Baicalin loaded in folate-PEG modified liposomes for enhanced stability and tumor targeting

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Bioavailability of baicalin (BAI), an example of traditional Chinese medicine, has been modified by loading into liposome. Several liposome systems of different composition i.e., lipid/cholesterol (L), long-circulating stealth liposome (L-PEG) and folate receptor (FR)—targeted liposome (L-FA) have been used as the drug carrier for BAI. The obtained liposomes were around 80 nm in diameter with proper zeta potentials about −25 mV and sufficient physical stability in 3 months. The entrapment efficiency and loading efficiency of BAI in the liposomes were 41.0–46.4% and 8.8–10.0%, respectively. The morphology details of BAI liposome systems i.e., formation of small unilamellar vesicles, have been determined by cryogenic transmission electron microscopy (cryo-TEM) and small angle X-ray scattering (SAXS). In vitro cytotoxicity of BAI liposomes against HeLa cells was evaluated by MTT assay. BAI loaded FR-targeted liposomes showed higher cytotoxicity and cellular uptake compared with non-targeted liposomes. The results suggested that L-FA-BAI could enhance anti-tumor efficiency and should be an effective FR-targeted carrier system for BAI delivery.

1. Introduction

In China and several Asian countries the dry root of Scutellaria is one of the most popular and multi-purpose herbs. Baicalin (BAI, 5,6-dihydroxyflavone-7-O-D-glucuronic, Fig. S1), the single active ingredient extracted from the dry root of Scutellaria, is a flavonoid used to treat acute icteric, acute non-icteric and chronic hepatitis. It has been reported that BAI has an anti-tumor effect on bladder, prostate, colorectal, ovarian, lung, breast, and liver cancer cells [1–8]. And it has also been proved that BAI can protect the liver from drug-induced injuries [9–11]. However, BAI has low hydrophilic properties and is poorly absorbed after administration, which leads to low bioavailability and severely limits its anti-tumor efficacy and clinical application [12–14]. The absolute bioavailability of baicalin has been found to be only 2.2 ± 0.2% in rats after administration [15]. In order to improve the absorption and bioavailability of BAI, different drug delivery systems for treatment of tumor have been studied extensively. BAI loaded solid lipid nanoparticle (SLN) was prepared by using the coacervation method [16]. The observed optimized formulation had entrapment efficiency of 86.29 ± 1.43%, mean particle size of 343.7 ± 7.07 nm. In some studies, baicalin-polyvinylpyrrolidone coprecipitate was prepared to improve the solubility and absorption of BAI in blood plasma [17]. A nanocrystal (or nanosuspension) drug delivery system was used to improve bioavailability of BAI. The mean particle size of baicalin-nanocrystals was 336 nm [18]. BAI liposomes with 350 nm mean particle size and 60% entrapment efficiency were also successfully prepared in a previous study [19]. Sterically stabilized spongosomes for multidrug delivery of BAI and Brucella javanica oil was recently established for clinical applications [20,21]. Compared with the traditional drug delivery systems, polysaccharide capsules [22], stimuli-responsive protein-based biomimetic protocells [23]...
and dipeptide-based enzyme-responsive nanoparticles [24] will be new possibility for BAI delivery in the future. Overall, the particle sizes of the previous BAI delivery systems were larger than 200 nm. The new delivery system with smaller particle size for BAI is expected to improve the solubility and absorption of BAI.

Through the enhanced permeability and retention (EPR) effect, nanocarriers preferentially accumulate in tumors [25]. This results in higher concentrations than in plasma or in other tissues. Thus, nanoparticles can achieve passive targeting of a tumor through the EPR effect. In general, nanoparticles smaller than 100 nm are considered excellent for tumor targeting [26]. Recently, some researchers studied the effect of nanoparticle size on tumor accumulation in a mouse cancer model [27]. They found that the optimal nanoparticle size was between 60 and 80 nm. They also considered that particles larger than 100 nm tend to have low permeation into tumors. Nanoparticle size also affects the intracellular trafficking, which can affect tumor accumulation [28,29]. However, the EPR effect varies depending on the tumor model and patient, and there can be a huge variation between different areas of a single tumor [30,31]. To overcome the above drawbacks related to passive targeting, "active targeting" was developed. Folate receptors (FRs) are frequently overexpressed in a wide range of tumor types, and thus, present an attractive target for tumor-selective drug delivery [32,33]. Folic acid (FA) has high binding affinity to tumor-associated folate receptors (FR), providing a selective delivery of drug to FR-positive tumor cells [34,35]. Due to its small size and ready availability, FA has become one of the most investigated targeting ligands for tumor-specific drug delivery [36–42]. It can be incorporated into nanoparticles via FA conjugation that tends to bind tightly to the FR and trigger cellular uptake by endocytosis [43].

