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Peptide ligand-mediated liposome distribution and targeting to EGFR expressing tumor in vivo

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ABSTRACT

Epidermal growth factor receptor (EGFR) is an important anti-cancer therapy target that is applicable to many cancer types. We had previously reported the screening and discovery of a novel peptide ligand against EGFR named GE11. It was shown to bind to EGFR competitively with EGF and mediate gene delivery to cancer cells with high-EGFR expression. In this study, we conjugated GE11 on to liposome surface and examined their binding and distribution to EGFR expressing cancer cells in vitro and in vivo using fluorescence imaging techniques. GE11 liposomes were found to bind specifically and efficiently to EGFR high-expressing cancer cells. In vivo in H1299 xenograft mouse model, GE11 liposomes also extravasated and accumulated into the tumor site preferentially, and demonstrated better targeting and drug delivery capacities.

1. Introduction

Liposomes have been increasingly developed as preferred drug carriers in anti-tumor treatments (Bangham et al., 1965; Bangham, 1968). PEG-conjugated liposomes can somewhat escape from the capture of reticuloendothelial system to circulate longer than conventional liposomes. So they have a tendency to traverse leaky vascular and passively accumulate in tumor tissues based on the so-called enhanced permeability and retention (EPR) effect (Allen et al., 1991; Lasic et al., 1991; Woodle and Lasic, 1992; Wu et al., 1993). Such stealth liposome formulations had been used to deliver anti-cancer drugs and attained significant therapy effect (Mayhew et al., 1992; Vaage et al., 1992). In addition, modification of liposomes with antibody or antibody fragment, or small molecular ligands has also been developed a promising strategy for tumor targeting. Drug-loaded liposomes with active targeting were shown to have more enhanced anti-cancer efficacy (Lopes de Menezes et al., 1998; Asai et al., 2002; Gabizon et al., 2003).

The epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase over-expressed on many human cancer cells surface. It is regarded as a significant target for tumor-targeted therapy. EGFR-targeted immunoliposomes were shown to promote efficient intracellular delivery of doxorubicin to tumor cells, and therefore resulted in superior anti-tumor effects in a series of animal xenograft models (Mamot et al., 2003, 2005, 2006). In addition to antibodies and antibody fragments, peptide ligands having specific interaction with receptors over-expressing in tumor or tumor neovasculature were utilized to direct chemotherapeutics, proteins, and gene constructs (Kok et al., 2002). Some peptides such as the RGD-derived peptides were widely used (Takikawa et al., 2000; Schiffelers et al., 2003; Maeda et al., 2004). Many other studies had attempted to find other effective ligands for various targets based on phage display screening, comparative sequence/structure analysis and so on (Morpurgo et al., 2002; Wu et al., 2004). We had previously reported the screening of a noval peptide ligand (GE11) for EGFR (Li et al., 2005). It was shown to bind effectively to EGFR over-expressing cancer cells-mediated target specific gene transfection when conjugated to a PEI vector.

In this study, we used this peptide ligand to construct an active targeting liposome drug delivery system towards EGFR positive cancer cells. We also examined the liposome distribution in vivo in tumor bearing mice. The aim of this study is to explore the feasibility of using peptide ligand-directed liposome as target therapeutics for cancer.

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2. Materials and methods

2.1. Materials

The sequence of the peptide GE11 is YHWYGYTPQNVI as described in reference Li et al. (2005). It was custom synthesized by GL Biochem Ltd. (shanghai). Its structure and purity were confirmed by HPLC and MS. The irrelated peptide D11 is also synthesized for control. Recombinant hEGF was a gift from Dr. Li Z.P. (State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences).

The lipid 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) was from Avanti Polar Lipids (AL, USA). Eggphosphatidylcholine, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine–*N*-[methoxy(polyethylene glycol)2000] (DSPE–PEG2000), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine–*N*-[maleimide (polyethylene glycol)2000] (DSPE–PEG2000–Mal) were from NOF Corporation (Japan). Cholesterol was from Sigma. Lissamine™ rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (rhodamine DHPE) and *N*-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (fluorescein DHPE) were from Invitrogen Corporation (USA). *N*-Succinimidyl 3-(2-pyridyldithio) propionate (SPDP) and tris(2-carboxyethyl) phosphine (TCEP) were from Pierce Biotechnology, Inc. (USA). Cy5.5 mono NHS ester was supplied by GE Healthcare (USA). Doxorubicin hydrochloride was from Shenzhen Main Luck Pharmaceuticals Inc. MTT was from Shanghai Pufei Biotechnology Co., Ltd. All other chemicals in analytical grade were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Cell lines and animal models

