Micromixer Based Preparation of Functionalized Liposomes and Targeting Drug Delivery

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S Supporting Information

ABSTRACT: We present here a specific targeting nanocarrier system by functionalization of liposomes with one new type of breast cancer targeting peptide (H6, YLFFVFER) by a micromixer with high efficiency. Antitumor drugs could be successfully delivered into human epidermal growth factor receptor 2 (HER2) positive breast cancer cells with high efficiency in both in vivo and ex vivo models.

KEYWORDS: Nanocarrier, HER2, Antitumor, YLFFVFER

Breast cancer is the leading type of malignancy in female.^{1,2} Human epidermal growth factor receptor 2 (HER2, also known as neu or ERBB-2) positive breast cancer showed higher incidence of metastasis and recurrence than HER2 negative cancer, especially. HER2 is a member of the epidermal growth factor receptor family and plays an important role in biomarkers as well as targeting therapy for human cancers.^{3,4} Nowadays, chemotherapy remains the primary treatment strategy for patients with breast cancer. Advanced researches have applied nanomaterials to chemotherapy so that drug delivery efficiency could be improved.^{5,6} As reported, nanoparticles can easily reach the tumor sites and remain there for a long time, owing to the enhanced permeability and retention (ERP) effect.^{7,8} Among the nanosized drug delivery carriers, liposomes have some advantages. Liposome is a spherical vesicle that has a lipid bilayer and is composed of phospholipids. The phospholipid bilayer consists of hydrophilic heads and hydrophobic tails, which can form self-assembled nanostructures by hydrophobic interactions.^{9–11} Drugs can be encapsulated in either the cavity (hydrophilic drugs) or the lipid bilayer (hydrophobic drugs) of liposomes.^{12–14} Taking advantage of the good biocompatibility, good control, and release ability, liposomes are usually used as the carriers for drug delivery,¹⁵ and liposome drugs have been widely used in clinical therapy, such as paclitaxel liposome and doxorubicin (DOX) liposome.¹⁶ Currently, researchers have paid more attention to targeting drugs with the characteristics of active transportation and capability of achieving this at cancer cells specifically. Targeting therapy has gradually become the hot spot of breast cancer treatment.¹⁷ For example, trastuzumab

was the first targeting drug to obtain FDA approval for use in HER2 overexpressed breast cancer.¹⁸ Advancements in nanomaterial and nanotechnology have provided a promising strategy for cancer targeting drug delivery.^{19,20} Recent researches have shown that the drug delivery efficiency of nanocarriers could be dramatically enhanced by a modification of the specific recognition molecules.²¹ As promising interface molecules, peptides may be a good choice of recognition element to modify liposomes in order to increase the targeting specificity.^{22–26} For instance, one type of tumor metastasis targeting peptide was conjugated with the doxorubicin containing liposome to realize drug delivery.²⁷ The delivery efficiency was enhanced by TAT (a penetrating peptide) modified liposomes²⁸ and GE11 (a targeting peptide) modified liposomes to epidermal growth factor receptor (EGFR) positive nonsmall cell lung cancer.²⁹

Herein, H6-modified liposomes (H6-LS) were prepared by thin film hydration method. We hypothesized that the H6-modified nanocarrier may be a promising candidate for targeting delivery of antitumor drugs toward HER2 positive breast cancers. H6, a novel peptide (YLFFVFER) was proposed by high throughput library screening toward HER2 in our previous work.³⁰ This octapeptide (YLFFVFER) showed nanomolar affinity toward HER2. H6 could be conjugated with amphiphilic molecular 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol)-2000]-*N*-hydroxy-

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succinimidyl (DSPE-PEG₂₀₀₀-NHS) by the nucleophilic substitution reaction to obtain a targeting compound H6-PEG₂₀₀₀-DSPE. The reaction efficiency was improved by our micro-mixer. Furthermore, the specific nanoparticles modified by H6-PEG₂₀₀₀-DSPE (H6-LS) showed a good affinity toward human breast cancer (SKBR3, overexpressing HER2) cell line. Antitumor drugs encapsulated in the liposomes were successfully delivered into tumor cells in vitro. Additionally, we further demonstrate that our nanocarrier loading drugs could inhibit the growth of HER2 overexpressing tumor in vivo. As we expected, this nanocarrier loading drug system show high efficiency of drug targeting delivery.

