Stabilized liposomal nanohybrid cerasomes for drug delivery applications
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Experimental Section

Materials: Paclitaxel (PTX, 99.5%) were purchased from Shanghai Jinhe Bio-Technology. N-[N-(3-Triethoxysilyl)propylsuccinamoyl]dihexadecylamine was synthesized according to the reported method[1]. Dipalmitoylphosphatidylcholine (DSPC) were purchased from Avanti Polar Lipids, Inc. Tween 80 was obtained from Tianjin Institute of Fine Chemicals retrocession (Tianjin, China). Triton X-100 was purchased from Sigma-Aldrich. Dialysis membrane was obtained from Spectrum Laboratories Inc. (MWCO: 12-14000, California, US). Methanol was of high-performance liquid chromatography (HPLC) grade from Shandong Yuwang Co. (China). Water used for the cerasome preparation was distilled and deionized using a Milli-Q Gradient System.

Preparation of the paclitaxel-loaded cerasomes (PLCs)

The PLCs were prepared according to the method described by Bangham[2]. The proamphiphile cerasome-forming lipid is insoluble in water and cannot form liposomal bilayer unless its alkoxyisilyl head is partially hydrolyzed. The hydrolysis was performed by addition of HCl as an acid catalyst to an ethanol solution of the lipid and the followed incubation for an appropriate time at 25℃. Then, paclitaxel in chloroform was added into the obtained sol and dried in a rotary evaporator to form a thin film layer. The film was then dried overnight on vacuum drying oven. The lipid film was hydrated with water to produce suspensions of multilamellar vesicles (MLV). To obtain small and homogeneous vesicles, the suspension was ultrasonicated for 5 min with a probe-type sonicator.

The PLCs were subjected to centrifugation at 10000rpm for 10min. At this condition the cerasome remained suspended and the unencapsulated paclitaxel precipitated. The PLCs were stored in tight containers at 4℃ for further experiments. The conventional paclitaxel-loaded liposomes (PLLs) were prepared using similar procedure for
comparative studies.

**Fourier transform infrared (FT-IR) spectra**

FT-IR spectra of cerasomes were acquired using a Varian Resolution Fourier transform infrared spectrometer (Varian FTS 3100, USA). Samples were prepared in the forms of potassium bromide (KBr) disk. Approximately 1 mg sample and 99 mg of KBr powder was blended and triturated with agate mortar and pestle. The mixture was compacted using an IR hydraulic press at a pressure of 8 tons for 1 min. For each spectrum a 512-scan interferogram was collected with a 4 cm$^{-1}$ resolution from the 4000 to 500 cm$^{-1}$ region at room temperature with nitrogen gas.

**Morphology of the PLCs**

The PLCs solutions were deposited onto a carbon-coated copper grid and negatively stained with 2wt% of uranyl acetate. After being air-dried for about 1h at room temperature, the morphology of the PLC was observed by a Hitachi H-7650 transmission electron microscope (TEM) equipped with a CCD camera. The accelerating voltage is 120 KV. Samples for scanning electron microscopy (SEM, Hitachi S-4800, Japan) were prepared by casting an aliquot of the PLCs dispersion (0.5 mM) onto copper foil.

**Particle size distribution and zeta-potential**

The size distribution and zeta potential of the PLCs and PLLs was determined using a 90Plus/BI-MAS dynamic light scattering (DLS) analyzer (Brookhaven Instruments Co., U.S.A) at room temperature after appropriate dilution with distilled deionized water.

**Stability of the PLCs**

The stability of the PLCs was estimated by measuring the average size of the vesicles using DLS with the stepwise addition of Triton X-100 aqueous solution to 1ml vesicle solution at room temperature. To further confirm the stability of both PLCs and PLLs liposome systems, the morphology and size of the particles after addition of a given amount of Triton X-100 to two systems were characterized by TEM.

**In vitro release of paclitaxel**

Release of paclitaxel from and PLLs and PLCs were investigated using the dialysis method at 37°C. An aliquot of the dispersion (2mL) was placed in a dialysis tube and was tightly sealed. Then, the tube was immersed in 50mL of release medium, i.e., PBS (pH 7.4) containing 0.1% (v/v) Tween 80 to maintain sink condition. While stirring the
release medium using the magnetic stirrer at 300rpm, samples (2mL) were taken at predetermined time intervals from the release medium for 120 h, which was refilled with the same volume of fresh medium.

Concentration of paclitaxel was determined by HPLC equipped with a Waters 2487 Dual λ Absorbance Detector, 717 plus autosampler and 515 HPLC dual pumps. A reverse phase hypersil C-18 column (250mm×4.6 mm, 5μm, Merck, Germany) was used at room temperature and the detector wavelength was set at 227 nm. Mixture of methanol: water (70:30, v/v) was used as the mobile phase at a flow rate of 1.0 mL/min. The area under the peak was calculated using numerical integration (Simpson's rule). A standard curve was developed using known amounts of paclitaxel dissolved in methanol.

Cell culture

2774 cells (human ovarian cancer cell line 2774) were incubated in RPMI1640 cell culture medium under CO2 (5%) atmosphere at 37 °C. Cells were subcultured every 2~4 days depending on cell proliferation condition. At confluence, the cells were suspended by rinsing in PBS, followed by brief incubation with trypsin-EDTA solution (0.5%) for 5min. The cells were pelleted by centrifugation for 5 min at 800 rpm and resuspended in RPMI1640 complete medium. This wash procedure was repeated twice and then cells were collected before use.

