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Development of the Novel PEG-PE-Based Polymer for the Reversible Attachment of Specific Ligands to Liposomes: Synthesis and *in vitro* Characterization

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Abstract

Surface grafting of liposomes with the wide variety of ligands including antibodies and other proteins is a promising approach for targeted delivery of therapeutics. In this paper, we describe a simple method of synthesizing a hydrazine-functionalized polyethylene glycol-phosphatidylethanolamine (PEG-PE)-based amphiphilic polymer which can conjugate a variety of ligands via a reversible, pH-cleavable bond. In this method, the targeting ligand is attached to the distal end of the PEG chain, which facilitates its easy access to the targeted site of interaction. The reversible attachment of targeting ligands is useful especially in multifunctional liposomal systems, where after successfully performing the function of targeting to the specific site, the bulky ligands, such as proteins or antibodies, are cleaved off in response to an environmental stimulus to expose some other functionalities such as ligands for intracellular penetration or organelle-specific targeting.

To investigate the applicability of the protocol, the model ligands monoclonal antinucleosome antibody 2C5 and antimyosin antibody 2G4, and glycoproteins concanavalin A (Con-A) and avidin were conjugated to the synthesized polymer and incorporated into liposomes. *In vitro* assays including biochemical, enzyme-linked immunosorbent, fluorescence microscopy and flow cytometry were used to confirm three key characteristics of the modified and/or liposome-attached proteins: successful conjugation of the targeting ligands to the polymer, preservation of specific activity of the ligands after the conjugation and liposome attachment, and the facile pH-sensitive ligand detachment. Monoclonal mAb 2C5 and 2G4, immobilized on the liposome surface, retained their binding affinity to corresponding antigens as confirmed by ELISA. The Con A-bearing liposomes showed significantly higher agglutination in the presence of its substrate mannan compared to plain liposomes (PL) and avidin-functionalized liposomes bound specifically with biotin-agarose. The study on the pH-dependence showed almost 80 % of the hydrazone bond was cleaved after rather brief pre-incubation of the immunoliposomes at pH 5 for 0.5 to 1 h. Fluorescence microscopy and flow cytometry analysis of cancer cells (HeLa and MCF-7) treated with cancer cell-specific targeting ligand mAb 2C5-bearing liposomes, showed enhanced cellular binding. Studies at low pH clearly confirmed the easy cleavability of the targeting ligand from the liposome resulting in significantly less or virtually no cellular association.

Keywords

Liposomes; amphiphilic polymer; pH-sensitive; hydrazone bond; targeting ligands; bioconjugates; multifunctional

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INTRODUCTION

Liposomes have served as an efficient drug delivery system for enhanced efficacy and decreased systemic toxicity of various biologically-active molecules.^{1,2} However, one of the major disadvantages of liposomal drug delivery systems (DDS) is that circulating liposomes are rapidly cleared when uptaken by cells of the reticuloendothelial system (RES) in the liver and spleen. To engineer liposomes capable of delivering therapeutic cargo to sites other than the RES, long-circulating polymer-coated (PEGylated) liposomes have been developed.^{1,2} The modification of nanocarriers by conjugating PEG on the surface reduces also many undesirable side effects triggered *in vivo* by various biological recognition mechanisms.³ This property of prolonged systemic circulation results also in passive targeting of liposomes even in the areas with a compromised vasculature such as infarcts and tumors by the enhanced permeability and retention (EPR) effect.⁴⁻⁹ Active targeting of liposomes to specific disease sites, such as tumors and infarcts, could be achieved by surface modification of the liposomes with various ligands, such as tumor-specific molecules including cancer cell-specific antinucleosome antibody (mAb 2C5), proteins (transferrin), peptides (RGD), and small molecules such as receptor ligands (folate) for anticancer therapy and antimyosin mAb (2G4) for the therapy of the myocardial infarction.¹⁰⁻¹⁶ Thus, pharmaceutical nanocarriers can be endowed with the properties of both, active and passive targeting.^{17,18}

In the case of active targeting of PEGylated liposomes, the targeting moiety should be attached “above” the protective polymer layer by coupling it to the distal end of a PEG chain to provide accessibility of the ligand to the target organ or tissue.^{9,17,19} Liposomes can also be endowed with other functionalities such as enhanced intracellular penetration by surface attachment of cell-penetrating peptides (CPPs) as well as enhanced intracellular organelle recognition after cellular internalization by addition of specific ligands that target subcellular organelles such as mitochondria, lysosome or nuclei.²⁰⁻²¹