The human non-small cell lung carcinoma cell line H1299 and the human lung adenocarcinoma cell line SPCA1, both expressing EGFR were used in this study. The cells were cultured in RPMI1640 culture medium, supplemented with 10% fetal bovine serum (Gibco) at 37 °C in humidified atmosphere containing 5% CO_2.

H1299 xenograft mouse models were prepared by the animal experimental center of Shanghai Cancer Institute. They were used for in vivo experiments about 3–4 weeks after tumor cell inoculation (tumor size about 5 mm diameter), and humanely sacrificed afterwards. The animal study protocols were approved by the Animal Study Committee of Shanghai Jiaotong University School of Pharmacy.

2.3. Liposomes preparation

Liposomes were prepared using the thin filmhydration and extrusion method containing EPC:CHOL:DSPE–PEG at 10:5:0.5 molar ratios. The size distribution of the liposomes were routinely examined by photon correlation spectroscopy (PCS) using a Zetasizer3000H (Malvern Instruments).

For fluorescence labeled liposomes, the fluorescent lipids (rhodamine DHPE, fluorescein DHPE or Cy5.5 DSPE) were incorporated at about 0.5–0.64 mol% total lipids.

The doxocubicine-loaded liposome was prepared by the pH loading method (Mayer et al., 1990; Haran et al., 1994). Briefly, dry lipids were hydrated in 125 mM ammonium sulfate, 20 mM HEPES buffer at pH 4.0, followed by extrusion to reach 100 nm particle size. The solution was then dialyzed in HEPES buffer (pH 7.2) and diluted with 20 mM HEPES, 150 mM NaCl (pH 7.5) to the required concentration. Doxorubicin was added and incubated for at 60 °C for 10 min. Encapsulation efficiencies of over 90% were consistently obtained.

2.4. Conjugation of ligands to maleimide–PEG–DSPE and incorporation into liposomes

For the synthesis of ligand–PEG–DSPE molecule (Roberts et al., 2002), GE11 or EGF was dissolved in PBS–EDTA and mixed at 1:1.2 molar ratio with *N*-succinimidyl 3-(2-pyridyldithio) propionate dissolved in DMSO. After 1 h in room temperature, the mixture was lyophilized, and dissolved in the solution containing tris(2-carboxyethyl) phosphine under nitrogen to expose the –SH group. The thiolated protein or peptide was then added to the MAL–PEG2000–DSPE micelle solution at 5:1 molar ratio while maintaining mixing under nitrogen at 10 °C overnight. HPLC analysis confirmed that most of MAL–PEG–DSPE molecules were conjugated with the ligands after such reactions.

Ligand-conjugated lipids were transferred into preformed liposomes based on the procedure developed by Ishida et al. with minor modifications (Ishida et al., 1999). Briefly, ligand–PEG–DSPE solution was added into the preformed liposome solution at 9:100 molar ratio and incubated at 60 °C for 1 h (50 °C and 30 min for doxorubicine containing liposomes). The solutions were then dialyzed against PBS using SnakeSkin™ Pleated Dialysis Tubing, 10,000 MWCO (Pierce Chemical Company) for 4 h to remove unconjugated ligands. Non-targeted liposomes were prepared similarly by substituting ligand-conjugated lipid with mPEG2000–DSPE and used as controls.

2.5. In vitro cell binding and drug delivery studies

EGFR high-expressing H1299 cells were seeded at 2×10^5 cm^{-2} density on 35 mm-diameter culture dishes and cultured in RPMI1640 medium overnight. After the cell culture reached about 80% confluence, ligand-conjugated rhodamine labeled liposomes were diluted in RPMI1640 and added into the culture dish at the dose of 0.2 mg total lipids per well. In the binding competition experiments, 50-fold molar excess of free GE11 or EGF were added into the medium 2 h before the addition of ligand-conjugated liposomes. After 4 h incubation at 37 °C, the cells were washed six times with PBS (pH 7.4) to remove unbound liposomes. The bound fluorescent liposomes were visualized using Confocal Laser Scanning Microscope (CLSM, Zeiss LSM 510, Germany).