The aim of this study was to design stable liposome formulations for BAI with an appropriate size, high drug loading and tumor-targeted function. PEG-cholesteryl hemisuccinate (PEG-CHEMS) and folate-PEG-cholesteryl hemisuccinate (F-PEG-CHEMS) were synthesized and then incorporated into the liposome system formed by hydrogenated soybean phosphatidylcholine and cholesterol to obtain BAI loaded long-circulating stealth liposomes (L-PEG-BAI) [44] and folate receptor—targeted liposome (L-FA-BAI). Cellular uptake was investigated using rhodamine B (RB) as a probe for the FR-targeted liposome and non-targeted liposome formulations. The HeLa human cervical cancer cells, which have high level of FR-expression, were selected as model cancer cells [45]. To the best of our knowledge, this is the first report about successfully loading BAI into a tumor-targeted liposome formulation. The results of this study imply that FR—targeted liposome improves bioavailability of BAI and could enhance its efficacy in clinical applications for systematically, orally or regionally-administrations [46].

2. Materials and methods

2.1. Materials

Chemicals were obtained from commercial suppliers: baicalin (BAI 95%, Aladdin Chemical Reagent Co., Ltd., China); hydrogenated soybean phosphatidylcholine (HSPC 95%, Shanghai Taiwei Pharmaceutical Co., Ltd., China); cholesterol (CHOL 95%, J&K Scientific Co., Ltd., China); cholesteryl hemisuccinate (CHEMS ≥ 95%, Tokyo Chemical Industry Co., Ltd., Japan); polyoxyethylene bis-amine (NH2-PEG-NH2 99%, MW = 2000, Shanghai Yuanye Biotechnology Co., Ltd., China); monomethoxy-polyethylene glycol (MPEG 99%, MW = 2000, Shanghai Yuanye Biotechnology Co., Ltd., China); rhodamine B (RB ≥ 99%, Acros Organics, USA); Folic acid (FA ≥ 98%, Aladdin Chemical Reagent Co., Ltd., China); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT 98%, Sigma–Aldrich, MO, USA); double distilled water.

2.2. Synthesis of PEG-CHEMS and folate-PEG-CHEMS

The structures of CHEMS and PEG-CHEMS are shown in Fig. S2. As described previously [47,48], CHEMS, EDC, NHS, and TEA were dissolved in dichloromethane. The pre-dried MPEG was also dissolved in dichloromethane and consequently, added drop by drop. The reaction mixture was stirred overnight at room temperature. The solution then evaporated and the residue was re-dissolved in 50 mM Na2CO3 to form micelles. Afterwards, the micelles were then dialyzed against double distilled water using a dialysis membrane with a molecular weight cut-off (MWCO) of 14 kDa to remove low molecular weight by-products.

The structure of folate-PEG-CHEMS is shown in Fig. S2. The synthesis of folate-PEG-CHEMS has been done via reaction of folate-PEG-amine with CHEMS-NHS [49]. First, folate-PEG-amine and CHEMS-NHS were synthesized [50]. For synthesis of folate-PEG-amine, folic acid and PEG-bis-amine were dissolved in DMSO. Then NHS, EDC and TEA were added to the solution, and the reaction allowed proceeding at room temperature in darkness overnight. The reaction mixture was dissolved in freshly prepared double distilled water. This was then dialyzed against double distilled water using a dialysis membrane with a molecular weight cut-off (MWCO) of 1 kDa to remove low molecular weight by-products. Folate-PEG-amine was then dried by lyophilization and gave a yield of 60%. For synthesis of CHEMS-NHS, CHEMS was reacted with NHS and EDC in tetrahydrofuran at room temperature overnight. The CHEMS-NHS product was purified by recrystallization. Finally, for synthesis of folate-PEG-CHEMS, folate-PEG-amine and CHEMS-NHS were dissolved in CHCl3, and reacted at room temperature overnight. The solvent was then removed by rotary evaporation and the residue was re-dissolved in 50 mM Na2CO3 to form micelles. After that, the micelles were dialyzed against double distilled water using a dialysis membrane with a molecular weight cut-off (MWCO) of 14 kDa to remove low molecular weight by-products.