Therefore, it is important to optimize such multifunctional nanocarriers by proper combination of several of the properties including longevity in the circulation, targetability, intracellular penetration and organelle recognition to improve its efficacy.²² Another approach of preparing “smart” multifunctional liposomes is to introduce the property of stimuli-sensitivity.²³⁻²⁴ In our earlier study, we came up with a novel stimuli-sensitive multifunctional nanocarrier, a PEGylated TAT-p-modified pH-sensitive liposome.²³⁻²⁴ The PEG chains which provide the longevity in systemic circulation for accumulation in a tumor or infarcted tissue by passive targeting were cleaved off at lowered pH environment of hypoxic areas to expose the previously hidden non-specific cell penetrating function, such as TAT-p.

In our present study, we demonstrated a simplified synthesis of hydrazine-functionalized PEG-PE-based amphiphilic polymer, which could conjugate variety of ligands via the reversible, pH-cleavable bond. Although, the concept of end-group-hydrazine-functionalized PEG-lipid conjugate has been reported,²⁵ in our study, we report a novel scheme of the modification of PEG-PE via only two facile reaction steps to prepare hydrazine-functionalized PEG-PE. The ligand-polymer conjugate was easily incorporated into the liposomes via its PE fragment. The targeting ligands were attached to the distal end of the PEG-chain to have a sufficient freedom for various cellular interactions. Introducing a pH-sensitive linkage between bulky targeting ligand and liposome in the multifunctional liposomal system could be especially useful for drug delivery into tumors or infarcted regions with the lowered pH. After the successful target accumulation, lowered pH in such areas could cleave off the targeting ligands (and possible PEG) and expose other previously

hidden (shielded) non-specific functionalities. The model ligands used to confirm the applicability of our protocol were mAbs 2C5 and 2G4, and proteins Con-A and avidin. In this paper, we confirm that the suggested method of using stimuli-sensitive polymer for the ligand attachment can be successfully applied for the ligand conjugation, preservation of its specific activity and further pH-dependent cleavage of these ligands attached to the liposomal surface.

EXPERIMENTAL PROCEDURES

Materials

1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (PEG_{2K}-PE), 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B Sulfonyl)(ammonium salt, Rh-PE), L- α -phosphatidylcholine (egg, chicken), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino(polyethylene glycol)2000] (ammonium salt) (DSPE-PEG_{2K}-Amine) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL) and used without further purification. Cholesterol, chloroform-*d*, triethylamine, Con-A, avidin, α -methyl mannopyranoside, 5,5'-Dithio-bis (2-nitrobenzoic acid) (DTNB), N-acetyl-L-cystein were purchased from Sigma (St. Louis, MO). Sephadex G 25 superfine medium was from GE Healthcare Biosciences Corp. (Piscataway, NJ, USA). 2-Iminothiolane-HCl (Traut's reagent), 4-(4-N-maleimidophanyl) butyric acid hydrazide-HCl (MPBH), fluorescein isothiocyanate (FITC-NHS), biotin-agarose, 2,4,6-trinitrobenzene sulfonic acid (TNBSA), 5 % w/v solution in methanol, were purchased from Pierce (Rockford, IL). Paraformaldehyde was purchased from Electron Microscopy Sciences (Hatfield, PA). Fluoromount-G was from Southern Biotech (Birmingham, AL). The mAb 2C5 was produced in ascites via the i.p. injection of 1.5×10^6 hybridoma cells into pristane-primed 4 week old BALB/c male mice. The production and purification of the mAb 2C5 were carried out by Harlan Bioproducts (Indianapolis, IL) using the cells from our laboratory. Cardiac myosin-specific mAb 2G4 of the immunoglobulin IgG class available in our laboratory was collected and purified from murine ascites according to the standard ammonium sulfate precipitation method.^{15,26}

Cell culture

Human cervical carcinoma (HeLa) cells and breast cancer cell line (MCF-7) were purchased from ATCC (Manassas, VA). Dulbecco's modified Eagle's media (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin solution were from CellGro (Kansas City, MO). Cells were grown in DMEM supplemented with 10 % FBS, 100 IU/mL of penicillin, streptomycin and 250 ng/mL amphotericin-B at 37 °C and 5 % CO₂.