At the first stage, the micromixer was fabricated with two slices of bonded polished chrome glasses (the substrate and the cover) using the conventional lithography strategy. (The fabrication details were shown in [Supporting Information](#).) Briefly, the designed microstructure was displaced to the chrome glass by undergoing a series of treatments as described in the literature, i.e., exposure, development, dechroming, and etching to get the substrate plate. The size of the micromixer chip is 75 mm (L) × 25 mm (W) × 2 mm (H). The chip mainly consists of a reaction chamber with the size of 600 μm (W) × 43 mm (L), three inlets, and one outlet. Inside of the reaction chamber, an uneven structure was fabricated, and the about 20 bulges were formed in order to make the disturbance of the reaction liquid. Synthesis of the H6-PEG₂₀₀₀-NHS was carried out in the fabricated micromixer. H6 peptide (M.W. 1119 Da) was synthesized ([Figure S1](#)) and conjugated with 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol)-2000]-*N*-hydroxysuccinimidyl (DSPE-PEG₂₀₀₀-NHS M.W. 3000 Da) in the micromixer chamber to obtain the targeting compound H6-PEG₂₀₀₀-DSPE. As shown in [Scheme 1a](#), peptide H6 dissolved in newly distilled *N,N*-dimethylfor-

Scheme 1. Schematic of Microfluidic Device and Liposomes: (a) Structure of the Micromixer and (b) Structure of H6 Modified Liposomes (Encapsulated with FITC and DOX)

mamide (DMF) (2 mg/mL) was introduced from inlet A, and DSPE-PEG₂₀₀₀-NHS in DMF (3 mg/mL) was introduced from inlet C at the same flow rate of 0.56 μL/min. *N,N*-Diisopropylethylamine (DIPEA) in DMF (1:100) was introduced from inlet B to adjust the base condition. After 15 h, targeting compound was collected from the outlet.

We used high-performance liquid chromatography (HPLC) to monitor the micromixer based reaction. As shown in [Figure 1a](#), the peak of peptide was decreased and the peak of peptide-PEG₂₀₀₀-DSPE was increased along the time. After 15 h, the conversion rate was achieved 90%. Compared to the conventional vessels ([Figure S2](#)), the micromixer reaction is low consumption and high efficiency. Then we used matrix-assisted

Figure 1. Monitoring of the conjugation between peptide and DSPE-PEG₂₀₀₀-NHS. (a) HPLC spectra of reaction mixture at different times. (b) MALDI-TOF-MS spectra of NHS-PEG₂₀₀₀-DSPE. (c) MALDI-TOF MS spectra of H6-PEG₂₀₀₀-DSPE.

laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) to identify the products. The mass peak of the reactants 3019.58 (DSPE-PEG₂₀₀₀-NHS, [Figure 1b](#)) and the mass peak of 15 h product is 4088.02 (H6-PEG₂₀₀₀-DSPE, [Figure 1c](#)), indicating a successful and effective conjugation based on the micromixer.

