Preparation and evaluation of 2-methoxyestradiol-loaded pH-sensitive liposomes

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The development and clinical application of 2-methoxyestradiol (2-ME) as a new type of antitumor drug are limited due to its poor solubility, rapid metabolism *in vivo*, and large oral dosage. 2-ME-loaded pH-sensitive liposomes (2-ME-PSLs) was prepared containing the lipids, Lipoid E-80 (E-80), cholesteryl hemisuccinate (CHEMS), and cholesterol (CHOL) via thin-film ultrasonic dispersion. First, preparation conditions of 2-ME-PSLs were optimized by orthogonal test. Then 2-ME-PSL was characterized, and the release behavior and stability of 2-ME-PSL *in vitro* were evaluated. The optimal preparation conditions for 2-ME-PSLs were as follows: 2-ME : E-80+CHEMS 1:15; CHOL : E-80+CHEMS 1:5; ultrasonication time 20 minutes. The mean particle size, PDI, zeta potential, and entrapment efficiency (EE) of 2-ME-PSLs were 116 ± 9 nm, 0.161 ± 0.025, -22.4 ± 1.7 mV, and $98.6 \pm 0.5\%$, respectively. As viewed under a transmission electron microscope, 2-ME-PSLs were well dispersed and almost spherical. They exhibited significant pH-sensitive properties and were fairly stable when diluted with a physiological solution. In conclusion, 2-ME-PSLs were successfully prepared and possessed a favorable pH sensitivity and good dissolution stability with a normal solution.

Keywords: 2-Methoxyestradiol. pH sensitive liposomes. *In vitro* release. Stability.

INTRODUCTION

2-Methoxyestradiol (2-ME) is the physiological metabolite of 17β-estradiol, catalyzed by catechol-*O*-methyltransferase (COMT) *in vivo* (Dubey, 2017). It exhibits antitumor effects against a broad spectrum of tumors, especially breast cancer (Salama *et al*., 2011; Zhao *et al*., 2017), by anti-angiogenesis, inhibiting proliferation, and/or inducing apoptosis (Massaro *et al*., 2017; Zou *et al*., 2018). As a new type of antitumor drug, 2-ME is effective by oral administration. The use of 2-ME to treat breast cancer is currently in phase II clinical research. However, due to its poor water solubility, rapid metabolism *in vivo*, and a large oral dosage (Guo *et al*., 2017; Said *et al*., 2017), the development and clinical application of its products are limited. Therefore, it is important to focus on the development of new delivery systems for 2-ME.

A liposome, as an artificially prepared spherical vesicle composed of a lamellar phase lipid bilayer, has great potential application in drug delivery, especially for entrapping hydrophobic drugs to increase drug solubility and targeting (Benson, 2017; Giacometti *et al*., 2017; Zabielska-Koczywas, Lechowski, 2017). However, liposomes easily leak drugs in physiological environments, which is not a conducive phenomenon because it could not leads to drug aggregation in tumor sites (Wang *et al*., 2017). In order to improve the liposome therapeutic efficacy, multifunction liposomes have been designed (Dai *et al*., 2014; Chen *et al*., 2017; Chi *et al*., 2017; Dai *et al*., 2017). Considering that the pH value of the tumor tissue (about 5.0) is lower than that of the normal tissue, pH-sensitive liposomes have been developed as an efficient system for controlled drug release to improve drug efficacy after intravenous administration (Liang *et al*., 2017; Lee, Thompson, 2017).

In this study, we prepared 2-ME-loaded pH-sensitive liposomes (2-ME-PSLs), which were stable in the physiological environment, and fused intelligently into the acidic environment of tumor tissues. After optimizing preparation conditions and characterizing the 2-ME-PSLs, we investigated their stability and *in vitro* release behavior for pH sensitivity. The study lays down a foundation for

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the future development and application of 2-ME in the treatment of various cancers..

