μm (Nuclepore). When necessary, the initial lipid mixture was supplemented with various quantities of PEG-DOPE, pNP-PEG-DOPE, or their mixture in a required molar ratio. When PEG-DOPE- and/or pNP-PEG-DOPE-containing liposomes were prepared, the lipid/polymer film was dissolved in citrate, pH 5.1 (slightly acidic pH prevents the pNP residue from fast hydrolysis) with an excess of OG, and the solution was dialyzed against Na-citrate. Additionally, 1 mol% of DTPA-stearylamine (DTPA-SA) was added to all lipid compositions, when subsequent radiolabeling of liposomes with ^{111}In was required.

2.2.3. Preparation of radioactive labels and labeling of liposomes with ^{111}In

When necessary, liposomes were radioactively labeled with ^{111}In via the liposome-incorporated amphiphilic chelating agent DTPA-SA. DTPA-SA was synthesized according to recommendations of [46] with some changes. Briefly, 1.55 g of SA and 2.5 g of DTPA cyclic anhydride were mixed with 250 ml of dry chloroform. The mixture was refluxed for 1 h, the top outlet of the system being coated with foil. Then, 3 ml of TEA was added, and the mixture was refluxed for an additional 48 h. Chloroform was evaporated on a rotary evaporator, its traces were removed by heating with water at 50°C. 100 ml of 0.1 N HCl was added to the dry product, and the mixture was stirred with heating at 80°C for 10 min and incubated overnight at room temperature. The precipitate was separated by centrifugation, washed three times with 100 ml of 0.1 N HCl with stirring and then lyophilized. The lyophilized product was washed twice with 100 ml of methanol, and then recrystallized twice from boiling methanol and dried. To label DTPA-SA-containing liposomes with ^{111}In, the liposomal suspension (normally, 2 ml) was supplemented with 30 μl of 1.0 M Na-citrate and incubated for 1 h with a required quantity of citrate complex of ^{111}In (added as $InCl_3$) at room temperature, and then dialyzed overnight against HBS at 4°C to remove unbound ^{111}In.

2.2.4. Characterization of liposomes

Liposome size and size distribution were determined immediately after preparation using a standard method of dynamic light scattering with a Coulter N4+ MD Submicron Particle Size Analyzer (Coulter Electronics). For all polymer-containing liposomes, the efficacy of polymer incorporation into the liposomal membrane was monitored by gel chromatography and/or HPLC in order to check for possible formation and presence of micelles composed of free non-incorporated polymer. The separation is based on the fact that the size of micelles is usually within the 15–60 nm limits, whereas liposomes are approx. 150 nm and up in size.

2.2.5. Proteins

Proteins that were attached to liposomes in our experiments by interaction with the pNP group of liposome-incorporated pNP-PEG-DOPE or by preliminary modification of antibodies with a hydrophobic residue of NGPE [47] and subsequent co-incorporation into liposomes together with PEG-DOPE include ConA, WGA, avidin, mon2G4, and mon2C5.

2.2.6. Antibody modification with NGPE

For incorporation directly into the liposomal membrane, mon2G4 and mon2C5 antibodies were preliminarily modified with the hydrophobic 'anchor', NGPE, as described in [47]. Briefly, 0.3 mg of NGPE were dried with argon from chloroform solution and then solubilized in 0.5 ml of 0.016 M OG in 50 mM MES. The solution was supplemented with 12 mg of EDC and 15 mg of NHS. The mixture was incubated for 5 min and then added to the solution of 2 mg of the antibody in 0.1 M HEPES, pH 7.6. pH was adjusted to 8.0 with 1 M NaOH, and the mixture was incubated overnight at 4°C. The modified antibody was purified by dialysis in a Spectrum dialysis bag (MWCO 1000) overnight against HBS at 4°C and stored at the same temperature until use.

2.2.7. Attachment of proteins to liposomes

To attach proteins to liposomes, two different protocols were used. According to one protocol, protein was pre-modified with NGPE and then hydrophobically anchored into the liposomal membrane during liposome preparation [8,9,47]. This protocol was used with mon2G4 and mon2C5 only. In this case, NGPE-modified antibody ('cold' or ^{111}In-labeled) was added to the dried lipid or lipid/polymer film

of a required composition (approx. 1 mg of modified protein per 10 mg of lipid or lipid/polymer mixture) in OG-containing solubilization buffer. In the process of detergent dialysis and liposome formation, NGPE antibody was quantitatively anchored into the membrane via the hydrophobic NGPE group [8,9]. Antibody incorporation into the membrane is usually quantitative. However, liposomes were additionally purified by gel filtration on Sepharose CL-6B to exclude any non-incorporated antibody from the preparation.

Alternatively, non-modified proteins (ConA, WGA, avidin, mon2G4, and mon2C5) were attached via pNP groups to the distal ends of liposome-grafted DOPE-PEG-pNP chains. For this purpose, proteins were added (approx. 1 mg of protein per 10 mg of lipid/polymer mixture) to pNP-PEG-DOPE-containing liposomes stored at pH 5.1 (Na-citrate buffer), and pH was raised to 8.5. Incubation for about 2 h resulted in a sufficient protein binding and simultaneous hydrolysis of non-reacted pNP groups. Protein-containing liposomes were purified by gel filtration on a Bio-Gel A15M column.