The PEG-CHEMS and F-PEG-CHEMS products were then dried by lyophilization giving yields of 54 and 34%, respectively. The identities of the products were confirmed by 1H NMR in D-ChlCl2 and D2O, respectively. The purity of the product was confirmed using a UV–vis spectrophotometer (UV–1800; Shimadzu, Japan) as described previously [51].

2.3. Liposomes preparation

Liposomes were prepared by thin film hydration followed by high-pressure homogenization [52]. The lipid mixture was dissolved in chloroform and placed in a flask. The lipids were dried by rotary evaporation until a thin film of the lipids was deposited. Residual organic solvents were dried under vacuum overnight. The lipid film was hydrated with the PBS buffer (pH 7.4) containing BAI by sonication in a water bath. These dispersions were processed through a high-pressure homogenizer (HPH ATS Engineering, Canada) with 5 homogenization cycles at 600 bar. Fluorescent liposomes were prepared using the same method except that the lipid film was initially hydrated in RB solution [50]. Fluorescent FR-targeted liposome (L-F-RB) and non-targeted liposome (L-PEG-RB) were prepared, respectively.

After preparation, all liposome dispersions were left to stand for at least 1 h at a temperature above the lamellar liquid crystalline melting temperature (Tm), in order to avoid stiff gel formation [53]. Un-entrapped baicalin was removed by a Sephadex G-50 column.

2.4. Dynamic lights scattering (DLS) spectrophotometry

The liposomes were assessed at proper intervals (after 1, 30, and 90 days) for changes in mean particle size and zeta potential by dynamic light scattering (DLS) spectrophotometry using a Delsa™
Nano C Particle Analyzer (Beckman Coulter, USA) at a fixed angle of 165° and a temperature of 25 °C. Each test was repeated three times.

2.5. Entrapment efficiency (EE) and drug loading (DL)

The entrapment efficiency of baicalin in the liposomes was determined by the centrifugal-ultrafiltration method. An aliquot (2 mL) of the sample was placed in the upper chamber of a centrifuge tube matched with a centrifugal-ultrafiltration tube (Amicon® Ultra-4Centrifugal Filter Units, Millipore, USA, MWCO = 14 kDa) and was centrifuged for 30 min at 3500 rpm at 4 °C [54]. The ultrafiltrate in the ultrafilter contained the un-entrapped BAI. Next, the ultrafiltrate was diluted in PBS (pH 7.4) and the free drug concentration in sample was determined by a UV–vis spectrophotometer (UV-1800; Shimadzu, Japan) at 315 nm. The calibration curve of the baicalin concentration against absorption was 

\[ y = 0.0424x + 0.0069 \quad (R^2 = 0.9999) \]

All experiments were conducted at 25 °C.

The entrapment efficiency (EE%) and drug loading (DL%) were calculated by the following formulas:

\[ \text{EE} = \left(1 - \frac{C_U}{C_T} \right) \times 100\% \]

\[ \text{DL} = \left( \frac{C_T - C_U}{C_T + C_U} \right) \times 100\% \]

where \( C_U \) is the amount of un-entrapped BAI, \( C_T \) is the total amount of BAI added into the liposome system, and \( C_L \) is the total amount of lipids.

2.6. Cryogenic transmission electron microscopy (cryo-TEM)

For cryo transmission electron microscopy studies, a sample droplet of 2 μL was put on a lacey carbon filmed copper grid (Science Services, Muenchen), which was hydrophilized by air plasma glow discharge (Solarus 950, Gatan, Muenchen, Germany) for 30 s. Subsequently, most of the liquid was removed with blotting paper leaving a thin film stretched over the lace holes. The specimens were instantly shock frozen by rapid immersion into liquid ethane cooled to approximately 90 K by liquid nitrogen in a temperature-controlled freezing unit (Zem Cryobox, Carl Zeiss Microscopy GmbH, Jena, Germany). The temperature was monitored and kept constant in the chamber during all the sample preparation steps. After freezing the specimens, the remaining ethane was removed using blotting paper. The specimen was inserted into a cryotransfer holder (CT3500, Gatan, Muenchen, Germany) and transferred to a Zeiss/Leo EM922 Omega EFTEM (Zeiss Microscopy GmbH, Jena, Germany). Examinations were carried out at temperatures around 90K. The TEM was operated at an acceleration voltage of 200 kV. Zero-loss filtered images (DE = 0 eV) were taken under reduced dose conditions (100–1000 e/nm2). All images were registered digitally by a bottom mounted CCD camera system (Ultrascan 1000, Gatan, Muenchen, Germany) combined and processed with a digital imaging processing system (Digital Micrograph GMS 1.9, Gatan, Muenchen, Germany).

