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Enhanced anticancer efficacy and tumor targeting through folate-PEG modified nanoliposome loaded with 5-fluorouracil

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Abstract

Cancer targeted therapies have attracted considerable attention over the past year. Recently, 5-fluorouracil (5-FU), which has high toxicity to normal cells and short half-life associated with rapid metabolism, is one of the most commonly used therapies in the treatment of cancer. In this study the folic acid-conjugated pegylated nanoliposomes were synthesized and then loaded into them with 5-FU to improve the anti-tumor efficacy. The average size of liposomes (LPs) was about 52.7 nm which was identified by TEM. In the liposome uptake studies, the level uptake of folate-conjugated liposomes has increased compared to non-conjugated LPs according to LPs concentration, incubation time and presence of concentration of free folic acid (FA). The MTT assay and apoptotic test were carried out in HCT116 and MCF-7 cells for 24 or 48 h. The results revealed that the folate-PEG modified 5-Fu loaded nanoliposomes had strong cytotoxicity to cancer cell compared to pure 5-FU or PEG modified 5-FU loaded liposomes in a concentration- and time-dependent manner, and mainly enhanced the cancer cell death through folate-mediated endocytosis. Hence, the folate-PEG modified nanoliposome is a potential targeted drug-delivery system for the treatment of FR-positive cancers.

Keywords: targeted drug delivery, folate receptor, 5-fluorouracil, nanoliposomes

Classification numbers: 2.05, 5.08, 5.09

1. Introduction

Chemotherapy is a general approach for cancer treatment. Until now 5-fluorouracil (5-FU) has been used clinically for

colon cancer [1], breast cancer [2, 3], gastrointestinal cancers [4]. Unfortunately, serious toxic side effects and rapid degradation have limited its further clinical use [5, 6]. There are some researches about encapsulation of 5-FU in liposomal nanocarrier to improve biological half-life and/or reduce side effect [7, 8]. Liposomes are non-toxic and biocompatible. They can be incorporated both hydrophilic and hydrophobic drugs either in the aqueous phase or the lipid bilayer of the



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liposome, respectively [9, 10]. Due to these characteristics, liposomes has received FDA approval and are widely applied as drug carriers or diagnostic agents in clinic [11, 12].

In order to achieve tumor-targeted drug delivery, folic acid (FA) was commonly employed as a targeting ligand to modify nanocarriers [13–19]. FA plays a critical role in DNA and RNA synthesis, one-carbon metabolism and cellular growth. Cellular uptake of folate is mediated by cell membrane folate receptor (FR) that have a low expression level in normal cell, but is overexpressing within the tumor cells [20, 21].

Here, we aimed at development of 5-FU loading with FA modified nanoliposomes for active targeting to tumor tissues. Then, the liposome targeting ability and cytotoxicity were investigated by evaluating the cellular uptake, MTT assay, and apoptosis test in HCT116 and MCF-7 cells.

2. Materials and methods

2.1. Materials

Hydrogenated soya phosphatidylcholine (HSPC) was obtained from Shanghai Taiwei Pharmaceutical Co. Ltd (China). Cholesterol (Chol) was ordered from Hubei prosperity Galaxy Chemical Co. Ltd (China). Monomethoxy-poly(ethylene glycol) (mPEG, MW = 2000, 99%), 5-fluorouracil (5-FU) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Cholesteryl hemisuccinate (CHEM, Tokyo Chemical Industry Co. Ltd, Japan), rhodamine B (RhB, Acros Organics, USA) and folic acid (FA, Aladdin Chemical Reagent Co. Ltd, China) were obtained from commercial suppliers. All other reagents were of at least analytical grade and used without further purification.

Human breast adenocarcinoma cell line (MCF-7) and human colon cancer cell line (HCT116) were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Cell Biology of Shanghai Institute, Shanghai, China). Roswell park memorial institute (RPMI) 1640 media, Dulbecco's modified eagle medium (DMEM) 1% penicillin/streptomycin were purchased from Gibco-Invitrogen (Carlsbad, CA). Fetal calf serum was purchased from Hyclone Laboratories Inc., Australia.