2.2.8. Determination of the rate of hydrolysis and chemical conversion of the p-nitrophenylcarbonyl group in pNP-PEG-DOPE

To investigate a comparative rate of hydrolysis and chemical conversion of pNP groups in pNP-PEG-DOPE, a suspension of pNP-PEG-DOPE micelles with a concentration of 0.4 mg/ml was prepared by addition of 10 mM Na-citrate, 140 mM NaCl, pH 4.0 (CBS) to the dried film of pNP-PEG-DOPE and extensive vortexing. After vortexing in citrate buffer, the lipid-modified polymer formed a transparent system typical for micelles. A 0.5 ml aliquot of the micellar suspension obtained was mixed with 1.5 ml of 2 mM diaminobutane (DAB, a model amino-containing compound capable of chemical interaction with pNP groups) in 100 mM borax, 140 mM NaCl, pH 8.5 (BBS), or with DAB-free BBS within 10 min after the preparation of micelles in CBS. The releasing *p*-nitrophenol (pNP) was registered spectrophotometrically at 405 nm (OD_{405}), with suspensions of the micelles prepared from DOPE-PEG-OH in CBS/BBS with or without DAB used as the blanks (all concentrations were the same as in working suspensions). The molar concentration of the pNP released during the hydrolysis or coupling reactions at different time points was calculated from OD_{405} values using a standard 10 mM pNP solution diluted with the appropriate buffer mixtures.

2.2.9. Radiolabeling of proteins with ^{111}In and determination of the quantity of liposome-bound proteins

To estimate the quantity of a protein bound to a single liposome, proteins (non-modified or, in the case of mon2G4 and mon2C5 antibodies, after the modification with NGPE) were preliminarily radiolabeled with ^{111}In. For this purpose, all proteins were initially modified with DTPA using the DTPA anhydride according to [48]. Aliquots of protein solutions (5–10 mg/ml) in 0.1 M $NaHCO_3$ pH 8.0 were supplemented with 50–100-fold molar excess of DTPA anhydride. After overnight incubation at 4°C, samples were dialyzed against a large excess of 0.1 M Na-phosphate buffer pretreated with Chelex-100 at 1 g resin per liter to better ensure removal of any contaminating trivalent cations. If necessary, the number of moles of DTPA covalently linked to a given protein may be assessed by TNBS assay to determine free amino groups of a protein [49]. Then, all DTPA-modified proteins were labeled with ^{111}In using citrate at pH 5.5–6 as the transchelator. For this purpose, 100 μg of DTPA-protein was supplemented with 1 mCi of $^{111}InCl_3$ in 0.1 M Na-citrate, pH 5.5, and incubated at room temperature for 30 min. Free ^{111}In was removed from the labeled protein by Sephadex G-10 column chromatography or by dialysis against a large volume of HBS. The radioactivity bound with the protein was then determined. Since the quantities of protein and lipid were always known, as well as the number of phospholipid molecules forming a liposome of a given size [50], we could estimate the exact quantity of a given protein associated with a single liposome by measuring the liposome-associated ^{111}In radioactivity after protein attachment to liposomes (whatever attachment protocol was used) and removal of non-bound protein.

2.2.10. Agglutination of lectin-containing liposomes

The carbohydrate binding activities and specificities of liposome-attached lectins were examined using an aggregation assay [51]. The multivalent sub-

strates for ConA (mannan) or WGA (glycophorin) were dissolved in PBS buffer at different concentrations and 100 μl aliquots of the solution obtained were added to suspensions of lectin-containing liposomes (total lipid concentration was adjusted to approx. 1 mg/ml). The mixtures were vortexed, incubated for 20 min at room temperature and then transferred to the UV/VIS spectrophotometer. Liposome aggregation was followed by the turbidity (optical density, OD) increase at 400 nm. The preservation of carbohydrate specificity of liposome-bound lectins was examined by the same agglutination assay after the incubation of the lectin-bearing liposomes with 100 μmol of corresponding inhibitors (α-methyl mannoside for ConA-liposomes and L-fucose for WGA-liposomes).

2.2.11. Binding of avidin-liposomes to biotin-agarose

To examine the preservation of the specific binding activity of the liposome-attached avidin to biotin, we have used a binding assay on biotin-agarose. For this purpose, pNP-PEG-DOPE-containing ^{111}In-labeled liposomes (see above) with attached avidin were applied to the column of biotin-agarose and eluted with HBS. After elution, the column was additionally washed with a large volume of HBS, and then the radioactivity remaining associated with the column was measured. ^{111}In-labeled PC:Chol liposomes, protein-free pNP-PEG-DOPE-liposomes (after complete hydrolysis of pNP) and pNP-PEG-DOPE-liposomes with attached ConA were used as controls.

2.2.12. Determination of antibody-liposome immunoreactivity by direct binding of radiolabeled antibody-liposome with antigen monolayer

To determine the preservation of the specific activity of pNP-PEG-DOPE-liposome-associated mon2G4, we estimated the direct binding of ^{111}In-labeled mon2G4-bearing liposomes with a monolayer of a corresponding antigen: dog cardiac myosin [42,43]. Antigen-coated microtiter plates were prepared following the same procedure as was used for the ELISA protocol (see below). Mon2G4-bearing ^{111}In-labeled liposomes were added to the antigen-coated wells. The maximum count used per 50 μl aliquot was 2×10^6 cpm. The reaction was allowed to proceed until equilibrium, for 4 h at room temperature or overnight at 4°C. The wells were extensively washed to remove loosely bound radioactivity, cut and counted in a γ-scintillation counter for ^{111}In activity. The binding of ^{111}In-labeled mon2G4-liposomes was compared with the binding of ^{111}In-labeled PC:Chol liposomes, protein-free pNP-PEG-DOPE-liposomes (after complete hydrolysis of pNP groups), and mon2C5-liposomes.

2.2.13. Immunological activity of free, modified and liposome-attached antibodies

Generally, the immunological activity of starting antibodies, NGPE-modified antibodies, and antibodies attached to the liposome surface by two different methods was tested by sandwich ELISA. For the myosin-antimyosin assay, 96-well U-bottomed microtiter plates were coated with 50 μl of 10 μg/ml dog cardiac myosin overnight at 4°C. Plates were washed three times with PBS containing 0.05% Tween (PBST, pH 7.4) and blocked with 10% heat-inactivated horse serum albumin to saturate the remaining non-specific binding surfaces and minimize non-specific binding. Serial 10-fold dilutions of various preparations were added to the wells and incubated at 37°C for 1 h. The plates were then washed three times and HRP-tagged goat anti-mouse IgG was added to each well. The plates were again incubated at 37°C for 1 h and washed with PBST. The bound material was revealed by treating with *o*-phenylenediamine hydrochloride as a colored substrate in citrate buffer and OD was read on an ELISA reader at 405 nm. ELISA for nucleosome-antinucleosome antibody was similarly carried out in microtiter plates coated with nucleohistone at 10 μg/ml. All other steps were as described above.