Doxorubicin-loaded liposomes were used to test the drug delivery efficiency of the targeted liposomes to EGFR high-expressing H1299 and SPCA1 cells. Free doxorubicin, GE11-modified doxorubicin liposome and non-targeting doxorubicin liposomes were added into cells for 2 h at 37 °C. The cells were then washed and let grown for another 48 h. The cell viabilities after different treatments were determined using a MTT assay. IC50 were calculated with ±95% confidence intervals (CIs) using Micromath Scientist 2.0.

2.6. In vivo distribution and targeting study

The peptide itself was labeled with the near infrared dye Cy5.5 by reacting its amine group with the succinimidyl ester of Cy5.5. The fluorescent peptides were then injected through the tail vein in H1299 xenograft mice and imaged using an Optix in vivo fluorescence imaging system (GE Health).

Similarly, DSPE was also labeled with Cy5.5 and incorporated into the liposome to examine its in vivo distribution in xenograft tumor bearing mice.

At least three mice were included in each group and series of images were taken at various time points after injection. The images were then processed using the fluorescence lifetime gating for Cy5.5 to remove most autofluorescent interferences.

Table 1
The particle size distribution of GE11-conjugated liposomes

Liposome formulation	Preformed liposome size (nm)	Ligand-conjugated liposome size (nm)
Rhodamine liposome	116.3 ± 11.9	125.7 ± 15.5
FITC liposome	127 ± 7.2	133.3 ± 17.0
Doxorubicin liposome	134 ± 32.6	178.3 ± 23.7
Cy5.5-labeled liposomes	115 ± 10.3	158.4 ± 9.6

3. Results

3.1. Preparation of peptide ligand-conjugated liposomes

All the liposomes were prepared and sized by extrusion through 100 nm membranes. The ligand-conjugated DSPE–PEG2000 molecules were then inserted into preformed liposomes based on the method developed by Ishida et al. (Ishida et al., 1999). The process had little disturbance to the integrity of the liposomes. The liposome size distribution before and after the insertion were shown in Table 1.

3.2. Binding and endocytosis of ligand-modified liposomes by EGFR expressing cells

Both EGF- and GE11-modified liposomes and control mPEG–DSPE liposomes were added to cultured EGFR expressing H1299 cells. All liposome formulations has total 9% ligand–PEG–DSPE and mPEG–DSPE combined. For EGF-modified liposomes, the binding was optimum with 6% of EGF–PEG–DSPE (plus 3% mPEG–DSPE). For GE11-modified liposomes, the binding was the optimum with 9% of GE11–PEG–DSPE. The representative confocal fluorescence microscopy images were shown in Fig. 1A-a and b. In contrast, the control liposome with 9% mPEG–DSPE had very limited binding (Fig. 1A-c).

Adding free EGF to the GE11 liposome bound cells diminished the fluorescence significantly (Fig. 1B-a). The fluorescence remaining was mostly inside cells, presumably in endocytic vesicles. The Z-stack scan of GE11 liposome bounded cells also suggested active endocytosis of GE11 liposomes (Fig. 2). We also examined GE11 liposome binding at 4 °C (Fig. 1B-b). As expected, the fluorescences were now only seen on cell surfaces. Similarly, the binding fluorescence of GE11 liposomes could be largely competed off by excess unlabeled free GE11 (Fig. 1B-c).

3.3. In vitro doxorubicin delivery studies

For cytotoxicity studies, doxorubicin-loaded liposomes was conjugated by peptide ligand GE11 for targeted delivery to EGFR high-expressing cells. We detected the different cell killing effects of free doxorubicin, targeted and non-targeted liposomal doxorubicin using MTT assay after 4 h treatment. IC50 were calculated based on the MTT data and listed in Table 2. In both H1299 and SPCA1 cell lines, the GE11-modified liposomal doxorubicin showed much higher cytotoxic efficiency than mPEG liposomes, although the IC50s were both a bit lower than free doxorubicin which could readily traverses cell membranes in vitro.

