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LHRHa aided liposomes targeting to human ovarian tumor cells: preparation and cellular uptake

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In this study, a synthetic nonapeptide similar to luteinizing hormone-releasing hormone (LHRHa), the ligand of an extracellular membrane receptor specific to ovarian tumor cells, was selected as targeting moiety and electrically adsorbed to the negatively charged liposomes composed of phospholipid and monocholesterolsuccinate. Docetaxel, as the first line chemotherapy for ovarian tumor, was chosen to be encapsulated into the liposomes. And a high encapsulate efficiency (93%) and drug loading efficiency (20%) of liposomes were achieved via central composite design. In order to investigate the targeting efficiency of the drug delivery system, *in vitro* cell uptake was determined and the results showed an increasing uptake of LHRHa aided liposomes compared to normal ones.

1. Introduction

In order to avoid side effects and increase the efficacy of chemotherapy, a series of tumor targeted drug delivery systems were established, such as liposomes, nanoparticles and microemulsions. Among them, liposomes are an optimal choice due to their bioaffinity and the ease of preparation. However, conventional liposomes composed of phospholipids and cholesterol were more prone to be swallowed by macrophages and result in the aggregation of drugs loaded in liver and spleen, which may also lead to lower blood drug concentration. To avoid the uptake of liposomes by liver and spleen and to maintain a lasting high blood concentration, a kind of PEGylated liposomes were prepared by Immordino et al. (2003). Nevertheless, when other organs and spleen except the liver were targeted, site-specific liposomes will be more appreciated.

Luteinizing hormone-releasing hormone (LHRH) is a linear decapeptide, acting on the pituitary gland to stimulate the release of luteinizing hormone and follicle-stimulating hormone, which in turn regulates ovulation and spermatogenesis in the gonads (Benatzi et al. 2001). The natural LHRH is unstable *in vivo*, and therefore, a number of synthetic LHRH analogues (LHRHa) with better bioactivity were synthesized and are currently in therapeutic use. According to Peter Völker's study, 70% ovarian cancer cell lines expressed luteinizing hormone-releasing hormone receptors (Völker et al. 2002). In short, LHRHa will be a fine targeting moiety for delivering drugs to ovarian tumors. Miyazaki's study revealed that when adriamycin was linked with LHRH covalently to form an AN-207 a high targeting efficiency was achieved (Miyazaki et al. 1999). Dharap synthesized a paclitaxel-PEG-LHRH which also showed a satisfactory result (Dharap et al. 2003).

Docetaxel, a semi-synthetic taxoid antineoplastic agent, which is now selected as the first line chemotherapy for ovarian cancer (Markman et al. 2001), is more soluble in water than paclitaxel. However, the clinically used docetaxel preparation still needs ingredients like Tween 80 and ethanol, which results in a series of adverse side effects as fluid retention. Thereby, a Tween 80-free delivery system with increased safety has become a research focus. Among the various drug delivery systems, the preparation of liposomes is a relatively mature technology with high encapsulation efficiency of both lipophilic and hydrophilic drugs.

Strategies for drug targeting by targeting moieties include: 1) combine targeting moiety covalently with drugs, the so called targeting prodrugs, and; 2) combine targeting moiety covalently with drug delivery systems, which would carry drugs loaded specifically to targeting organs, and; 3) targeting moiety being electrically adsorbed on the surface of the drug delivery system, which could also deliver drugs or therapeutic genes to targeting organs (Tros de Ilarduya and Düsgünez 2000, Tros de Ilarduya et al. 2002).

In the present study, negatively charged lipid materials – monocholesterolsuccinate – were synthesized in our laboratory, and used to form anionic liposomes which can adsorb positively charged LHRHa by electrostatic interaction. To achieve high entrapment efficiency (EE%) and drug loading efficiency (DL%), central composite design (CCD) was carried out according to the result of single-factor experiment, and an *in-vitro* cellular uptake study was carried out using fluorescence liposomes, to investigate the targeting efficiency of this newly developed delivery system.

2. Investigations, results and discussion

The synthesis of monocholesterolsuccinate (Scheme) is described in 3.2. The resultant monocholesterolsuccinate were white needle crystals with a recovery rate of 85%. The melting point of the product (180 °C) and the character of ^{1}H NMR conformed to the reference (Wu et al. 2005) exactly.