2.2.14. Stability of calcein-loaded liposomes in mouse serum

Liposomes for serum-provoked destabilization experiments were prepared by sonication. A solution of a mixture of PC:Chol (7:3 molar ratio) in chloroform and a required quantity of PEG-DOPE or pNP-PEG-DOPE was dried under argon and additionally freeze-dried using a Freezone 4.5 Freeze Dry System (Labconco). The film obtained was hydrated with 60 mM calcein solution (self-quenching concentration) in HBS, pH 7.4. After sonicating the mixture using a bath-type sonicator (Laboratory Supplies) for 10 min, the calcein-loaded liposomes were sepa-

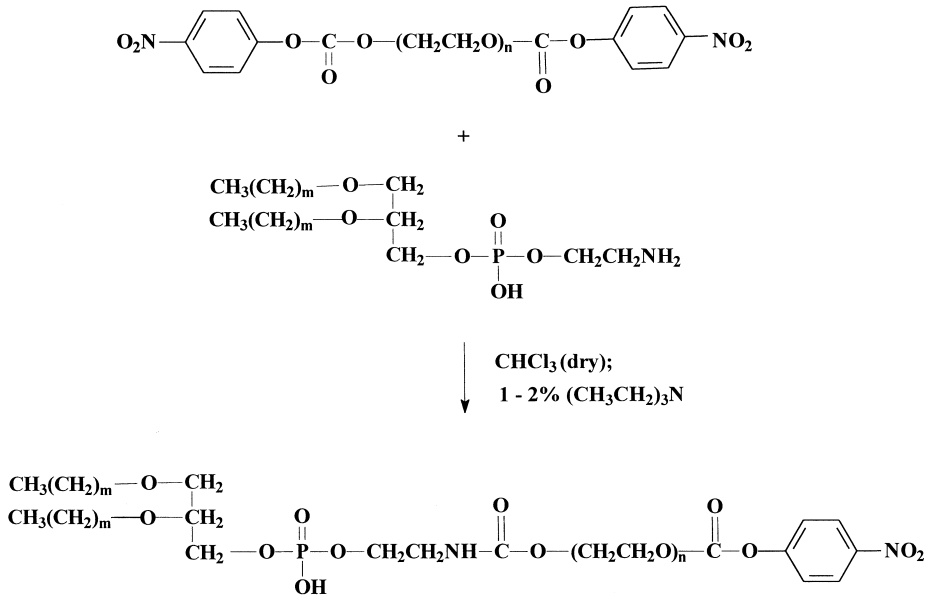

Fig. 1. General reaction scheme for the synthesis of pNP-PEG-PE.

rated from free calcein by column chromatography (Sephadex G-25; 0.8×5.2 cm), and additionally dialyzed overnight at 4°C against HBS. In case when mon2C5-pNP-PEG-DOPE-liposomes were obtained, 60 mM calcein in Na-citrate buffer (pH 5.1) was used for film hydration. After antibody attachment via pNP groups (see above), liposomes were separated from unbound antibody on a Sepharose CL-4B column.

Calcein-loaded liposomes of various compositions were incubated in HBS or in HBS with 50% mouse serum at 25°C for certain time intervals, and the fluorescence intensity of the released calcein was measured using a F-2000 Fluorescence Spectrophotometer (Hitachi Instruments) at an excitation wavelength of 490 nm and an emission wavelength of 530 nm. To determine the total 'releasable' fluorescence, each liposomal preparation was treated by Triton X-100 in order to destroy liposomes and release all the intraliposomal calcein.

3. Results and discussion

3.1. Ligand attachment via free terminal p-nitrophenylcarbonyls and hydrolysis of non-reacted pNP groups

The new single terminus-reactive PEG derivative, pNP-PEG-DOPE, was synthesized according to the general scheme presented in Fig. 1. The derivative obtained is quite stable at slightly acidic pH values (around 5); however, its pNP group easily interacts with amino groups of low molecular weight and high molecular weight ligands at pH values from 8 and up, with the formation of a stable carbamate (urethane) bond (see the reaction scheme in Fig. 2). Since the pNP group itself undergoes hydrolysis at pH values above neutral, one has to be sure that the attachment of amino group-containing ligands to the pNP group in pNP-PEG-DOPE proceeds faster than, or at least at a comparable rate to, pNP hydrolysis. With this in mind, we have investigated a comparative rate of hydrolysis and chemical conversion of pNP groups in pNP-PEG-DOPE at pH 8.5. For this purpose, pNP-PEG-DOPE micelles were prepared, and the rates of pNP group spontaneous hydrolysis and interaction with the model amino group-containing ligand, DAB, were determined following the release of the free *p*-nitrophenol, characteristic of both reactions. The molar concentrations of the pNP released at different time points were calculated from OD_{405} values using as a standard 10 mM solution of the pNP.