Methods

Synthesis, purification and characterization of synthesized polymers 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[thio(polyethylene glycol)2000](ammonium salt) (DSPE-PEG-_{2K}-SH)—6.2 mg (45.05 μ M) of Traut's reagent dissolved in 50 μ L of methanol were added into the chloroform solution (25 mg/mL) of DSPE-PEG-_{2K}-amine (25 mg, 8.95 μ M) at room temperature (RT). The reaction mixture was stirred at RT for 2 hrs. Chloroform was evaporated completely, the residue dissolved in PBS and dialyzed using a cellulose ester membrane (MWCO. 1 kDa, Spectrum Medical Industries, Houston, TX) overnight. The dialysate was freeze-dried, weighed, dissolved in chloroform at 5 mg/mL and stored at -80 °C. The quantification of sulfhydryl groups was performed by DTNB assay using N-acetyl cysteine for the preparation of the standard SH calibration curve.²⁷ The yield of the reaction was calculated to be 60 %. The freeze-dried product (5 mg) was dissolved in deuterated (*d*)-chloroform (1 mL) and analyzed by ¹H-NMR using Varian 500 mHz.

1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[hydrazido(polyethylene glycol)2000](ammonium salt) (DSPE-PEG-2K-CONHNH₂)—MPBH (13.1 mg, 36.9 μ M) dissolved in PBS was added into the solution of DSPE-PEG-2K-SH (17.7 mg, 6.2 μ M) in PBS (pH 7.4). The reaction mixture was stirred at RT for 4 hours and put for dialysis using cellulose ester bag (molecular weight cut off 2 kDa) against water overnight before freeze-drying. The hydrazide content of the freeze-dried product was determined by the TNBSA assay following the manufacturer's protocol and using the MPBH for the preparation of a standard calibration curve.²⁸ The freeze-dried fluffy solid was dissolved in chloroform at 10 mg/mL and stored at -80°C . The solid product (5–10 mg) was dissolved in *d*-chloroform (1 mL) and characterized by ¹H-NMR using Varian 500 MHz spectroscopy. The conjugates were not chromatographically isolated after the coupling steps. All polymers were purified by dialysis after the conjugation.

Liposome Preparation

Activation of antibodies (mAb 2C5, mAb 2G4) and proteins (Concanavalin A, Avidin):

Antibodies (mAbs 2C5, 2G4) and glycoproteins Con-A and avidin were aldehyde-activated using freshly prepared Na-periodate. 100 μ L of Na-periodate (0.1 M) in water was added to 1.0 mL of the antibody or protein solution (10 mg/mL) in the dark and stirred for 10 min. The reaction was quenched by the addition of sodium sulfite at a 2X molar excess over the periodate added. The aldehyde-activated antibodies or proteins were purified by gel filtration using a desalting spin column (Pierce) and used immediately.

Plain Liposomes (PL): Liposomes were prepared by the lipid film hydration technique. Briefly, a dry lipid film was prepared by rotary evaporation of a chloroform solution of all the ingredients including eggPC/cholesterol or eggPC/cholesterol/Rh-PE (in molar ratios of 65/30 or 64.5/30/0.5 with 5 mole % of DOPE-PEG-2K) followed by the freeze drying (Labconco, Freeze dry system, Freezone) for at least 4 h to remove the traces of the solvent. The dry lipid film was hydrated in PBS, pH 7.4, at lipid concentration of 4 mg/mL. The hydrated mixture was vortexed vigorously for 5 min to form multilamellar vesicles and then extruded through the 200 nm pore-sized polycarbonate membrane filters (Avanti Polar Lipids) for 20 times to yield liposomes with the uniform particle sizes of 150–200 nm mean diameter.