3.4. In vivo peptide ligand-mediated tumor targeting

Cy5.5-labeled GE11 peptides, Cy5.5-labeled unrelated peptide D11 and free Cy5.5 dye molecules were injected though tail vein

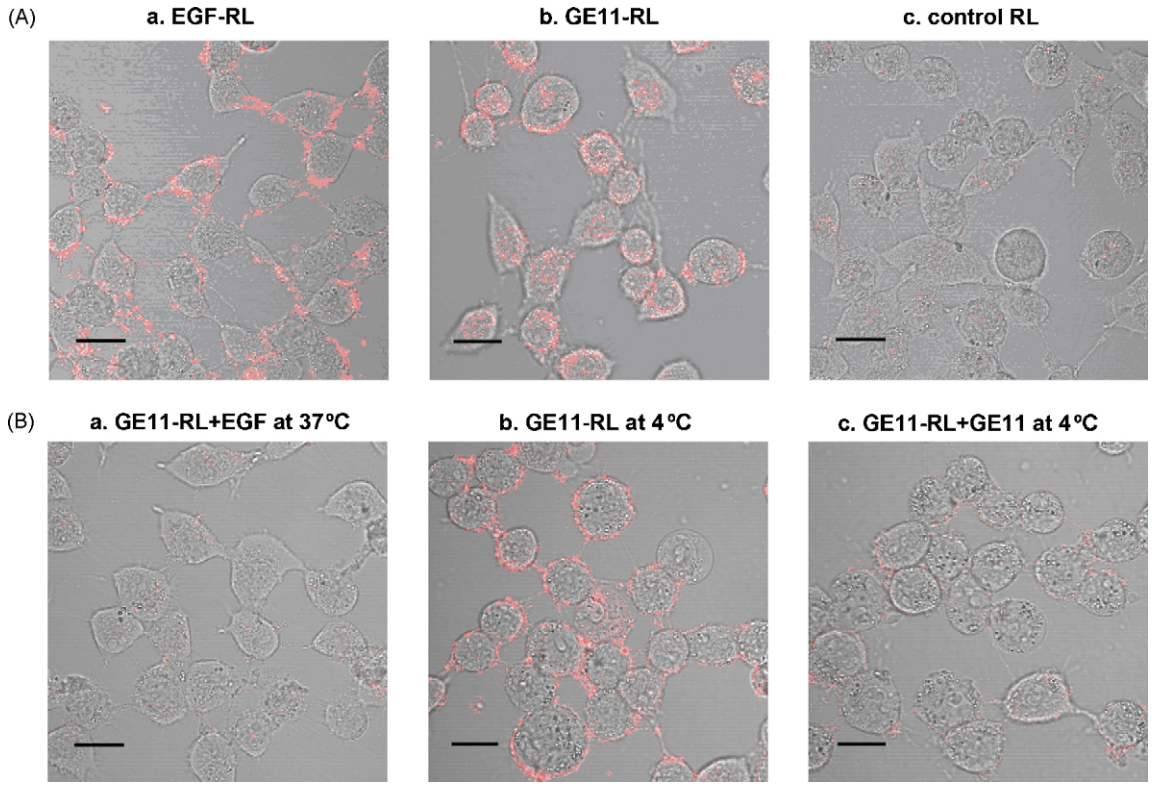

Fig. 1. Fluorescence microscopy studies of ligand-directed rhodamine liposome (RL) binding to EGFR high-expressing H1299 cells. Panel (A) Binding of EGF, GE11-targeted liposomes and non-targeted liposome to H1299 cells at 37 °C: (a) EGF liposomes; (b) GE11 liposomes; (c) control liposomes. Panel (B) Competition of GE11-targeted liposomes to H1299 at 37 °C or 4 °C in the presence of 50× mole excess free ligands: (a) binding at 37 °C with excess free EGF; (b) binding at 4 °C without free ligand; (c) binding at 4 °C with excess free GE11. Scale bar is 20 μm.