The results obtained are presented in Fig. 3. In both cases (in pure BBS and in BBS with DAB) the release of free pNP followed a single exponential function ($R^2 > 0.99$). The half-times of pNP release

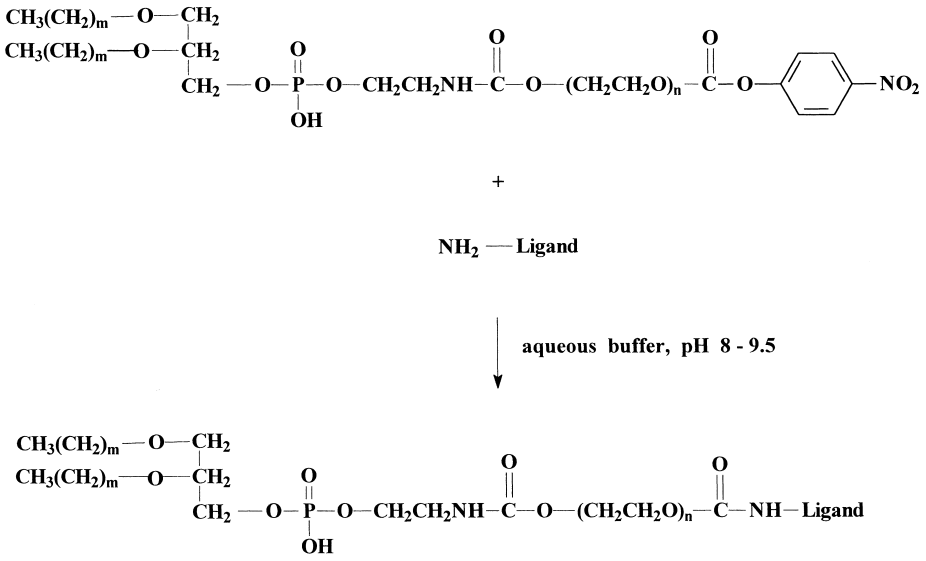

Fig. 2. General reaction scheme for the attachment of amino group-containing ligands to the pNP group.

in BBS, pH 8.5, in the presence of DAB and in BBS alone were 20 and 31 min, respectively. It shows that in the presence of DAB, about one third of the active pNP groups react with the primary amino groups of the ligand. The remaining pNP groups undergo hydrolysis. At higher pH values the fraction of pNP groups reacting with primary amino groups increases (at pH 9.3 about 65% of pNP groups bind the ligand, while only 35% of pNP groups hydrolyze; data not shown). The complete conversion or hydrolysis of pNP groups at pH values above 8 takes between 1 and 2 h. It is worth mentioning that the concentration of free pNP, upon completion of the reaction, was approx. 20 μM, which is about 85% of pNP-PEG-DOPE concentration in the systems. It means that at least 85 mol% of the initial polymer synthesized by us, following the scheme shown in Fig. 1, contained active pNP groups.

Concluding, pNP groups in pNP-PEG-DOPE effectively interact with the primary amino groups of a ligand at pH values of 7.5 and above, while non-reacted pNP groups are eliminated from the system by spontaneous hydrolysis. The relative rates of the coupling and hydrolysis processes can be controlled by the pH value of the reaction system. Even at pH values only slightly above neutral, quite a sufficient number of pNP groups is available for ligand binding.

3.2. *Liposome preparation and characterization; quantitation of liposome-attached protein*

The method of detergent (OG) dialysis yields preparations of similar size (approx. 200 nm) and size distribution for all types of liposomes used – plain PC:Chol (7:3) liposomes, PEG-DOPE-liposomes, pNP-PEG-DOPE-liposomes, and protein-pNP-PEG-DOPE-liposomes (see Fig. 4) – as was shown by

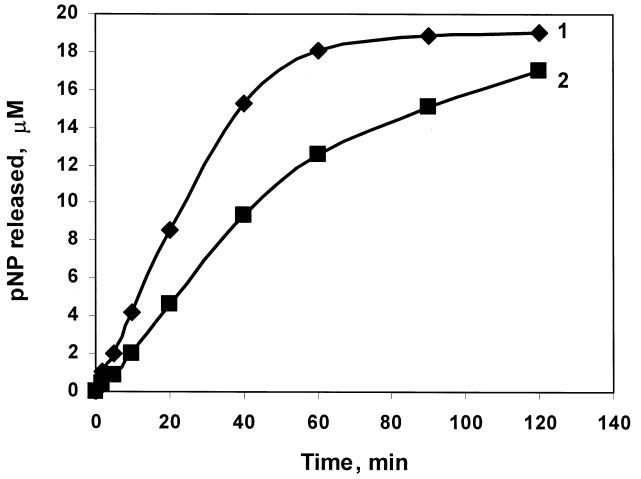

Fig. 3. Comparative rates of spontaneous hydrolysis of the pNP group (2) and its interaction with the amino group-containing ligand (1). Diaminobutane was used as a model reagent. See Section 2.2 for details.