2.7. Small angle X-ray scattering (SAXS)

The SAXS experiments were performed using the Incoatec™ X-ray source IpS with Quazar Montel optics at the Institute of Physical Chemistry, Hamburg University. The samples were filled in a holder for liquids, equipped with Kapton®-windows. The distance between the sample and the detector was 1.6 m. This distance allowed measurements in the q-range interval from 0.008 to 0.32 [Å⁻¹]. The beam diameter at the sample position was 1 mm. The X-ray exposure time was 1 h. The background subtraction and all necessary normalizations were performed using the Scatter package [55]. The final intensity, \( I(q) \), was displayed as a function of the modulus of the scattering vector \( q = (4\pi/\lambda) \sin(\theta) \), where \( \lambda = 1.54 \text{Å} \) is the X-ray wavelength and \( 2\theta \) is the angle between the incident and scattered X-rays.

2.8. In vitro baicalin release studies

Release of baicalin from the liposome was evaluated using dialysis method. An aliquot of each liposome solution (1 mL) was placed in a dialysis tube (MWCO = 14 kDa). The tube was then immersed in 100 mL of PBS (pH 7.4) [56] at 37 °C and placed in a rotary shaker. Samples (3 mL) were taken from the release medium at predetermined time intervals. Subsequently, the same volume of fresh medium was refilled into the release medium. The concentration of baicalin was assayed by using a UV–vis spectrophotometer (UV-1800; Shimadzu, Japan) operating at 315 nm. All experiments were carried out in triplicate.

2.9. Cell culture

The human cervical cancer cell line (HeLa) was purchased from American Type Culture Collection (Manassas, VA, USA), which has amplified FR expression. HeLa cells were seeded onto cell culture dishes containing folate-free RPMI 1640 media with 10% fetal bovine serum, L-glutamine (5 mM), non-essential amino acids (5 mM/L), penicillin (100 U/mL), and streptomycin (100 U/mL) (Invitrogen, Carlsbad, CA, USA), at 37 °C in a humidified 5% CO2 atmosphere in the incubator.

2.10. In vitro cytotoxicity assay

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay. HeLa cells were plated in 96-well plates with 100 μL medium at a density of 5 × 10^3 cells per well. After 24 h, cells were exposed to various concentrations of pure BAI, L-BAI, L-PEG-BAI and L-FA-BAI with or without 1 mM free folate acid for 44 h. Then, the culture media were discarded and replaced with fresh culture medium containing 1 mg/mL MTT and continue cultured for another 4 h at 37 °C. The resulting formazan crystals were solubilized with 100 μL DMSO. The absorbance was measured using an enzyme-linked immunosorbent assay reader 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.

2.11. Uptake of FR-targeted fluorescent liposomes by HeLa cells

HeLa cells were washed three times with pH 3.5 saline (130 mM NaCl, 20 mM NaAc) and PBS at 4 °C to remove receptor-bound free folate. For liposome uptake studies, the cells were incubated with L-FA-RB and L-PEG-RB in a folate-free media for 60 min at 37 °C. To determine the effect of FR blockade, 1 mM free folic acid was added to the incubation media as a FR blocking group. At the end of incubation, the cells were washed three times with cold PBS and then visualized and photographed by using a laser scanning confocal microscope (A1, Nikon, Japan).

3. Results and discussion

3.1. Liposomes preparation and characterization

In order to select the optimal composition of liposomes, 5 samples with different lipid and drug mass ratio were prepared (Table
According to the data of samples 1–3, a higher drug/lipid ratio did not increase entrapment efficiency but it increased drug loading. It was also found that samples with higher HSPC/CHOL ratios had smaller sizes and higher entrapment efficiencies. Cholesterol could reduce the fluidity of the membrane and plays an important role in stabilizing and controlling the drug permeability properties of the liposomal membrane bilayer\[57\]. However, adding too much cholesterol would cause the HSPC membrane to become broken, which may result in lower entrapment efficiency\[58\]. Combined with the results of particle size, entrapment efficiency and drug loading, the formula of sample 5 was selected as optimal for the further investigations.

