International Journal of Pharmaceutics 374 (2009) 145–152

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Tumor-targeted PE38KDEL delivery via PEGylated anti-HER2 immunoliposomes

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ARTICLE INFO

Article history:
Received 22 November 2008
Received in revised form 8 March 2009
Accepted 11 March 2009
Available online 24 March 2009

Keywords:
Breast cancer
Cytotoxicity
PE38KDEL
PEGylated immunoliposomes
RhuMAbHER2

ABSTRACT

We previously reported the development of PE38KDEL-loaded anti-HER2 poly(lactic-co-glycolic acid) (PLGA) nanoparticles that bind and internalize in HER2-overexpressing breast cancer cells, enabling potent anti-tumor activity. To overcome the problems associated with the short half-lives of this drug delivery system, we have constructed PE38KDEL-loaded anti-HER2 PEGylated liposomes (PE-HER-liposomes). PE-HER-liposomes were constructed with Fab′ of recombinant humanized anti-HER2 monoclonal antibody (anti-HER2 Fab′) covalently linked to PEGylated liposomes containing PE38KDEL (PE-liposomes). We attached anti-HER2 Fab′ to the terminus of PEG (polyethylene glycol) on PEGylated liposomes. Incorporation of pyridylthiopropionoylamino-PEG-distearoylphosphatidylethanolamine (PDP-PEG-DSPE) into PEGylated liposomes followed by mild thiolysis of the PDP groups resulted in the formation of reactive thiol groups at the periphery of the liposomes. Efficient attachment of maleimide-derivatized anti-HER2 Fab′ took place under mild conditions. The characterization of PE-HER-liposomes, such as particle size, was evaluated by dynamic light-scattering detector. The Micro BCA method was used to determine the encapsulation efficiency of PE38KDEL and the quantity of conjugated Fab′. Flow cytometry and confocal microscopy showed that PE-HER-liposomes possessed receptor-specific binding and internalization for HER2-overexpressing SK-BR3 cells. Remarkably, PE-HER-liposomes were more cytotoxic than non-targeted PE-liposomes in HER2-overexpressing breast cancer cells. In conclusion, PE-HER-liposomes could serve as a promising therapeutic candidate for the treatment of HER2-overexpressing breast cancers.

1. Introduction

Immunotoxins are composed of antibodies or antibody fragments linked to a toxin, such as ricin or Pseudomonas exotoxin A (PE) (Pastan et al., 2006). Currently, PE-based immunotoxins have been widely applied to clinical trials. However, the clinical use of PE-based immunotoxins is limited by their severe non-specific toxicity (Pastan et al., 2007). PE38KDEL is a 38 kDa mutant form of PE and exhibited superior anti-tumor activity and less non-specific toxicity (Gao et al., 2008). We previously reported the development of PE38KDEL-loaded anti-HER2 poly(lactic-co-glycolic acid) (PLGA) nanoparticles (PE-NPs-HER) that bind and internalize in HER2-overexpressing breast cancer cells, enabling potent anti-tumor activity (Chen et al., 2008). The results showed that PE-NPs-HER were well tolerated in mice with a much higher MTD (maximally tolerated dose) than the control immunotoxins PE-HER constructed by chemically coupling PE38KDEL to rhuMAbHER2 (recombinant humanized anti-HER2 monoclonal antibody). Moreover, PE-NPs-HER was shown to exhibit a much better therapeutic efficacy in HER2-overexpressing breast cancer bearing mice.

However, the further development of PE-NPs-HER in clinical application in breast cancer treatment was still hampered by several problems. For example, the nanoparticles without PEG (polyethylene glycol) coating would be rapidly cleared from the circulation in a short time (Moghimi and Szebeni, 2003). Furthermore, the initial burst effect occurred frequently in PLGA nanoparticles, resulting in a rapid drug release. Although PLGA is a kind of biodegradable and biocompatible components that was previously approved by the Food and Drug Administration (FDA) for clinical use, there have not been any drug-loaded PLGA nanoparticles that are under clinical application. Liposomes (phospholipid bilayer vesicles) are the most advanced of the particulate drug carriers

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doi:10.1016/j.ijpharm.2009.03.018

(Sapra and Allen, 2003). In 2007, FDA granted approval to doxorubicin HCl liposomes injection (Doxil, Alza Corporation) for use in combination therapy in patients with multiple myeloma. Many other liposomal anticancer drugs are also in clinical trials (Sapra and Allen, 2003). However, one of the major drawbacks of classical liposomes is their rapid clearance from the circulation (Allen and Cullis, 2004). Several reports showed that stealth liposomes (e.g. PEGylated vesicles) could avoid the uptake of RES (reticuloendothelial system), thus resulting in a much longer time in circulation (Moghimi and Szebeni, 2003; Allen and Cullis, 2004). Moreover, PEGylated liposomes are stable in the blood circulation and could even be stable in water solution for more than 1 week, indicating that PEGylated liposomes meets the requirement for an effective drug delivery system (Lv et al., 2005; Erjavec et al., 2006; Song et al., 2006). Hence, long-circulated PEGylated liposomes represent a novel strategy for cancer targeted therapy.

Immunoliposomes, in which monoclonal antibody (mAb) fragments are conjugated to liposomes, represent the next generation of molecularly targeted drug delivery systems (Sapra and Allen, 2003). By combining the tumor targeting properties of mAbs with the pharmacokinetics and drug delivery advantages of liposomes, immunoliposomes offer the promise of selective drug delivery to tumor cells, including internalization and intracellular drug release within targeted cells. In the present study, we developed a novel PE38KDEL-loaded PEGylated liposomes conjugated with anti-HER2 Fab′ (PE-HER-liposomes). The results showed that PE-HER-liposomes (less than 200 nm) have a high drug loading and antibody conjugation efficiency. Flow cytometry and confocal study demonstrated that PE-HER-liposomes could be efficiently bound to and were internalized into HER2-overexpressing tumor cells, resulting in potent cytotoxicity. Thus, the generated PE-HER-liposomes could represent a novel strategy for HER2-overexpressing breast cancer therapy.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (EPC) and methoxy (polyethylene glycol) (2000) distearoylphos-phatidylethanolamine (mPEG-DSPE) were purchased from Lipoid GmbH (Ludwigshafen, Germany). PDP-PEG-DSPE, pyridyldithiopropionoylamino poly(ethylene glycol) distearoylphosphatidylethanolamine were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol (CHOL), dithiothreitol (DTT), N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES), N-succinimidyl-4-(*p*-maleimidophenyl)-butyrate (SMPB) and 2-(N-morpholino)ethanesulfonic acid (MES) were purchased from Sigma (St. Louis, MO, USA). RhuMAbHER2 was kindly provided by the National Engineering Research Center for Antibody Medicine (Shanghai, China). Fab′ fragments of rhuMAbHER2 (anti-HER2 Fab′) were prepared as described previously (Martin et al., 1981). PE38KDEL was purified and analyzed as reported before (Song et al., 2005). Three human breast cancer cell lines SK-BR3, MDA-MB-231 and MCF-7 were maintained in the cell culture facility of International Joint Cancer Institute, the Second Military Medical University (Shanghai, China). All chemicals used in this study were of analytical grade.