MATERIAL AND METHODS

Material

2-methoxyestradiol was purchased from Aikeda Chemical Reagent Co., Ltd. (Sichuan, China), Lipoid E-80 from Lipoid GmbH (Ludwigshafen, Germany), cholesteryl hemisuccinate (CHEMS) and cholesterol (CHOL) from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and high-performance liquid-chromatography (HPLC)-grade methanol from Tianjin Kermel Chemical Reagent Co., Ltd. (Tianjin, China). All other reagents were of analytical grade.

Preparation of 2-ME-PSLs

The 2-ME-PSLs wasWe prepared via thin-film ultrasonic dispersion method, using the lipids E-80, CHOL and CHEMS in the formulation. Briefly, 2-ME (10 mg) and lipids were dissolved in chloroform and then evaporated at 40° C under reduced pressure until the organic solvent was removed to form a thin lipid film. The dried lipid film was hydrated with phosphate-buffered saline (PBS; 10 mL) and then sonicated it in a bath-type sonicator at 100 W.

Orthogonal experiment

In the study, the preparation conditions for 2-ME-PSL was optimized PSLs by orthogonal test on the basis of the pre-experiment; the ratios of 2-ME: lipid (A), lipid: cholesterol (B), and ultrasonic time (C) as factors; and particle size as the evaluation index. The lipid was a mixture of E-80 and CHEMS (1:1). The independent factors and the levels were shown in Table I. In order to reduce systematic errors, the experiments were replicated for 3 times and completely randomized. The particle size was expressed as a mean intensity diameter.

Characterization of 2-ME-PSLs

Particle size and zeta potential

The particle size, size distribution and zeta potential of 2-ME-PSLs were measured by dynamic light scattering (DLS) analysis using a Malvern Zetasizer Nano ZS90 (Malvern Instruments, Malvern, UK). Each test was performed in triplicate. Results were expressed as mean ± standard deviation (SD).

TABLE I - Factor and level

Level	Factor		
	2-ME: E80+CHEMS	CHOL: E80+CHEMS	Sonication time (min)
1	1:10	1:4	20
2	1:12	1:5	30
3	1:15	1:6	40

Morphology investigation

The morphology of 2-ME-PSLs was observed using a JEM-2010 transmission electron microscope (TEM; JEOL Co., Ltd., Japan) with the negative-staining method. After diluted with an appropriate amount of distilled water, a drop of the sample was deposited on a dedicated copper grid and then stained with 1% (w/v) uranyl acetate. The grid was positioned at room temperature and allowed to dry before observation.

Entrapment efficiency

The encapsulation efficiency (EE) of 2-ME in PSLs was determined according to the literature (Xu *et al.*, 2017) with a little modification. Briefly, 2-ME-PSLs (400 μL) was centrifuged in an ultrafiltration tube (50 kDa; Millipore, Massachusetts, US) at 3000 rpm for 30 minutes to separate the water phase. Non-entrapped 2-ME in the water phase was determined directly and total 2-ME in the PSLs was determined after lysing the liposomes with methanol (1:5; v/v). 2-ME in the various samples were detected by HPLC using an LC-20AT pump and SPD-20A UV/VIS detector (Shimadzu Corp., Kyoto, Japan) and a Diamonsil C18 analytical column (200 × 4.6 mm, 5 μm; Dikma Technologies, Inc., Foothill Ranch, California, US). The mobile phase was a mixture of acetonitrile and double-distilled water (50/50, v/v) at a flow rate of 1.0 mL/min and the detection wavelength was 205 nm. The HPLC method was validated by the studies on precision, accuracy and standard curve (data not shown). The entrapment efficiency (EE) was calculated according to the equation (1):

$$EE(\%) = \frac{C_{total}V_{total} - C_{water}V_{water}}{C_{total}V_{total}} \times 100\% \qquad (1)$$