The liposomes were prepared via a film-ultrasonic method as described in 3.3. To obtain liposomes with satisfying characteristics, several preparation associated parameters were determined, among which encapsulation efficiency (EE%) and drug loading efficiency (DL%) are of great significance for the screening of preparation methods and for the quality control of the drug delivery system. The encapsulation efficiency was calculated as follows: encapsulation efficiency (EE%) = [drug encapsulated/(drug encapsulated + drug unencapsulated)] × 100%, while, the drug loading efficiency (DL%) = [weight of encapsulated drug/(the weight of encapsulated drug + weight of lipid material)] × 100%.

As an efficient quantitative measurement, HPLC was involved for the determination of EE% and DL%. Since the particle size and PDI also influence the quality and stability of the product, they were also taken into account (Xu et al. 2006).

According to the former optimization standard, a series of single-factor experiment were carried out to investigate the influence factors, including: the ultrasonic conditions, the ratio of phospholipid to monocholesterolsuccinate (molar to molar), the amount of drug (docetaxel) addition, the amount of lipid materials and the amount of TES buffer addition. Since EE%, size (nm) and PDI were the most critical parameters, they were defined as the response variables to screen those factors. According to the single-factor experiment, the most influencing factors were chosen for the further optimization studies, known as the central composition design (CCD). In terms of central composite design (CCD), a factor is defined as an input variable whose value can be set during the experiment, while the response variable is the measured quantity whose value is affected by levels chosen for the factors. CCD reduces the experimental runs that are necessary to establish a mathematical trend in the experimental design region. In this study, according to the single-factor experiment result, EE%, size and PDI stayed constant while ultrasonication treatment (200W × 10 s) were more than 10 times and the amount of the TES buffer addition seemed hardly diversify those response variant. Conversely, other three factors revealed having close relationship with the response variant. Accordingly, the amount of drug (Doc) addition, the amount of lipid material (phospholipids and monocholesterolsuccinate) addition and the ratio of phospholipids to monocholesterolsuccinate were chosen as process variables for the CCD study.

Table 1: Independent variables and their levels investigated in the central composite design

Factors	Factor level in code form				
	$-\sqrt{3}$	−1	0	1	$\sqrt{3}$
X1	0.66	1.64	2.97	4.31	5.28
X2	8.26	20.18	37.16	54.14	66.06
X3	1	2.9	5.5	8.1	10

X1: Docetaxel addition (μmol) for 5 ml liposomes suspension
X2: Total lipid material addition (μmol) for 5 ml liposomes suspension
X3: Ratio of phospholipids: monocholesterolsuccinate (molar to molar)

In general, CCD were constructed in such a way that $2^n + 2n + 1$ experiments were required where n represents the number of factors to be studied, and it affects experimental results. Accordingly, the three-factor CCD requires 15 experimental points, each of which being a result of different formulations of 5 ml liposomes suspensions. The experimental run was shown in Table 1. In order to estimate the pure experimental uncertainty of CCD, it is important to measure repeatedly the response function to the conditions determined by the central points. In this study, three repeated experiments were performed. The determined results are presented in Table 2.

STATISTICA 6.0 was used to obtain the regression equations (listed in Table 3), fit the response model and predicate result. The response model map was mapped against two process variables while the third was held constant at its middle level. According to the response model maps, the final optimized formulation of 5 ml liposomes suspension could be predicated as: $X_1 = 4.5$ μmol, $X_2 = 18$ μmol and $X_3 = 9$. That's to say, preparing 5 ml liposome suspension needs 3.6 mg of docetaxel, 12.1 mg of phospholipids and 0.69 mg of monocholesterolsuccinate. The predicted and observed values of the optimized liposomes are illustrated in Table 4, which showed an excellent optimization.

According to Table 4, the values of those response variants are ideal for the preparation of liposomes containing docetaxel, and the values of EE% and DL% were higher than reported (Immordino et al. 2003). Moreover, the predicated values were close to the observed values, which indicated that the optimization for the liposomes loading docetaxel by CCD was successful.