2.2. Preparation of immunoliposomes

2.2.1. Preparation of PEGylated liposomes

PEGylated liposomes, composed of EPC:CHOL:mPEG2000-DSPE (2:1:0.08 molar ratio), were prepared using the thin-film hydration method as described previously (Allen et al., 1995). Briefly, CHOL and lipids (EPC and mPEG2000-DSPE) were dissolved in chloroform and dried in a rotary evaporator under vacuum to form a thin-film layer, which was re-suspended in a 2 mg/ml PE38KDEL solution (pH 7.0) until completely hydrated. Then, the liposomes dispersion was serially passed through 0.8, 0.4 and finally 0.2 μm polycarbonate membranes (Avestin, Ottawa, Canada) under nitrogen gas with an extruder (Avestin, Ottawa, Canada). Un-entrapped PE38KDEL was removed by passing the liposomes over Sepharose CL-4B column with PBS (pH 7.4) and the collected liposomes were freeze-dried. The resultant PEGylated liposomes were stored in tight containers at 4 °C for further experiments.

2.2.2. Preparation of PEGylated immunoliposomes

2.2.2.1. Preparation of maleimidophenylbutyrate-Fab′ (MPB-Fab′). Anti-HER2 Fab′ was dissolved in HEPES buffered saline (25 mM HEPES, 140 mM NaCl, pH 7.4) at concentration of 10 mg/ml. SMPB (25 mM in dimethylformamide) was slowly added to the Fab′ solution at a molar ratio of 20:1 (SMPB/Fab′) for 30 min at room temperature. Unbound SMPB was removed by passing the solution over a Sephadex G50 column in HEPES and MES buffered saline (25 mM HEPES, 25 mM MES, 140 mM NaCl, pH 6.7) (Allen et al., 1995), accompanying with the pH was lowered. Meanwhile, MPB-BSA (bovine serum albumin) was prepared as a control.

2.2.2.2. Antibody conjugation. PEGylated immunoliposomes were prepared as described as follows. First, liposomes were prepared with EPC/CHOL/PDP-PEG-DSPE at a molar ratio of 2:1:0.02 with 4 mol% mPEG-DSPE (total PEG lipid, 5 mol% of PL). After the liposomes were hydrated with a 2 mg/ml PE38KDEL solution (pH 7.0), the pyridyldithio groups were reduced by the addition of dithiothreitol (DTT) to a final concentration of 20 mM for 30 min at room temperature. DTT was removed by Sephadex G50 column, using HEPES and MES buffered saline as the elution buffer. Thiolated liposomes (20 μmol PL) were incubated overnight at room temperature with MPB-Fab′ (15 nmol) at Fab′/PL molar ratio 1:1330, at a final PL concentration of 54 mM. Unbound MPB-Fab′ was removed by passing the liposomes over a Sepharose CL-4B column with PBS (pH 7.4) (Allen et al., 1995). The amount of the unbound MPB-Fab′ was determined by the Micro BCA method according to the manufacturer's protocols. The following designations are used: PE38KDEL-loaded PEGylated liposomes conjugated with anti-HER2 Fab′ (PE-HER-liposomes), PE38KDEL-loaded PEGylated liposomes (PE-liposomes), PE38KDEL-loaded PEGylated liposomes conjugated with BSA (PE-BSA-Liposomes). Additionally, the control immunotoxins PE-HER (PE38KDEL:rhuMAbHER2 = 1:1 molar ratio) was constructed by chemically coupling PE38KDEL to rhuMAbHER2 as described previously (Gao et al., 2008).

2.3. Characterization of PEGylated immunoliposomes

2.3.1. Particle size and zeta potential

The average size and zeta potential of PEGylated liposomes and PEGylated immunoliposomes with or without PE38KDEL were analyzed using a dynamic light-scattering detector (Zeta sizer ZS, Malvern, UK). At least three different batches were analyzed to give an average value and standard deviation for the particle diameter and zeta potential.

2.3.2. Evaluation of drug contents

Drug loading determination was carried out as follows. First, PE38KDEL was labeled with FITC according to a standard protocol (van Dam et al., 1991). FITC-PE38KDEL (FITC labeled PE38KDEL)-loaded PEGylated liposomes (FITC-PE-liposomes) were prepared as described above. Afterwards, the liposomes were put into the 1,000,000 Da MWCO ultra centrifugal filter devices (Sartorius, Goettingen, Germany) and were centrifuged for 30 min at 1000 rpm at

4 °C. The flow-through liquid was collected and the entrapped liposomes were freeze-dried. Then the freeze-dried liposomes were dissolved in 3 ml ethanol and ethanol was evaporated under N_2 gas. After that, the dissolved liposomes were re-dissolved in 3 ml PBS, vortexed and centrifuged at 12,000 rpm for 30 min. Finally, the supernatant was collected and detected as described below. The concentration of FITC-PE38KDEL was determined by a LS 55 Fluorescence Spectrotrometer (PerkinElmer, Waltham, MA) at λ_{ex}: 490 nm and λ_{em}: 510 nm. A standard curve of FITC-PE38KDEL concentration was constructed using known concentrations of FITC-PE38KDEL (10–100 μg/ml). The entrapment efficiency (EE) was calculated from the following equation: EE (%) = amount of FITC-PE38KDEL in the liposomes (μg) × 100/amount of total FITC-PE38KDEL added to the liposomes (μg).

