

The Preparation of Cationic Folic Acid and Its Application in Drug Delivery System^{*}

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Abstract The cationic folic acid (CFA) was prepared by introducing triethylenetetramine into folic acid with EDCI/NHS and characterized by IR, NMR and mass spectra. It was found that approximately one of two carboxyls in the folic acid molecule was substituted to form CFA. The conversion of γ -carboxyl is found to be 59% higher than 30% of α -carboxyl. The CFA and doxorubicin hydrochloride can be loaded on the ionic shell of PTX-encapsulated micelle to form CFA loaded binary drug carrier *via* static interaction in aqueous solutions. The successful loading was demonstrated by zeta potential measurement and the drug load amount (DLA) of CFA was measured by HPLC. *In vitro* cytotoxicity results revealed the CFA drug carrier showed higher cytotoxicity to cancer cell MDA-MB-321 than the binary drug carrier without CFA and the positive control, while it showed lower cytotoxicity to normal cell HUVEC than the positive control, and similar cytotoxicity with the binary drug carrier without CFA. These results as well as confocal laser scanning microscopy observation indicate the synthesized CFA drug carrier possesses active tumor-targeting property.

Keywords: Folic acid; Cationize; Tumor-targeting; Drug carrier; Cytotoxicity.

INTRODUCTION

Chemotherapy plays an important role in anti-tumor treatment till now, but it still remains some challenges^[1–3]. First, most anti-tumor drugs are hydrophobic and difficult to be absorbed by human body leading to low bioavailability. Secondly, the drug usually had high cell cytotoxicity and killed the tumor cell and normal cell without selectivity. However, the polymer based drug delivery systems including micelle, vesicle, nanogel, macromolecular drug precursor and so on show significant potential for solving these problems^[4–6]. For example, polymer micelles can increase bioavailability *via* encapsulating drugs in their hydrophobic core, and achieve sustainable drug release *via* diffusion effect, so that it can increase the pharmaceutical effect, depress the side effect, and ease the pains of the patients in chemotherapy. Furthermore, it is also possible for polymers to endow tumor targeting ability which can orientationally deliver drugs to tumor sites so as to kill tumor cells more effectively *via* some chemical modification. Thus, polymer based drug carrier was considered as promising candidate for the future drug delivery system^[7–9].

These progresses also encourage the scientists to develop various smart drug carriers with different functions in recent years^[10–13], and among these, tumor-targeting ability aroused many interests^[14–17]. The tumor

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targeting ability is usually divided into active and passive tumor targeting ability. The passive tumor targeting is based on EPR (enhanced permeability and retention) effect^[17], which pointed out that the micelle with certain diameter ranges (50–200 nm) can leak from the blood vessel and accumulate in the tumor site during drug's *in vivo* circulation because the blood vessels of tumor tissue usually have a large pore size than normal blood vessels. On the other hand, because some special antigens were rarely expressed on normal cells but over-expressed on tumor cells, some micelles modified with their corresponding antibody are able to selectively accumulate on tumor site *via* specific antigen-antibody interaction. Folic acid (FA) is a typical antibody for active tumor targeting application^[15, 16] because FA receptor was widely distributed and highly expressed on various tumor cells including uterus, breast, brain, lung and kidney cancer cells and hardly expressed on normal cells. Furthermore, it was also reported that the FA molecules covalently bounded on the micelles still retained its ability to specifically combine with FA receptor as same as free FA molecules.

However, it's easy for polymer drug carrier to get passive tumor targeting ability since the micelle's diameter can be easily controlled by adjusting its component and composition. But in case of the incorporation of active tumor targeting ability, it usually needs tedious synthetic strategy. Thus, it remains a challenge for polymer micelles to incorporate active tumor targeting ability *via* simple and mild methods. In our previous work^[18], a novel binary-drug loaded micelle based on PCL (poly(ϵ -caprolactone))-heparin conjugate where the hydrophobic paclitaxel (PTX) can be encapsulated in the core and hydrophilic doxorubicin hydrochloride (DOX·HCl) can be absorbed on the shell was prepared. Moreover, the pH sensitive release behavior of DOX·HCl was found, and the micelle size was also controlled around 100 nm which is consistent with the requirement of EPR effect. These advantages as well as easy preparation showed great potential for PCL-heparin conjugate micelles as smart drug delivery systems. However, this micelle is still lack of active tumor targeting ability.