Liposomes were then prepared by the conventional thin lipid film method (the preparation details were shown in [Supporting Information](#)). Briefly, soy phospholipids/cholesterol/H6-PEG₂₀₀₀-DSPE (molar ratio 20:10:2) were dissolved in dichloromethane/methanol mixed dissolvent. The mixed solvent was evaporated under reduced pressure at 40 °C to form a lipid film, which was subsequently hydrated with phosphate-buffered saline (PBS) and sonicated for 30 min with a bath type sonicator to obtain liposomes. The morphologies of liposomes loading doxorubicin (LS-DOX) and H6-liposomes loading doxorubicin (H6-LS-DOX) were shown by TEM. Both LS-DOX and H6-LS-DOX were spherical in shape and had good dispersion ([Figure 2](#)). The average hydrodynamic diameter and surface charge were characterized by measuring the size and zeta potential with dynamic light scattering (DLS), as shown in [Figure 2a,b](#). The diameter of LS-DOX was around 90 nm, and the zeta potential in PBS was around −14.0 mV, while the diameter of H6-LS-DOX was around 130 nm and the zeta potential was around −20 mV. It is indicated that after modification both the size and systematic stability were increased. Furthermore, encapsulated efficiency was assessed. Both LS and H6-LS-DOX showed high encapsulation efficiencies ([Figure S3](#)). To investigate the DOX release in vitro, the required quantity of DOX-loaded liposomes was transferred into a dialysis bag. All of the release rates of DOX from loaded liposomes were much slower than that of DOX-sol ([Figure S5](#)). Then, the stability of DOX-loaded liposomes in serum was investigated using a filtration test. The fluorescence

Figure 2. Characteristics of different liposomes. (a) Size distribution of unmodified liposomes loading doxorubicin. (b) Size distribution of DSPE-PEG₂₀₀₀-H6 modified liposomes loading doxorubicin. (c) Transmission electron microscopy (TEM) graph and zeta potential of unmodified liposomes loading doxorubicin. (d) TEM graph and zeta potential of DSPE-PEG₂₀₀₀-H6 modified liposomes loading doxorubicin.

of LS-DOX, SP-LS-DOX, and H6-LS-DOX did not show obvious changes after incubation in serum at 37 °C for 24 h followed by membrane filtration (Figure S4). In other words, most of liposomes appeared to retain their state without aggregation. These results indicated the substantial stability of DOX-loaded liposomes.

Then we continued to observe the bioactivity of the prepared liposomes. Confocal microscopy (Olympus FV1000-IX81 confocal-laser scanning microscope) was used to investigate cellular uptake of the liposomes (Figure 3). We employed FITC (fluorescein isothiocyanate) as the mimic of the hydrophilic drug. When lipid film was hydrated with phosphate-buffered saline (PBS), FITC could be loaded in liposomes. Human breast cancer cells SKBR3 (HER2 over-expression cancer cells) and Human Embryonic Kidney 293A (HER2 nonexpression cells) were applied as the positive cell and control cell models, respectively. SP (EFVYFLRF), a sequence scrambled peptide was served as negative control peptide. The above cells were incubated with H6-LS-FITC (15 $\mu\text{g/mL}$, 200 μL), LS-FITC (15 $\mu\text{g/mL}$, 200 μL), and SP (15 $\mu\text{g/mL}$, 200 μL) as well as Hoechst 33342 (nucleus indicator, 10 $\mu\text{g/mL}$). The confocal images (60 $\times$, oil-immersion objective) were obtained in the excitation wavelength of both 488 nm (FITC) and the 405 nm (Hoechst 33342). After 15 min of incubation, the SKBR3 cells treated with LS-FITC (liposome loading FITC) and SP-LS-FITC exhibited very low fluorescence intensity (Figure 3a,c). However, H6-LS-FITC demonstrated much more fluorescent intensity in SKBR3 Cells in Figure 3b, revealing the higher uptake in SKBR3 cell line. Again, very low fluorescence was observed in 293A cell lines in Figure 3d, revealing that the enhanced uptake of H6-LS could

Figure 3. Confocal microscopy images of liposomes toward cells. Green fluorescence represents FITC, and blue fluorescence is for Hoechst 33342. (a) SKBR3 cells were incubated with LS-FITC. (b) SKBR3 cells were incubated with H6-LS-FITC. (c) SKBR3 cells were incubated with SP-LS-FITC. (d) 293A cells were incubated with H6-LS-FITC.

be mediated by HER2, the H6 receptor, very specifically. These results have further proved that liposomes modified with H6 peptide have a remarkable specific recognition ability toward HER2 positive breast cancer cells.