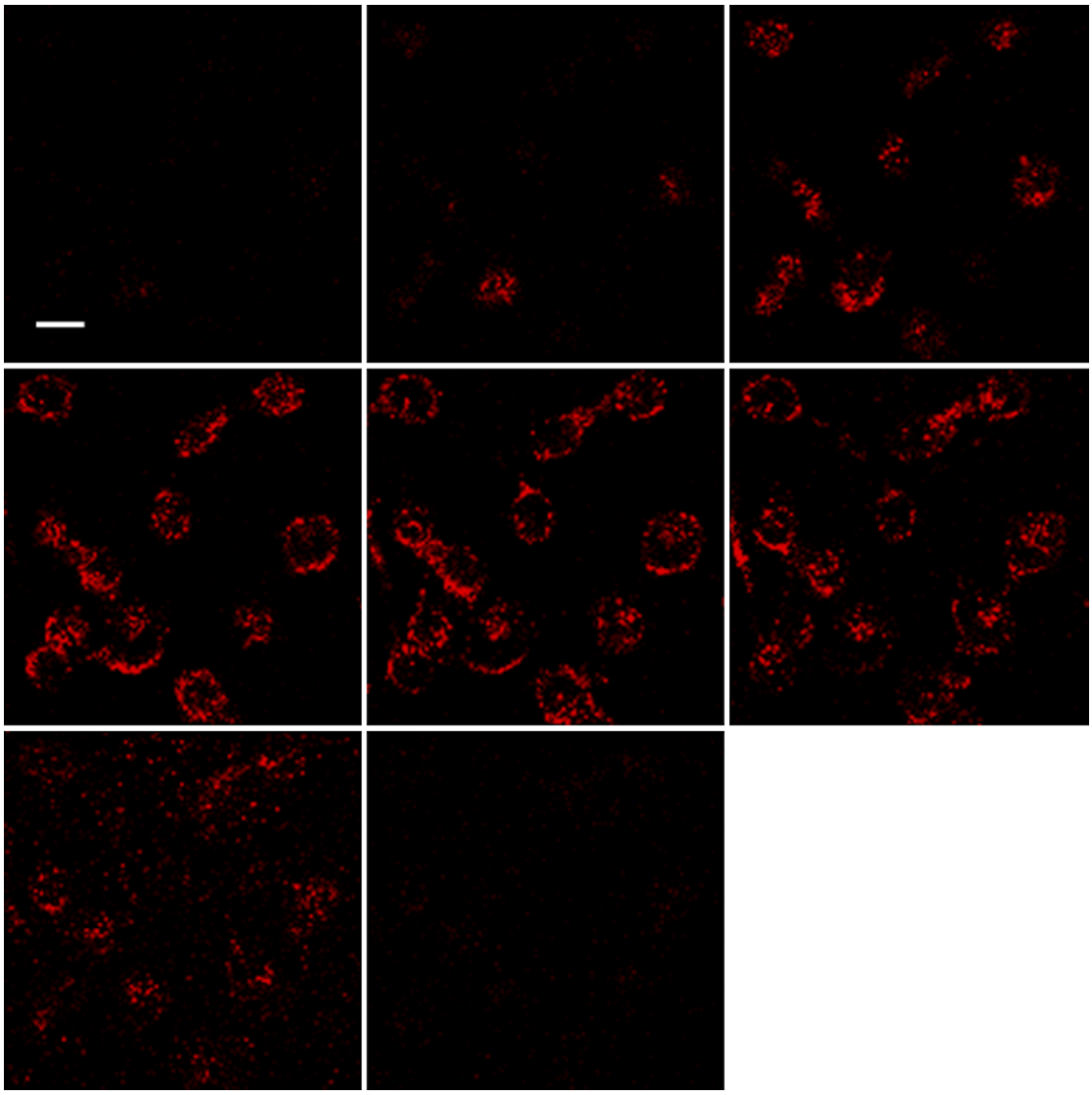

Fig. 2. Internalization of GE11-conjugated liposome by H1299 cells. Eight silices fluorescence images from the top to the bottom of the cells were shown using the Z-stack scan mode of confocal fluorescence microscope. Scale bar is 20 μm.

into H1299 xenograft tumor bearing mice and imaged for fluorescence distribution at various time points. In these groups, the fluorescence signals were the strongest in the kidney and bladder, as their main excretion organ. We had to limit the scanning area to the tumor and surrounding areas for avoid the interference from kidney and bladder. As shown in Fig. 3, only in the animals injected with GE11–Cy5.5, fluorescence signals were shown to accumulate in tumor tissues from 12 to 24 h after the injection.