Determination of toxicity of paclitaxel using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazoliumbromide (MTT) assay

2774 cells were seeded in 96-well culture plates at a density of 1×10⁴ cells per well in 0.2 mL of growth medium and allowed to attach for 24h. The culture medium was replaced with either the suspension of free PTX or PLCs at different concentrations and cells were incubated for 24h or 72h. After incubation, 20 μl of MTT solution (5 mg/ml) was added to each well, followed by 4 h incubation. Wells were then aspirated and 150 μl dimethylsulfoxide (DMSO) was added to each well to dissolve the dark blue crystals thoroughly. The absorbance was measured at 492 nm using a microplate reader (Thermo, Multiskan MK3). The IC₅₀ values (i.e., concentration resulting in 50% growth inhibition) were calculated by the curve fitting of the cell viability data, considering the optical density of the control well as 100%.

Statistics
The Student's t-test (two tailed) was used to determine significant differences between IC\textsubscript{50} or drug concentrations in experiments. P-values less than 0.05 were considered significant.

**Results**

The formation of siloxane bonds on the cerasome surface was proved by Fourier transform infrared spectroscopy (Fig. S1). Stretching bands assigned to the Si-O-Si and Si-OH groups were observed around 1100 and 920 cm\textsuperscript{-1}, respectively. The former peak intensity was much stronger than the latter in cerasomes in the dry state. Thus it is suggested that the cerasomes have a silicalike surface with siloxane frameworks.

![Fig. S1 FT-IR spectroscopy of cerasomes](image)

In Fig. S2, the TEM micrograph showed that the paclitaxel-loaded cerasomes were spherical and the vesicular size was well consistent with the hydrodynamic diameter as evaluated by dynamic light scattering (DLS) measurements.
Fig. S2 TEM image of the paclitaxel-loaded cerasomes

All the PLLs and PLCs were sealed in vials and stored at 4°C for 3 months, respectively. As shown in Fig. S3, it was much different from PLLs that PLCs showed almost no change in particle size after long-term storage, indicating no aggregation or fusion of the PLCs.

Fig. S3. The hydrodynamic diameter ($D_{\text{hy}}$) of the PLCs and PLLs after storage 3 months

Fig. S4 shows the resistance of PLCs and PLLs against Triton X-100 as determined by the DLS of the vesicles. When three equivalents of Triton X-100 were added to the PLLs, the $D_{\text{hy}}$ decreased drastically, indicating a collapse of the vesicles. In contrast to liposomes, the cerasomes exhibited remarkable morphological resistance toward Triton X-100. The $D_{\text{hy}}$ of the PLCs did not change at all even in the presence of 40 equivalents.
of Triton X-100. Such surprising morphological stability of cerasome was also confirmed by TEM image. The cerasome vesicles retained their vesicular structure upon the addition of 40 equiv of Triton X-100.

**Fig. S4** Hydrodynamic diameters ($D_{hv}$) and TEM image of PLCs upon the addition of Triton X-100

The stability of the PLCs was investigated in detail regarding pH variations after the PLCs were prepared for 15 min and 24 h. The PLCs prepared after 15 min were exposed to HCl solution (pH=2) and NaOH solution (pH=9), respectively, allowed for two different incubation times (30 min and 24 h) before the DLS measurements. (Fig. S5)

**Fig. S5** The diameter of the paclitaxel loaded cerasomes under various pH value and incubation time
The freshly prepared cerasomes had an average diameter of 205.2±28.6nm. When they were subjected to acidic conditions (pH=2), the diameter remarkably increase to 1.9±0.23μm for 30 min, and 3.9±0.32μm for 24 h, respectively. In comparison, the light scattering intensity of the PLCs exhibited almost no changes in alkaline condition (pH=9). The particle sizes were evaluated to be 195.3±28nm for 30 min, 198.5nm±29nm for 24h, respectively. The results concluded that cerasomes freshly prepared were stable in alkaline condition, but sensitive to acid because inorganic siloxane networks were not well developed on the surface of the freshly prepared cerasomes.

There were plenty of Si-OH groups on the surface of the freshly prepared cerasomes.

\[
R\text{-Si-OH} \rightleftharpoons R\text{-Si-O}^- + H^+ \quad (1)
\]

When the freshly prepared cerasomes are immersed in the acidic solution (pH=2), the equilibrium (1) shifts to the left so the zeta potential is nearly neutral (+5.3eV). The hydrodynamic diameter increases drastically due to the loss of charges on the cerasome surface and the formation of the vesicular aggregates. When immersed in alkaline solution (pH=9), the equilibrium (1) shifts to the right. The zeta potential is negative charge(-60.5ev), so the particles are stable. At neutral pH, such cerasomes acts as a polyanionic vesicular particle(-30.2ev) which is also stable.

Interestingly, the PLCs prepared after 24 h exhibited remarkable morphological resistance toward both acidic and alkaline conditions. The particle size of the cerasome had almost no change even after 24 h incubation in acidic solution. Nevertheless, the same acidic and alkaline treatment could destroy conventional liposomes.

It is clear that remarkable stability of the cerasome comes from the development of the siloxane network on the vesicular surface. At Kikuchi’s pioneering work cited as Reference 8, it was found that the siloxane network grew with increasing incubation time. While the monomer, dimer and trimer species were detected in the sample prepared after 15 min, oligomers with higher molecular weight such as tetramers and pentamers were additionally detected for the sample during prolonged incubation. The well-developed siloxane network on the cerasome surfaces prepared after 24 h resulted in high stability.

As shown in Fig. S6, PLCs were physically stable for more than 2 months in the hydrated state at 4°C and retained 88% of their initial drug content over that period. On
the contrary, DSPC liposomes retained only 45% of their initial PTX. The results indicate that the storage stability of the PLCs is much higher than that of the conventional phospholipid liposomes.

![Graph showing PTX encapsulation over time](image)

**Fig. S6.** The percentage of PTX encapsulated in the liposomes and cerasomes after long-term storage