We estimated that the endocytosis would be different between the H6-LS-FITC and LS-FITC. We designed the cell assays to monitor the endocytosis and release of both the liposomes along with time in different cell lines. SKBR3 were incubated with H6-LS-FITC (15 $\mu\text{g/mL}$, 200 μL) and LS-FITC (15 $\mu\text{g/mL}$, 200 μL) as well as Hoechst 33342 (10 $\mu\text{g/mL}$). Confocal image was also captured at the time point of 0, 5, 10, and 15 min, respectively. As show in Figure 4, after 5 min incubation, the green fluorescence of H6-LS-FITC has appeared with little fluorescence of LS-FITC (Figure 4a,b,e,f). When it came to 10 min incubation of H6-LS-FITC, fluorescence intensities on the SKBR3 cell membrane are getting stronger and showing the tendency to diffuse into cells (Figure 4g). After 15 min incubation, H6-LS-FITC has been endocytosed and released into the cytoplasm (Figure 4h). After the H6-LS-FITC incubation time of 20 min or longer, the situation is similar as the one of 15 min. We estimated the endocytosis has achieved saturation at about 15 min. However, after incubation with LS-FITC for 15 min a low green fluorescence appears on the cell membrane Figure 4a–d. Green fluorescence intensities in cells treated with H6-LS-FITC were significantly higher than those who exposed to LS-FITC at all test time. Obviously, the HER2 targeting peptide H6 plays a key role as the interface molecules between the biosystems and the liposomes. It demonstrates that H6 peptides could enhance tumor targeting and penetration of drug encapsulated nano-carriers and is a promising candidate for developing targeted drug delivery systems.

Figure 4. Fluorescence confocal images of SKBR3 incubated with LS-FITC and H6-LS-FITC cellular uptake at different times.

Furthermore, we investigated the antitumor activity against SKBR3 cells in vitro with H6 modified liposomes in which DOX was encapsulated. LS-DOX, H6-LS-DOX, and SP-LS-DOX with different DOX concentration of 0.001, 0.01, 0.1, 1, 10, and 100 $\mu\text{g/mL}$ (150 μL) incubated SKBR3, respectively. After 24 h treatment with LS-DOX, H6-LS-DOX, and SP-LS-DOX, the cell viabilities were measured by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. As shown in Figure S7, at the DOX concentration of 0.01 $\mu\text{g/mL}$, the cell viability of SKBR3 is over 60%, while H6-LS-DOX was lower than LS-DOX and SP-LS-DOX. When DOX concentrations turned to 100 $\mu\text{g/mL}$, the cell viability of SKBR3 decreased to 20%. Furthermore, the half maximal inhibitory concentration (IC_{50}) values at 24 h of H6-LS-DOX was 5.2-fold, 6.5-fold, and 17-fold lower than that of LS-DOX, SP-LS-DOX, and DOX. The difference may be attributed to the improved targeting efficacy of the H6-modified liposomes. As expected, H6-LS-DOX displayed a significantly greater efficacy relative to LS-DOX and SP-LS-DOX. In other words, H6-LS-DOX was more effective to kill the HER2 overexpressed breast cancer cells than LS-DOX, which might be promising in specific delivery of therapy or imaging agents to breast cancers.