An aliquot (50 mg each) of the freeze-dried liposomes powder were dissolved with the ethanol (2 ml) to determine the content of FITC-PE38KDEL in the freeze-dried liposomes powder using the following equation: drug loading = amount of FITC-PE38KDEL in the liposomes (μg)/amount of the liposomes (mg).

2.3.3. *In vitro release of PE38KDEL from the conventional liposomes and PEGylated immunoliposomes*

Ultrafiltration–centrifugation method is used to evaluate the in vitro release of the liposomes as described below. Briefly, 2 ml of the PE38KDEL-loaded conventional liposomes (non-PEGylated), PEGylated liposomes or the control blank liposomes were put into the 1,000,000 Da MWCO ultra centrifugal filter devices. After different times of incubation at 4 or 37 °C, the liposomes were centrifuged for 30 min at 1000 rpm at 4 °C and the flow-through liquid was collected. Meanwhile, fresh medium was added to replenish the medium which had been partially depleted. The released PE38KDEL in the collected liquid was quantitatively determined by Micro BCA method. At 4 °C, this procedure was repeated on days 1, 2, 3, 7, 14, 21 and 28. At 37 °C, this procedure was repeated at 1, 2, 4, 6, 9, 12 and 24 h. The percentage of the released proteins from total protein loading was calculated for every sample from the different incubation periods and presented as a cumulated curve.

2.3.4. *Determination of anti-HER2 Fab′ on the surface of PEGylated immunoliposomes*

The following methods were used to confirm anti-HER2 Fab′ on the PEGylated immunoliposomes surface.

2.3.4.1. Gel chromatography analysis. PE-HER-liposomes were prepared with EPC/CHOL/PDP-PEG-DSPE and MPB-Fab′ as described above. Meanwhile, the PEGylated immunoliposomes used as controls were also prepared with EPC/CHOL/mPEG-DSPE and MPB-Fab′. Separation of MPB-Fab′ from the PEGylated immunoliposomes was performed as follows. Briefly, 2 ml liposomes were applied onto a Sepharose CL-4B column (diameter 1.5 cm, bed volume 44 ml) and eluted with PBS (pH 7.4). Eluted fractions were collected, concentrated and analyzed by SDS–PAGE.

2.3.4.2. SDS–PAGE analysis. To evaluate the conjugation efficiency of Fab′ and the amount of unconjugated Fab′ present in the PEGylated immunoliposomes, SDS–PAGE followed by Coomassie blue staining was used. All the gels were run under non-reducing conditions in a Tris/glycine/SDS buffer by using Mini-Protean II electrophoresis unit from BioRad. The gels were stained with Coomassie blue to reveal protein, destained and dried.

2.3.4.3. Protein assay. The amount of conjugated Fab′ present in PE-HER-liposomes was quantified as described below. PE-HER-liposomes were prepared with EPC/CHOL/PDP-PEG-DSPE and MPB-Fab′ as described above. Separation of MPB-Fab′ from the PEGylated immunoliposomes was performed as follows: 2 ml liposomes were applied onto Sepharose CL-4B column and eluted with PBS (pH 7.4). Eluted fractions were collected and concentrated. The unconjugated MPB-Fab′ included in the post-peak was quantified by the Micro BCA method as previously described (Park et al., 1998; Kocbek et al., 2007). Briefly, 500 μl of Micro BCA working solution was added to 500 μl of the liquid of the post-peak, which contains the unconjugated MPB-Fab′. After 60 min of incubation, the absorbance was measured at 562 nm using a microplate reader (FLx800TB, BioTek, USA). The results were compared to a standard curve of MPB-Fab′ solution in PBS (pH = 7.4) ranged from 0.5 to 20 μg/ml. MPB-Fab′ density and coupling efficiency were calculated from the following equations. Amount of coupling MPB-Fab′ (μg) = amount of total added MPB-Fab′ (μg) − amount of the MPB-Fab′ in the post-peak (μg); MPB-Fab′ density = amount of coupling MPB-Fab′ (μg)/amount of phospholipid in freeze-dried liposomes (mg); coupling efficiency (%) = amount of coupling MPB-Fab′ (μg) × 100/amount of total added MPB-Fab′ (μg).

2.4. *In vitro experiments*

2.4.1. *Cell culture*

Three human breast cancer cell lines SK-BR3, MDA-MB-231 and MCF-7 were cultivated in monolayers to 80% confluence in RMPI 1640 medium supplemented with 10% fetal bovine serum at 37 °C in humidified environment of 7.5% CO_2. The medium was replenished every other day and the cells were subcultured after reaching confluence.

2.4.2. *Recognition properties of PEGylated immunoliposomes*

To examine the binding affinity and specificity of PEGylated immunoliposomes to HER2-overexpressing and negative breast cancer cells, 1×10^6 cells were treated with various concentrations (0 and 100 μg/ml) of rhuMAbHER2 for 30 min at 4 °C, before incubation with 1 mg FITC-PE-HER-liposomes (FITC-PE38KDEL-loaded PEGylated immunoliposomes) in 1 ml culture medium for 45 min at 4 °C or for 1 h at 37 °C, then cells were pelleted by centrifugation. Thereafter, the cells were then washed and analyzed by flow cytometry analysis (FCM). FCM was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

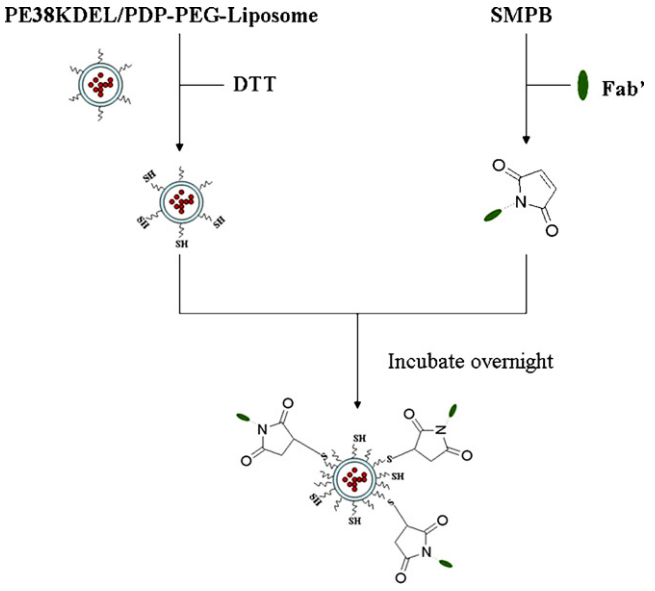

Fig. 1. Schematic diagram of the coupling of a maleimide-activated antibody with PEGylated liposomes containing PDP-PEG-DSPE.