PEG-CHEMS and F-PEG-CHEMS were incorporated into liposome systems to prepare BAI loaded long-circulating stealth liposome and FR-targeted liposome, respectively. Cholesteryl hemisuccinate is a cholesterol derivative, which can also improve the stability of a liposome system\[47\]. In addition, it was proven that cholesterol and its derivative anchored PEG-modified liposomes were easily incorporated into the liposomal membranes compared to phospholipids-anchored PEG-modified liposomes (PEG-DSPE, PEG-PE)\[59\]. L-BAI, L-PEG-BAI and L-FA-BAI liposome (Table S2) were prepared for the further experiments.

As shown in Fig. 1A, the mean particle sizes of L-PEG-BAI and L-FA-BAI were 70.9 ± 1.4 nm and 68.4 ± 3.6 nm, respectively, and thus, slightly smaller than L-BAI (87.6 ± 1.6 nm). After lyophilization, the mean particle sizes and PDI of all samples remained unchanged during 90 days storage. The mean particle sizes of the liposomes prepared in this study were much smaller than those in previous studies, the sizes of which ranged from 250 to 350 nm\[16–19\].

Surface charge is an important indication for the stability of a colloidal nanoparticle system in a medium. The repulsion between
nanoparticles with the same sign of surface charge provides extra stability. The zeta-potential values of L-BAI, L-PEG-BAI and L-FA-BAI are shown in Fig. 1A and indicate negative charges on the liposome surfaces, which may be due to the overall negative charge of HSPC. Negatively charged lipids improved electrostatic and steric stabilization of the liposomal formulation [80]. The absolute zeta potential values of L-FA-BAI and L-PEG-BAI were lower than that of L-BAI. The reason for this might be the hydrophilic PEG chains could stretch out on the surface of the bilayers to form a barrier, which shield the negative charge of CHEMS [47]. There was a higher decrease of L-BAI in the zeta potential from $-23.2 \pm 1.2$ mV to $-19.2 \pm 3.4$ mV after 90 days storage, when compared to L-PEG-BAI and L-FA-BAI. Despite the decrease of zeta potential values during storage, all samples still exhibited a stable potential of about $-18$ to 25 mV. It can be concluded that L-BAI, L-PEG-BAI and L-FA-BAI displayed good stability for at least 90 days.

From Fig. 1B, it can be seen that the EE values of freshly prepared L-BAI, L-PEG-BAI and L-FA-BAI were 46.4 $\pm$ 0.7%, 43.9 $\pm$ 1.3% and 41.0 $\pm$ 1.6%, respectively. Owing to the slightly larger particle size of the L-BAI liposome, more BAI would be entrapped in the conventional liposome than in the PEGylation liposome or the FR-targeted liposome. The DL values of L-BAI, L-PEG-BAI and L-FA-BAI were 10.0 $\pm$ 0.6%, 9.4 $\pm$ 0.6% and 8.8 $\pm$ 0.7%, respectively, indicating similar loading and entrapment characteristics. After 90 days storage no significant changes in drug loading and entrapment efficiency were observed for all the liposomes. However, the EE values of all samples were not as high as expected due to the very low solubility of BAI in water and oil. It has been reported that a phosphate buffer solution can increase the solubility of BAI to a certain extent [61,62], and it was supposed that only some BAI could be entrapped in the inner aqueous compartments of liposomes. From Fig. 1 it is interesting to note that the DL in this study has increased a lot compared to the previous studies in which drug loading values ranged between 2.5 and 5.7% [19,63,64]. It should be mentioned that L-FA-BAI had the lowest EE value while L-BAI had the highest EE value. The reason for this could be that L-FA-BAI had the smallest particle size, which resulted in the smallest inner aqueous phase of liposome. Then the least BAI could be entrapped in the inner aqueous phase of liposome.