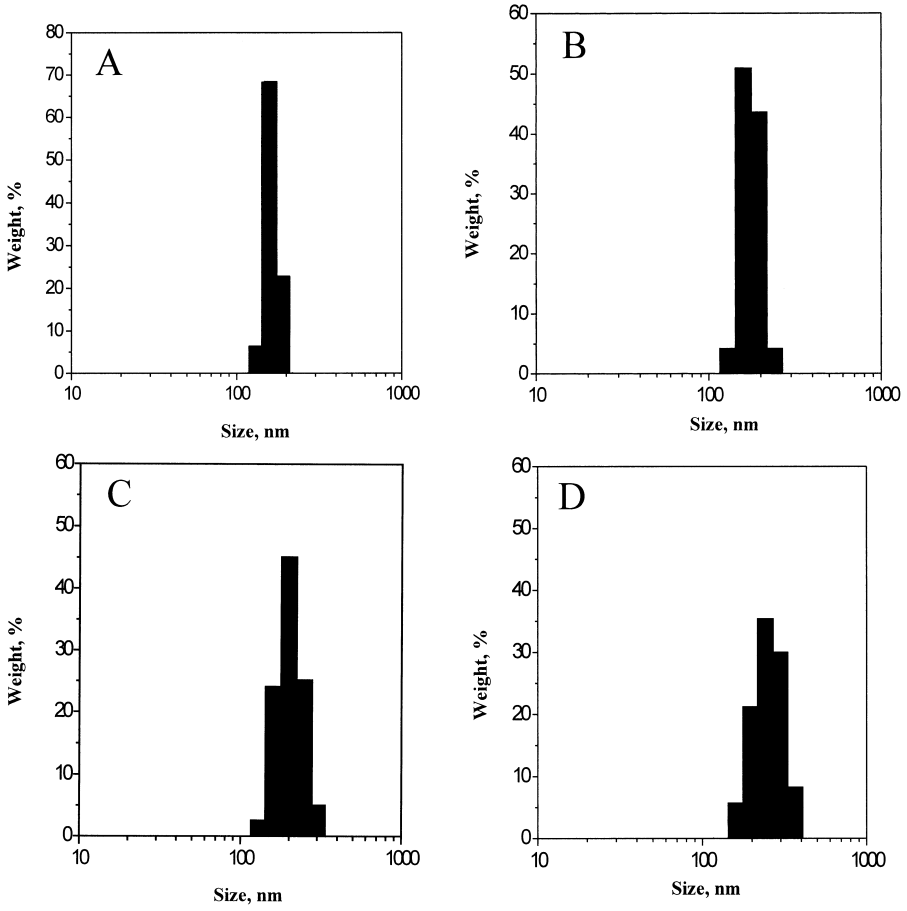

Fig. 4. Liposome size and size distribution in different preparations. (A) Plain PC:Chol liposomes; (B) PEG(2000)-PE-coated PC:Chol liposomes (6 mol% of PEG-PE); (C) pNP-PEG-DOPE-coated PC:Chol liposomes (6 mol% of pNP-PEG-DOPE); (D) pNP-PEG-DOPE-coated PC:Chol liposomes (6 mol% pNP-PEG-DOPE) with attached mon2C5 coupled to distal pNP groups. See Section 2.2 for details.

dynamic light scattering, the most frequently used method of liposome size determination, which provides the most consistent and accurate measurements [52–54].

In all cases, the incorporation of polymers in liposomes was complete, since HPLC did not reveal any micelles of free polymer in the system. To estimate the quantity of a protein bound to a single pNP-PEG-DOPE-liposome, proteins were radiolabeled with ^{111}In prior to their coupling to liposomes (see Section 2.2). Since the quantity of a protein and radiolabel was always known, we could estimate the exact quantity of a protein on the liposome surface by measuring liposome-associated ^{111}In radioactivity after protein attachment to liposome and removal of free protein. From the radioactivity data, we determined that, depending on the protein and starting lipid-to-protein ratio, between approx. 80 and 200 protein molecules can be bound to a single 200 nm liposome, whatever protein is used (ConA, WGA, avidin, or an antibody). In general, the increase in the molar fraction of pNP-PEG-DOPE in liposomes and the increase of the protein-to-pNP ratio results in a certain increase in the number of bound protein molecules, when pNP-PEG-DOPE concentration in the lipid mixture was below 1 mol%. However, at a constant concentration of pNP-PEG-DOPE in liposomes and constant protein-to-pNP ratio, no regularities were found for the binding efficacy of different proteins. The binding yield for the same or different proteins in separate runs usually varied by a factor of about 2, with the total number of protein molecules bound with a single liposome being within the limits described above. From a practical point, it

means that, even upon incubation under conditions where some hydrolysis of pNP residues has to occur, pNP-PEG-DOPE-modified liposomes still demonstrate the ability to bind substantial quantities of proteins, sufficient for further liposome targeting in vivo [7]. It is important that practically the same quantity of protein can be bound to liposomes containing 6 mol% of pNP-PEG-DOPE and 6 mol% of the mixture of PEG-DOPE and pNP-PEG-DOPE in 5:1 molar ratio. It means that even as little as approx. 1 mol% (of total lipid) of pNP-PEG-DOPE can provide a sufficient number of reactive groups on the liposome surface to bind approx. 100 protein molecules to a single 200 nm liposome. Only when the fraction of pNP-PEG-DOPE goes below 1 mol%, does the quantity of an attached protein begin to decrease.

3.3. Agglutination of lectin-pNP-PEG-DOPE-liposomes

To investigate the preservation of the specific activity of lectins coupled to liposomes via distal pNP groups in pNP-PEG-DOPE-liposomes, we, similar to [51], have performed an agglutination analysis. The turbidity changes of suspensions of lectin-liposomes in the presence of their corresponding substrates are presented in Fig. 5. Upon the addition of polyvalent substrates (mannan for ConA-liposomes and glycophorin for WGA-liposomes), the turbidity of the suspensions increases with the increase in substrate concentration, indicating increasing agglutination of lectin-liposomes. However, in the presence of lectin-specific low molecular weight competitive monovalent substrates (α-methyl mannoside for ConA-liposomes and L-fucose for WGA-liposomes), practically no changes in turbidity were observed, even at high concentrations of the polyvalent substrates. At the same time, when monovalent substrates were reversed (i.e. L-fucose was used with ConA-liposomes and α-methyl mannoside with WGA-liposomes), virtually no inhibition of liposome agglutination with polyvalent substrates was observed (data not shown). Together, these data show that both lectins immobilized on pNP-PEG-DOPE-liposomes via the distal pNP groups preserve their functional binding activity and carbohydrate specificity.

