Supplementary Information

A pH-sensitive T7 peptide-decorated liposome system for HER2 inhibitor extracellular delivery: An application for the efficient suppression of HER2+ breast cancer

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1. Synthesis and characterization of T7-PEG_{2000}-DSPE

T7-PEG_{2000}-DSPE was synthesized through the covalent combination of sulfhydryl and maleimide. Mal-PEG_{2000}-DSPE was dissolved in dichloromethane, and the solvent was removed through rotary evaporation. Then, pH 7.4 PBS buffer was added for the preparation of a micellar solution. T7-Cys was dissolved in pH 7.4 PBS buffer and was slowly dripped into the micellar solution and slightly stirred at room

temperature for 48 h with the protection of N_2. The molar ratio of Mal-PEG_{2000}-DSPE to T7-Cys was 1:1.2. The obtained solution was dialyzed (3500Da cutoff) for the removal of excess T7-Cys. The freeze-dried T7-PEG_{2000}-DSPE powder was stored at −20 °C. T7-PEG_{2000}-DSPE was characterized by ^{1}H-NMR and MALDI-TOF mass spectrometry.

Figure S1a shows the ^{1}H-NMR spectra of Mal-PEG_{2000}-DSPE and T7-PEG_{2000}-DSPE. The red mark is the characteristic peak of maleimide at 6.7 ppm. This peak disappeared in the spectra of T7-PEG_{2000}-DSPE, indicating that the maleimide group completely reacted with the terminal sulfhydryl group on T7-Cys [1]. Figure S1b presents the mass spectrum results of Mal-PEG_{2000}-DSPE and T7-PEG_{2000}-DSPE. The difference between the highest peaks of T7-PEG_{2000}-DSPE and Mal-PEG2000-DSPE was 939, which was close to the mass of T7-Cys, further indicating that T7 peptide was successfully grafted to PEG_{2000}-DSPE.