3.2. Cryogenic transmission electron microscope (cryo-TEM)

Cryo-TEM measurements were used to study the morphology of BAI loaded liposomes. From Fig. 2 it is obvious that all three samples mainly present the small unilamellar vesicle (SUV). The uniquely recognizable phospholipid bilayers of the vesicle membrane and empty core observed in Fig. 2 are good indications of a SUV structure. Besides the SUV structure, some “onion-like” (circle in circle) multilamellar vesicles (MLVs) are also found in three liposome images. From Fig. 2 it can be seen that all three liposomes were spherical or oval in shape. L-BAI, L-PEG-BAI and L-FA-BAI had similar characters, which demonstrate that the incorporation of PEG-CHEMS and F-PEG-CHEMS does not significantly influence the morphology of the liposomes. The above results were in accor-
dance with the previously discussed DLS results, where the mean particle size of L-BAI, L-PEG-BAI and L-FA-BAI were 87.6 ± 1.6 nm, 70.9 ± 1.4 nm and 68.4 ± 3.6 nm, respectively.

3.3. Small angle X-ray scattering (SAXS)

SAXS was used to investigate the mean or global features of the samples since cryo-TEM, as a method, can only image a small part of the bulk volume. Representative SAXS curves, before and after the BAI loading for the conventional liposome (PEGylation liposome and FR-targeted liposome), are shown in Fig. 3. The characteristic scattering from a monodisperse system of spherical shells (as a rough model of vesicles) could give rise to notable bumps on the curve that is related to the radius of the vesicles. In polydisperse systems, such bumps are smeared to a smooth decaying curve due to the superposition of many different vesicle sizes. The observed SAXS polydispersity reconfirms the DLS results. The absence of peaks indicates that well-defined multilamellar vesicles or other periodically nanostructured nanoparticles, such as cubosomes, were not present. As shown in Fig. 2, the samples were composed of polydisperse unilamellar vesicles. The increased scattering of PEGylation samples at small angles could be attributed to the addition of polymeric components, i.e., PEG chains, to the system. After loading with BAI, the SAXS intensity increased at \( q \sim 0.02 \) [Å\(^{-1}\)] for conventional liposome and PEGylation liposome, while for the FR-targeted liposome the intensity decreased. The corresponding distances at \( q \sim 0.02 \) [Å\(^{-1}\)] are around 30 nm, which could represent formation of smaller vesicles attributed to the loading of BAI in the liposomes and PEG-liposomes. In the case of FA-liposomes the trend was opposite, i.e., BAI loading decreased the population of ~30 nm in diameter liposomes.

3.4. In vitro baicalin release studies

Profiles of BAI released from the PEG-CHEMS and F-PEG-CHEMS liposomes (L-PEG-BAI and L-FA-BAI) were studied in vitro using the dialysis method in a medium of PBS (pH 7.4) at 37 °C in a water bath rotary shaker. BAI released from the conventional liposome (L-BAI) and pure BAI solution was also investigated as controls. The in vitro accumulative BAI released from liposomal systems of different compositions are given in Fig. 4.

More than 95% of the BAI was rapidly released from the pure BAI solution within 10 h, and the accumulative release value reached 100% in the following hours. The in vitro BAl released from L-BAI showed an initial burst in the first few hours then gradually leveled off after 5 h, with about 83.4% of BAI released after 48 h. At the same time, the BAI release rate of L-PEG-BAI and L-FA-BAI decelerated so that only about 58.3% and 56.9% BAI was released, respectively. L-FA-BAI showed a similar smooth release curve compared to the L-PEG-BAI because PEG-CHEMS molecular section was introduced in both formation. The final amount of BAI released from both L-PEG-BAI and L-FA-BAI was less than that from L-BAI. This indicates that the liposomes with PEG chain could be more effective to prolong the in vitro release of BAI. The above results suggest that the in vitro release of BAI from both PEG-CHEMS and F-PEG-CHEMS liposomes is significantly lower than that of conventional liposome, and reflects the prolonged-release property of the two liposomes.

3.5. In vitro cytotoxicity assay

In order to assess the activity of BAI loaded liposomes on tumor cells, the cellular cytotoxicity was evaluated using an MTT assay. Fig. 5 shows that the viability of HeLa cells decreased with increasing BAI dose in the HeLa cells. The viability of HeLa cells treated with 6.25 \( \mu \)g/mL and 100 \( \mu \)g/mL pure BAI for 48 h was 100 ± 1.7% and 21.8 ± 1.6%. The cytotoxicity of L-PEG-BAI against HeLa cells was slightly higher than L-BAI at a concentration ranging from 25 to 100 \( \mu \)g/mL. This indicates that the sustained release characteristics
of L-PEG-BAI delivered prolonged and effective concentrations of BAI due to the existence of PEG-CHEMS. L-BAI and L-PEG-BAI both showed a slightly lower inhibition potential than pure BAI solution, while L-FA-BAI possessed more inhibition potential than pure BAI solution at concentrations from 12.5 to 100 μg/mL. For example, the viabilities of HeLa cells treated with 100 μg/mL of pure BAI and L-FA-BAI were 21.8 ± 1.6% and 14.9 ± 1.0%, respectively. Moreover, it should be pointed out that Blank-L-FA did not show any obvious cytotoxicity to HeLa cells even at a high concentration up to 4 mg/mL and after 48 h exposure.