2.2. Methods

2.2.1. Preparation of folate-modified PEG-liposomes. Liposomes were prepared by thin film hydration technique followed by high-pressure homogenization, previously described by Mourtas *et al* [22] and Chen *et al* [23]. In brief, the lipid mixture composed of CHEMS/NHS/TEA/PEG (PEG-CHEM or folate-PEG-CHEM, synthesized as reported by Wu *et al* [24]) were dissolved in a chloroform/methanol (2:1 v/v) mixture in a pear-shaped flask. The lipids were subsequently dried by rotary evaporation until a thin film was formed. Residual organic solvents were further dried under vacuum at room temperature overnight. The lipid film was hydrated with the appropriate volume of phosphate-buffered saline (PBS) buffer (pH 7.4), which

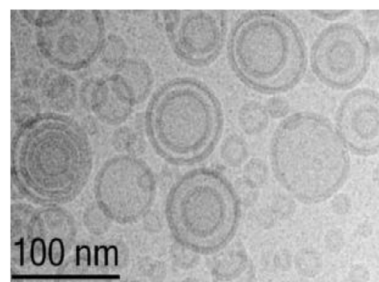


Figure 1. Transmission electron micrographs of the folate modified PEGylated liposomes.

contained 5-fluorouracil by sonication in a water bath. The drug was passively loaded. Non-encapsulated drug was removed by centrifugation at 16000rpm for 30 min. FR-targeted liposome rhodamine B (F-RhB/PEG/LPs) and 5-FU-loaded folate-PEG modified nanoliposomes (F-5-FU/PEG/LPs) were prepared by the same method as previously reported [23, 24]. These liposome suspensions were passed through high-pressured homogenizer (HPH ATS Engineering, Canada) at 600 bar homogenization cycles. All the samples were lyophilized for long stability and frozen at -20°C for 12h before being lyophilized for 48h. The freeze-dried powders were rehydrated with double distilled water when required for further experiments.

2.2.2. Dynamic lights scattering and zeta potential measurements. The mean particle sizes, polydispersity index (PDI), and zeta potentials were measured by dynamic light scattering (DLS) using the DelsaTM Nano C Particle Analyzer (Beckman Coulter, USA) at a fixed angle of 165°C and 25°C . The mean diameter was calculated from the volume distribution curves given by the particle analyser.

2.2.3. Encapsulation efficiency and drug loading in liposomes. The concentration of 5-FU incorporated into liposome vesicles was monitored by centrifugation method. The samples were centrifuged at 16000rpm for 30 min at 4°C . The ultrafiltrate contained the non-entrapped 5-FU was diluted in PBS and determined using a UV-Vis spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) at 265 nm. The drug content (DL) was calculated using the standard calibration curve and the percent of the drug encapsulation (EE) was calculated using the following formulas:

$$\text{DL}(\%) = \frac{W_T - W_{UL}}{W_L} \times 100,$$

$$\text{EE}(\%) = \frac{W_T - W_{UL}}{W_T} \times 100,$$

where W_T is the total amount of 5-FU added to the system, W_{UL} is the amount of the non-entrapped drug, W_L is the total weight of liposomes.

2.2.4. Cell culture. The HCT116 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 media; MCF-7 cells were cultured in DMEM. The culture medium was supplemented with 10% heat-inactivated fetal bovine serum