Table 1
Characterization of the PEGylated liposomes with or without PE38KDEL.

Formulations	PE38KDEL	Particle size (nm)	Zeta potential (mv)	EE (%)	Drug loading (μg/mg TL)
PEGylated liposomes	With	181.40 ± 4.59[a]	−34.90 ± 4.16	89.32 ± 3.48[c]	10.46 ± 1.20[c]
	Without	178.43 ± 5.56	−36.43 ± 3.23	ND[b]	ND
PEGylated immunoliposomes	With	194.47 ± 3.20	−32.47 ± 3.07	90.49 ± 6.39	10.17 ± 1.39
	Without	190.40 ± 3.69	−33.60 ± 2.76	ND	ND

[a] All data are expressed as the mean ± SD ($n = 3$).
[b] ND, not done.
[c] $P > 0.05$; there was no significant difference between PE-liposomes and PE-HER-liposomes in the EE and drug loading using Student's unpaired t-test.

2.4.3. Binding and internalization of PEGylated immunoliposomes

Binding and internalization of FITC-PE-HER-liposomes in HER2-overexpressing and negative cell lines were examined by LSCM. SK-BR3 and MCF-7 cells were incubated with 1 mg FITC-PE-HER-liposomes for 2 h at 37 °C. Then the cells were washed twice with PBS, followed by fixation with 4% *p*-formaldehyde (PFA) for 30 min. Afterwards, 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen) was used to counterstain the nuclei of the cells. Finally, cells were imaged by Leica TCS SP2 Confocal Spectral Microscope (UV–vis) and the images were analyzed with the Leica Confocal Software.

2.4.4. In vitro cytotoxicity

Cytotoxicity was analyzed using Cell Titer 96 non-radioactive cell proliferation assay kit according to the manufacturer's protocol (Promega, Madison, WI) as we described before (Chen et al., 2008). Briefly, breast cancer cells (1×10^4) were cultured in the presence or absence of PEGylated liposomes, PEGylated immunoliposomes or free PE38KDEL for 2 days at 37 °C in a CO_2 incubator. The concentration of PE38KDEL used in this assay was varied from 1 to 20,000 pM (equivalent to TL (total lipid) concentration of up to 0.1 mg/ml). Cytotoxicity of PEGylated liposomes and PEGylated immunoliposomes without PE38KDEL was evaluated at 2 days at the highest TL concentration (0.10 mg/ml) used in the cytotoxicity assays. Then, 20 μl of MTS/PMS solution was added to each well. After incubation for 2 h at 37 °C, the absorbance of each well was measured at 490 nm using a microplate reader.

3. Results and discussion

3.1. Characterization of the liposomes

3.1.1. Particle size and zeta potential

Preparation of PEGylated immunoliposomes was carried out as summarized in Fig. 1. The resultant PE38KDEL-loaded PEGylated liposomes were sized at 181.40 ± 4.59 nm (mean ± SD; $n = 3$) (Table 1), which was not much larger than drug-free PEGylated liposomes ($P > 0.05$, Student's unpaired t-test). That PE38KDEL encapsulation did not increase the size was also observed in PEGylated immunoliposomes. However, compared with the size of