In addition, the synthesized monocholesterolsuccinate was negatively charged, the use of such anionic lipid material could increase the zeta potential of the liposomes and enlarge the interval of the lipid double-layer. The former can make the liposomes more stable with a relatively small

Scheme Synthesis of monocholesterolsuccinate

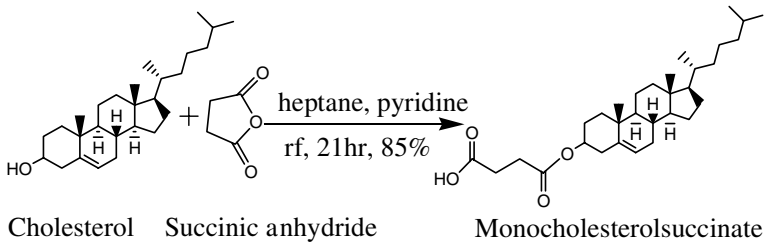

Table 2: Observed response for the CCD design

No.	Doc (mg)	PC (mg)	CHS (mg)	EE	DL	Size (nm)	PDI
1	1.325	11.284	2.002	0.8421	0.0774	273	0.425
2	1.325	13.507	0.858	0.7057	0.0611	238	0.479
3	1.325	40.258	5.372	0.7190	0.0204	210	0.292
4	1.325	36.239	2.302	0.7789	0.0260	211	0.256
5	3.482	11.284	2.002	0.9298	0.1959	328	0.312
6	3.482	13.507	0.858	0.8667	0.1736	328	0.278
7	3.482	40.258	5.372	0.8379	0.0601	256	0.287
8	3.482	36.239	2.302	0.9750	0.0809	222	0.312
9	0.533	23.645	2.212	0.6193	0.0126	209	0.338
10	4.266	23.645	2.212	0.9085	0.1303	275	0.296
11	2.4	5.256	0.492	1.0411	0.3030	470	0.321
12	2.4	42.034	3.933	0.9264	0.0461	206	0.276
13	2.4	13.972	7.19	0.9657	0.0987	306	0.412
14	2.4	25.403	1.307	1.0308	0.0847	208	0.320
15	2.4	23.645	2.212	0.8935	0.0765	244	0.382
16	2.4	23.645	2.212	0.8553	0.0735	234	0.391
17	2.4	23.645	2.212	0.8480	0.0729	256	0.286
18	2.4	23.645	2.212	0.9416	0.0803	271	0.398

No. 1–15 were 15 experimental points for different formulations of 5 ml liposome suspensions, No. 16–18 were the repeated measurements for the central point to estimate the pure experimental uncertainty of CCD.

Table 3: Regression equations for the response

$$EE\% = 99.14779 + 20.24153\ X1 - 1.2581\ X2 - 9.00455\ X3 + 0.03682\ X1\ X2 + 0.54146\ X1\ X3 + 0.11229\ X2\ X3 - 3.17802\ X12 + 0.00583\ X22 + 0.32047\ X32$$
$$R = 0.9151$$

$$Size = 374.7279 + 59.0603\ X1 - 8.2251\ X2 - 3.0725\ X3 - 0.4822\ X1\ X2 + 0.0089\ X1\ X3 + 0.0057\ X2\ X3 - 4.0824\ X12 + 0.0883\ X22 - 0.3360\ X32$$
$$R = 0.9202$$

$$PDI = 0.522186 - 0.037104\ X1 - 0.001654\ X2 - 0.000154\ X3 + 0.002011\ X1\ X2 - 0.00096\ X1\ X3 - 0.000088\ X2\ X3 - 0.008461\ X12 - 0.000076\ X22 + 0.000195\ X32$$
$$R = 0.8515$$

$$DL\% = 18.25579 + 7.64604\ X1 - 0.938\ X2 - 0.90684\ X3 - 0.07499\ X1\ X2 + 0.03353\ X1\ X3 + 0.01845\ X2\ X3 - 0.37196\ X12 + 0.0099\ X22 + 0.00196\ X32$$
$$R = 0.9582$$

X1: docetaxel addition (μmol) for 5 ml liposomes suspension
X2: total lipid material addition (μmol) for 5 ml liposomes suspension
X3: ratio of phospholipids: monocholesterolsuccinate (molar to molar)
The three equations represented the quantitative effect of process variables (X1 and X2 and X3) and their interactions on their response variant EE, size, PDI and DL. According to those equations, the response variants achieved good regression coefficients

PDI, and the later directly improve the EE% and DL% for the liposoluble drugs such as docetaxel.