Cy5.5 DSPE liposomes were modified with GE11 and injected through the tail vein into H1299 tumor bearing mice. The tumor bearing areas were scanned at various time points after injection. The fluorescence intensity images were shown in Fig. 4 after normalization of the whole body fluorescence using the eXplore Optix OptiView analysis software. Both the GE11-modified liposomes and the control mPEG–DSPE liposomes showed distribution and accumulation at the tumor site. But the fluorescences in GE11 liposome injected mice were relatively stronger and lasted longer.

Table 2
IC50 of drug in EGFR high-expressing cells

IC50 (μg/ml)	Doxorubicin	GE11 doxorubicin liposome	Doxorubicin liposome
H1299	2.68 ± 0.92	3.25 ± 0.37	9.85 ± 1.7
SPCA1	4.55 ± 1.06	6.89 ± 1.23	42.75 ± 12.81

4. Discussion

For targeted drug delivery towards tumor, small molecule ligands, peptides, antibody and antibody fragments had all been used (Ahmad et al., 1993; Woodle et al., 2001; Kok et al., 2002; Gabizon et al., 2003; Schiffelers et al., 2003; Pastorino et al., 2006; Saul et al., 2006; Oba et al., 2007). EGFR is an important target for its overexpressed in many cancers (Schmidt et al., 1997; Lutsenko et al., 2002; Mamot et al., 2003). In our previous study (Li et al., 2005), peptide GE11 was identified as a novel ligand with high affinity towards EGFR. Radiolabeled peptide was found to have broad tissue distribution in vivo, with some preferential accumulation in tumor. But the distribution pattern was only studied at two-time point: 0.5 and 4 h after injection, because of the radioactivity assay limitation. In this study, we used near infrared fluorescence label and in vivo fluorescence imaging techniques, which enabled us to study the distribution pattern over time on the same animal. The

