Effect of lipid composition on incorporation of trastuzumab-PEG-lipid into nanoliposomes by post-insertion method: physicochemical and cellular characterization

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Abstract

Context: Anti-HER2 immunoliposomes are promising nanotechnology based systems for active targeting of breast tumors, which depends on the amount of incorporated antibody.

Objective/Aim: In this work, we investigated the possible effect of lipid composition on the incorporation of trastuzumab-PEG-PE micelles into nanoliposomes and on their subsequent specific cellular targeting.

Materials and methods: Trastuzumab (anti-HER2 monoclonal antibody) was monothiolated and conjugated to maleimide-PEG-PE micelles. Liposomes of different lipid compositions were prepared by the thin layer hydration. Trastuzumab-PEG-PE micelles were incorporated into the liposomes by the post-insertion method. The percentage of lipid mixing was determined based on fluorescence resonance energy transfer. Cellular binding and uptake of rhodamine-labeled immunoliposomes were studied in SKBR-3 (HER2+++ ) and MCF-7 (HER2+) cells. Also, antitumor cell activity of the immunoliposomes was compared to free trastuzumab and the liposomes.

Results: The lipid mixing of trastuzumab-PEG-PE micelles depended on the liposome composition. The immunoliposomes containing DPPC, cholesterol and PEG-PE showed prominent lipid mixing. The lipid mixing was consistent with the cell binding results which showed an efficient and specific binding of the immunoliposomes to SKBR-3 cells. Antitumor cell activity of the immunoliposomes in SKBR-3, unlike MCF-7 cells, depended on the content of trastuzumab.

Discussion: Cholesterol and PEG-PE in the liposome composition are prerequisites for a successful lipid mixing due to their ability to facilitate fusion. The higher lipid mixing results in higher antibody incorporation and consequently higher targeted cell binding.

Conclusions: The lipid mixing depends on the liposome composition, which reflects targeted cell binding of the immunoliposomes.

Keywords
HER2, immunoliposomes, lipid mixing, specific cell binding

Introduction

The human epithelial growth factor receptor-2 (HER2/ErbB2) possesses an important role in the development and also progression of different types of cancers (Hynes & Stern, 1994). This membrane receptor HER2 is a member of the tyrosine-specific protein kinase family, the EGFR (epidermal growth factor receptor) family, which consists of four EGF receptors: ErbB1, ErbB2, ErbB3 and ErbB4 (Steinhauser et al., 2006). HER2 receptor contains an intracellular (cytoplasmic), a transmembrane, and an extracellular domain, which the latter is involved in ligand binding (Chen et al., 2003; Steinhauser et al., 2006). This oncogen receptor is overexpressed in 20–30% of breast and ovarian cancers as well as some other adenocarcinomas while its expression in normal adult tissue is weak (Hynes & Stern, 1994; Tokuda, 2003). This overexpression of oncogenic HER2 receptors causes poor prognosis in patients (Hynes & Stern, 1994). Many studies have also shown HER2 to be a direct contributor to oncogenesis, metastasis and angiogenesis (Park et al., 2001; Steinhauser et al., 2006). Among tumor markers, HER2 is one of the most promising and attractive targets for immunotherapy (Camirand et al., 2002). It acts as a biomarker and a therapeutic target in HER2 positive breast cancers.

Trastuzumab (Herceptin®, rhu mAb), a recombinant humanized IgG1 monoclonal antibody with high affinity for the extracellular part of HER2 receptor, was approved by the FDA in 1998 for therapy against metastatic breast cancers overexpressing the HER2 receptors (Tripathy, 2005). Numerous clinical trials have constantly shown that trastuzumab possesses effectiveness and antitumor activity in
HER-2-overexpressing metastatic breast cancers (Baselga, 2001; Cobleigh et al., 1999; Vogel et al., 2002). Trastuzumab can elevate the response rate to 50% and extent the survival of women with HER-2-overexpressing metastatic breast cancers in combination with chemotherapy (Slamon et al., 2001). The most important toxicity related to trastuzumab is cardiotoxicity which probability was highest in patients who received it concurrently with anthracyclines (Cobleigh et al., 1999).

Trastuzumab has shown several promising properties. It has been demonstrated to have antitumor activity by reversing the consequences of overactive HER2 such as uncontrolled cell growth and differentiation in tumor cells, survival and metastasis (Baselga et al., 1998; Hudziak et al., 1989; Lewis et al., 1993; Niehans et al., 1993; Pietras et al., 1998). Also, trastuzumab can serve as a specific ligand by binding to the HER2 receptor and targeting chemotherapeutic agents or drug carriers actively to tumor cells (Nahta & Esteva, 2003). Trastuzumab as the homing ligand can be served for targeted cellular drug delivery and gene therapy using due to the internalizing characteristics of the HER2 receptor.