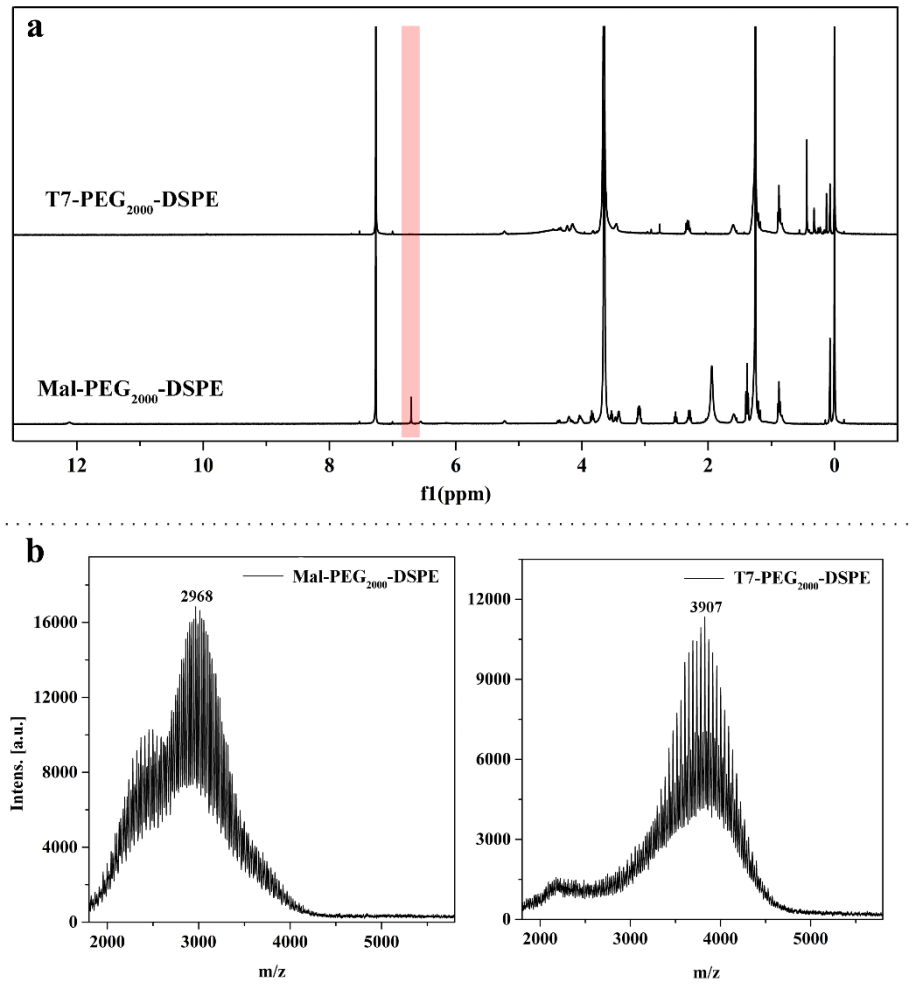

Figure S1. (a) Characterization of Mal-PEG_{2000}-DSPE and T7-PEG_{2000}-DSPE in $CDCl_3$ by ^{1}H-NMR spectrometry. (b) Characterization of Mal-PEG_{2000}-DSPE and T7-PEG_{2000}-DSPE by mass spectrometry.

2. Optimization of pH-sensitive formulations

The liposomes were prepared with the ranging dosage of α-TOS and T7-PEG_{2000}-DSPE. Drug release experiments at pH 6.8 and 7.4 were carried out according to the operations in Section 2.4. The release characteristics of T7-LP@LAP prepared with different formulations are shown in Figure S2. When the ratio of the stabilizer (α-TOS) was 30%, LAP was released from liposomes in pH 7.4 PBS, indicating that the

liposomes were labile in normal physiological environments. However, as the ratio reached 80%, the liposomes were stable and were not pH sensitive. When the ratio was 55%, the liposomes remained stable at pH 7.4 and released LAP at pH 6.8. With the addition of T7-PEG_{2000}-DSPE, the liposomes showed a sustained release characteristic.

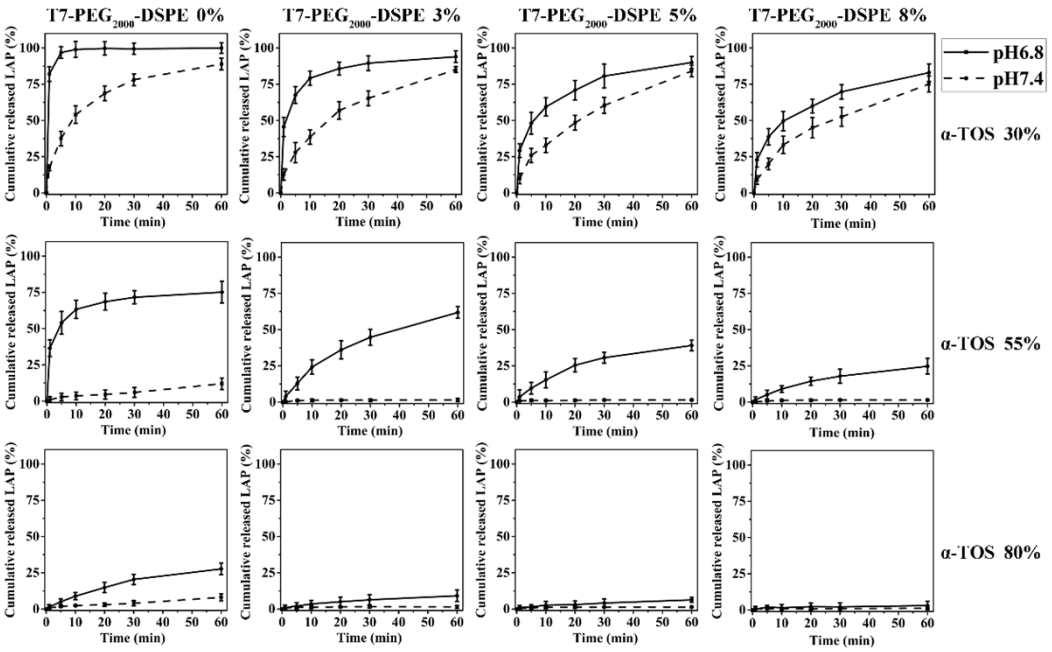

Figure S2. Cumulative release rate of LAP from different liposomes at pH 6.8 and pH 7.4, respectively.