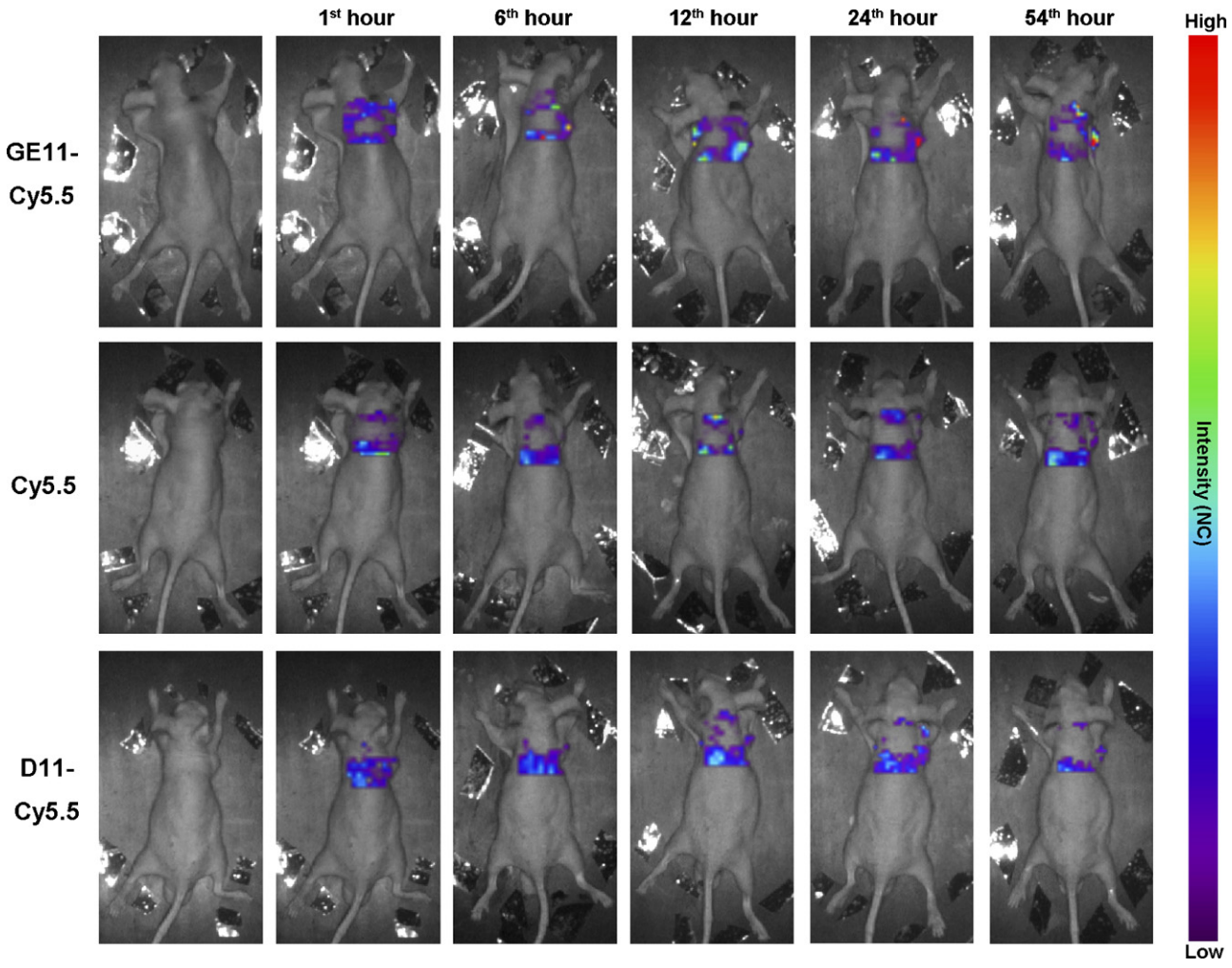

Fig. 3. The fluorescence image of Cy5.5-labeled GE11, Cy5.5-labeled unrelated peptide D11 and Cy5.5 in tumor bearing mice. Images shown were taken at 1, 6, 12, 24 and 54 h after injection.

method is very straightforward and the data sets had much less variability.

Furthermore, we used the peptide ligand GE11 to modify a widely used drug delivery vehicle (liposomes) and studied its binding and uptake by tumor cell and tumor tissue in vitro and in vivo. To conjugate the peptide ligand to liposome surfaces, we adopted the post-insertion method developed by Ishida et al. (Ishida et al., 1999). The peptide ligand was coupled to the distal end of the polyethylene glycol chain of the lipid, and then inserted into the preformed liposome membrane after incubation. The method is very efficient and reliable with high-ligand loading efficiencies (Hansen et al., 1995; Bohl Kullberg et al., 2002) and preserved stability of doxorubicin-loaded liposomes (Iden and Allen, 2001; Moreira et al., 2002). The linkage of the ligands to the distal PEG ends helped to minimize the sterical interference when binding to target cells (Hansen et al., 1995; Maruyama et al., 1995). But the ligand and mPEG density presented were found to be critically important. In our studies, we optimized the ligand and mPEG density to target cells and eliminate nonspecific binding. 9% molar percent total PEG–lipid was found to be preferable for H1299 cells, and 6% of EGF–PEG–lipids and 9% of GE11–PEG–lipids resulted maximum binding in vitro. The optimum conditions may vary among different tumor models and between in vitro and in vivo environments too. In this study, we adopted the same ligand density formulation determined in vitro in animal studies. In reality, it would need to be further optimized.

To examine the in vivo distribution of the peptide and peptide-modified liposomes after tail vein injection, many methods had been used, including radiolabel and radioactivity counting, histology and immunohistochemistry, and reporter drug or reporter gene assays. Small animal in vivo fluorescence imaging is a newly developed tool for monitoring the biodistribution in live animals. Cy5.5 is one of the most commonly used dye because of its superior tissue penetration and low background (Ntziachristos et al., 2003; Cheng et al., 2006). The main advantage of the in vivo imaging technique is that it allows continuous monitoring of the same live animals throughout the course of the study.

In those mice injected intravenously by Cy5.5-labeled peptide GE11, there was specific fluorescent accumulation in tumor region from 1 to more than 54 h after injection, compared to Cy5.5-labeled unrelated peptide D11 and Cy5.5 dye itself which was quickly showed up in the kidney and bladder region and eliminated. The distribution pattern was a bit different for liposomes, with or without the ligand GE11. Both liposome formulations showed the so-called enhanced permeability and retention effect. Because the liposome sizes were close to the vascular opening (Yuan et al., 1995; Hobbs et al., 1998), they could leak preferably through out the tumor vasculature. The EPR effect would be similar with