Liposomes are spherical vesicles made of lipid bilayers with an aqueous core. They are the most common and well-defined carriers and represent a successful platform for drug delivery (Hamilton & Piccart, 2000). Liposomal drug formulations have several advantages, such as biodegradability, biocompatibility and low cytotoxicity. During the last decades, liposomes have attracted increased interest as an excellent model for biological membranes as well as efficient carriers for drugs, diagnostics, vaccines, nutrients, cosmetics, and other bioactive agents (Chen et al., 2003; Shmeeda et al., 2009). Although liposomal formulations are one of the promising approaches in drug delivery, they may possess some problems. Rapid clearance of the conventional liposomes is identified as one of their major drawbacks in anticancer drug delivery (Park et al., 2001, 2002; Yang et al., 2007a). They are rapidly captured by reticuloendothelial system (RES) (Fredika & Mauro, 2006; Steinhauser et al., 2006). Their rapid clearance from the body can be overcome by using flexible hydrophilic polymers, such as polyethylene glycol (PEG) anchored to phospholipids such as phosphatidylethanolamine in the liposomal formulation (Sawant & Torchilin, 2012). Surface modification of liposomes with PEG leads to sterically stabilized (stealth) liposomes which show longer circulation half lives (Millella et al., 2004; Nahta & Esteva, 2006; Waterhouse et al., 2003). They are also useful for passive targeting of the tumor microenvironment and preferentially and spontaneously accumulate in tumor tissues through the enhanced permeability and retention (EPR) effect (Cirstoiu-Hapca et al., 2009). Moreover, the presence of PEG chains on the liposome surfaces provides a stronger inter-bilayers repulsion that can help overcome the attractive van der Waals forces, hence stabilizing liposomes by avoiding aggregation (Garbuzenko et al., 2005; Immordino et al., 2006).

The non-specific properties of liposomes are another problem related to liposomes which can be overcome by targeting approaches (Jerome et al., 2006). Active targeting relies on ligand-directed binding of nanoparticles (Nahta et al., 2006) to tumor-specific or tumor-associated antigens in the tumor environment (Daugherty & Mrsny, 2006; Nahta & Esteva, 2006). Targeted liposomes increase drug accumulation in the desired tissues and cause higher and more selective therapeutic activity. Among different targeting moieties, monoclonal antibodies have the highest potential of specificity and variability (Elbayouni & Torchilin, 2008).

Immunoliposomes are among the actively targeted carriers which can deliver their cargo to the specific tumor tissues by attaching antibodies against tumor antigens to the liposomal surface (Immordino et al., 2006; Yang et al., 2007a). There are different preparation techniques for immunoliposomes such as surface adsorption (Price et al., 2001), covalent direct attachment of antibody to a functionalized liposome (Hansen et al., 1995; Mcmullen & McElhaney, 1996; Sulkowski et al., 2005), and the newer method of post-insertion (Cheng & Allen, 2008; Pan et al., 2007). Surface adsorption is not efficacious since it leads to destabilization after exposure to serum protein in vivo. The efficiency of antibody attachment is also relatively low and the liposome aggregation is frequently observed. Moreover, the amount of attached ligand is not simply controllable, and the correct orientation of the antibodies is not ensured (Nobs et al., 2004).

Covalent conjugation of antibody either directly or by the post-insertion method are among the easiest strategies. The covalent conjugation is usually performed by different reactions, such as the conjugation of two thiol groups to form a disulfide bond (Mercadal et al., 1998), the conjugation of carboxylic acid with primary amines (amide linkage) (Maruyama et al., 1997), the conjugation of carbohydrate with hydrazide groups (hydrazone bond) (Harding et al., 1997; de Menezes et al., 1998), and the conjugation between thiol groups in ligands and maleimide groups (thioether bond) (Allen et al., 1995; Cheng & Allen, 2008; Nielsen et al., 2002; Sapra et al., 2004; Steinhauser et al., 2006; Yang et al., 2007). The reaction between thiol-bearing monoclonal antibody and maleimide group is a highly efficient reaction that leads to a stable thioether bond (Ansell et al., 2000; Nobs et al., 2004).

In the post-insertion method, the reaction occurs between the ligand and an anchor, and the resulting conjugate is introduced into the liposome structure by simple mixing (Nobs et al., 2004). The post-insertion technique has many advantages including: the acceptable position of ligands on the outer surface of the liposomes, high-insertion efficiencies in optimized co-incubation conditions (temperature and duration) (Sawant & Torchilin, 2012), and a decrease in non-specific interactions between the lipids and liposomes (Moreira et al., 2002). The post-insertion method is a simple and rapid technique by which a variety of ligands can be incorporated into a variety of pre-formed liposomes (Allen et al., 2002). Although the post-insertion technique can be very simple and efficacious, it is affected by different factors. It has been shown that lipid composition is effective on the incorporation of liposomes into biological membranes (Chernomordik, 1996). However, the effect of liposome composition on the incorporation of immunomicelle into the liposomal bilayer structure and the consequent effect on target cell association have not studied clearly yet. Therefore, here we developed anti-HER2 immunoliposomes by the post-insertion method with the aim to take advantage of the
active targeting against HER2 receptors in breast cancer cells and more importantly to investigate the effect of different liposomal compositions on the incorporation of trastuzumab-PEG-PE micelles (immunomicelles) into liposomes and their cellular association with SKBR-3 (HER2+++ versus MCF-7 (HER2+) cells).

Materials and methods

Materials

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-sn-glycero-3-phospho-rac-(glycerol) (DPPG) were obtained from Lipoid (Ludwigshafen am Rhein, Germany). 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-(maleimide(polyethyleneglycol)-2000) (Mal-PEG-DSPE), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(methoxy(polyethylene glycol)-2000) (PEG-DOPE), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rh-DOPE), 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2,1,3-benzoxadiazol-4-yl) (NBD-PE) and cholesterol (Chol) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Trastuzumab (Herceptin<sup>®</sup>) was purchased from Roche (Mannheim, Germany). 2-Iminothiolane (Traut’s reagent) and 5,5'-dithio-bis-(2-nitrobenzoic acid) (Ellman’s reagent) were obtained from Sigma (St. Louis, MO), Bradford reagent and glutathione (GSH) from Fermentas (St. Leon-Rot, Germany) and Merck (Darmstadt, Germany), respectively. MCF-7 and SK-BR-3 cell lines were purchased from the Pasteur Institute (Tehran, Iran).