In vitro release study

The *in vitro* release of 2-ME in PSLs was investigated using dialysis in 2 different PBS media (0.3% Tween-80, pH 7.4 ± 0.1 and 0.3% Tween-80, pH 5.5 ± 0.1) at 37 °C (Seong, Yun, Park, 2017). Briefly, 2.0 mL of 2-ME-PSLs

was put into a dialysis bag (molecular-weight cutoff [MWCO] = 8,000-14,000 Da), which was soaked in double-distilled water for 24 hours before use. The end-sealed dialysis bags were then immersed in 100 mL release media stirred at 100 rpm at 37 °C. At pre-determined time intervals (1, 2, 4, 6, 8, 12, 24 h), an aliquot of 0.5 mL release media was withdrawn and replaced by an equal volume of pre-warmed fresh media. Concentrations of drug in the samples were determined by the HPLC method described above.

Stability study

Generally, 2-ME-PSLs was given by intravenous injection after dilution with 5% glucose or 0.9% sodium chloride injection. Thereforefor, the stability of 2-ME-PSLs after diluted with 5% glucose or 0.9% sodium chloride injection was evaluated. The physicochemical properties, including appearance, droplet size and drug content, were then investigated.

RESULTS AND DISCUSSION

Preparation and optimization of 2-ME-PSLs

The main results of the orthogonal test and range analysis are shown in Table II. The major–minor order that affected particle size was A > C > B, that is, the ratio of 2-ME : E-80+CHEMS > sonication time > CHOL: E-80+CHEMS. The optimal levels of 2-ME : E-80+CHEMS and sonication time were 1:15 and 40 minutes, respectively. Particle size decreased as the ratio decreased and as the sonication time increased. The optimal level of CHOL : E-80+CHEMS was 1:5. E-80, CHEMS, and CHOL as the part of the double molecular film affected the formation of liposomes. The optimal conditions for preparing 2-ME-PSLs were as follows: 2-ME, 10 mg; E-80+CHEMS (1:1), 150 mg; CHOL, 30 mg; distilled water, 10 mL; sonication, 40 minutes.

In this experiment, the liposomes exhibited pH sensitivity when the acid-responsive CHEMS were anchored to the lipid bilayer. The amount of CHEMS (E-80 : CHEMS = 1:1) without optimization was according to the literature (Lee, Thompson, 2017). In the orthogonal experimental design, we selected particle size rather than EE as the evaluation index, mainly because 2-ME is a hydrophobic drug: if the EE was too low, it resulted in drug precipitation, which was visible to the naked eye. Therefore, the particle size was suitable to be as the evaluation index.

TABLE II - The results of orthogonal test

No.	A	B	C	d/nm
1	1	1	1	218
2	1	2	2	175
3	1	3	3	165
4	2	1	2	148
5	2	2	3	135
6	2	3	1	174
7	3	1	3	117
8	3	2	1	128
9	3	3	2	139
K_1	186	161	173	
K_2	152	146	154	
K_3	128	159	139	
R	58	15	34	

Characterization of 2-ME-PSLs

Droplet size distribution and zeta potential are shown in Figure 1. DLS analysis showed the mean particle size, polydispersity index (PDI), and zeta potential of 2-ME-PSLs to be 116 ± 9 nm, 0.161 ± 0.025, and -22.4 ± 1.7 mV, respectively. Figure 2 shows that 2-ME-PSLs were almost spherical in shape and well dispersed without any clustering or aggregation. The particle size was consistent with that determined by DLS analysis, and EE was as high as 98%.