In this paper, a simple strategy that can incorporate active tumor targeting ability very easily was carried out. First, cationic folic acid (CFA) was prepared by covalently connecting with triethylenetetramine *via* EDCI/NHS because triethylenetetramine is easy to be cationized even at physiological environment. Then, CFA was further absorbed onto the shell of PCL-heparin conjugate micelle as same as DOX·HCl. Finally, the *in vitro* cytotoxicity results showed absorbed CFA still retained significant tumor targeting ability.

EXPERIMENTAL

Materials and Measurements

Paclitaxel (PTX) and folic acid were both bought from Shanghai Jinhe Biotech Co., Ltd, and doxorubicin hydrochloride (DOX·HCL) was purchased from Beijing Huafeng United Technology Co., Ltd. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI), *N*-hydroxysuccin-imide (NHS) were both bought from Sigma-Aldrich, USA and used as received. The breast cancer cell MDA-MB-321 and human umbilical vein endothelial cell (HUVEC) were purchased from Jiangyin Kangzhongkangming Bio-tech Ltd. Company, Jiangsu, China. HPLC grade methanol and acetonitrile were bought from Shanghai Anpu Chemical Company. All of other chemical reagents and solvents used were of analytical grade.

FTIR measurement was conducted on a Shimadzu IR Prestige21 spectrometer and ¹H-NMR was carried on Bruker ARX 400 apparatus with TMS as internal standard. MS spectrum was measured by a Micromass Quattro Premier XE instrument. UV spectra were measured on a HITACHI U-280 UV-Vis spectrometer. Zeta potential and the average micelle diameter were measured on a NANO ZS ZEN3600, Malvern Instruments, and the micelle morphology was observed on a JEM-3010 Transmission Electron Microscope. The dynamic laser scattering (DLS) measurement was carried on a D-63225 Langen/Hessen Laser Goniometer System, Germany. Confocal laser scanning microscopy (Leica TCS-SP5) was used to visualize the cellular uptake and intracellular distribution of CFA loaded micelle.

The Synthesis of Cationic Folic Acid (CFA)

1 g FA was dissolved in 100 mL DMF and 260 mg NHS (0.0025 mol) and 440 mg EDCI (0.0025 mol) were added into the DMF solution successively and stirred for 30 min under ice-water bath. After then, 0.643 g trethylenetetramine (0.0044 mol) was also added into the solution and reacted for 24 h under room temperature. After the reaction, 100 mL deionized water was added to form pale yellow precipitate in the solution. The precipitate was collected by filtration and washed by water and acetone three times respectively. Then, it was dissolved in 0.1 mol/L HCL solution and the solution was lyophilized to get CFA.

The Encapsulation of PTX

The synthetic protocol of PCL-heparin conjugate was reported in our previous publications^[19–22]. The conjugate (10 mg) and PTX (10 mg) were dissolved in 10 mL mixture solution (THF:water = 1:3, vol). The solution was under ultrasonic for 10 min. Then the solution was placed in a dialysis bag (MWCO 3500) and dialyzed with distilled water for 24 h. After dialysis, the precipitated PTX was removed from solution by centrifuge (4000 r/min, 5 min). Finally, the PTX loaded nanoparticles were obtained by lyophilization.