To examine whether the molecular delivery effects were viable in the in vivo conditions, we first performed optical imaging experiments in tumor xenograft model by using HER2-overexpressed SKBR3 cells. 1,1-Dioctadecyl-3,3,3,3-tetramethylindotricarbonylamine iodide (DiR) was encapsulated in the liposomes to realize the in vivo imaging. Similar to the above, H6-LS-DiR and LS-DiR were both prepared and injected from the tail vein (1 mg/mL, 200 μL) at a dose corresponding to 1 $\mu\text{g/mL}$ of DiR. Mice treated with PBS only were served as negative control. Figure 5a,b shows the real-time biodistribution and tumor accumulation of the different liposomes at 4 and 8 h postinjection. After 4 h, the tumor fluorescence intensity in H6-LS-DiR treated mice was slightly higher than LS-DiR. After 8 h, H6-LS-DiR showed a good targeting efficiency and accumulated continuously in tumor sites, while LS-DiR showed a relatively low fluorescence intensity. After 8 h, tumor tissues were excised and their fluorescence images were recorded. As shown in Figure 5c,d, the fluorescence levels in the livers of nanocarriers-treated mice were very high, which might be attributed to the high macrophage uptake nature of the liver. H6-LS-DiR showed higher tumor target ability and

Figure 5. In vivo and ex vivo imaging of tumor targeting delivery by H6-LS-DiR and LS-DiR. (a,b) Real-time biodistribution and tumor accumulation of H6-LS-DiR, LS-DiR, and PBS treatment at 4 and 8 h. (c) Ex vivo fluorescence imaging of tumor accumulation and biodistribution. (d) Quantification of the fluorescence signals ex vivo. Fluorescence intensity was measured in counts/energy/area and is presented as an average ($n = 4$). (e) Excised tumor photograph after LS aided drug delivery. (f–h) H&E stained excised tumor of the mice after H6-LS-DOX, LS-DOX, and PBS treatment. (i) The tumor size changing curves in the monitored 9 days. (j) The mice weight changing curves in the monitored 9 days.

the fluorescence intensity than LS-DiR, which indicated that the target peptide H6 plays an important role in the molecular delivery process.

Next we did in vivo drug delivery and cure assays. Tumor inhibition studies were carried out to examine the efficiency of H6-LS-DOX, LS-DOX, and saline. SKBR3 cells were also xenografted in nude mice to construct the tumor models. After the tumors had been allowed to develop approximately 50–120 mm^3 the mice were randomly divided into three groups ($n = 4$) in order to minimize the random errors of the weights and tumor-sizes. H6-LS-DOX, LS-DOX, and PBS were intravenously injected into tumor-bearing mice via tail veins. The mice were treated with the nanocapsules (300 μL) each day for six consecutive days at a dose corresponding to 100 $\mu\text{g/mL}$ of DOX. The tumor sizes and the mice weights were monitored in the 6 days and in the following 3 days. Therefore, observation was carried out in 9 consecutive days. The results were determined and are shown in Figure 5d–j. The tumors treated with H6-LS-DOX are much smaller than those treated with LS-DOX and those untreated (Figure 5e,i), which indicated an efficient therapeutic effect. After cure assays, the tumor tissue sections were analyzed by H&E (hematoxylin–eosin) staining for evaluation of the morphology. Compared to the control group, H6-LS-DOX treatment caused the loose spaces between

tumor tissues. The obvious changes of the tumor morphology indicated that the efficient tumor apoptosis (Figure Sf–h). The body weight of the mice treated with H6-LS-DOX, LS-DOX, and PBS were also shown in Figure Sj. It revealed that the nanocapsules have delivered the drugs to the tumor site in an effective way by the modification of H6 with low side effects.

H6-LS loaded drugs may be prospects for drug delivery. Because the high affinity of H6-LS and SKBR3 cells high inhibition of H6-LS-DOX, our nanocarrier drug delivery is promising in clinical targeting chemotherapy for killing human breast cancer. For further study, we ought to do more effort to realize our goal, applying our nanocarrier drug delivery to clinical therapy.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.6b00028.

Some experimental details, encapsulation efficiency, inhibition studies, and some additional figures (PDF)

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Notes

The authors declare no competing financial interest.

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