Trastuzumab thiolation

In order to conjugate trastuzumab to maleimide-bearing micelles via the thioether linkage, a thiol group was introduced into trastuzumab through a ring opening reaction between the primary amine of trastuzumab and 2-iminothiolane. Accordingly, trastuzumab was dissolved in 25 mM HEPES-buffered 145 mM saline solution (HBS, pH = 7.4) and mixed with 87.5 mM EDTA and 2-iminothiolane solution for producing a monothiolated antibody. Three-level factorial design was carried out to optimize the degree of trastuzumab thiolation as a function of different factors including different pH values (6.3, 7.3 and 8.3), the molar ratio of 2-iminothiolane/trastuzumab (11, 43 and 75) and the reaction time (5, 60 and 90 min). The thiolated trastuzumab was purified by Amicon 30 kDa ultra centrifugation device (Millipore, Billerica, MA) at 12000g and 8 °C.

The degree of thiolation was calculated from the molar ratio of the recovered thiol to trastuzumab concentration. Trastuzumab recovery was calculated from Bradford protein assay (Bradford, 1976). Briefly, five micro liters of the purified thiolated trastuzumab was mixed with 250 μl of Bradford reagent and the light absorbance was measured at 595 nm with a microplate spectrophotometer model Power Wave XS (Biotek, Seattle, WA). The thiol concentration was calculated relative to Ellman assay standard curve plotted for GSH as presented in Ellman assay section (Ellman, 1959).

Purity of the thiolated trastuzumab was verified by an isocratic high-performance size exclusion chromatography (HPSEC) using a HPLC system (Knauer, Berlin, Germany). The analysis was performed in TSKGEL5000 PWXL column (30 cm × 7.8 mm) (Tosoh Bioscience, Tokyo, Japan), HBS as the mobile phase at flow rate of 0.5 ml/min and at UV wavelengths of 280 and 245 nm for the detection of trastuzumab and 2-iminothiolane, respectively.

Bioconjugation of trastuzumab to Mal-PEG-PE micelles

The solutions of Mal-PEG-PE and PEG-PE in chloroform were mixed at a molar ratio of 1:4. Then, the solvent was thoroughly removed under vacuum by rotary evaporation model Q01 (Heidolph Instruments, Schwabach, Germany) for 4 h under ice-bath condition. The resulting dry film was further hydrated by a vortex for 5 min with an appropriate volume of HBS to obtain a 10 mM concentration. The micelles were sonicated for 3 min by a probe sonicator (VP200H; Hielscher, Berlin, Germany). The hydrodynamic diameters of the micelles were determined using a laser light scattering particle size analyzer (SALD-2101; Shimadzu, Japan) calibrated with nominal 100 nm polystyrene. SPAN index was calculated for determination of the polydispersity.

In order to link the thiolated trastuzumab from the previous section to the maleimide-functionalized micelles, the conditions of the bioconjugation reaction were optimized using a factorial design technique. Accordingly, micelles were incubated with GSH at four maleimide/thiol molar ratios (1/2, 1/1, 3/1 and 10/1) each for three time periods (15, 120 min, and overnight) at 4 ºC. All the samples were prepared in triplicate. The bioconjugation yields were assessed by the Ellman assay and are presented in the next section. A lower molar ratio of maleimide/thiol which resulted in a higher yield was recognized as the optimum condition for bioconjugation of trastuzumab to Mal-PEG-PE micelles.

Ellman assay

Ellman reagent, also known as DTNB, is a very useful compound used for measuring free thiol (sulphydryl) groups in aqueous solutions. This reaction produces a measurable yellow product after reacting with sulphydryls that can be photometrically quantified (Ellman, 1959). The solution of 400 μg/ml Ellman reagent in HBS was added to 100 μl of different concentrations of GSH prepared by serial dilution. After 15 min, light absorbance was measured at 412 nm by the microplate spectrophotometer for the standard solutions of GSH in range of 25–400 μM and HBS plus Ellman reagent as the blank (linear regression coefficient = 0.0032 ± 0.0009 μM<sup>−1</sup>, R<sup>2</sup> = 0.99). Ellman assay was performed for the thiolated trastuzumab before and after conjugation to maleimide-micelles. The bioconjugation yield was determined from Equation (1), where A<sub>mAb-SH</sub> and A<sub>mAb-SH/Mal</sub> are light absorbance at 412 nm for thiolated trastuzumab alone and after mixing with maleimide functionalized micelles, respectively. A<sub>0</sub> is blank containing Ellman reagent and HBS.

Bioconjugation yield (%) = \( \left( \frac{A_{mAb-SH} - A_{mAb-SH/Mal}}{A_{mAb-SH} - A_0} \right) \times 100 \) (1)
Preparation of nanoliposomes

Different liposomal formulations were prepared by the thin layer hydration method. Briefly, lipid solutions in chloroform including DPPC (20 mg/ml), DPPG (20 mg/ml), CHOL (50 mg/ml), PEG-DOPA (20 mg/ml) and Rh-DOPA (0.5 mg/ml) were mixed at different molar ratios as indicated in Table 1. Rh-DOPA and NBD-DSP were incorporated in the lipid composition as the fluorescent probes if indicated. A thin lipid film was formed by rotary evaporation under vacuum at 150 rpm for 4 h. The film was hydrated by HBS overnight to obtain 5 mM total lipid concentration. The preparation was done at a temperature above critical temperature required for liposome preparation as indicated in Table 1. The liposomes were extruded 10 times through double-stacked 80 nm polycarbonate filters at 50–200 psi nitrogen gas pressure and at the same temperature as the preparation step using a thermo barrel-equipped extruder (Northern lipids, Burnaby, Canada).