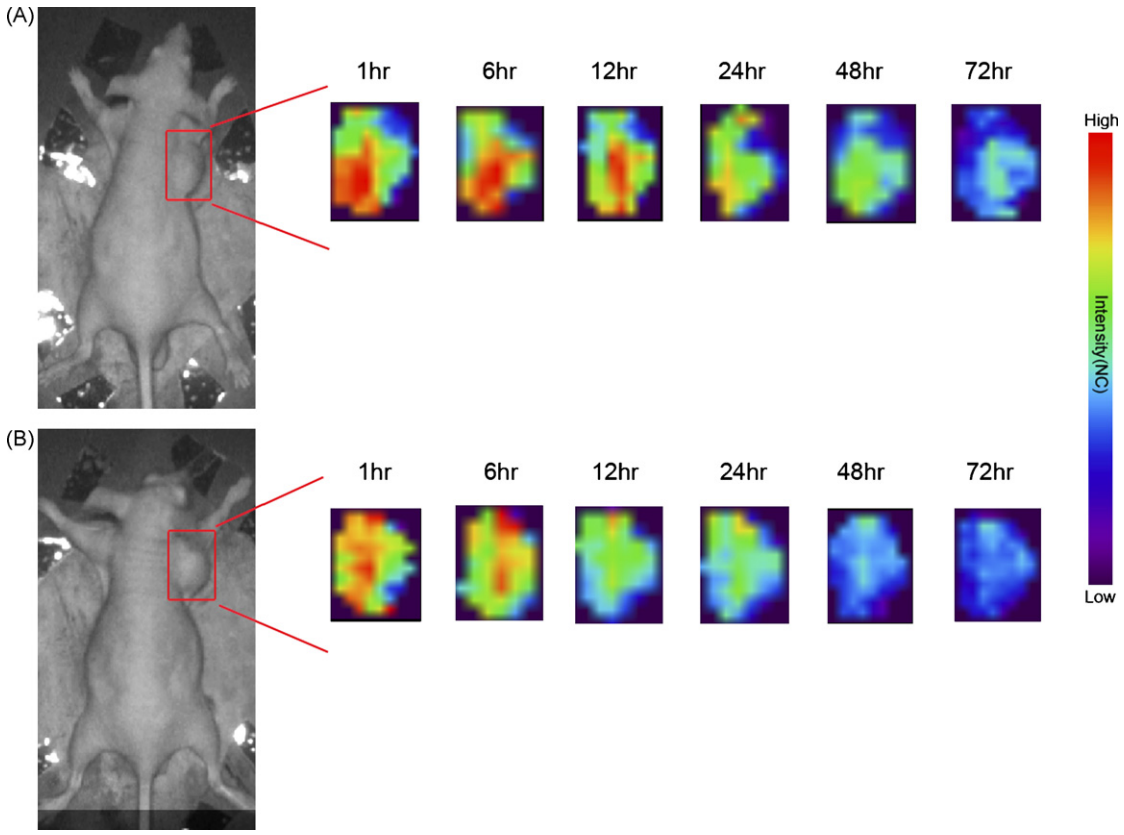

Fig. 4. The fluorescence images of ligand GE11-directed liposome distribution and accumulation in tumor tissues. Images shown (from left to right) were the light picture of the mouse, fluorescence images of tumor site taken at 1, 6, 12, 24, 48 and 72 h after the injection of Cy5.5-liposomes. (A) GE11 liposomes and (B) non-targeted liposomes.

or without the ligand, because it is mainly a particle size effect. Therefore we see fluorescence accumulation in the tumor region in both mPEG- and ligand-modified sample. But the extend and duration of the tumor accumulation could be different. Specific binding to the cancer cell and rapid internalization would definitely help drug delivery and efficacy (Wu et al., 1993; Tanaka et al., 2004; Kirpotin et al., 2006). As shown in Fig. 4, this is exactly the case for GE11 liposomes, as compared to mPEG liposomes.

In summary, we used fluorescence imaging techniques to examine peptide ligand GE11-directed binding to EGFR expression cancer cells in vitro and in vivo. We demonstrated that the GE11 peptide is an effective EGFR ligand and can mediate targeted liposome delivery to EGFR positive tumors in vivo after tail vein injection. Further investigations on the therapeutic efficacies of this delivery system in various preclinical models are undergoing.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2008.07.012.

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