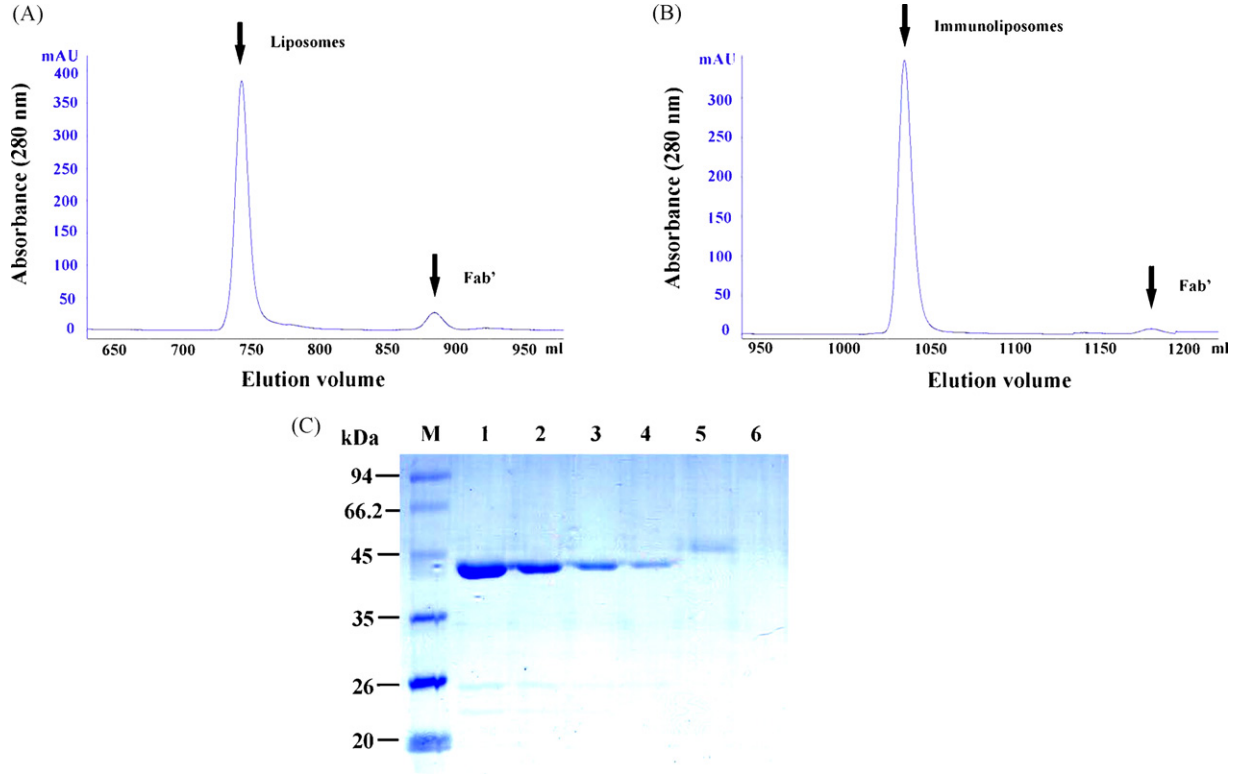

Fig. 2. (A) and (B) Separation of liposomes from the Fab′ by the Sepharose CL-4B gel filtration. PEGylated immunoliposomes were prepared with EPC/CHOL/PDP-PEG-DSPE and MPB-Fab′ as described in Section 2. Meanwhile, the control PEGylated immunoliposomes were also prepared with EPC/CHOL/mPEG-DSPE and MPB-Fab′. Separation of MPB-Fab′ from the PEGylated immunoliposomes was performed as follows. Briefly, 2 ml liposomes were applied onto a Sepharose CL-4B column (diameter 1.5 cm, bed volume 44 ml) and eluted with PBS (pH 7.4). Eluted fractions were collected, concentrated and analyzed by SDS–PAGE. The arrows indicated the eluted liposomes or MPB-Fab′. (C) SDS–PAGE analysis. Lane M: protein marker; lane 1: 4 μg Fab′; lane 2: 2 μg Fab′; lane 3: eluted MPB-Fab′ from (A); lane 4: eluted MPB-Fab′ from (B); lane 5: PEGylated immunoliposomes; lane 6: PEGylated liposomes.

PE-liposomes, there was an increase of 13 nm in the size of PE-HER-liposomes, presumably owing to the presence of anti-HER2 Fab′ fragments on the liposomes surface. It is well known that the particle size plays an important role on the alternation of pharmacokinetics by affecting the tissue distribution and clearance. Liposomes with small particle size (<200 nm) are known to have increased drug accumulation in the tumor via enhanced permeability and retention (EPR) effect (Moreira et al., 2001; Xu et al., 2005). Thus, our immunoliposomes maintaining size of <200 nm would be expected to have a satisfactory drug accumulation in the tumor. The zeta potential of PEGylated immunoliposomes was slightly higher than PEGylated liposomes (Table 1), indicating incorporation of anti-HER2 Fab′ to the liposomes resulted in an increase in the zeta potential. The increase in zeta potential after the incorporation of anti-HER2 Fab′ was consistent with our results obtained previously (Chen et al., 2008).

3.1.2. Evaluation of drug contents

To obtain large drug loading to meet therapeutic application, high concentration of PE38KDEL (≈2 mg/ml, the solubility of PE38KDEL was about 10 mg/ml) was chosen as the concentration of hydration solution. As a result, the prepared PE-HER-liposomes have a drug loading of 10.17 ± 1.39‰ (mean ± SD; $n = 3$) and an encapsulation efficiency of 90.49 ± 6.39% (mean ± SD; $n = 3$) (Table 1). Except otherwise stated, these fresh-prepared liposomes was used in subsequent experiments.

3.1.3. Determination of Fab′ on the surface of liposomes

Gel chromatography was a common method used to separate unconjugated mAbs or antibody fragments from nanoparticles (Shi et al., 2001; Ito et al., 2004). MPB-Fab′ and PEGylated liposomes which were separated on the Sepharose CL-4B column were analyzed by spectrophotometrical analyses. The two peaks were completely resolved (Fig. 2A), which implied that the PEGylated liposomes can be separated from the MPB-Fab′. Compared with the control PEGylated liposomes prepared with EPC/CHOL/mPEG-DSPE and MPB-Fab′, PEGylated immunoliposomes prepared with EPC/CHOL/PDP-PEG-DSPE and MPB-Fab′ have a much lower post-peak. Considering the post-peak contained the unconjugated MPB-Fab′, the results indicated that the Fab′ was indeed conjugated to the PEGylated immunoliposomes via PDP-PEG-DSPE.

The presentation and integrity of anti-HER2 Fab′ on the PEGylated immunoliposomes was confirmed by the SDS–PAGE under non-reduced condition. The molecular weight of conjugated anti-HER2 Fab′ was larger than free Fab′ (<45 kDa), indicating that anti-HER2 Fab′ was efficiently incorporated into the PEGylated liposomes in intact form (Fig. 2B).

The protein assay was used to quantify the amount of bound Fab′ on liposomes surface. According to the protein assay, the amount of conjugated Fab′ of PE-HER-liposomes was shown to be approximately 27.83 ± 3.13 μg anti-HER2 Fab′/mg phospholipid (mean ± SD; $n = 3$), and the coupling efficiency of conjugated Fab′ of PE-HER-liposomes was 66.94 ± 4.43% (mean ± SD; $n = 3$).

3.1.4. In vitro release of PE38KDEL from the liposomes

As shown in Table 1, there was no significant difference between PE-liposomes and PE-HER-liposomes in the EE and drug loading ($P > 0.05$). Furthermore, the conjugated anti-HER2 Fab′ would interfere the quantitation of released PE38KDEL in vitro. As a result, we used PE-liposomes, but not PE-HER-liposomes, to perform in vitro release assay of PE38KDEL. In this study, the release profile of PE38KDEL from the liposomes was determined by cumulative percentage of protein release. The release profiles of PE38KDEL were characterized by a sustained release of the proteins over a 28-day period at 4 °C or over a 24-h period at 37 °C. As shown in Fig. 3, the release rate of PE38KDEL of the conventional liposomes (non-PEGylated) was much higher than PEGylated liposomes, which was consistent with the previous study (Yang et al., 2007). At 4 °C, PEGylated liposomes released only 1.75% of PE38KDEL within 2 days, and 4% with 7 days. Furthermore, at 37 °C, the PEGylated liposomes released only 32% of PE38KDEL within 24 h, whereas the conventional liposomes released nearly 100%, suggesting that the PEGylated liposomes exhibit a slow release rate and meet the requirement for an effective drug delivery system.