Lipid recovery by Stewart assay method

Phospholipids were extracted from liposomes by the Bligh and Dyer method (Bligh & Dyer, 1959). Briefly, to 0.8 volume parts of samples, three parts of chloroform/methanol mixture (1:2 v/v) were added to give one phase. After vigorous mixing and incubation at room temperature, one volume part of chloroform and water was added separately and mixed by a vortex after each addition. The samples were centrifuged and the lower chloroform phases containing lipids were separated. To determine the phosphate content of phospholipids, the Stewart assay was performed (Stewart, 1980). First, ferrothiocyanate reagent was prepared by dissolving ferric chloride hexahydrate and ammonium thio-cyanate in the deionized water. The reagent was then mixed with different concentrations of standard solutions of DPPC as well as the extracted samples in chloroform. After incubation at room temperature, the optical densities of standards and samples were read at 485 nm using an UV spectrophotometer (UV-1650pc; Shimadzu, Japan). The concentrations of phospholipid in the test samples were calculated from the standard curve plotted for DPPC. Consequently, the lipid recovery percentage for each formulation was calculated by Equation (2), where $M_0$ and $M_f$ are initial total phospholipid amount used and the amount of phospholipid in the chloroform phase calculated from the corresponding calibration curve of DPPC, respectively.

$$\text{Lipid recovery (\%)} = \left( \frac{M_0 - M_f}{M_0} \right) \times 100 \quad (2)$$

Immunoliposome preparation by post-insertion method

Immunoliposomes can be generated by two methods, through coupling antibody directly to the liposome surface or by insertion of the antibody coupled to micellar lipids into the liposomes. Trastuzumab-PEG-liposomes (Stealth immunoliposomes) were prepared using the post-insertion method. Accordingly, trastuzumab-PEG-PE micelles were incubated with different formulations of freshly prepared nanoliposomes at a molar lipid ratio of 1/25 (micelle/liposome) overnight at 4 °C while stirring at 100 rpm.

Particle size analysis

Particle sizes of different immunoliposome formulations at micelle to liposome mole ratio of 1/25 were measured by the laser light scattering particle size analyzer (Shimadzu, Japan). This technique measures particle size distributions by measuring the angular variation in intensity of light scattered as a laser beam passes through a dispersed particulate sample. After the instrument set to zero by HBS buffer, the samples which were diluted to an appropriate concentration with HBS buffer were transferred to a quartz cell for measurement at room temperature. The polydispersity index (SPAN index) was determined as a measurement of the distribution of immunoliposome population, which refers to the level of homogeneity of the sizes of the particles. SPAN index was calculated for all of the liposomal formulations by Equation (3), where $D_{0.9}$, $D_{0.5}$ and $D_{0.1}$ are the respective particle diameters determined at 90th, 50th and 10th percentile of particles undersized. A small value of SPAN index (<1) indicates a homogenous vesicle population.

$$\text{Span} \text{ index} = \left( \frac{D_{0.9} - D_{0.1}}{D_{0.5}} \right) \quad (3)$$

To determine stability of the immunoliposomes, their particle sizes were measured after 6 month storage in a refrigerator at 4 °C.

Table 1. Lipid composition, the molar ratio and preparation temperature related to different formulations of nanoliposomes.

| Formulation | Composition          | Molar ratio | Temperature (°C) |
|-------------|---------------------|-------------|-----------------|
| F1          | DPPC:PEG-PE         | 95:5        | 40              |
| F2          | DPPC:PEG-PE:Fluor-PE | 94:5:1      | 40              |
| F3          | DPPC:Chol:PEG-PE    | 50:45:5     | 60              |
| F4          | DPPC:Chol:PEG-PE:Fluor-PE | 49:45:5:1 | 60              |
| F5          | DPPC:Chol           | 55:45       | 60              |
| F6          | DPPC:Chol:Fluor-PE  | 54.5:44.5:1 | 60              |
| F7          | DPPC:DPPG           | 55:45       | 40              |
| F8          | DPPC:DPPG:Fluor-PE  | 54.5:44.5:1 | 40              |

*Fluor-PE denotes Rh-PE and NBD-PE in 1:1 molar ratio for lipid mixing assay. Instead, Rh-PE alone was used for cellular studies.
Lipid mixing assay

The nanoliposomal and immunoliposomal (1/25 micelle/liposome molar ratio) formulations were diluted to 750 μM total lipid concentration with HBS buffer. Fluorescence intensity of the dilutions was determined prior and after liposome solubilization with 10% Tween-20 and 5 min sonication in a bath sonicator (Teca3; Cividate Camuno, Italy). Fluorescence intensities were determined with a fluorescence plate reader (infinite M200; Tecan, Gro¨dig, Austria) at the excitation and emission λmax of 460 and 535 nm for NBD-PE, respectively. The fluorescence recovery was calculated according to Equation (4), where I1Liposome, I1Liposome+Tween 20 and I0 are the fluorescence intensities of the liposomes prior to and after the Tween-20 addition and the HBS buffer blank, respectively.

\[
\% \text{ Fluorescence recovery} = \frac{(I_{\text{Liposome}} - I_0)}{(I_{\text{Liposome}} + \text{Tween}20) - I_0} \times 100
\]