To meet the requirement of being stable under physiological condition and release drug at pathological sites simultaneously, the liposome was required low release at pH 7.4 and high release at pH 6.8. Therefore, the average ratio (R) of the release rate at pH 6.8 and 7.4 corresponding to the six time points was used as the evaluation index for the optimization of the liposomes. The calculation formula was as follows:

$$R = 1/6\sum\frac{r_{pH6.8}}{r_{pH7.4}}$$

The *R* values for different liposomes is listed in Table S1. pH sensitivity increases with the value. According to the data and the curves in Figure S2, the stability of the liposomes was enhanced upon the addition of α-TOS, and the stability was appropriate when the ratio of α-TOS was ≥55%. The increase in T7-PEG_{2000}-DSPE weakened the pH sensitivity of the liposomes. The largest value of *R* appeared when the ratio of α-TOS and T7-PEG_{2000}-DSPE were controlled at 55% and 3%. Simultaneously, the liposome showed a sustained release effect.

Table S1. *R* values for the liposomes with different formulations.

Content of α-TOS	Content of T7-PEG_{2000}-DSPE			
	0%	3%	5%	8%
30%	7.0	9.4	8.1	8.0
55%	17.8	25.4	15.9	10.2
80%	3.6	3.2	2.9	2.0

Table S2. Particle sizes, zeta potentials, and entrapment efficiency and drug loading of LAP in different LAP-loaded liposomes (n = 3, mean ± SD).

Formulation	Size (nm)	PDI	Zeta potential (mV)	EE (%)	DL(%)
LP@LAP	116±8	0.28±0.04	-4.0±0.5	92.7±3.3	6.1±0.5
T7-LP@LAP	144±3	0.15±0.02	-4.8±0.6	79.1±5.1	5.5±0.4

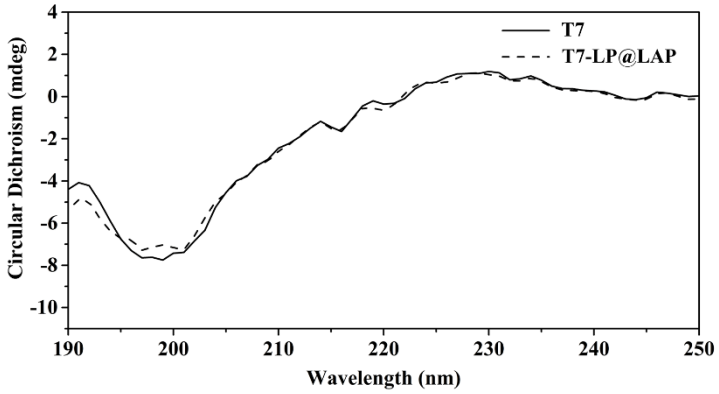

Figure S3. Circular dichroism spectra of T7 peptide on the surface of liposome and free T7 peptide.

3. Quantification of LAP, DOPE, T7 peptide, α-TOS and RhoB

LAP was quantified with an internal standard method on a LCMS/MS system. Separation was performed using an ultimate liquid chromatography system (Shimadzu, Japan) with an Acquity UPLC®BEH C18 analytical column (2.1 mm × 100 mm, 1.7 μm). The mobile phase was composed of acetonitrile and ultra-pure water containing 0.1% formic acid. The flow rate was 0.5 mL/min, and the injection volume was 1 μL. The volume percentage of acetonitrile increased from 10% to 90% at the first 1 min, then remained 90% until 2 min. Detection was carried out on a QTRAP® 5500 mass spectrometer (AB SCIEX, USA) in positive electron spray ionization (ESI) mode. The working parameters of ESI were as follows: curtain gas, 20 psi; ion spray voltage, 5500 V; temperature, 500 °C; gas 1, 60 psi; and gas 2, 60 psi. Declustering potential was 100 V, and collision energy was 51 eV.