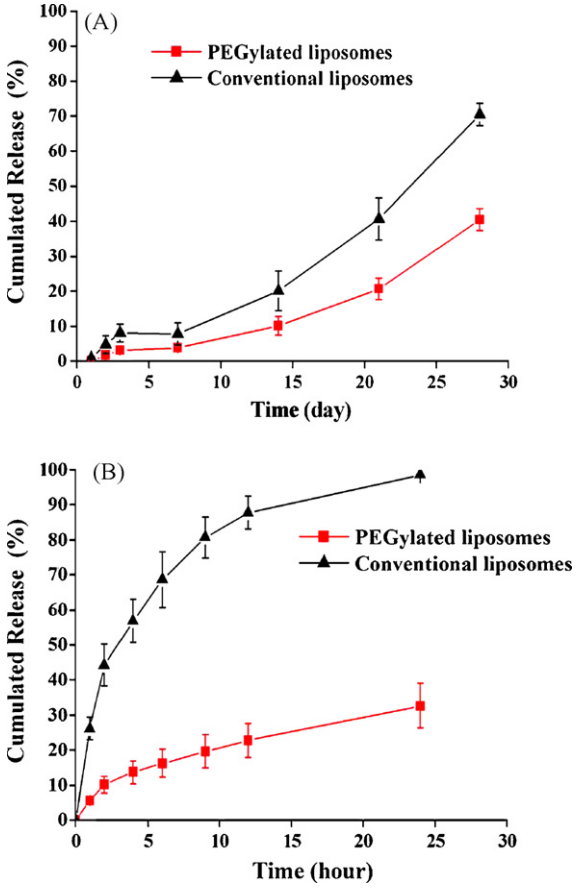

Fig. 3. Protein release profiles from PE38KDEL-loaded PEGylated liposomes. For the release profile, 10 mg PEGylated liposomes containing 100 μg PE38KDEL were incubated in PBS at 4 °C (A) or 37 °C (B). Points (mean ± SD, $n = 4$), expressed as a percentage of cumulated released protein from total protein loaded in the liposomes.

3.2. In vitro experiments

3.2.1. Recognition properties of antibody modified liposomes

We performed flow cytometry to evaluate the recognition properties of FITC-PE-HER-liposomes to HER2 antigen at 4 °C (Fig. 4A) or 37 °C (Fig. 4B). As shown in Fig. 4A, PE-HER-liposomes was shown to possess potent binding affinity to HER2-overexpressing SK-BR3 cells, but nearly no binding affinity to HER2-negative MCF-7 cells. To confirm the specificity of the interaction of FITC-PE-HER-liposomes with the target cells, a competition assay was performed. The binding of PE-HER-liposome to SK-BR3 cells was inhibited by excessive rhuMAbHER2 (100 μg/ml). Similar results were obtained in MDA-MB-231 cells. In contrast, the slight binding of PE-HER-liposome observed in MCF-7 cells was not inhibited by rhuMAbHER2 and was considered as nearly background. Furthermore, the results obtained at 37 °C were very similar to those obtained at 4 °C. The present data confirmed anti-HER Fab′ was effectively conjugated to liposomes surface and still preserved its biological activity during the covalent conjugation procedure.

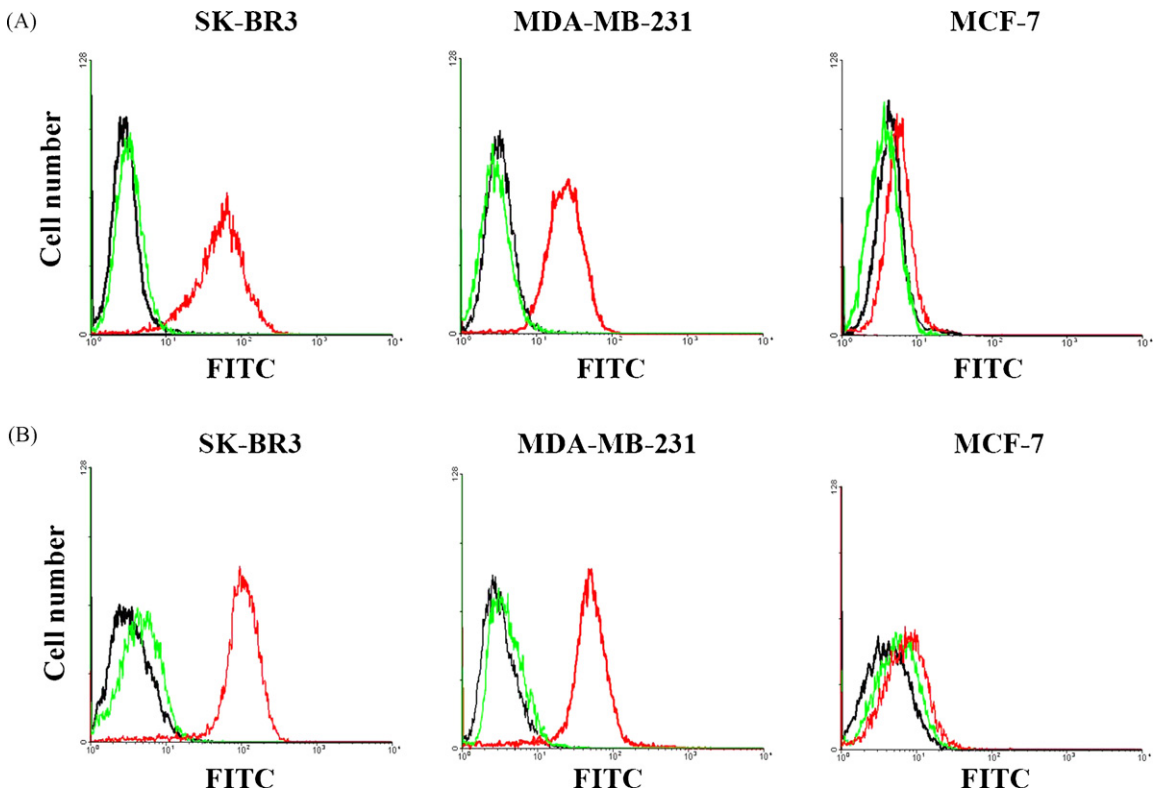

Fig. 4. Binding of PEGylated immunoliposomes to SK-BR3 cells. 1×10^6 SK-BR3 cells were treated with various concentrations (0 and 100 μg/ml) of rhuMAbHER2 for 30 min at 4 °C, before incubation with 1 mg FITC-PE-HER-liposomes in 1 ml culture medium for 45 min at 4 °C (A) or 60 min at 37 °C (B), and then the cells were pelleted by centrifugation. Thereafter, the cells were assessed by FCM. The cells pre-treated with 100 μg/ml rhuMAbHER2 (green line) exhibited no detectable fluorescence. Untreated cells (black line) and cells not pre-treated with 100 μg/ml rhuMAbHER2 (red line) were used as negative and positive controls. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.2.2. Binding and internalization of PEGylated immunoliposomes

To confirm the internalization efficiencies of antibody modified liposomes, SK-BR3 and MCF-7 cells incubated with FITC-PE-HER-liposome were analyzed by laser-scanning confocal microscopy. Internalization of FITC-PE-HER-liposome would lead to cytoplasmically localized green FITC staining. After 2 h incubation, FITC fluorescence was detected in the plasma membrane and the cytoplasm of the cells (Fig. 5). In contrast, when incubated with HER2-negative MCF-7 cells for 2 h, no significantly FTIC fluorescence was detected.