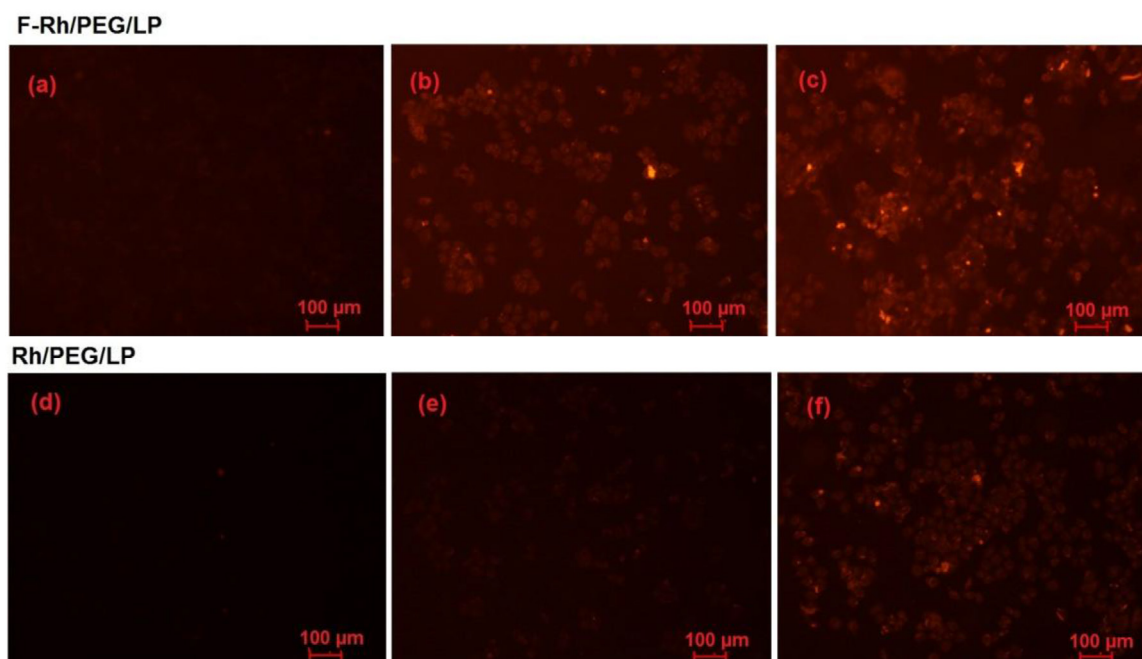


Figure 2. Fluorescence images of MCF-7 cells after incubation of F-Rh/PEG/LP (a)–(c) and Rh/PEG/LP (d)–(f) at different concentration of rhodamine 123 of $0.5 \mu\text{g ml}^{-1}$, $5 \mu\text{g ml}^{-1}$, $50 \mu\text{g ml}^{-1}$, respectively, for 2 h.

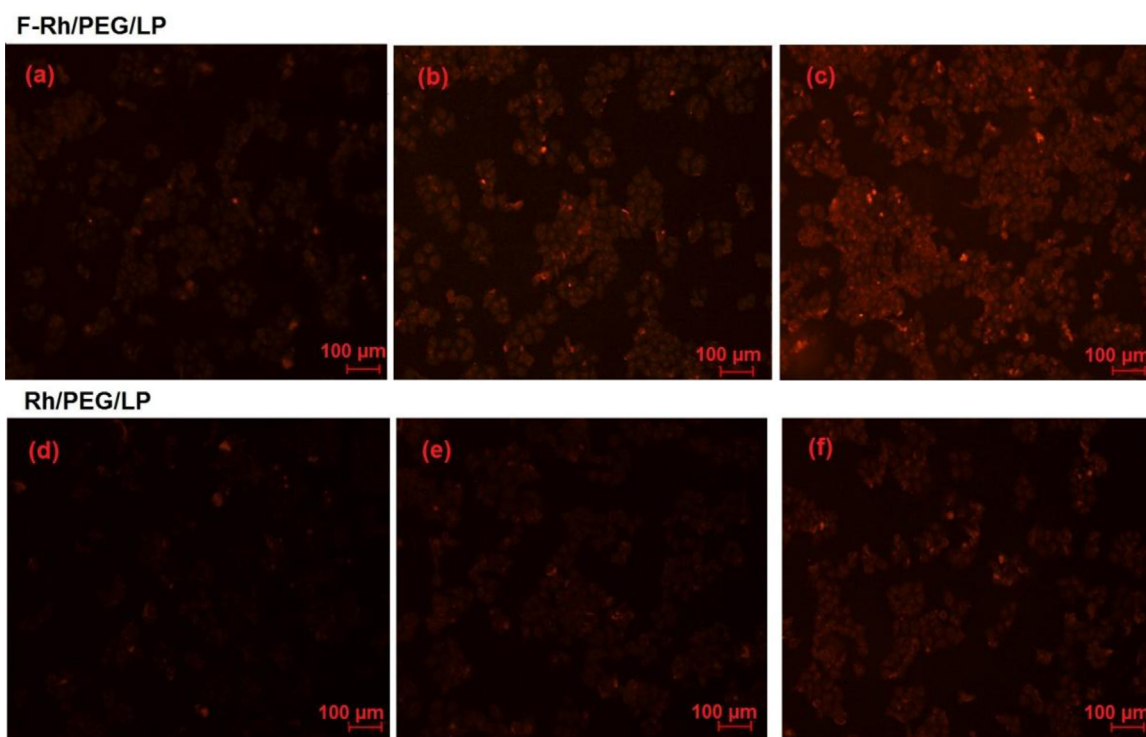


Figure 3. Fluorescence images of MCF-7 cells after incubation F-Rh/PEG/LP (a)–(c) and Rh/PEG/LP (d)–(f) for 2, 6 and 24 h, respectively, at $0.5 \mu\text{g ml}^{-1}$ of rhodamine 123.