Antibody/protein-grafted liposomes: Antibodies or proteins containing reactive aldehyde groups were incubated with DSPE-PEG-2K-CONHNH₂ in PBS (pH 7.4) overnight at 0°C to produce polymer-lipid conjugate-modified products. The freshly prepared loose micelles of antibody/protein conjugates (5 mol % of the total liposomal lipid) were incubated with pre-formed PL overnight at pH 7.4 for the transfer of the micelle components to liposomes via the post-insertion technique. The antibody or protein-grafted liposomes, 2C5-Hz-PEG-2K-DSPE-modified liposomes (2C5-L) and 2G4-Hz-PEG-2K-DSPE-modified liposomes (2G4-L) as well as avidin-Hz-PEG-2K-DSPE-modified liposomes (avidin-L) and concanavalin-A-Hz-PEG-2K-DSPE-modified liposomes (Con-A--L) were separated from unbound antibody/protein by extensive dialysis using cellulose ester membranes (MWCO 300 KDa) against PBS, pH 7.4, at 4°C overnight.

Characterization of liposomes: Zeta plus dynamic light scattering equipment (Brookhaven Instruments, Holtsville, NY) was used to determine the liposome size and size distribution. The zeta potentials of formulations were measured by a zeta Phase Analysis Light Scattering (PALS) with an ultrasensitive zeta potential analyzer instrument (Brookhaven Instruments, Holtsville, NY). The liposome suspension was diluted as needed with water or a 1 M KCl solution to measure the particle size or zeta potential, respectively.

Determination of immunoreactivity 2C5-L or 2G4-L by ELISA: An indirect ELISA (using an enzyme-tagged secondary Ab) was performed to assay the ability of the immunoliposomes to recognize the target antigens. ELISA micro-plates were coated with 50 μ L of 10 μ g/mL nucleosomes for testing 2C5-L, or with 50 μ L of 10 μ g/mL cardiac myosin for testing 2G4-L overnight at 4°C. The plates were rinsed with the TBST buffer (Tris-buffered saline supplemented with 0.05 % w/v Tween-20) and coated with 10 % FBS in Tris-buffered saline. To the antigen-coated plates, 50 μ L of 2C5-L or 2G4-L was added at antibody concentration of 10 μ g/mL and incubated for 1 h at room temperature. Then, the plates were washed with TBST and coated with 50 μ L/well horseradish peroxidase-anti-mouse IgG conjugate (ICN) at 1:5000 dilution. The conjugate was removed after 1 h incubation at room temperature, the plates were again washed and incubated with enhanced Kblue TMB peroxidase substrate (Neogen Corporation, Lexington, KY) for 15 min. The microplates were read at dual wavelengths of 620 nm and at 492 nm using a Labsystems Multiskan MCC/340 microplate reader installed with GENESIS-LITE Windows-based microplate software.

Interaction of avidin-L with biotin: To examine the binding of Rh-PE-labeled avidin-L with biotin, two columns were prepared with the same quantity of biotin-agarose. The column was washed with PBS. Rh-labeled PL and avidin-L of same fluorescence intensity were passed through the column and first PBS, and then avidin-denaturing guanidine-HCl buffer, pH 1.5, were used as an eluent. The eluent was collected in 1 mL fractions. The fluorescent intensity of each fraction was measured using a multi-detection microplate reader (Bio-Tek, Winooski, VT) at the 540/625 nm excitation/emission wavelengths. The degree of retention of the corresponding preparation on the column indicative of the preservation of the functional activity of the avidin to interact with biotin was estimated by following the decrease in the rhodamine fluorescence in the eluent.

Aggregation assay for Con-A-L: The carbohydrate binding activity and specificity of the Con-A in Con-A-L was examined using an aggregation assay. The substrate for Con-A, mannan was dissolved in PBS, pH 7.4, at different concentrations, and 100 μ L aliquots of the solution were added to a 25 μ L suspension of Con-A-L and PL in 96-well plates. The plates were shaken, incubated for 20 min at room temperature and transferred to a Labsystems Multiskan MCC/340 microplate reader to be read at OD450 nm. Con-A in presence of mannan aggregates, and the increase in the turbidity of the system is indicative of the preservation of Con-A's functional activity after the conjugation and immobilization on the liposome surface. The experiment was also performed in presence of 100 μ M of the competitive inhibitor, α -methyl mannopyranoside.