For the formulation of the LHRHa-liposomes complex, the amount of LHRHa added was a critical parameter. If there is not enough LHRHa addition, the targeting efficiency would be directly decreased. In contrast, when in excess, the remnant LHRHa with less space steric hindrance, rather than LHRHa-liposomes, can be more easily combined with the receptor site on the targeting cell, and it may further decrease the targeting efficiency. Furthermore, two parameters – zeta potential and size – were investigated to determine the ideal addition quantity of ligand. In fact, size was not a critical parameter, because the tiny nonapeptide (LHRHa) had little influence on the size of the complex.

In this study, a series of LHRHa: monocholesterolsuccinate ratios were studied for the optimization of LHRHa addition (see 3.3) and the changes of zeta potential are shown in Fig. 1, which revealed that the addition of LHRHa should be of 1/2 molar ratio of monocholesterolsuccinate and this needs to be further investigated. The Transmission electron microscopy of the final LHRHa-Liposomes complex is shown in Fig. 2.

LHRHa-fluorescein-liposomes with different molar ratios of LHRHa/monocholesterolsuccinate were prepared and their targeting efficiency to SKOV3 cells, which had the LHRHa receptor on the cell surface, was determined following the method mentioned in 3.5. Figure 3 directly shows the relationship between the molar ratios of LHRHa/monocholesterolsuccinate and the targeting efficiency. The cell uptake experiment revealed the same result as the zeta potential detection mentioned above.

Table 4: Comparison of the observed and predicted values of the response variants of the liposomes prepared under optimum conditions

Response variables	Predicted value	observed value	Bias%
EE%	93.14	91.34 ± 2.25	−1.93
DL%	21.72	20.49 ± 0.51	−5.66
Size (nm)	345.74	342 ± 21	−1.08
PDI	0.2537	0.255 ± 0.03	0.51

Bias% = (observed value – predicated value)/predicated value × 100%

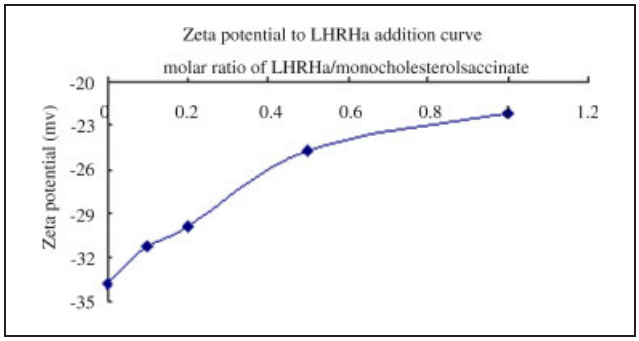

Fig. 1: Zeta potential to LHRHa addition curve. According to Fig. 1, after the value of LHRHa/monocholesterolsuccinate (molar ratio) exceeded 1/2, the zeta potential remained almost unchanged, which indicated the ideal molar ratio of LHRHa to monocholesterolsuccinate was 1/2.