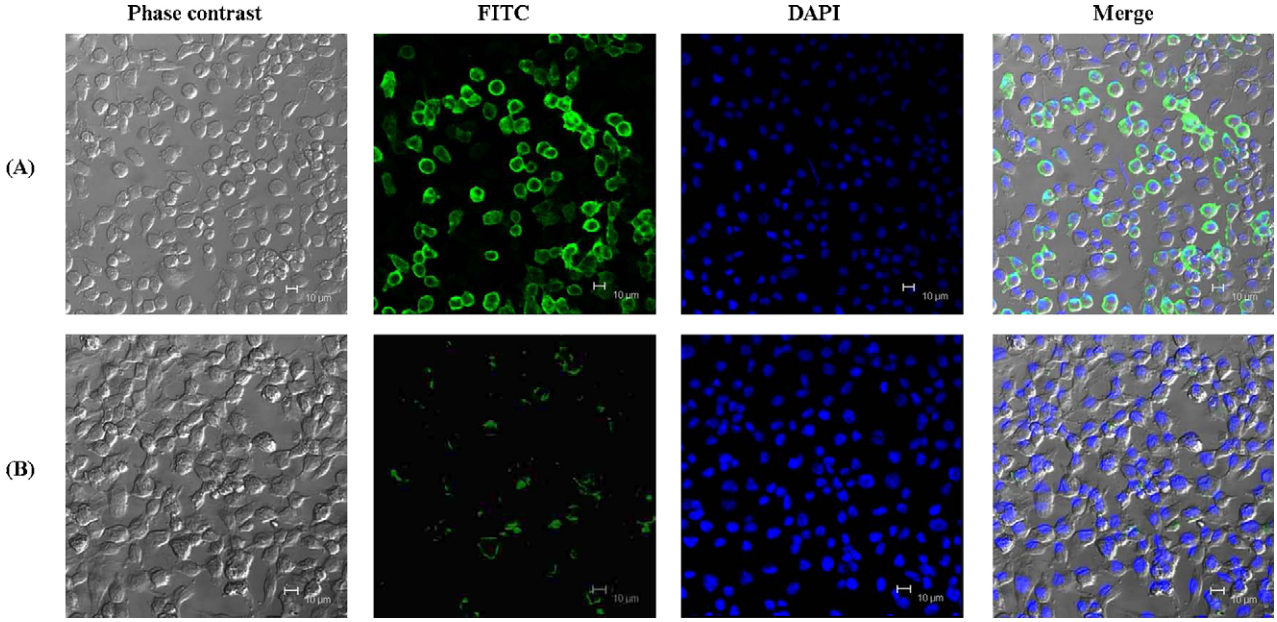

Fig. 5. Internalization analysis of PEGylated immunoliposomes by confocal laser-scanning microscopy. SK-BR3 cells (A) and MCF-7 cells (B) were incubated with FITC-PE-HER-liposomes for 2 h. Then the cells were counterstained with DAPI. Finally the cells were imaged by Leica TCS SP2 Confocal Spectral Microscope (UV–vis) and the images were analyzed with the Leica Confocal Software. Bar represents 10 μm.

Table 2
IC_{50} (pM) of various agents in breast cancer cell lines.

	PE-HER-liposomes	PE-HER	PE-liposomes + anti-HER2 Fab′	PE-BSA-liposomes	PE38KDEL
SK-BR3	36.63 ± 11.67[a]	83.98 ± 18.68*	>2000	>2000	>2000
MDA-MB-231	291.92 ± 89.36	413.71 ± 68.28*	>2000	>2000	>2000
MCF-7	>2000	>2000	>2000	>2000	>2000

[a] Data are expressed as the mean ± SD ($n = 3$).
* $P < 0.05$; comparison of IC_{50} of PE-HER-liposomes and PE-HER in SK-BR3 or MDA-MB-231 cells showed significant differences using Student's unpaired t-test.