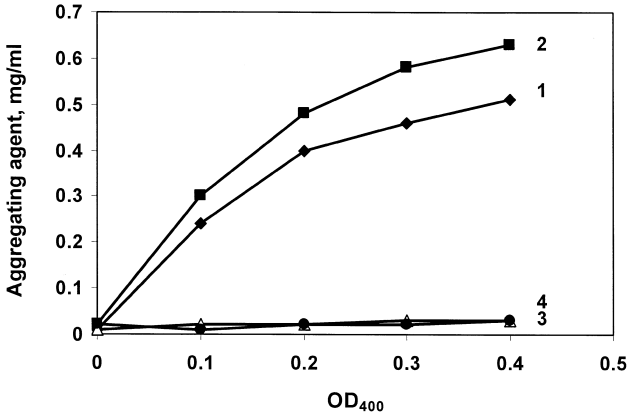

Fig. 5. Agglutination of lectin-pNP-PEG-PE-liposomes with and without inhibition. Agglutination of WGA-liposomes with glycophorin: (1) in the absence of the competitive low molecular weight ligand L-fucose; (3) in the presence of excess of L-fucose. Agglutination of ConA-liposomes with mannan: (2) in the absence of the competitive low molecular weight ligand α-methyl mannoside; (4) in the presence of α-methyl mannoside. See Section 2.2 for details.

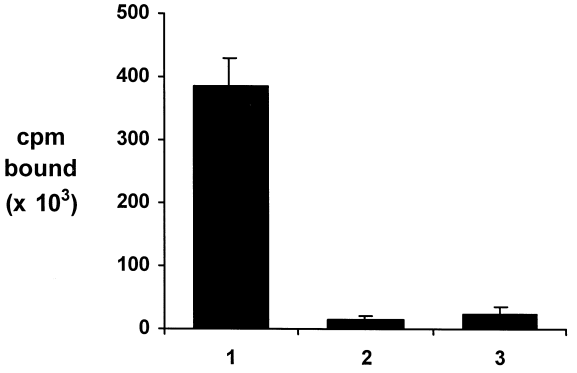

Fig. 6. 1, binding of ^{111}In-labeled avidin-pNP-PEG-DOPE-liposomes on a biotin-agarose column. Controls: 2, binding of protein-free ^{111}In-pNP-PEG-DOPE-liposomes (after the complete hydrolysis of pNP groups) on a biotin-agarose column; 3, binding of ^{111}In-labeled ConA-pNP-PEG-DOPE-liposomes on a biotin-agarose column. Presaturation of the biotin-agarose column with the excess of free avidin completely abolished the binding of avidin-liposomes (data not shown). See Section 2.2 for details.

3.4. Binding of avidin-pNP-PEG-DOPE-liposomes with biotin-agarose

To prove the preservation of the specific activity of avidin coupled to liposomes via distal pNP groups in pNP-PEG-DOPE-liposomes, we have investigated

the binding of avidin-liposomes on a biotin-agarose column. The data presented in Fig. 6 clearly show that avidin-liposomes strongly bind to biotin-agarose, while control avidin-free pNP-PEG-DOPE-liposomes and pNP-PEG-DOPE-liposomes with attached ConA demonstrate only marginal binding. The specificity of avidin-liposome binding on biotin-agarose was further confirmed by the fact that pretreatment of the biotin-agarose column with an excess of free avidin almost completely abolished the binding of the avidin-liposomes (not shown). Thus, similar to lectins, avidin covalently bound to the distal tips of PEG-liposomes via pNP groups of liposome-incorporated pNP-PEG-DOPE preserves its functional activity and specificity.

3.5. Activity of liposome-bound mon2G4

The antimyosin mon2G4 was used to study the interaction of ^{111}In-labeled mon2G4-pNP-PEG-DOPE liposomes with a monolayer of the antigen, dog cardiac myosin, in a direct binding assay, as well as for the comparison of two different methods of antibody immobilization on liposomes – by direct membrane incorporation of an antibody modified with a hydrophobic group of NGPE [8,9,47] and by covalent attachment of a non-modified antibody to the distal pNP groups in pNP-PEG-DOPE-liposomes. Fig. 7 presents the data of the direct binding assay of various preparations of ^{111}In-labeled liposomes with the monolayer of dog cardiac myosin. It is clearly seen that ^{111}In-labeled mon2G4-liposomes bind to the monolayer of myosin 20–25 times better than control protein-free ^{111}In-pNP-PEG-DOPE-liposomes (after the complete hydrolysis of pNP groups) and non-specific ^{111}In-labeled mon2C5-pNP-PEG-DOPE-liposomes. ^{111}In-Labeled plain PC:Chol liposomes demonstrated background binding on the level of pNP-PEG-DOPE-liposomes. The preincubation of the monolayer with an excess of free mon2G4 completely abolished the binding of mon2G4-liposomes (data not shown). These results clearly evidence the preservation of antibody-specific activity towards the antigen after covalent coupling to the distal ends of polymer chains of PEG-liposomes via pNP groups.

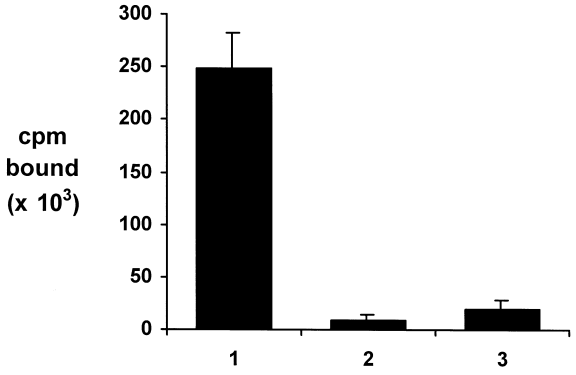

Fig. 7. 1, binding of ^{111}In-labeled mon2G4-pNP-PEG-DOPE-liposomes with a monolayer of the antigen (dog cardiac myosin). Controls: 2, binding of protein-free ^{111}In-pNP-PEG-DOPE-liposomes (after the complete hydrolysis of pNP groups) on a myosin monolayer; 3, binding of ^{111}In-labeled mon2C5-pNP-PEG-DOPE-liposomes on a myosin monolayer. See Section 2.2 for details.