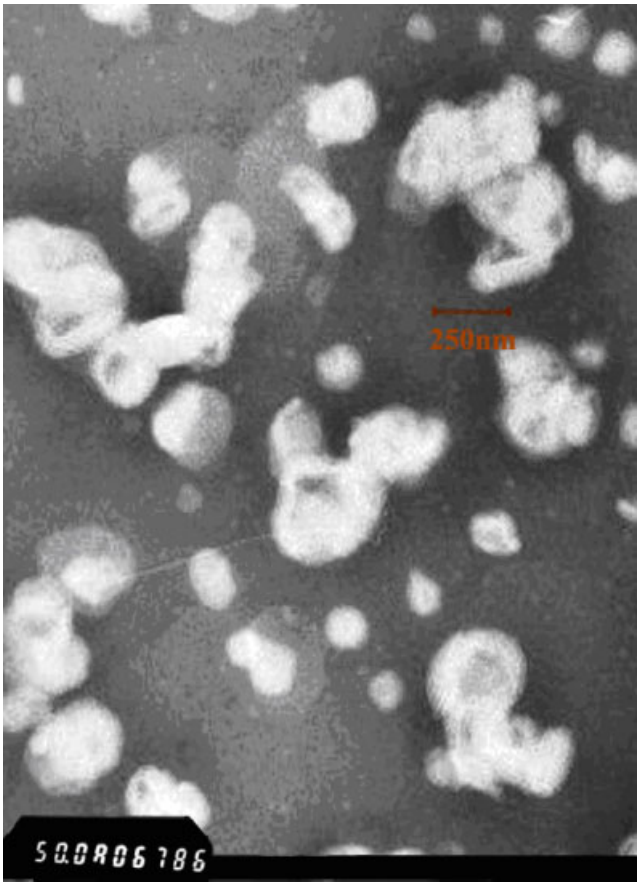

Fig. 2: Transmission electron microscopy of LHRHa-Liposomes complex (× 50,000)

The preparation of LHRHa-fluorescein-liposomes was described in 3.5. In order to verify the specific targeting efficiency, human ovarian tumor cells SKOV3 (human ovarian tumor cell line), C26 (mouse colon tumor cell line), Hela (human cervix tumor cell line) and A549 (human lung tumor cell line) were chosen for cell uptake study and the results were compared. Different concentrations of liposomes or LHRHa-Liposomes complex were added into the cell plates for cell uptake determination (see 3.5). For the controlled comparison, an excess amount of free LHRHa was added in order to pre-saturate the LHRHa receptors to see if this will prevent the uptake priority of LHRHa-Liposomes.

Results shown in Figs. 4–7 revealed that SKOV3 cell uptake of LHRHa-fluorescein-liposomes were $1.5 \sim 2$ fold to normal liposomes while other tumor cells showed no significant difference. As for SKOV3 cells, the cells which were pre-saturated, less LHRHa-liposomes were phagocytized than those not being pre-treated, for the LHRHa receptors on the SKOV3 surface were barricaded with the dissociative LHRHa.

With respect to target-oriented drug delivery systems, the receptors on the cell surface are an ideal targeting site of the active targeting preparations, because of the specific

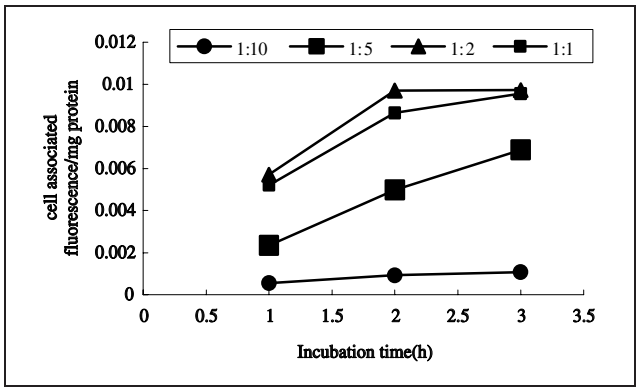

Fig. 3: Effect of different ratio of LHRHa: monocholesterolsuccinate on cell uptake of liposomes in different time course (SKOV3 cell line). According to Fig. 3, the uptake efficiency (cell associated fluorescence/mg protein) got the highest value when the mole ratio was 1/2, and under such ratio, the SKOV3 cell phagocytosis for the LHRHa-fluorescein-liposomes came to the saturation point when the cell incubated with liposomes for 2 h.