(FBS) (Hyclone Laboratories Inc., Australia) and 1% penicillin/streptomycin. The cells are incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37°C .

2.2.5. Tumor targeted fluorescent liposomes uptake assay. Cells were seeded into 24-well plates and allowed to adhere 24 h in RPMI 2640 media with 10% serum. And then, the medium was removed and replaced by RPMI 1640 without

serum. The cells were incubated with different concentrations of rhodamine encapsulated liposomes (F-Rh/PEG/LP or Rh/PEG/LP) for indicated time periods in the presence or absence of folate ligand at 37°C . After that, cells were washed three times with cold PBS (pH 7.4, 4°C) to remove unbound-Rh encapsulated liposomes and free FA. The cell finally were visualized and photographed under an inverted fluorescence microscope (Nikon Eclipse Ti-S, MA, USA).

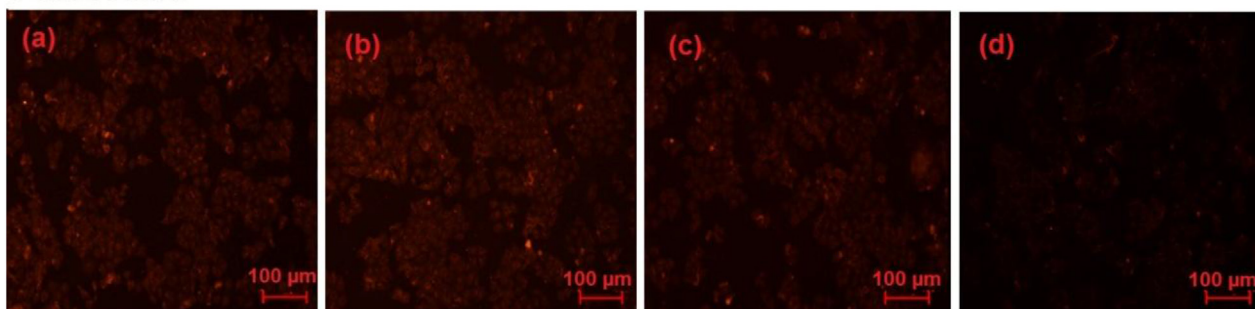
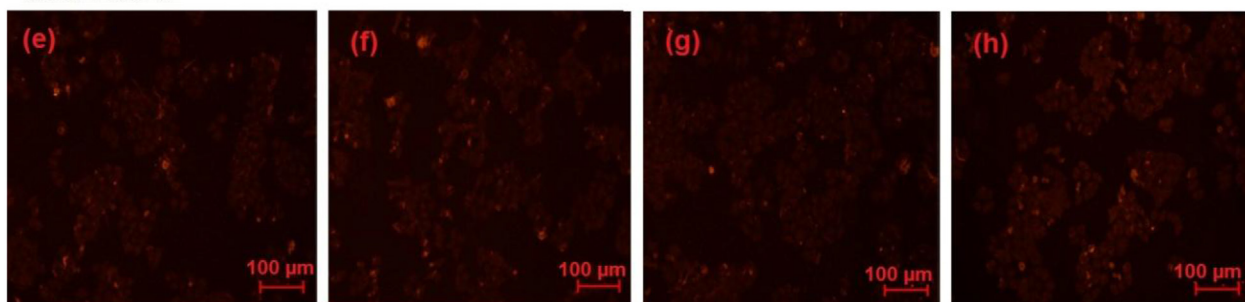
F-Rh/PEG/LP**Rh/PEG/LP**

Figure 4. Effect of free FA on uptake of F-Rh/PEG/LP (a)–(d) and Rh/PEG/LP (e)–(h) at different FA concentrations of 0 $\mu\text{g ml}^{-1}$, 1 $\mu\text{g ml}^{-1}$, 10 $\mu\text{g ml}^{-1}$ and 200 $\mu\text{g ml}^{-1}$, respectively, in MCF-7 cells. The cells were incubated with rhodamine 123-loaded liposomes of 5 $\mu\text{g ml}^{-1}$ for 4 h and photographed using the reverse fluorescence microscopy.