The comparison of two different protocols of antibody immobilization (see the results of the ELISA experiment in Fig. 8) shows that, at the intermediate PEG concentration used (6 mol% of total lipid), there is a very small difference in the activity of two different PEG-immunoliposomes (compare curves 2 and 3), and both preparations, in terms of their immunoreactivity, do not differ much from the preparation of polymer-free liposomes containing the same quantity of membrane-incorporated NGPE-mon2G4. Still, the attachment of the antibody via pNP groups does not require any preliminary chemical modification of the antibody and makes the whole coupling procedure more simple and speedy.

3.6. Activity of liposome-bound mon2C5; effect of PEG quantity

The benefit of pNP-mediated attachment of antibodies to the distal ends of liposome-grafted polymer chains over NGPE-mediated membrane incorporation at high concentrations of the liposomal PEG was shown in experiments involving antinucleosome mon2C5. The ELISA data presented in Fig. 9 clearly show that when NGPE-modified mon2C5 is co-incorporated into the liposomal membrane together with PEG-DOPE, the antigen-binding activity of the liposomal antibody gradually decreases with the increase in the concentration of PEG-DOPE (compare curves 1–5 for liposomes with 0–15 mol% of PEG-DOPE). At 15 mol% PEG-DOPE, mon2C5 binding with nucleosomes is completely abolished

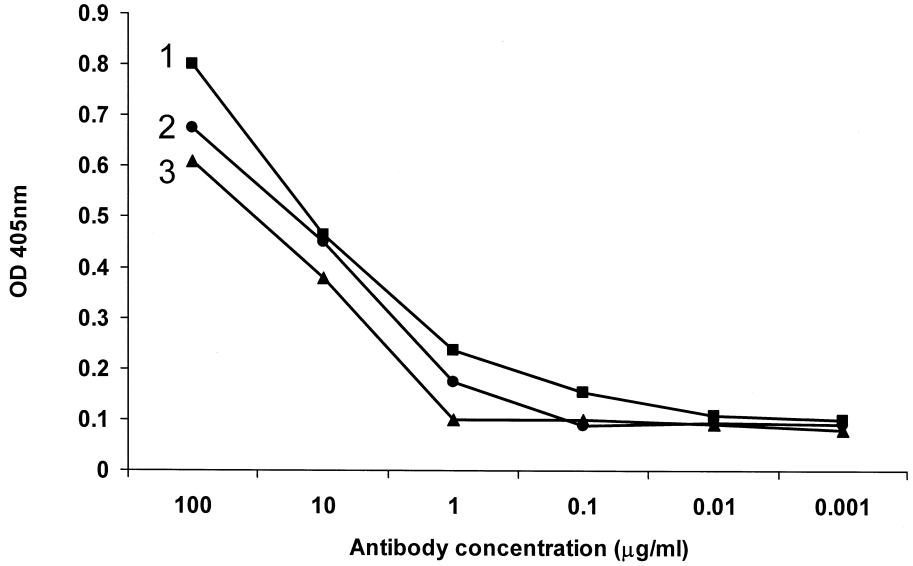

Fig. 8. ELISA results for various preparations of liposome-bound antimyosin mon2G4. 1, NGPE-mon2G4 incorporated into the membrane of PC:Chol liposomes; 2, mon2G4 immobilized on the distal ends of polymer chains of pNP-PEG-DOPE-liposomes (polymer concentration 6 mol% of total lipids); 3, NGPE-mon2G4 co-incorporated into PC:Chol liposomes together with 6 mol% of PEG-DOPE. See Section 2.2 for details.

because of steric hindrances created by neighboring polymeric chains [8,9,29,30]. The data correspond well to the observations made in [31,32]. However, if the antibody was attached to liposomes containing the same high concentration of PEG-DOPE via covalent coupling to the distal ends of polymeric chains via pNP groups (in this case liposomes contained 15 mol% of a 5:1 mixture of PEG-DOPE and pNP-

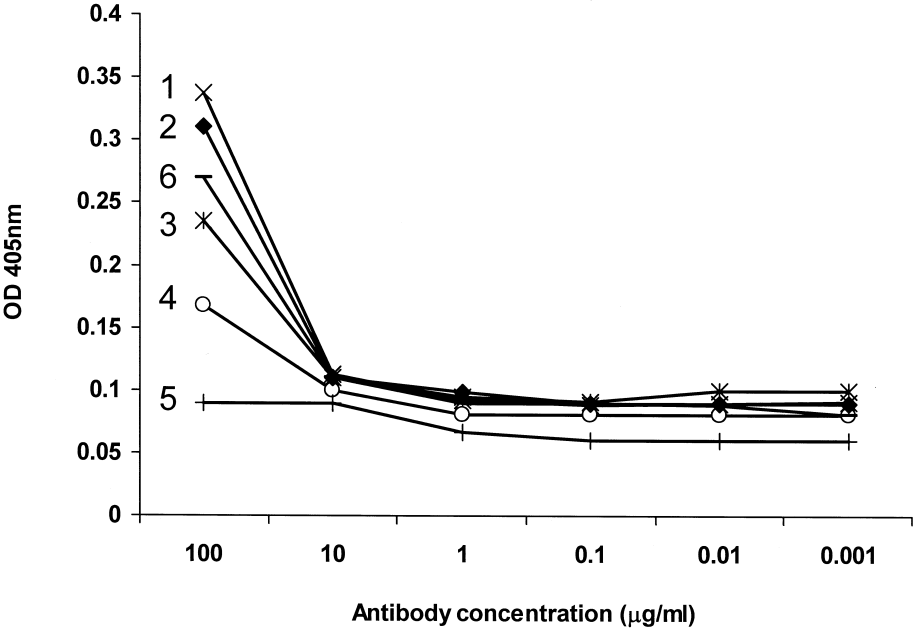

Fig. 9. ELISA results for various preparations of liposome-bound antinucleosome mon2C5. 1, NGPE-mon2C5 in PC:Chol liposomes without PEG; 2, as in (1) with 2 mol% PEG-DOPE; 3, as in (1) with 8 mol% PEG-DOPE; 4, as in (1) with 11 mol% PEG-DOPE; 5, as in (1) with 15 mol% PEG-DOPE; 6, unmodified mon2C5 covalently attached (with active site protection) via pNP groups to liposomes containing 15 mol% pNP-PEG-DOPE. See Section 2.2 for details.