Labeling of antibody (2C5) with carboxyfluorescein (CF): A 50 μ L aliquot of 2 mM 5-CF succinimidyl ester (Molecular Probes) in DMSO was added to 2 mL of a 12 μ M solution of 2C5 in 50 mM TBS, pH 9.0. The mixture was incubated overnight at 4°C and free CF was removed by the dialysis against TBS. The CF-labeled antibody was used to prepare 2C5-liposomes (CF-2C5-L) following above procedure. The pH of 200 μ L of CF-2C5-L (1.2 μ g of lipid/mL) in PBS, pH 7.4, was lowered to pH 5 by the addition of HCl (0.1 N). The mixture was incubated at the room temperature for 1 h and dialyzed against PBS using a 300 KDa MWCO cellulose ester membrane. Fluorescence intensity of the same amount of lipids in immunoliposomes was measured at pH 7.4. Both the solutions after dialysis at pH 7.4 and pH 5 were measured using a multi-detection microplate reader (Bio-Tek, Winooski, VT) at 485/528 nm excitation/emission wavelengths.

Cell experiments

Interaction of 2C5-L with cancer cells *IN VITRO* and pH-dependent cleavability of mAb 2C5:

After the initial passage in tissue culture flasks, HeLa cells were grown on cover slips placed in 6-well tissue culture plates. After the cells reached the 60–70 % confluence, the plates were washed with PBS and incubated with 1 % BSA in DMEM for 1 h at 37°C, 5 % CO₂. Rh-PE (Lissamine-rhodamine B-phosphatidyl ethanolamine)-labeled 2C5-L and Rh-PE-labeled PL, pre-incubated at pH 7.4 or pH 5 for 1 h, were added to a final lipid concentration of 0.1 mg/mL and incubated for 90 min at 37°C, 5 % CO₂. After the incubation, the cells were washed with PBS and the cover slips were mounted cell-side down on glass slides with fluorescence-free glycerol-based mounting medium (Fluoromount-G; Southern Biotechnology Associates) and studied with a Nikon Eclipse E400 microscope under the bright light or under the epifluorescence with a rhodamine filter.

FACS analysis: Approximately 200,000 HeLa and MCF-7 cells were seeded in 6-well tissue culture plates. After the cells reached 60–70 % confluence, the plates were washed with PBS and incubated with 1 % BSA in DMEM for 1 h. The immunoliposomes were pre-incubated for 1 h at pH 7.4 or pH 5 and treated with formulations at lipid concentration of 0.1 mg/mL for 90 min. The cells were centrifuged, washed with ice-cold PBS at least 3 times before analysis using a BD FACS Caliber flow cytometer. The cells were gated using forward-versus side-scatter to exclude debris and dead cells before analysis of 10,000 cell counts. The data were analyzed with BD Cell Quest Pro Software.

Statistical analysis: The data were tested for statistical significance using the paired student's t-test using GraphPad prism 4 (GraphPad Software, Inc.; San Diego, CA). Any p value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Synthesis and characterization of 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[thio(polyethylene glycol)2000](ammonium salt) (DSPE-PEG-_{2K}-SH) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[hydrazido(polyethylene glycol)2000](ammonium salt) (DSPE-PEG-_{2K}-CONHNH₂)

The terminal hydrazine group-containing polymer DSPE-PEG-_{2K}-CONHNH₂ was synthesized following the scheme presented in the Figure 1. The starting polymer containing amine group was transformed into a thiol-modified polymer by the reaction using the Traut's reagent. The reaction yield was 60 % by the DTNB assay. The next reaction with the cross-linking agent MPBH yielded 89 % of DSPE-PEG-_{2K}-CONHNH₂ calculated from the TNBSA assay.

The polymers were characterized by ¹H NMR in d-chloroform using Varian 500 MHz spectrophotometer (see the data in the Figure 2).

¹H-NMR of DSPE-PEG-_{2K}-SH: δ 0.87–0.89 (t, 6H, (-CH₂CH₃)₂), 1.25 (s, H from long chains of PE), 1.59–1.65 (m, 5H), 1.95–2.05 (m), 2.27–2.35 (m), 2.51–2.70 (m, -CH₂CH₂CH₂SH), 3.14–4.4 (m), 5.2–5.24 (m, 1H), 7.89 (bs, 1H, -NHCO).

¹H-NMR of DSPE-PEG-_{2K}-CONHNH₂: δ 0.87–0.89 (t, 6H, (-CH₂CH₃)₂), 1.25 (s), 1.58 (s), 2.02–2.69 (m), 3.14–4.4 (m), 5.21 (bs, 1H), 7.17–7.19 (m, 2H, Ar-H), 7.48–7.60 (m, 2H, Ar-H), 7.9 (bs, 1H, -NHCO).