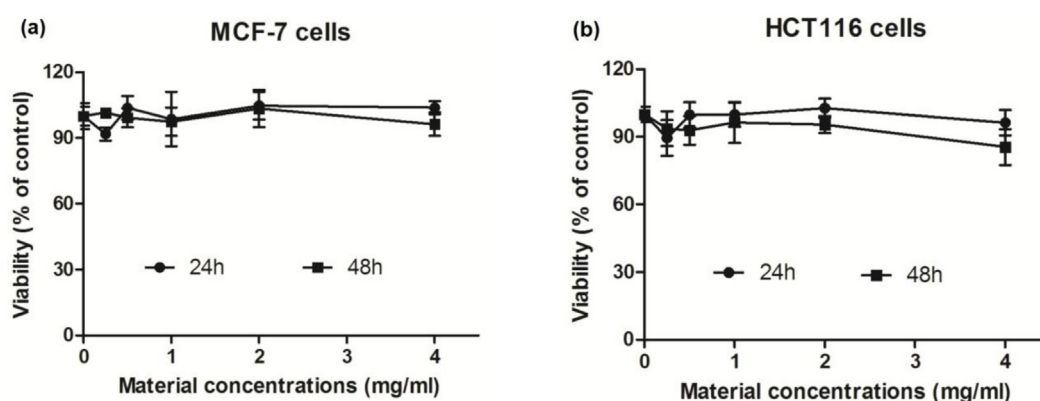


Figure 5. The toxicity evaluation of folate-PEG modified nanoliposome by MTT methods. The cells MCF-7 (a) and HCT116 (b) were incubated with different concentrations of FA/PEG/LP for 24 or 48 h.

2.2.6. *In vitro* cytotoxicity assay. The cells were plated at a density of 1×10^4 cells per well in 96-well plates and pre-incubated for 24 h. Then, the cells were treated to blank liposomes (FA/PEG/LP), 5-FU/LP, PEG/5-FU/LP, FA/PEG/5-FU/LP and pure 5-FU at different concentrations for 48 h, respectively. Cell viability was measured using the MTT method by Plate Reader (EnSpire[®] Multimode Plate Reader, Waltham, Massachusetts) at wavelengths of 492 and 630 nm. After exposure, the media were discarded. Then MTT solution with concentration of 1 mg ml^{-1} was directly added into each well, and incubated for 4 h at 37 °C. The resulting formazan crystals were solubilized with 100 μl of dimethyl sulfoxide (DMSO). The absorbance was measured at 570 nm, with the absorbance at 630 nm as the background correction. The effect on cell proliferation was expressed as the percent cell viability. Untreated cells were taken as 100% viable.

2.2.7. Apoptosis determine. MCF-7 cells were plated into 96-well plates and allowed to adhere overnight at 37 °C in incubator. Cells were subsequently treated with 3 $\mu\text{g ml}^{-1}$ of different 5-FU liposome formulations for 24 h. Then, cells were washed three times with PBS (pH 7.4) and stained with Hoechst 33342 (5 $\mu\text{g ml}^{-1}$) for 10 min at room temperature. In the end of culture periods, culture cells were washed three times with pre-cold PBS to remove excess dye and cell morphology was captured by a fluorescence microscope.

2.2.8. Analysis. All data were performed triplicates in three individual experiments and expressed as mean \pm SD. The student's *t*-test analysis was performed to compare the means of two groups. The GraphPad Prism (GraphPad Software Inc., CA, USA) and excel (Microsoft) software were utilized to obtain graphs and statistics.