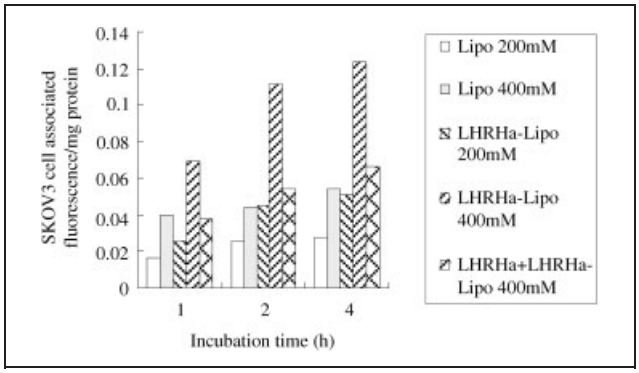

Fig. 4: SKOV3 cell line uptake of different fluorescein-liposomes in different time course. Figure 4 showed for SKOV3 cell line containing the LHRHa receptor, the uptake of LHRHa-liposomes was better than normal liposomes on different concentration levels, and the cells pre-saturated with LHRHa were not sensitive to the LHRHa-liposomes, and its uptake of LHRHa-liposomes were close to the normal liposomes. All this indicated the specific targeting efficiency.

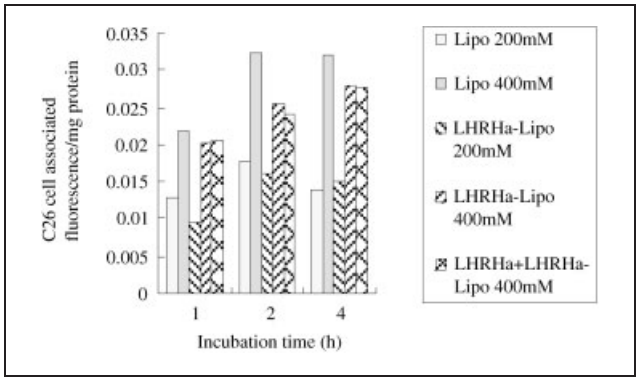

Fig. 5: C26 cell line uptake of different fluorescein-liposomes in different time course

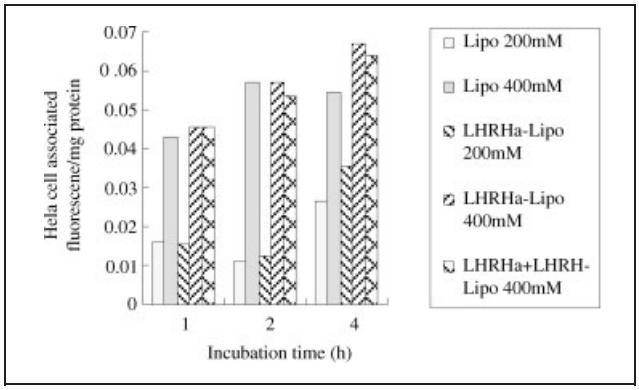

Fig. 6: Hela cell line uptake of different fluorescein-liposomes in different time course

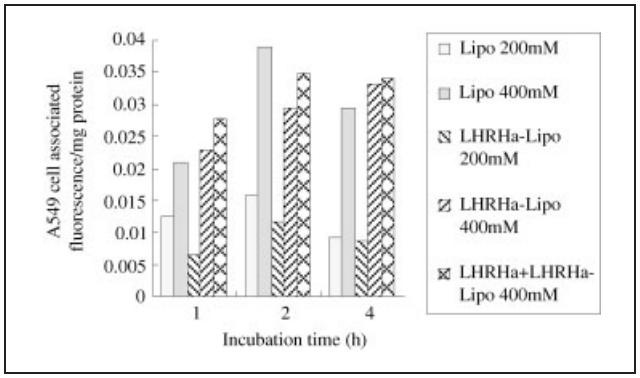

Fig. 7: A549 cell line uptake of different fluorescein-liposomes in different time course

interaction between the receptor and the ligand. The receptor-identifying drug delivery system can let the therapeutic drugs concentrate in the target organ, tissue or cells, and efficiently decrease side effects. Both chemical coupling and electrostatic adsorption were utilized for coupling the ligand with drug delivery systems (Dharap et al. 2003). Although the chemical coupling proved more stable, the process for preparation was either complicated or the steric structure of the complex might be changed. By direct chemical coupling, the attachment might be adversely affected the recognition properties of ligand for the target antigen (Kocbek et al. 2007). Static adsorption was commonly used in gene delivery systems. A successful example was implemented by linking the transferrin (TF) with liposomes. The absolute value of negative zeta potential of the liposomes was decreased after adding LHRHa, which showed that the LHRHa had been linked with the liposomes successfully. Still, further studies need to be carried out on the stability of the complex as well as its targetability *in vivo*.