Lipid mixing as a determinant of immunomicelle incorporation into liposomal structures is based on the fluorescence resonance energy transfer (FRET) phenomenon (Struck et al., 1981). In this assay, the various liposomal membranes labeled with a combination of fluorescence energy transfer donor and acceptor lipid probes, NBD-PE and Rh-PE, diluted with HBS to 750 μM total lipid concentration and then were mixed with Trastuzumab-PEG-PE micelles at 1/25 micelle/liposome molar ratio. Fluorescence intensity was determined before and after liposome solubilization with 10% Tween-20 and 5 min sonication in an ultrasonic bath sonicator. Fluorescence intensities were determined with the fluorescence plate reader at the excitation and emission λmax for NBD-PE, 460 and 535 nm, respectively. The lipid mixing percentage was calculated according to Equation (5), where I1, I2 and Imax are the fluorescence intensities of liposomes prior to and after addition of the immunomicelle, and after solubilization by Tween-20, respectively.

\[
\text{Lipid mixing (}) = \frac{(I_2 - I_1)}{(I_{\text{max}} - I_1)} \times 100
\]

Cell culture

Cellular studies were performed using SK-BR-3 (HER2 receptor over expressing, HER2+++) and MCF-7 (HER2 receptor low expressing, HER2+) cells. The cells were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS; PAA, Australia) and 1% antibiotic–antimycotic (Penicillin–Streptomycin, Australia) in an incubator at 37°C and 5% CO2.

Fluorimetric assay of cell binding

To characterize if the immunoliposomes were able to bind to the HER2 receptors, SK-BR-3 and MCF-7 cells were treated individually with the liposomes and the corresponding immunoliposomes at different concentrations. The cell-associated fluorescence intensity of Rh-PE probe was studied by fluorimetry. The cells were seeded in 96-well plates (density of 10 000/well) and placed in the incubator at 37°C and 5% CO2 for 24 h. The cells were washed twice with sterile PBS solution. Each liposome or immunoliposome was diluted with HEPES buffer (25 mM) to obtain the concentrations of 750 μM. The cells were incubated with 100 μl of each sample at 4°C for 1 h. Then, the medium was removed and the cells were washed twice with sterile PBS. The cell-associated fluorescence intensity was measured with the fluorescence plate reader at the respective rhodamine λmax of excitation and emission of 550 and 580 nm, respectively, following cell disruption with 100 μl of 10% Tween-20 and a 1-h incubation at room temperature while shaking.

Cellular internalization and competitive binding assay by flow cytometry

To characterize if the immunoliposomes were able to target HER2 receptor specifically, the SKBR-3 cells were treated with F4-immunoliposomes at three different molar ratios of trastuzumab to liposome (1/250, 1/625 and 1/2500) and F4-liposomes. The cells were seeded in six-well plates (at the density of 300 000/well) and incubated at 37°C and 5% CO2 for 24 h. The cells were washed twice with sterile PBS and incubated with the samples. To verify specificity of the immunoliposome binding to the HER2 receptors, the experiments were individually performed for the cells with or without pre-incubation with 2.5 μg/ml trastuzumab for 30 min at 4°C. After incubation for 4 h at 37°C, the cells were harvested, washed and resuspended in cold PBS (pH 7.4) for determination of the cell-associated rhodamine fluorescence at the emission wavelength of 580 nm (channel FL2) for total of 10 000 events (cells/sample) using a flow cytometer [Becton-Dickinson (BD) Biosciences, Heidelberg, Germany]. The analysis was performed subsequently with FlowJo software version 7.6.2 (Tree Star Inc., San Carlos, CA).

Fluorescence microscopy

SK-BR-3 cells were plated at 100 000 cells/cm² density in Petri dishes and maintained at 37°C in a CO2 incubator. After 2 days, the culture medium was completely aspirated and the cells were harvested. The cell suspension (1,000 000 cells/ml) was prepared in normal saline. The cells were harvested by centrifugation at 1000 rpm for 5 min. The supernatant was discarded and the cells were incubated with 100 μl of F4-liposome and F4-immunoliposome formulations (containing 1 mol% Rh-PE, 750 μM total lipid concentration) for 1 h at 4°C. The cells were centrifuged again and the pellets were washed twice with PBS solution. Then, the cell suspensions were mounted on glass slides and were imaged using epifluorescence microscope model Eclipse E600 (Nikon; Kanagawa, Japan) equipped with G-2A filter at 10,000× magnification.

Cell antitumor activity

The antitumor activity of free trastuzumab at the antibody concentration corresponds to 1/250 immunoliposomes, the F4-liposome and the F4-immunoliposomes were examined at different molar ratios of trastuzumab/liposome (1/250, 1/625 and 1/2500) and the total lipid concentration of 750 μM using MTT assay (Twentyman & Luscombe, 1987). Briefly, MCF-7 and SKBR-3 cells were seeded in 96-well plates at the density

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of 25,000 cells/well and incubated for 24 h at 37 °C and 5% CO₂. Then, the culture medium was replaced with the samples diluted in the fresh medium. After 24 h incubation, the culture medium was supplemented with 100 μl MTT (0.5 mg/ml). After 3 h further incubation at 37 °C, 100 μl DMSO was added to each well. The absorbance was read at the light wavelengths of 570 and 650 nm by the microplate reader. Cell viability was calculated from the Equation (6):

\[
\text{Cell viability} (\%) = \left( \frac{A_{570} - A_{650}}{A_{570}} \right) \times 100
\]

(6)

Where \( A_{570} \) and \( A_{650} \) are the sample absorbance at the respective light wavelengths of 570 and 650 nm; similarly, \( A_{c570} \) and \( A_{c650} \) are the light absorbance of control samples.