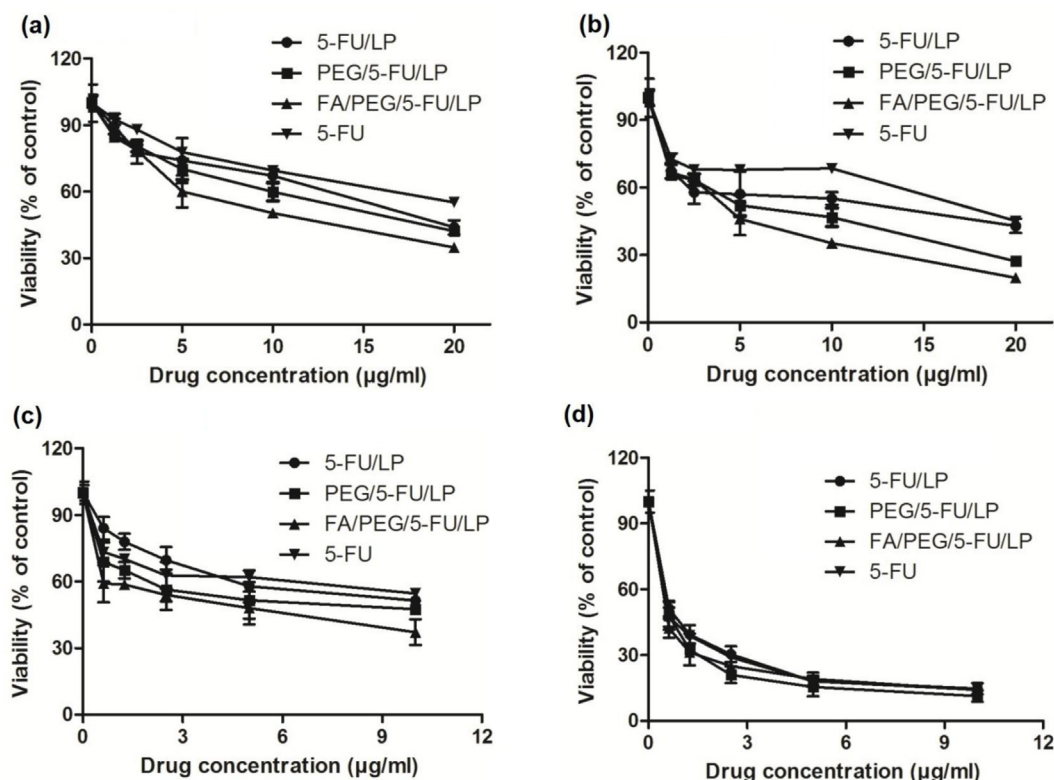


Figure 6. Cytotoxicity of different 5-FU-loaded nanoliposomes against HCT116 and MCF-7 cells after treatment for 24 or 48 h, respectively. MCF-7 cells treated with 5-FU-loaded nanoliposomes for 24 h (a) and 48 h (b). HCT116 cells treated with 5-FU-loaded nanoliposomes for 24 h (c) and 48 h (d). Data are presented as mean \pm SD.

3. Results

3.1. Preparation and characterization of 5-FU liposomes

The PEGylated liposome and folate-modified liposome was prepared. Figure 1 shows the high-resolution transmission electron micrographs of the folate modified PEGylated liposomes. The 5-FU liposomes were found to have a spherical shape with one or two layer of lipid molecules. The mean diameter of 5-FU liposomes formulation was about 52.7 nm. The PDI is 0.181. The drug encapsulation efficiency and drug loading of FA/PEG/LP are 16.14% and 1.189%, respectively. Nano-size of drug-loaded liposomes have been prepared and modified with folate ligand to obtain tumor targeting.

3.2. Liposomes uptake by tumor cells in vitro

In order to understand the uptake mechanism of liposomes into cells via folate-mediated endocytosis, the cellular uptake study was evaluated in MCF-7 at different conditions. As show in figure 2, increasing the incubated concentration of Rh-loaded PEGylated liposomes (Rh/PEG/LP) resulted in an increase in the actual amount internalized. The internalization degree of folate-targeted Rh/PEG/LP (F-Rh/PEG/LP) is higher than that of Rh/PEG/LP (non-targeted liposome) after 2 h of incubation in MCF-7 cells.

To investigate the effect of time on the folate-targeted liposomes, the FR-overexpressing cells, MCF-7, were

Table 1. The IC_{50} values of pure 5-FU and various formulation of 5-FU-loaded liposomes against MCF-7 or HCT116 cells for 24 and 48 h.

Drugs	MCF-7 cells ($\mu\text{g ml}^{-1}$)		HCT116 cells ($\mu\text{g ml}^{-1}$)	
	24 h	48 h	24 h	48 h
5-FU/LP	17.3	13.6	>10	0.703
5-FU/PEG/LP	15.5	7.15	6.73	0.600
F-5-FU/PEG/LP	10.2 ^{c,d}	4.47 ^{c,d}	4.07 ^{b,d}	0.527 ^{a,d}
5-FU	>20	17.9	>10	0.600

^a $p < 0.05$ compare to 5-FU

^b $p < 0.01$ compare to 5-FU

^c $p < 0.001$ compare to 5-FU;

^d $p < 0.05$ compare to 5-FU/PEG/LP.

incubated with non-targeted liposome (Rh/PEG/LP) and FR-targeted liposomes (F-Rh/PEG/LP) for 2 h, 6 h and 24 h (figure 3). The F-Rh/PEG/LP showed distinctively increase of cell uptake degree compared to Rh/PEG/LP for various periods incubation with MCF-7 cells. The Rh/PEG/LP uptake just had a little change during incubations.