The calibration curve was constructed by plotting the peak area ratio (LAP/IS) against LAP concentrations (1, 3, 10, 30, 100, 300, and 1000 ng/mL) including LLOQ. The regression equation was fitted as follows: $Y = 0.0181\ X - 0.2626$ ($r = 0.994$).

The prepared plasma samples (30 μL) were mixed with 120 μL of acetonitrile containing IS (10 ng/ml) by vortexing for 30 s. Then, the samples were centrifuged at 13000 rpm for 15 min, and the supernatant layers were used for testing.

The content of DOPE was measured with phosphorus assay. Ferric chloride (27.03 g) and ammonium thiocyanate (30.4 g) were dissolved in purified water, and the resulting solution was used as the chromogenic reagent. DOPE reacts with ferric ammonium thiocyanate to form a coordination compound that has a characteristic UV absorption at 485 nm. The liposomes were diluted with chloroform, then mixed with an equal volume of chromogenic reagent. After shaking for 10 min, the mixed solution was centrifuged. The lower layer was taken out for UV measurement. The standard curve was $Y = 125.5X - 8.91$ ($R^2 = 0.998$, X and Y represent the concentrations of DOPE and absorbance, respectively).

Primary amine was quantified with a fluoresceamine assay for the determination of the content of T7 peptide on the surfaces of the liposomes [2, 3]. The liposomes were incubated with fluoresceamine acetone solution (0.1 mg/mL) at room temperature for 10 min. Then, the fluorescence of the samples was measured with a microplate reader (Thermo Scientific Varioskan Flash, USA) with the excitation and emission wavelength setting at 390 and 480 nm. Standard curve ($Y = 4091X + 56.8$, $R^2 = 0.997$, where X is the concentration of T7 peptide and Y is fluorescence intensity) was constructed using

the known amount of T7 peptide and used in determining the correlation between fluorescence signal and the amount of primary amines.

α-TOS was quantified with an external standard method on a LCMS/MS system. Separation was performed in a Watets C18 analytical column (2.1 mm × 100 mm, 1.7 μm). The mobile phase was 0.1% formic acid. The flow rate was 0.5 mL/min, and the injection volume was 1 μL. Detection was carried out on a QTRAP® 5500 mass spectrometer (AB SCIEX, USA) in positive electron spray ionization (ESI) mode. The working parameters of ESI were as follows: ion spray voltage, 5500 V; electricity, 0.3 μA; temperature, 500 °C; gas velocity, 60L/h; and collision energy was 19 eV. The calibration curve was constructed by plotting the peak area against α-TOS concentrations (0.63, 1.25, 2.50, 5.00, and 10.00 μg/mL) including LLOQ. The regression equation was fitted as follows: Y = 5.1993 X + 2.4886 (r = 0.999).

RhoB was quantified with an external standard method on UV-Vis spectrophotometer. RhoB has strong characteristic absorption at 554 nm, while other components in liposomes have no absorption at this wavelength, so the characteristic absorbance can be used for quantitative analysis. The calibration curve was constructed by plotting the absorption at 554 nm against RhoB concentrations (0.5-8 μg/mL). The standard curve was Y = 0.1284X – 0.0014 (R^2 = 0.999, X and Y represent the concentrations of RhoB and absorbance, respectively).

References

[1] J. Tang, Q. Wang, Q. Yu, Y. Qiu, L. Mei, D. Wan, X. Wang, M. Li, Q. He, A stabilized retro-inverso peptide ligand of transferrin receptor for enhanced liposome-based hepatocellular carcinoma-targeted drug delivery, Acta Biomater, 83 (2019) 379-389.

[2] Jennifer, C., Rea, Annelise, E., Barron, Lonnie, D., Shea, Peptide-mediated lipofection is governed by lipoplex physical properties and the density of surface-displayed amines, Journal of Pharmaceutical Sciences, (2008).

[3] S. Udenfriend, S. Stein, P. Bohlen, W. Dairman, W. Leimgruber, M. Weigele, Fluorescamine: A Reagent for Assay of Amino Acids, Peptides, Proteins, and Primary Amines in the Picomole Range, Science, 178 (1972) 871-872.