**Statistical analysis**

Statistical analysis of the data was done using GraphPad Prism software version 5.04 (GraphPad Software Inc., La Jolla, CA) for Microsoft Windows. Data were compared using analysis of variance (ANOVA) followed by student’s post hoc t-test with Bonferroni correction. Differences were considered significant when \( p \) values were < 0.05 (confidence interval of 95%). Data was represented as the mean ± standard deviation (SD). All the experiments were run in triplicate.

**Results**

**Trastuzumab thiolation**

Thiolation of trastuzumab was considered as the first step for bioconjugation to the maleimide-functionalized liposomes. As shown in Figure 1, the effect of pH and the mole ratio of Traut’s reagent was determined very significant (\( p < 0.001 \)). Trastuzumab was successfully monothiolated at the certain condition (pH = 7.3, Traut’s reagent to trastuzumab mole ratio = 43 and incubation time = 1 h). Trastuzumab was completely purified from Traut’s reagent with an ultrafiltration centrifugal device (supplementary information). The trastuzumab recovery yield after purification and the degree of thiolation were determined to be 90 ± 3.1% and 1.03 ± 0.06, respectively.

**Immunomicelle preparation**

Immunomicelles were developed by conjugation of the purified thiolated trastuzumab to Mal-PEG-PE micelles. In order to achieve maximum conjugation yield, Mal-PEG-PE micelles were incubated with thiolated trastuzumab at four levels of maleimide/thiol mole ratios and three levels of incubation times. Figure 2 shows that the incubation time had no statistically significant effect on the yield of reaction (\( p > 0.05 \)), which indicates that the reaction was completed very rapidly, whereas the mole ratio had a very significant effect (\( p < 0.0001 \)). The reaction was almost completed at a maleimide/thiol mole ratio above three even at the early incubation time of 15 min; therefore, the best conjugation condition was determined to be for the mole ratio above three and a 15-min incubation time.

**Lipid mixing assay**

Different liposome formulations similarly containing 1% mole fluorescent lipid probes (Rh-PE/ NBD-PE 1:1) were prepared using the thin layer hydration method with the lipid recoveries ranging from 87.49 ± 1.26% to 94.24 ± 2.12% (Table 1). Then, to determine the level of incorporation of the immunomicelles into the liposomes, a lipid mixing assay was performed. Figure 3(A) shows the fluorescence intensities before and after solubilization of the nanoliposomes. The highest fluorescence intensity was found for F2 followed by F4, F6 and F8, but after the solubilization step, they showed similar fluorescence.

Figure 1. 3D plot for the effect of pH and Traut’s to trastuzumab mole ratio on desirability (trastuzumab degree of thiolation) after 1 h incubation at room temperature.
intensities ($p > 0.05$). Following the mixing of each liposome formulation with the immunomicelle, the highest intensity was found for F4 followed by F2, F8 and F6 (Figure 3B). Again no significant difference was found between them after solubilization ($p > 0.05$). As shown in Figure 4, F8 formulation (DPPG liposomes) had the maximum lipid mixing (48.5 ± 4.94%), F4 (cholesterol and PEG-PE containing liposomes) had the modest mixing of 26.7 ± 8.83%, F2 (PEG-PE containing liposomes) and F6 (classic DPPC liposomes) showed the lowest degrees of 4.6 ± 9.69% and 13 ± 3.06%, respectively.

**Particle size**

The immunoliposomes had sizes in the range of 53–76 nm that did not change significantly after 6 month storage at 4°C in a refrigerator except for the F6-immunoliposome formulation (Table 2).

**Cell binding assay**

To determine the cell binding of the immunoliposomes in comparison to the corresponding liposomes, the percentages of cell-associated fluorescence to total fluorescence of the liposomes/immunoliposomes were compared in SK-BR-3 (HER2$$^{++}$$) and MCF-7 (HER2$$^{+}$$) cells. Both the cell type and the formulation had very significant effects on cell binding ($p < 0.0001$). Differences of the cell binding between two cell lines were significant for the immunoliposomes (Figure 5A, $p < 0.001$), whereas they were not significant for the liposomes (Figure 5B, $p > 0.05$). Moreover, the differences between the liposomes and the immunoliposomes were significant in SK-BR-3 cells ($p < 0.001$). Among different immunoliposomes, the highest cell binding was found for F4-immunoliposome in SK-BR-3 cells followed by F2, F8 and F6 (Figure 5A).
Flow cytometry

The specific targeting potential of immunoliposomes was assessed by comparing the binding of liposomes conjugated to trastuzumab at different molar ratios with and without antibody block in SK-BR-3 cells. The cell-associated fluorescence intensity increased following the antibody conjugation that is more evident at the molar ratio of 1/250 (Figure 6, Table 3). The percentage of cell binding increased in a trastuzumab concentration-dependent manner, but it decreased while the cells were formerly blocked with anti-HER2 antibody. Inhibition of the specific cell binding was more pronounced for 1/250 immunoliposome (Table 3). To investigate the cell uptake efficacy, the immunoliposome-treated cells were further incubated at 37°C. The cell uptake progressively increased by increasing the molar ratio of trastuzumab-PEG-PE micelles to the liposome (Figure 7).

Epifluorescence microscopy

Epifluorescence microscopy was used to compare cell association of Rh-PE labeled F4-liposomes with the corresponding immunoliposomes. As presented in Figure 8 after 1-h incubation with SK-BR-3 cells at 4°C, the immunoliposomes showed more cellular association.