To represent the role of cell surface FRs in liposome binding, we were further performed free folate competition study. As showed in figure 4, the high folate concentration ($200 \mu\text{g ml}^{-1}$) significantly reduced the F-Rh-loaded liposome uptake in MCF-7 cells compared with low folate concentration (0, 1 and $10 \mu\text{g ml}^{-1}$). But, there is no effect in reducing Rh/PEG/LP uptake.

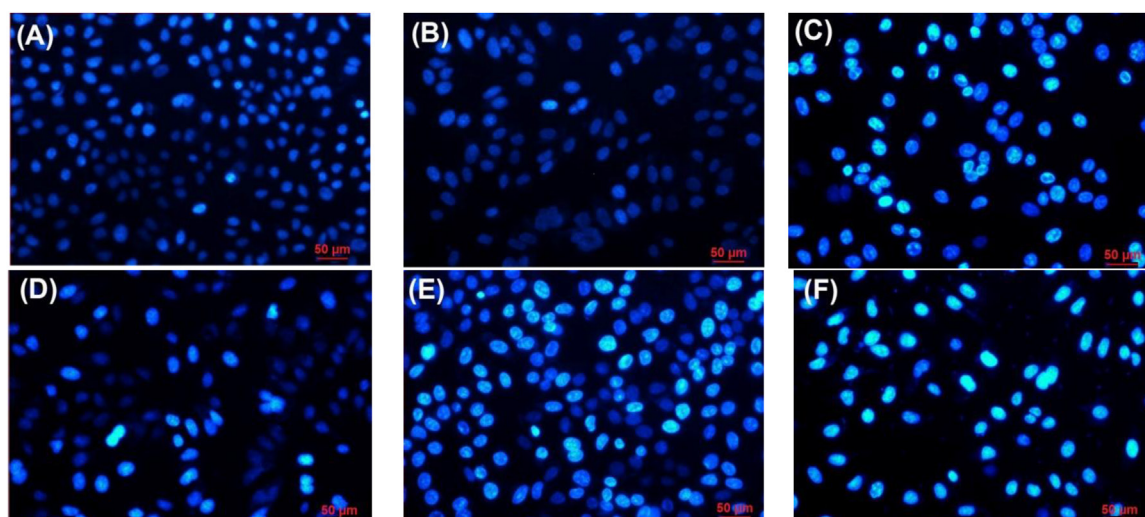


Figure 7. Tumor cell apoptosis assay. The MCF-7 cells were treated with different drug-loaded formulations containing 5-FU concentration of $3\mu\text{g ml}^{-1}$ for 24 h. The nuclear morphologies of apoptotic cells were stained with Hoechst 33342 dye and imaged by fluorescence microscope. (A) Control, (B) blank LPs, (C) pure 5-FU, (D) 5-FU/LPs, (E) 5-FU/PEG/LPs and (F) F-5-FU/PEG/LPs.

These experimental results indicated the folate-modified liposomes had a marked increase in cellular uptake compared to non-modified liposomes, and it was dependent on liposome concentration and incubated times. Furthermore, the uptake of folate-modified liposomes could be inhibited by the competition of high concentration of free FA. So, it suggested that the FA-modified liposome might be endocytosed via the FR in MCF-7 cell surface. In this study, we did not observe the saturation of the uptake capacity of the cells because of cell treatment with low concentration of liposomes.

3.3. Cytotoxicity study by MTT methods

Phospholipids are cell membrane component, so liposome made from lipid mixture (CHEMS/NHS/TEA/PEG) is well-known non-cytotoxicity [25]. As shown in figure 5, there was no significant difference in cell viability over the liposome concentration range of 0 to 4 mg ml^{-1} for 24 h or 48 h in MCF-7 cells (figure 5(a)) and HCT116 cells (figure 5(b)). In addition, in the cellular uptake study, tumor cells have been shown significantly increased uptake to the folate-modified liposome. Therefore, any side effects in drug delivery process can be associated with the encapsulated drug alone and FA/PEG/LP, on the other hand, only acts as vehicles for drug. It suggests that FA/PEG/LP is a safe drug delivery system for biomedical applications.