In vitro release study

In the *in vitro* release study, 0.3% Tween-80 contained in PBS was used to meet the sink condition because of 2-ME's poor solubility. Results are shown in Figure 3. A low level of 2-ME was released from the powder in PBS without Tween-80 at both pH 7.4 and 5.5 (<20% at 24 hours), further demonstrating the poor solubility of 2-ME. Under the sink condition, the level of 2-ME released from the crude powder increased significantly, but different pH conditions had no effect on this level. Less than 10% of 2-ME was released from PSLs in the pH 7.4 PBS medium within the initial 12 hours, but the level increased markedly after 12 until 24 hours. However, in the pH 5.5 PBS medium, more than 90% of 2-ME was released from the liposomes during the initial 6 hours, and 2-ME was almost completely released within 8 hours. These release profiles suggested that 2-ME-PSLs had good stability
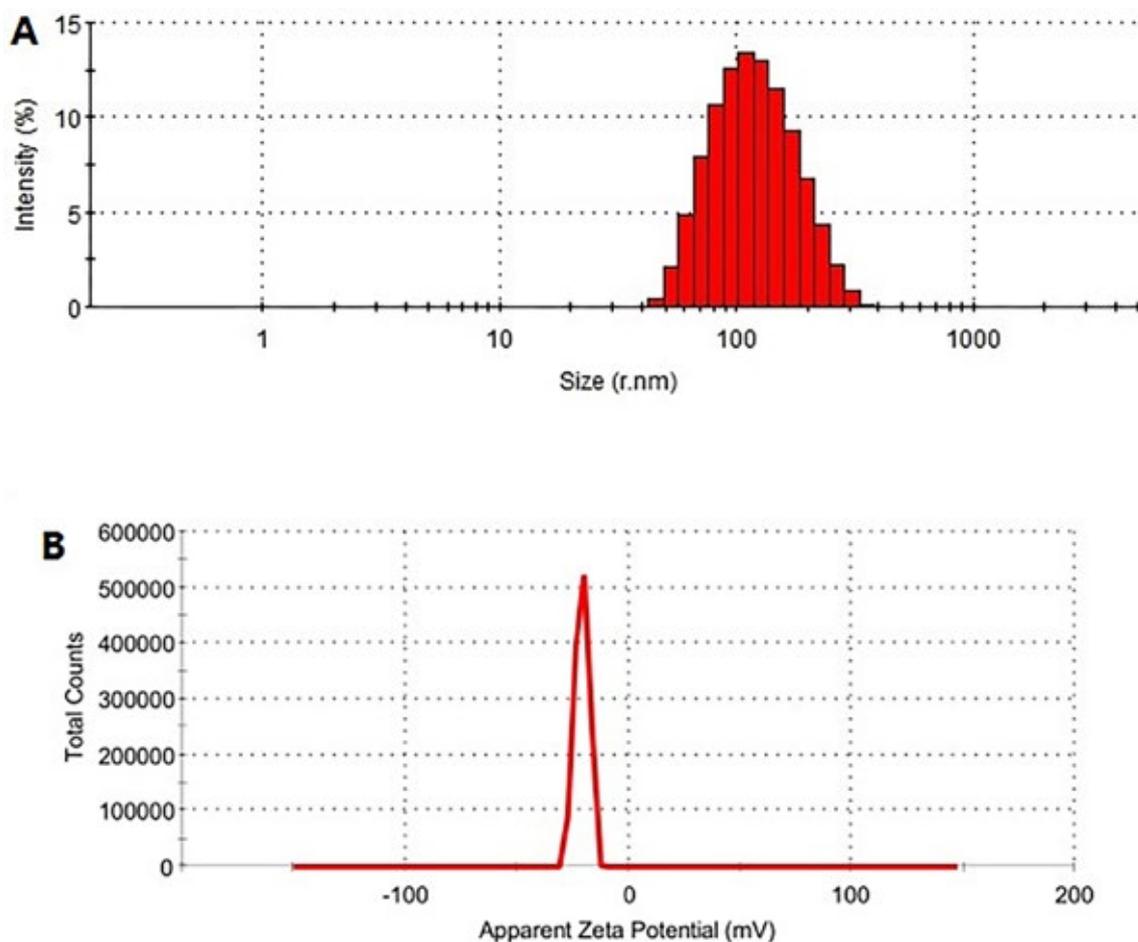
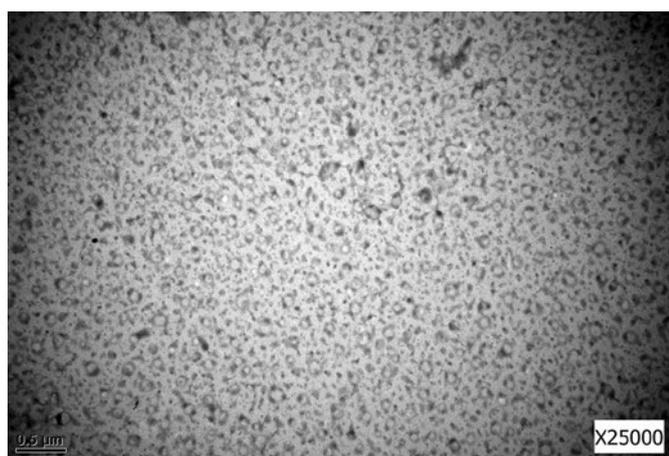**FIGURE 1** - Particle size and zeta potential of 2-ME-PSL.