Cell antitumor activity

Antitumor activity and cytotoxicity of the F4-liposome, the F4-immunoliposomes, and free trastuzumab in SKBR-3 and MCF-7 cells are presented in Figure 9. The immunoliposomes showed a cell-type specific antitumor activity ($p < 0.0001$). In SKBR-3 cells (expressing high level of HER-2 receptors), antitumor activity increased by increasing trastuzumab-PEG-PE content in the immunoliposomal formulations ($p < 0.001$). The immunoliposomes showed an identical antitumor activity to free trastuzumab as determined at the antibody concentration corresponds to 1/250 immunoliposomes. Unlike immunoliposomes, the corresponding liposomes did not show any cytotoxicity in SKBR-3 cells. In MCF-7 cells, the immunoliposomes as well as the liposomes and free trastuzumab did not show any significant cytotoxicity ($p > 0.05$).

Discussion

Active targeting is based on the association of overexpressed receptors in cancer cells with high affinity and specific ligands attached to nanocarriers (Torchilin, 2005). There is an increased interest in the use of active targeting as a strategy for increasing therapeutic effects of drugs. Immunoliposomes are among the most promising actively targeted carriers in which monoclonal antibodies against tumor-associated surface receptors are attached to the liposomal surface (Sapra et al., 2004). There are different methods to introduce antibodies to the liposome surface, one of which is the newer and the most efficient technique called the post-insertion method (Cheng & Allen, 2008; Pan et al., 2007).
This method is based on attachment of antibody to an anchor and then incorporation of the resulted conjugate to the liposome surface (Cheng & Allen, 2008; Nobs et al., 2004; Pan et al., 2007). There are different factors shown to be effective in the post-insertion method such as incubation time and temperature (Sawant & Torchilin, 2012). Moreover, the lipid composition can affect the fusion of liposomes to biological membranes (Chernomordik, 1996). However, the importance of lipid composition on post-insertion method and consequently the cellular association had not yet been studied. Therefore, in the present study, preparation of immunoliposomes of different compositions at preferable storage conditions by post-insertion method and cell association of the immunoliposomes were studied.

In the first step, among various antibody conjugation chemistries, it was far more preferred to conjugate Mal-PEG-PE micelles to the monothiolated trastuzumab through a rapidly forming very stable thioether linkage with a high reaction yield (Nielsen et al., 2002; Steinhauser et al., 2006; Yang et al., 2007a). Toward this approach, trastuzumab was successfully monothiolated by Traut’s reagent at the optimized condition (Traut/trastuzumab mole ratio of 40 for 1 h at pH of 7.30) (Figure 1) (Steinhauser et al., 2006). Then, the conjugation of thiolated trastuzumab to Mal-PEG-PE micelles was optimized for different factors (maleimide/thiol molar ratio and incubation time). It was shown that time had no significant effect on the conjugation process because the reaction of thiol-maleimide occurred very rapidly; however, the maleimide/thiol mole ratio had a determining effect on the yield of the conjugation reaction (Figure 2). If the maleimide/thiol mole ratio changes equivalent or more than three, an almost complete trastuzumab bioconjugation occurs because there are sufficient maleimide groups for the existed thiol groups of trastuzumab. As reported in the literature, the maleimide/thiol mole ratios of 4 (Nielsen et al., 2002) and 10 (Cheng & Allen, 2008) are effective.

Table 3. Binding efficiency % without and with pre-incubation with trastuzumab after 1h at 4°C in SK-BR-3 cells.

| Formulation          | Binding efficiency % without antibody block | Binding efficiency % with antibody block |
|----------------------|-------------------------------------------|----------------------------------------|
| 1/250 Immunoliposome | 48.11 ± 2.91                               | 24.01 ± 1.63                           |
| 1/650 Immunoliposome | 10.92 ± 1.43                               | 4.09 ± 0.60                            |
| 1/2500 Immunoliposome| 2.87 ± 0.11                                | 1.34 ± 0.04                            |
| Liposome (control)  | 0.21 ± 0.05                                | 0.20 ± 0.06                            |

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PEG-immunomicelles and the nanoliposomal formulations were prepared separately and consequently they were mixed together to form immunoliposomes spontaneously. As reported in previous studies using the post-insertion method (Cheng & Allen, 2008; Pan et al., 2007), the hydrophobic domain of DSPE is able to incorporate spontaneously into the preformed liposomal structure through a simple one-step incubation of the trastuzumab-PEG-PE micelles with preformed liposomes (Lu et al., 2006; Yang et al., 2007a); however, the effect of liposome composition on the level of antibody incorporation was unknown and was investigated by lipid mixing assay in this work.

As shown in Figure 4, the level of lipid mixing was dependent on the liposomal compositions. The F2 formulation (contains PEG-PE, but no cholesterol) showed the lowest degree of mixing. The level of mixing increased significantly for F4 which contains both cholesterol and PEG-PE that can be explained by the lipid bilayers stabilizing effect due to cholesterol (Gregoriadis & Davis, 1979; McMullen & McElhaney, 1996; Sulkowski et al., 2005). Nevertheless, the level of lipid mixing for the F6 formulation (contains cholesterol, but no PEG-PE) was not as high as F4.
documenting the positive role of PEG-PE. Hence, the combination of both lipids (cholesterol and PEG-PE) is a prerequisite for successful lipid mixing. It should be mentioned that the obtained results are in agreement with the previous studies showing inclusion of some lipids such as cholesterol and PE in the liposome composition facilitates biological fusion bilayer structures (Chernomordik, 1996). Among different liposome compositions, F8 formulation had the maximum lipid mixing probably because of the existence of negatively charged DPPG in the lipid composition that may facilitate liposome–antibody interaction (pI of trastuzumab = 9.2) (Wiig et al., 2005).