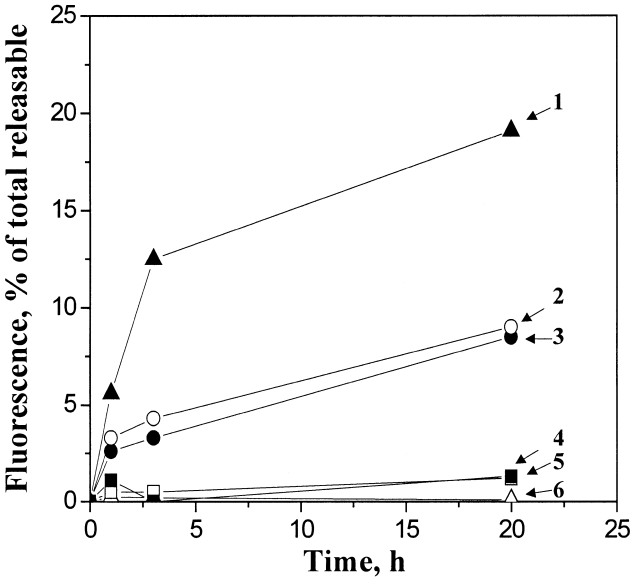

Fig. 10. The release of the fluorescent dye calcein from liposomes of various composition in the presence of 50% mouse serum. 1, plain PC:Chol liposomes; and liposomes with: 2, 0.5 mol% of pNP-PEG-DOPE; 3, 0.5 mol% mon2C5-pNP-PEG-DOPE; 4, 6 mol% of pNP-PEG-DOPE; 5, 6 mol% of mon2C5-pNP-PEG-DOPE; 6, 6 mol% of PEG-DOPE. See Section 2.2 for details.

PEG-DOPE), rather than by NGPE-mediated membrane incorporation, antibody activity was preserved to a very high extent (see curve 6 in Fig. 8). Thus, this fast and simple method of ligand attachment to the distal ends of liposome-grafted polymer chains developed by us permits the preservation of the specific activity of the immobilized ligand even with high concentrations of the protecting polymer. The data are in good agreement with earlier published observations involving more complex methods of antibody attachment to the distal ends of liposome-grafted polymers [31–37].

3.7. Stability of liposomes in mouse serum

Earlier, it was repeatedly shown that the attachment of amphiphilic PEG derivatives to the liposome surface does not cause leakage of a marker or drug from liposomes both in vitro and in vivo (see, for example, the early data in [20] and the recent findings in [55]). In the latter study, a successful targeting of doxorubicin entrapped in antibody-targeted PEG-liposomes to human myeloma cells in vitro and ex vivo was demonstrated with the drug being retained inside liposomes. Since the preparation of pNP-PEG-DOPE-containing liposomes follows standard procedures, and the reactive PEG derivative described here rapidly converts into a 'usual' liposome surface-attached PEG, one can hardly expect any abnormalities in the liposome stability both in vitro and in vivo compared to other PEG- or reactive-PEG-liposomes. Since the principal subject of our study is the development of new ligand attachment chemistry to PEG-liposomes, detailed in vivo studies are beyond the scope of this paper and constitute the subject of our ongoing investigation.

However, in order to additionally confirm the liposome stability and ability to retain an intraliposomal load (model drug), we have investigated the effect of mouse serum on the fluorescent dye (calcein) release from liposomes of different composition used in our experiments as well as from control liposomes. Preliminarily, it was shown that in the absence of serum, all liposomes used in these experiments do not release more than 2–4% of entrapped calcein after incubation in HBS for 20 h (data not shown, variations from sample to sample were insignificant). In the presence of mouse serum, however, the ability of liposomes to retain the entrapped fluorescent dye strongly depends on their composition.

The data presented in Fig. 10 clearly demonstrate that, in the presence of serum, calcein release proceeds at a high rate from plain PC:Chol liposomes (control) and liposomes containing small quantities of attached polymer or polymer-to-ligand conjugate, approx. 0.5 mol% of pNP-PEG-DOPE or mon2C5-pNP-PEG-DOPE (all pNP group-containing samples were preliminarily incubated for 2 h at pH 7.5 to hydrolyze free pNP groups). Still, in the last two cases the calcein release is somewhat slower, probably because of the partial steric protection still provided even with a small quantity of the attached polymer (or polymer-to-protein conjugate). At the same time, liposomes with 6 mol% of pNP-PEG-DOPE or mon2C5-PEG-DOPE demonstrate strongly inhibited calcein release very similar to that from sterically stabilized liposomes containing 6 mol% of PEG-PE (control). This result confirms a 'normal' protecting effect of pNP-PEG-DOPE, as well as the biological stability of pNP-PEG-DOPE-liposomes and their ability to retain the entrapped

compound (which might be considered a model drug).

4. Conclusion

A new amphiphilic PEG derivative, pNP-PEG-PE, is introduced, which easily incorporates into the liposomal membrane via its phospholipid residue and easily binds any amino group-containing ligands via its water-exposed pNP group. The reaction between the pNP group and ligand amino group proceeds easily and quantitatively at pH around 8, and remaining free pNP groups are easily eliminated by spontaneous hydrolysis. pNP-PEG-PE provides an easy and convenient tool for protein attachment to the distal ends of liposome-grafted PEG chains. pNP-PEG-PE-containing liposomes are able to bind substantial quantities of proteins via pNP groups located on the distal ends of PEG chains. Lectins, avidin, and antibodies bound with liposomes via pNP-PEG-PE retain their specific activity, even at high concentrations of liposome-grafted polymer.

Acknowledgements

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