FIGURE 2 - TEM image of 2-ME-PSL.

in a neutral environment (pH 7.4) and were released quickly in acidic environments (pH 5.5), demonstrating an obvious pH sensitivity. That is to say, rapid release during the delivery process could be avoided, and the effect of plasma on drug leakage was negligible. The release behavior of 2-ME-PSLs in different media was mainly caused by anchoring acid-responsive CHEMS

to the lipid bilayer, which was conducive to 2-ME-PSLs accumulating and exerting their effectiveness in tumor tissues (Monteiro *et al.*, 2017).

Stability study

As shown in Table III, when the 2-ME-PSLs were diluted with 1-10 times volume of normal saline soluiton, the appearance, particle size, and drug content showed no obvious changes. However, when 2-ME-PSLs were diluted with 5%, the samples were turbid, with subsequent occurrence of precipitation. The results suggested that 2-ME-PSLs were stable when diluted with normal saline solution rather than 5% glucose. This could be due to the different pH values of normal saline solution (6.5-7.5) and 5% glucose (3.5-6.5).

To evaluate the effect of pH on the stability of 2-ME-PSLs, we confirmed the change in liposome size after incubation with pH 7.4 PBS and pH 5.5 PBS. As shown in Figure 4, the size of liposomes gradually increased when incubated with pH 5.5 PBS, reaching about 1500 nm at 45 minutes. On the other hand, the
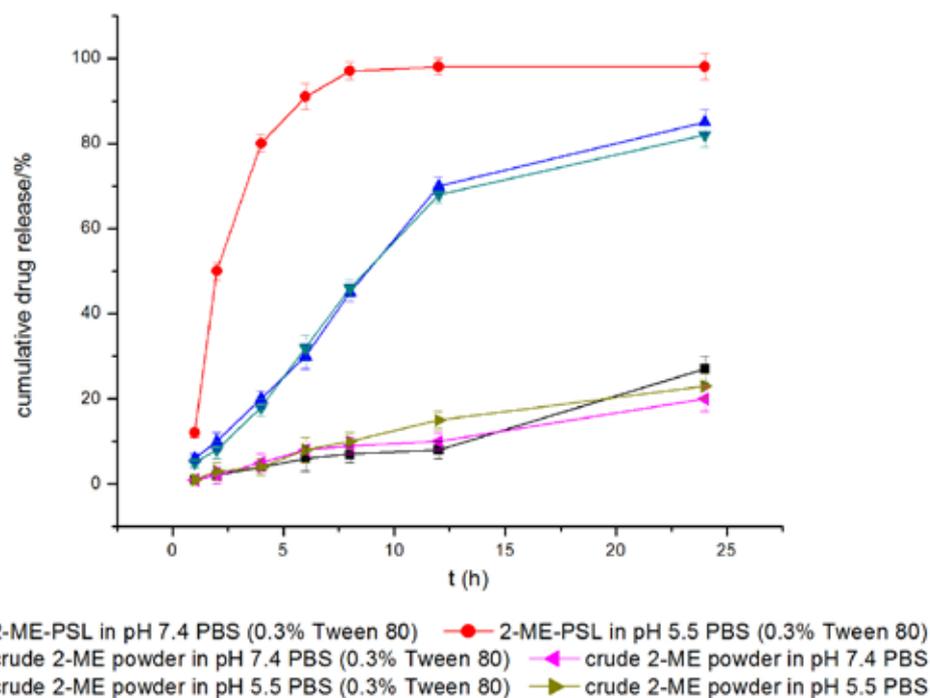
FIGURE 3 - *In vitro* release curves of 2-ME-PSL under different conditions (n=3).