Liposome preparation and characterization

Sizes of all types of liposomes prepared according to the general Scheme 1 (PL, 2C5-L, 2G4-L, Con-A-L, avidin-L) determined by the dynamic light scattering ranged between 150–200 nm (Figure 3). In all cases of the subsequent modification via the post-insertion technique, the incorporation of the added ligands into the liposomes was quantitative, since HPLC revealed no micelles of the free polymer-modified ligand in the system. To estimate the quantity of the protein bound to liposomes, the 2C5-L was used labeled with CF. Protein concentration per μg of lipid was measured by the associated fluorescence at the excitation wavelength of 490 nm and emission wavelength of 520 nm. From the fluorescence data, approx. 150–200 protein molecules of antibody were bound to the surface of a single 200 nm liposome. The calculations were based on the fact that while preparing the liposomes, the molar ratio of DSPE-PEG- $_{2K}$ -CONHNH $_2$ per antibody was 32/1 and that number of phospholipid molecules per 200 nm liposomes was estimated 25×10^4 .²⁹

Activity of mAbs 2C5 and 2G4 in 2C5-L and 2G4-L by ELISA

To investigate whether various targeting ligands could be successfully attached to the liposomal surface by this reversible pH-sensitive hydrazone bond without losing their specific activity, the ELISA assay was performed to determine the interaction of 2C5-L and 2G4-L with specific antigens for the liposome-attached antibodies, nucleosomes and cardiac myosin, respectively. Figure 4 represents the data of the direct binding assay of various preparations of liposomes with the corresponding antigens. The 2C5-L and 2G4-L bind to the mono-layers of their corresponding antigens with the same efficiency as native free antibodies. Control IgG2a isotype-matching mAb UPC10 was attached onto the liposome surface following the same protocol. All controls - PL, IgG-bearing liposomes (IgG-L) and native IgG demonstrated only a background binding to the antigens. These results indicate that the hydrazone bond used to attach the antibody was quite stable under the conditions used, and the antibodies' specific activity towards their antigens were completely preserved after covalent coupling to the distal tips of the PEG chains of liposomes via the hydrazone bonds.

Binding of avidin-L with biotin-agarose

To further confirm the preservation of the specific properties of proteins attached to liposomes using the suggested scheme, Rh-PE-labeled PL and avidin-L were passed through the biotin-agarose column (Figure 5). The results showed the free passage of PL through the column (the fluorescence increase in initial fractions of the eluent at 540/625 excitation/emission wavelength) and complete retention of avidin-L on the column. In other words, the avidin in avidin-L was capable of strong binding with biotin. However, the treatment of the column with bound avidin-L with avidin-denaturing buffer, pH 5, released the major part of the fluorescence from the column via two possible mechanisms. First, the buffer denatured the avidin protein, which compromised the binding of avidin with the biotin-agarose and resulted in the release of Rh-labeled liposomes. Second, the lowered pH of this buffer could also degrade the hydrazone bond between avidin and liposomes and also cause the liposome release (fluorescence increase in the eluent). The result demonstrated that the avidin was successfully attached to the distal end of the polymer on the liposome surface and the specific activity of the protein was completely retained after the conjugation.

Agglutination of Con-A-L

One more test was done with Con-A-L and was based on the agglutination analysis of Con-A-L in presence of ConA's polyvalent substrate mannan.^{30–31} Figure 6 shows the turbidity changes measured by the increase in absorbance at OD450 nm in the suspensions of Con-A-L compared to PL in presence of mannan. However, in presence of the Con-A-specific low

molecular weight competitive monovalent substrate, α -methyl mannopyranoside (100 μ M), the turbidity rise was significantly inhibited. These data also show that, similar to avidin-L, the Con-A immobilized on the surface of liposome via the DSPE-PEG-_{2K}-CONHNH₂ polymer preserved its functional binding activity and carbohydrate specificity.