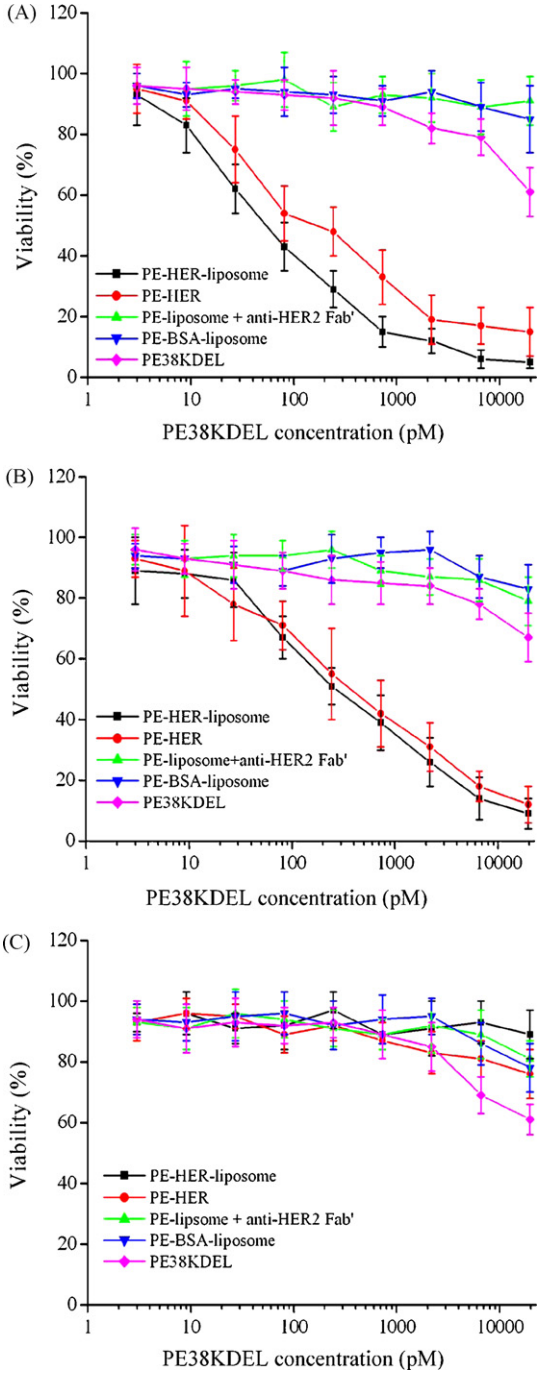

Fig. 6. In vitro cytotoxicity assays. Cytotoxicity was analyzed using Cell Titer 96 non-radioactive cell proliferation assay kit. Briefly, breast cancer cells (1×10^4) were cultured in the presence or absence of PE-HER-liposomes, PE-liposomes, PE-BSA-liposomes, PE-HER or free PE38KDEL for 2 days at 37 °C in a CO_2 incubator. Then, 20 μl of MTS/PMS solution was added to each well. After incubation for 2 h at 37 °C, the absorbance of each well was measured at 490 nm using a microplate reader. The cell viability was calculated as the relative absorbance of sample versus that of untreated control. (A) SK-BR3 cells; (B) MDA-MB-231 cells; (C) MCF-7 cells. Data represent mean ± SD of triplicate experiments.

3.2.3. *In vitro cytotoxicity assays*

PE-HER-liposomes were tested for cytotoxicity against SK-BR3, MDA-MB-231 and MCF-7 cell lines. As shown in Fig. 6, SK-BR3 cells, which expressed high level of HER2 antigens, were most sensitive to PE-HER-liposomes (IC_{50} = 36.63 pM). MDA-MB-231 cells expressing lower level of HER2 antigens than SK-BR3 were also sensitive to PE-HER-liposomes (IC_{50} = 291.92 pM). However, the HER2-negative MCF-7 cells were much less sensitive to PE-HER-liposomes, with a very high IC_{50} value (IC_{50} > 2000 pM). Similar results were obtained when the three cell lines were incubated with PE-HER, suggesting that the binding ability and specificity of PE-HER-liposomes and PE-HER to HER2 antigen were critical for the induction of cytotoxicity. It was clearly shown that PE-HER-liposome was more cytotoxic against SK-BR3 cells when compared with PE-HER (IC_{50} of PE-HER-liposome being 36.63 ± 11.67 pM vs. that of PE-HER being 83.98 ± 18.68 pM (mean ± SD, $n = 3$); $P < 0.05$) (Table 2). The similar results were obtained when MDA-MB-231 cells were used. In contrast to PE-based immunotoxins consisting of PE genetically fused to the antibody fragments, PE-HER-liposomes exploit the exponentially greater capacity of drug-loaded liposomes (Lopes de Menezes et al., 1998). Furthermore, owing to the multiple valency of binding of immunoliposomes (Park et al., 2004), PE-HER-liposomes had higher binding affinity and multivalency of immunoliposomes would also contribute to their higher binding affinity and cytotoxicity. However, mixture of PE-liposomes with free anti-HER2 Fab′ as the same amount as it conjugated to PE-HER-liposomes showed very high IC_{50} values for the three cell lines, indicating that the cytotoxicity of PE-HER-liposomes was dependent on the conjugated anti-HER2 Fab′ but not free anti-HER2 Fab′. Compared with other control groups, PE-HER-liposomes showed notably enhanced cytotoxicity against HER2-overexpressing breast cancer cells. We speculated that the antitumor mechanism of PE-HER-liposomes was due to their binding and getting internalized into tumor cells with subsequent intracellular release of PE38KDEL after liposomes degradation.

To eliminate the possibility that anti-HER2 Fab′ or liposomes formulated with lipid system was responsible for the cytotoxicity, we performed similar cytotoxicity assays with the PEGylated liposomes or the PEGylated immunoliposomes, which did not encapsulate PE38KDEL. Cytotoxicity of the PEGylated liposomes without PE38KDEL was evaluated at 2 days at the highest TL concentration used in the cytotoxicity assays. Cell viability of the PEGylated liposomes or PEGylated immunoliposomes without PE38KDEL was 95 ± 3% and 93 ± 2% (mean ± SD, $n = 3$), respectively. The results demonstrated clearly that the lack of cellular cytotoxicity of these materials due to the absence of PE38KDEL.

4. Conclusion

The clinical use of PE-based immunotoxins is severely hampered by the nonspecific toxicity of PE, which is usually characterized by hepatoxicity. Previously, we developed PE38KDEL-loaded anti-HER2 PLGA nanoparticles (PE-NPs-HER), which exhibited potent anti-tumor activity and less hepatoxicity. However, the short half-life, initial burst and biocompatibility restricted the clinical application of PE-NPs-HER. In the present study, we successfully constructed PE38KDEL-loaded PEGylated immuno-

liposomes conjugated anti-HER2 Fab′. The immunoliposomes (PE-HER-liposomes) fulfill many aspects of an effective drug delivery system. Firstly, PE-HER-liposomes have a size of <200 nm, making it may have a satisfactory drug accumulation in the tumor through the mechanism of EPR. Secondly, PE-HER-liposomes have a high drug loading of 10.17‰ and a high PE38KDEL encapsulation efficiency of 90.49%. Finally, the anti-HER2 Fab′ could be efficiently conjugated to the PEGylated liposomes. Flow cytometry demonstrated that PE-HER-liposomes possessed remarkable binding affinity and specificity towards HER2-overexpressing breast cancer cells. Confocal microscope study provided the direct evidence that our PE-HER-liposomes could be internalized in HER2-overexpressing breast cancer cells, resulting in potent cytotoxicity, which was further verified by the non-radioactive cell proliferation assay. In conclusion, our study might yield a kind of effective targeted nanomedicine of low toxicity to fight with HER2-overexpressing breast cancer. Our future studies will particularly focus on the pharmacokinetics and in vivo antitumor activity of this targeted nanomedicine.

Acknowledgements

We thank Ms. Yang Yang and Ms. Jing Xu for their technical assistance. This work was supported in part by the grants from National Natural Science Foundation of China, Shanghai Commission of Science & Technology, Ministry of Science and Technology of China (973&863 program projects), Pudong Commission of Science and Technology of Shanghai.

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