Table S3 shows that the IC_{50} value of the FR-targeted liposome (L-FA-BAI) was approximately 1.4 times lower compared to that of non-targeted liposome (L-BAI and L-PEG-BAI) and 1.1 times lower than that of pure BAI. This data suggests that FR-targeted liposomes containing F-PEG-CHEMS are more effective than non-targeted liposomes and pure BAI. However, this differential cytotoxicity between FR-targeted liposome and non-targeted liposome was eliminated in the presence of 1 mM free FA. The data shows that adding 1 mM free FA to L-FA-BAI increases the IC_{50} value from 58.3 ± 3.3 to 75.6 ± 3.6 μg/mL. This indicates that adding free FA could reduce the cytotoxicity of L-FA-BAI against HeLa cells. It could be inferred that drug uptake can be controlled by competition with free FA [65].

3.6. Uptake of FR-targeted fluorescent liposomes by HeLa cells

Uptake by HeLa cells of liposomes containing F-PEG-CHEMS was analyzed by confocal fluorescence microscopy. Fig 6A shows the cells uptake image of non-targeted RB liposome (L-PEG-RB), where the fluorescence is obvious. This result indicated that L-PEG-RB is taken up by cells due to its optimal nano size. Compared to Fig. 6A, B shows much stronger fluorescence. This indicates that

![Fig. 5. In vitro cytotoxicity of BAI liposomes against HeLa cells 48 h incubation. Cell viability is expressed as the percentage of untreated controls. Data were presented as mean ± SD (n = 6).](image)

![Fig. 6. Uptake of L-PEG-RB and L-FA-RB by HeLa cells. The HeLa cells were respectively incubated with L-PEG-RB and L-FA-RB with or without 1 mM of free folic acid. (A) Cells treated with L-PEG-RB; (B) cells treated with L-FA-RB; (C) cells treated with L-FA-RB plus 1 mM free folate.](image)
more RB accumulates in the cells when treated with FR-targeted liposome RB (L-FA-RB). Thus, L-FA-RB can be taken up by cells more easily than L-PEG-RB. By adding 1 mM free FA, less fluorescence is observed as shown in Fig. 6C. This demonstrates that less RB accumulated in the cells. It implies that free FA could block the interaction between L-FA-RB and the cellular folate receptor, thus impeding L-FA-RB entering the cells. Therefore, L-FA-RB shows a much higher cellular uptake and liposome internalization than L-PEG-RB, and the uptake could be blocked by 1 mM free FA. These results correlate well with the results from the above described cytotoxicity assay. It also shows that FR-targeted liposomes have efficient interactions with the cellular folate receptor, thus, they are potential to help a therapeutic agent entering the cell rapidly.

4. Conclusion

In this study, PEG-CHEMS and F-PEG-CHEMS were synthesized for preparation of PEGylation liposomes (L-PEG-BAI) and FR-targeted liposomes (L-FA-BAI). Formulations contain the liposomes with appropriate diameter around 80 nm and a significant fraction of small unilamellar vesicles (20–30 nm). The liposomes exhibited a high colloidal stability during storage. L-FA-BAI exhibits high drug loading and FR-targeting properties. The enhancement in cytotoxicity exhibited by L-FA-BAI was about 1.4 times (IC50 value) than the non-targeted control (L-BAI and L-PEG-BAI) and 1.1 times than pure BAI solution. Moreover, L-FA-BAI (demonstrated using L-FA-RB) showed a much higher cellular uptake than non-targeted liposome, which also can explain the higher cytotoxicity of L-FA-BAI compared to L-PEG-BAI. This liposome nanotechnology has potential use in Chinese medicine formulation. In vivo evaluations of BAI loaded FR-targeted liposome formulations are the next rational extension of this work.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2015.11.018.