IN VITRO assay with CF-labeled mAb 2C5 (pH-dependent degradation)

To investigate the reversibility of the ligand attachment to the liposome surface via a pH-sensitive degradable bond at lowered pH values (mimicking to a certain extent intratumoral and/or intracellular environment), the *in vitro* degradation study of the ligand-modified liposomes at pH 5 was performed (Figure 7A). CF-labeled mAb 2C5 was conjugated at the distal end of the DSPE-PEG-_{2K}-CONHNH₂ and incorporated into liposomes (CF-2C5-L). The incubation of this liposome preparation at pH 5 degraded the hydrazone bond in the linker polymer and removed the labeled mAb 2C5 from the liposome, which resulted in marked decrease in cell-associated Rh-PE fluorescence. The result indicated that almost 80 % of the hydrazone bond broke upon the incubation of the preparation at pH 5 resulting in removing the surface-attached mAb. Dialysis, when continued at pH 5 provoked even more degradation to around 90 %. This study proved the reversibility of the hydrazone bond between mAbs (or any other ligands) and liposomes, which is beneficial for the removal of the ligands attached via the proposed polymer linker from the liposome surface when it could become desirable at certain condition (for example, inside tumors or inside cells when other liposome-attached functionalities are required to become de-shielded).

Fluorescence microscopy and FACS analysis: Interaction of 2C5-L with cancer cells *IN VITRO*

Rhodamine-labeled 2C5-L were incubated with cervical cancer cells (HeLa) (Figure 7B). To enhance the cancer cell binding (eventual tumor accumulation), the mAb 2C5 was used, which recognizes the surface of numerous tumor, but not normal cells via the tumor cell surface-bound nucleosomes.^{15, 32–33} Because mAb 2C5 binds to a broad variety of cancer cells, it may serve as a specific ligand for the delivery of drugs and drug carriers into tumors. The fluorescence microscopy result clearly demonstrated the successful recognition of HeLa cells by 2C5-L, which resulted in the enhanced rhodamine fluorescence signal from the cell surface.

However, the pre-incubation of the 2C5-L at pH 5 resulted in the loss of the antibody from the liposome surface, therefore, preventing the liposome-to-cell association. PL didn't show any cell association being kept at both pH values. The comparison of panels a and c in Figure 7B clearly indicates the successful conjugation of the targeting moiety (mAb 2C5) on the liposome surface, which resulted in more cellular association compared to PL. At the same time, the comparison of panels a and b shows that the action of the lowered pH eliminates the recognition phenomena (i.e. detach the antibody from the liposome because of linker degradation).

To further confirm the successful conjugation, the preservation of the functional specificity of the antibody as well as the cleavability of the hydrazone bond at lowered pH, flow cytometry-based experiments were performed with two cancer cell lines (HeLa and MCF-7) (Figure 8). FACS analysis confirmed well the result obtained using the fluorescence microscopy and clearly demonstrated that the protocol used resulted in the successful conjugation of the antibody to liposomes and significantly better cellular association of 2C5-L compared to PL in both cell lines. And again, the pH-dependent linker degradation and antibody dissociation from liposomal surface at lowered pH (pH 5) was confirmed resulted in less cellular association compared to pH 7.4 in both cell lines.

Introducing a pH-sensitive linkage between a bulky targeting ligand and the liposome could be useful, for example, for drug delivery into tumors when decreased intra-tumoral pH after successful target accumulation could cleave off the targeting ligands and expose other functionalities such as ligands for enhanced cell penetration and intracellular trafficking to the organelles of interests.

In conclusion, an easy and simple method for synthesizing hydrazine-functionalized polyethylene glycol-phosphatidyl ethanolamine polymer was developed. The polymer can readily conjugate various targeting ligands to pharmaceutical nanocarriers, such as liposomes, easily via a pH-responsive reversible hydrazone bond. Proteins avidin, Con-A and monoclonal antibodies 2C5 and 2G4 were successfully immobilized on the liposome surface via the pH responsive hydrazone bond. Various *in vitro* assays confirmed that the targeting ligands retain their specific functional activity. At the same time, the exposure of the ligand-bearing liposomes to a lowered pH results in the degradation of the hydrazone bond and detachment of the ligand.

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Figure 1.
Synthesis of the novel polymer, DSPE-PEG-2K-CONHNH₂.

Figure 2.
 ^1H NMR spectra of DSPE-PEG-2K-NH₂, DSPE-PEG-2K-SH and DSPE-PEG-2K-CONHNH₂.