The *in vitro* antitumor activity of 5-FU-loaded liposomes and pure drugs were determined by MTT assay. Two cancer cells, MCF-7 and HCT116, were treated with pure 5-FU, 5-FU/LP, 5-FU/PEG/LP and F-5-FU/PEG/LP at different drug concentrations in two different periods of time (24 h and 48 h). MTT results showed a significant reduction in the cell viability of the treatment for both 24 h and 48 h in MCF-7 and HCT116 cell lines (figure 6). Here, the drug-loaded liposomes had similar activity to pure 5-FU in inhibiting the growth of HCT116 or MCF-7 cells.

The half maximal inhibitory concentration (IC_{50}) values of pure 5-FU, 5-FU/LP, 5-FU/PEG/LP and F-5-FU/PEG/LP against tumor cells were shown in table 1. There is little difference between pure 5-FU and other 5-FU-liposome formulations in HCT116 cell for 24 h or 48 h incubation, respectively (figures 6(c) and (d)). However, 5-FU-liposome formulations showed stronger cytotoxicity compared to pure 5-FU in MCF-7 cells (figures 6(a) and (b), and table 1). Furthermore, F-5-FU/PEG/LP exhibited greater cytotoxic activities to pure 5-FU or 5-FU/PEG/LP in MCF-7 cells. In addition, the MTT results also showed that 5-FU loaded-liposome formulations inhibited tumor cell growth in a time-dependent manner from 24 h to 48 h. This revealed that folate moieties in F-5-FU/PEG/LP would be conducive to enhancing a cytotoxic effect by binding of the liposomes on MCF-7 cells and subsequently increasing their intracellular uptake via FR-mediated endocytosis and accumulating in tumor cells.

3.4. Apoptotic analysis

Tumor cells generally underwent apoptosis in response to anti-cancer agents. To elucidate drug-loaded liposomes induced apoptosis in MCF-7 cells, cell nuclei were stained by Hoechst 33342 after different treatments. As shown in figure 7, the control and B-LPs groups seem to stain cytoplasm (figures 7(A) and (B)). But the cells treated with pure 5-FU or 5-FU-loaded liposomes exhibited chromatin condensation and nuclear fragmentation (figures 7(C)–(F)). Apoptotic features of 5-FU/PEG/LP and F-5-FU/PEG/LP treated groups notably higher than pure 5-FU or 5-FU/LP treated groups. It is indicated that the 5-FU loaded liposome had an enhanced apoptosis effect in cancer cells. The enhanced uptake of the folate-modified liposomes via FR-mediated endocytosis is one of the reasons that folate-modified liposomes had a higher effect than pure 5-FU or other liposome formulations. This result again demonstrated cytotoxicity or apoptosis effect of drug-loaded liposomes only attributed to the effect of the 5-FU release from it.

4. Conclusion

Some biocompatible polymers such as poly(lactide)-tocopheryl polyethylene glycol, chitosan and PEG, have been widely used for the tumor-targeted delivery and site-specifically release of anticancer drugs (paclitaxel, curcumin and doxorubicin) [26–28]. In recent years, the use of liposomes to increase the therapeutic index of chemotherapeutic drugs and to offers drug targeting and controlled release has emerged as a promising strategy against cancer [8, 29, 30]. PEGylated phospholipids are commonly used to protect the liposome surface from penetration and disintegration by plasma proteins or to avoid monocyte/macrophage clearance [31, 32]. In this case, liposomes can passively accumulate in tumor tissues due to the enhanced permeability and retention (EPR) effect. Here, 5-FU loaded folate-PEG modified nanoliposomes were prepared and evaluated for their *in vitro* antitumor activity in HCT116 and MCF-7 cells. The average size of this FR-targeting 5-FU-delivery system was about 52.7 nm, which can allow them preferentially accumulate at tumor sites [27, 33]. Additionally, the liposome with FA modification can provide active targeting capability to FR-positive cancer. The MTT assay showed that the blank liposomes had no significant toxic effect on the growth of two tumor cell lines (HCT116 and MCF-7). Further, the testing of cytotoxicity and apoptosis of 5-FU-loaded liposomes indicated that folate-modified liposomes effectively enhanced anticancer activity of drug through folate-mediated endocytosis. The F-5-FU/PEG/LP was found to have significantly greater cancer-inhibitory effect than without folate conjugation or pure 5-FU. Therefore, the results of this study illustrated this drug delivery system could be beneficial in the cancer treatment by targeting 5-FU to the tumor cells and reducing its toxicities. Moreover, further studies are in progress to improve drug loading of this system.

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