size of liposomes incubated with pH 7.4 PBS showed no obvious change. Furthermore, we could not observe a normal spherical morphology under TEM for the 2-ME-PSLs incubated with pH 5.5 PBS for 45 minutes. These results suggested that 2-ME-PSLs could lyse at pH 5.5 and then release 2-ME, thus meeting our primary goal that 2-ME-PSLs are stable in blood circulation and quickly release the drug after accumulating in tumor tissues. The structure of CHEMS, which possesses pH-sensitive properties, was the main reason for the pH sensitivity in 2-ME-PSLs. Because the pK_a of CHEMS was 5.8, when CHEMS participated in the formation of a liposome membrane, the zeta potential should have been negative in a neutral and/or alkaline environment, consistent with the zeta potential measured above. When CHEMS entered an acidic environment, it was protonated and the carboxyl group was oriented on the surface of the liposomes, resulting in liposome membrane instability and the decomposition of the lipid bilayer (Xiong *et al.*, 2011; Chen *et al.*, 2012). Therefore, 2-ME-PSLs could
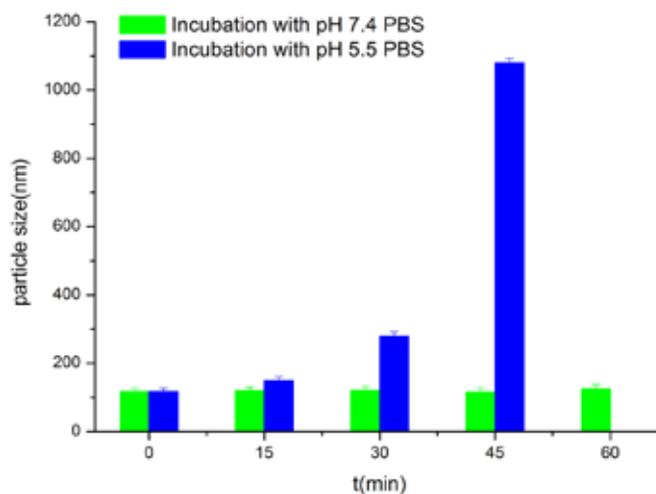

FIGURE 4 - The particle size of 2-ME-PSL incubation with different PBS (n=3).

exist stably in a neutral blood circulation environment. After the liposomes had entered the tumor tissues, their bilayer membrane became unstable and fusogenesis

TABLE III - The results of stability experiments (n=3)

Dilution times	Appearance	Particle size/nm	PDI	Drug content (%)
1	ivory, opalescence	129	0.21	99.1
2	ivory, opalescence	119	0.17	98.2
5	ivory, opalescence	117	0.19	97.1
10	ivory, opalescence	122	0.19	98.4

occurred, thereby promoting drug release and exerting a therapeutic effect. In conclusion, the stability study proved that pH-sensitive 2-ME-PSLs were stable when diluted with normal saline and were therefore suitable for clinical use.

CONCLUSION

In this study, 2-ME-PSL was successfully prepared with good stability diluted with normal saline and significant pH sensitivity. From preliminary study, we predicted that 2-ME-PSLs would be stable in blood circulation and would release the drug rapidly after reaching tumor sites, thus achieving the preferable antitumor effect. We intend to further study the pharmacokinetics and pharmacodynamics of 2-ME-PSLs *in vivo*.

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