Liposomes	Mean diameter $\pm$ SD (nm) (n=3)	Polydispersity Index $\pm$ SD (n=3)
Plain Liposomes	188.1 $\pm$ 45	0.098 $\pm$ 0.07
IgG Liposomes	187.5 $\pm$ 53.1	0.131 $\pm$ 0.07
2C5 Liposomes	203.1 $\pm$ 56.3	0.157 $\pm$ 0.14
Con-A Liposomes	205.1 $\pm$ 50.1	0.081 $\pm$ 0.02
2G4 Liposomes	221.1 $\pm$ 39.9	0.051 $\pm$ 0.05

Figure 3. Size distribution of 2C5-L (A); 2G4-L (B) and Con-A-L (C) by the dynamic light scattering technique. A slight increase in size was observed after antibody or protein attachment. The table represents mean diameter and the polydispersity index. Values are mean $\pm$ standard deviation.

Figure 4.

ELISA result for immunoliposomes. A. Binding of 2C5-L to the monolayer of the antigen (nucleohistone) in comparison to the native mAb 2C5; B. Binding of the 2G4-L to the monolayer of the antigen (porcine cardiac myosin) in comparison to the native mAb 2G4. The results show that the activity of the mAbs towards their antigens was retained after the conjugation on to the surface of liposomes.

Figure 5.

Binding of the Rh-PE-labeled avidin-L on biotin-agarose column compared to that of the Rh-PE-labeled PL. **A.** Fluorescence intensity of the eluent collected in 1 mL fractions using PBS, pH 7.4 as mobile phase. Strong interaction of the liposome surface-attached avidin with biotin resulted in the complete retention of avidin-L in the column compared to PL. **B.** Fluorescence intensity of eluent (avidin-denaturing buffer guanidine-HCl, pH 1.5) collected in 1 mL fraction. Slow release of avidin-L from the column is observed as the denaturation of the avidin progresses. The incubation of the column in denaturing buffer at room temperature resulted in an abrupt increase of the fluorescence in the eluent.

Figure 6.

Agglutination analysis of Con-A-L in the presence of mannan and α -methyl mannopyranoside. The turbidity (agglutination) increases significantly with increasing concentration of mannan at OD 450 compared to PL (** $p < 0.001$, $n = 5$). In the presence of conA-specific competitive monovalent substrate α -methyl mannopyranoside (100 μ M), a significant inhibition of the Con-A-L aggregation by the mannan was observed (** $p < 0.05$, $n = 5$). Result indicated the successful conjugation of Con-A on to the surface of liposome with full preservation of its specific activity.

Figure 7.

A. The cleavability of CF-labeled mAb 2C5 from the liposome surface at low pH. The CF-2C5-L were pre-incubated at pH 5 for 1 h at room temperature and dialyzed (MWCO. 300000) against PBS at pH 7.4 and pH 5. The result clearly shows significantly ($***p < 0.001$, $n=3$) less fluorescence after dialysis due to the hydrolysis of the hydrazone bond (around 80 % and 90 % fluorescence loss after the dialysis at pH 7.4 and 5, respectively) between mAb and liposome and release of free mAbs.

B. The effect of pre-incubation (at pH 5) of Rh-PE labeled 2C5-L on cellular association compared to 2C5-L at pH 7.4. Pre-incubation cleaved the antibody from the surface, thereby, causing the loss of the targeting potency of the liposomes, which resulted in virtually no cellular association. a and b. 2C5-L, pre-incubated at pH 7.4 and 5, respectively; c and d. PL, pre-incubated at pH 7.4 and 5. e. Untreated cells. Pictures of bright field and rhodamine channels are shown simultaneously for each treatment.

Figure 8.

Flow cytometry analysis of Rh-PE-labeled 2C5-L and PL at different pH in A. HeLa and B. MCF-7 cell lines. The 2C5-L and PL were pre-incubated at pH 5 and pH 7.4 for 1 h before the incubation with the cells for 1 h. The result shows less targeting and less cellular uptake of 2C5-L after the pre-incubation at pH 5 than after the pre-incubation at pH 7.4 in both cell lines because of detachment of cell targeting antibody from the surface of the liposomes (** $p < 0.05$, *** $p < 0.001$, $n = 3$).

Scheme 1.

Schematic representation of the preparation of antibody- or protein-grafted liposomes using DSPE-PEG-_{2K}-CONHNH₂ polymer.