The prepared immunoliposomes had favorable size distribution ranging from 53 to 76 nm (Table 2), with an almost 90% lipid recovery (Table 2). The sizes did not change significantly during the 6 months incubation in a refrigerator at 4°C except for the F6-immunoliposome. This suggests these immunoliposomes would be stable and non-aggregating in HBS (pH 7.4) with respect to their sizes at least for 6 months. The increased size of F6-immunoliposome after 6 months can be due to the lack of PEG-PE which exerts a stabilizing effect on the liposome formulation (Garbuzenko et al., 2005; Immordino et al., 2006).

Cell binding of immunoliposome formulations was shown to be dependent of cell line and liposome composition. Figure 5 shows binding of liposome was low and similar in two different cell lines, but the immunoliposomes showed higher cell binding depending on the liposome composition and cell line. The immunoliposomes exhibited a higher binding in SK-BR-3 cells (HER2+++) than MCF-7 (HER2+), so incorporation of trastuzumab into the liposomes preserves the affinity and recognition abilities of the antibodies. In other words, this suggested that the conjugation of trastuzumab by post-insertion method is an effective way for targeting HER2 positive breast cancer cells with this carrier. Similarly, the superior binding of the antibody conjugated carriers than native carriers in SK-BR-3 cells has been shown in different publications, though the method of antibody incorporation was different (Steinhauser et al., 2006) or the same (Nielsen et al., 2002, 2006; Yang et al., 2007a,b).

The lipid composition affects cell binding (Figure 5A). The F4-immunoliposome showed the highest cell binding in SK-BR-3 cells followed by F8, F2 and F6. Except for F8, cell bindings of other formulations are in agreement with the results of lipid mixing assay. It was suggested that higher antibody incorporation in the liposomal structures led to higher cell binding in SK-BR-3 cells, whereas DPPG (a negatively charged lipid) in F8 formulation instead of neutral lipids prevents cell binding. More than 5-fold increase in SK-BR-3 cell binding occurs following incorporation of the trastuzumab-PEG-PE into F4 liposome
formulations. Therefore, F4 was chosen as the appropriate formulation with high affinity for SK-BR-3 cells.

Flow cytometry assay in SK-BR-3 cell line (Figure 7) showed that F4-immunoliposomes had significantly higher cellular uptake in comparison with the corresponding F4-liposomes at 37°C since binding of the immunoliposomes against HER2 (an internalizing receptor) could increase cellular uptake through receptor-mediated endocytosis as indicated before (Steinhauser et al., 2006; Yang et al., 2007a). It was also recognized that cellular uptake was augmented by increasing the amount of trastuzumab incorporated to lipid. It seems that increasing antibody density of immunoliposomes results in higher cellular uptake as reported previously (Allen et al., 1995; Harding et al., 1997; Maruyama et al., 1997) since the higher density of antibody as a specific ligand is associated with higher chance of binding of immunoliposomes to target cells. As a confirmatory experiment, the cell binding of F4 immunoliposomes that were compared to the corresponding F4-liposomes in SK-BR-3 cells by fluorescence microscopy (Figure 8) showed again the higher cellular binding ability of the F4-immunoliposomes versus the F4-liposomes.

To investigate the specificity of the immunoliposomes, cell binding of F4-immunoliposomes were compared for SK-BR-3 cells pre-incubated with and without free trastuzumab by flow cytometry (Table 3). The pre-incubation experiment suggests that the cell binding was blocked with free trastuzumab similarly for different amount of trastuzumab to lipid. Therefore, the block of HER2 receptors with free trastuzumab led to the inhibition of the specific receptor-mediated binding of immunoliposomes as shown in similar studies (Benzinger et al., 2000; Kirpotin et al., 1997; Steinhauser et al., 2006; Wartlick et al., 2004).

As shown in Figure 9, the F4-immunoliposome produced a significant antitumor activity similar to free trastuzumab in SKBR-3 cells (HER-2+++). The cytotoxicity increased depending on the trastuzumab concentration that confirms preservation of trastuzumab antitumor activity after the bioconjugation reaction and incorporation of trastuzumab-PEG-PE into the liposomes. In similar studies, the antibody thiolation, the bioconjugation and the post-insertion processes have no adverse affect on the incorporated antibody activity. Hence, the immunoliposome formulations inhibited significantly the growth of tumor cells in vitro (Park et al., 2002; Yang et al., 2007b) or in vivo (Nielsen et al., 2002; Park et al., 2002). As the control experiment, no significant cytotoxic effect was found on MCF-7 cells (HER-2+), which is similar to free trastuzumab. Hence, the cytotoxicity profile of trastuzumab does not change after incorporation in the liposomes.

Conclusions

Trastuzumab-PEG-PE micelles were successfully incorporated at various extents into the liposomes of different compositions prepared by post-insertion method. The resulted immunoliposomes were capable to bind to SKBR-3 (HER2+++ ) breast tumor cell line efficiently and specifically. This effect was prominent for Stealth immunoliposomes (F4 formulation) which contains DPPC, cholesterol and PEG-PE as lipid composition. The F4-immunoliposomes did not show any significant cytotoxicity in MCF-7 cells. However, in SKBR-3 cells, the F4-immunoliposomes showed trastuzumab concentration dependant antitumor activity. The developed nanocarrier is proposed as a promising drug or gene delivery system with a biosensing property for HER2 receptor in breast cancer cells.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Supplementary material available online