3. Experimental

3.1. Materials

Docetaxel (Doc) was a gift from Mei Lian Co., Ltd (Chongqing P.R.C.). LHRHa was purchased from Chinese Peptide Company (Shanghai, P.R.C.). Authentic standards of docetaxel and paclitaxel (purity > 98%) were obtained from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, P.R.C.). Egg yolk phospholipid (Lecithin) was purchased from Shanghai Bio Life Science & Technology Co., Ltd (Shanghai, PRC). The Doc injection was prepared in injection workshop in Shenghe drug plant (Chengdu, P.R.C.). SKOV3, Hela, C26 and A549 cell lines were provided by the Institute of Biochemistry and Cell Biology, Chinese academy of sciences (Shanghai, P.R.C.). RPMI1640 Medium was purchased from Gibco (USA). BCA assay was purchased from Pierce Chemical. All other chemicals were of analytical reagent grade and purchased commercially.

In this study an Alltech HPLC (US) was used for the determination of docetaxel in liposomes. A Büchi R-114 rotary evaporator and a JY92-II probe ultrasonic producer (Ningbo Scientz Bio-tech Co. LTD) were involved in the preparation of liposomes. The fluorescence intensity was determined according to a Shimadzu RF-5301 fluorescence spectrophotometer and the phagocytosis pictures were taken with Carl Zeiss Axiovert 40 CFL phase difference/fluorescence/visible microscope.

3.2. Synthesis of monocholesterolsuccinate

Cholesterol 5.8 g and 1.5 g of succinic anhydride were added to 200 ml of normal heptane and refluxed for 21 hours with 15 ml pyridine as catalyst (see Scheme). The mixture was cooled and filtered and the resulting solid was recrystallized from acetone and dried under vacuum to remove the residual organic solvent (Wu et al. 2005).

3.3. Preparation of docetaxel loaded liposomes

Docetaxel, monocholesterolsuccinate and egg yolk phospholipids were dissolved in chloroform and then evaporated with a rotary evaporator under reduced pressure at room temperature to form a thin film. The film was kept in vacuum over night to remove the residual chloroform followed by a redispersion of the film to the added TES buffer (pH = 7.4), thus produced the multilamaller liposomes. Then the liposome suspensions were treated with a probe ultrasonic producer to form the single unilamelar liposomes.

3.4. Formation and optimization of LHRHa-liposomes complex

In order to investigate the LHRHa and liposome binding, it was necessary to calculate the ratio of the LHRHa binding to the negatively charged lipid material (molar to molar). Different amounts of LHRHa were added to the optimized liposomes and incubated for 30 min in a 37 °C thermostatic water bath to complete the static adsorption. The amount of LHRHa addition was controlled according to the ratio of LHRHa to monocholesterolsucinate (molar to molar). When positively charged LHRHa were adsorbed to the surface of the negatively charged liposomes, the zeta potential of the liposomes would increase until saturated. Besides, a minor change in particle size would also take place. Thus zeta potential and particle size change were taken into consideration in this study for the optimization of LHRHa addition in the preparation of LHRHa-liposomes complex.

3.5. LHRHa-fluorescein-liposomes preparation and cell uptake study

Fluorescein instead of docetaxel was encapsulated into liposomes according to the optimized formulation method, and then LHRHa-fluorescein-liposomes were prepared by interacting with LHRHa. The LHRHa-fluorescein-liposomes were added in to the cell plates and incubated with different cells. After incubating for a certain time, the supernatant in the cell plants were removed and the cells were rinsed with PBS buffer (pH = 7.4) for three times to stop the phagocytosis and the remnant unphagocytosed liposomes were removed. Each well of the cell plate was viewed by an Axiovert 40 CFL microscope to investigate the uptake of prepared LHRHa-fluorescein-liposomes, and then cells were disrupted and the fluorescence intensity was determined with RF-5301 fluorescence spectrophotometer while the total protein concentrations which represented the total amount of the cells were determined using BCA assay. The ratio of the fluorescence intensities to the total protein concentrations for each well indicated the uptake efficiency.

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