The amount of loaded PTX in the micelle was measured by HPLC (SHIMADZU LC-30A, Diamonsil TM C18 column (250 mm × 4.6 mm, 5 μm)) with the mixing elution of methanol and distilled water (80:20, volume ratio) at 30 °C. The flowing rate was set at 1 mL/min and the UV detector with wavelength of 227 nm was used. First, the standard curve of PTX was set by measuring pure PTX samples with different concentrations (1–100 μg/mL). Then, 3 mg lyophilized micelle was dissolved in 1 mL water and diluted to 50 mL by methanol in volumetric flask. The solution was under ultrasonic for 30 min and filtrated with 0.22 μm filter membrane before HPLC measurements. Drug loading content (DLC) and drug loading efficiency (DLE) were calculated according to the following equations.

$$\text{DLC (wt\%)} = \frac{\text{weight of loaded drug}}{\text{weight of polymer}} \times 100 \quad (1)$$

$$\text{DLE (wt\%)} = \frac{\text{weight of loaded drug}}{\text{weight of drug in feed}} \times 100 \quad (2)$$

The Loading of CFA and DOX

2 mL aqueous solution (1 mg/mL) of DOX·HCL with predetermined CFA according to Table 1 was added into 20 mL aqueous solution (1 mg/mL) of PTX-loaded nanoparticles slowly. The mixed solution was stood without stirring at room temperature for 4 h. Thereafter the solution was transferred into dialysis tubing (MWCO 3500) and dialyzed against distilled water for 12 h. The CFA loaded micelle was finally obtained by lyophilization. The DLE and DLC of DOX·HCL and FA can be measured by UV spectrometer and HPLC, respectively. The HPLC measurement was carried on Diamonsil TM C₁₈ column (250 mm × 4.6 mm, 5 μm) with the mixture elution of sodium dihydrogen phosphate solution (pH = 2.5, adjusted by phosphoric acid) and acetonitrile (50:50, vol) at the flowing rate of 1.0 mL/min under 30 °C.

Table 1. The feed amount of binary drug loaded micelle

Sample No.	PTX loaded micelle (mg)	DOX (mg)	Cationized FA (mg)
1	20	2	0
2	20	2	0.1
3	20	2	0.4

Evaluation of In vitro Anti-tumor Cytotoxicity

The cytotoxicity was studied using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium-bromide (MTT) assay. MDA-MB-321 and HUVEC cells were used respectively and the cells (8 × 10⁴ cell/well) were seeded in 96-well plates and incubated in DMEM media containing 10% FBS at 37 °C in 5% CO₂/95% air atmosphere for 24 h. The media were replaced with fresh media containing samples and incubated for 48 h. Thereafter, MTT solution was added. The plates were incubated at 37 °C for another 4 h. The intracellular metabolized product MTT formazan was then retrieved by an addition of dimethylsulfoxide (DMSO). The plates were read at 550 nm

by Microplate Reader (Thermo MK3), and the cell viability (CV) was calculated according to the following equation.

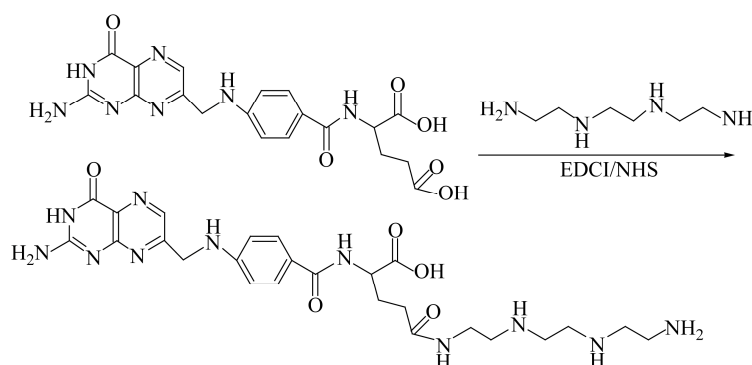
$$CV\% = \frac{E_a}{C_a} \times 100\% \quad (3)$$

where E_a and C_a are averaged absorptions of the experimental group and the control group, respectively.

RESULTS AND DISCUSSION

The Preparation and Characterization of CFA

Polyethyleneimide (PEI) is a typical cationic polymer which is very effective in gene delivery but its high cytotoxicity prevents its further application in biomedical field. However, the PEI oligomer such as triethylenetetramine was thought to be non-cytotoxic and suitable for preparing CFA. As depicted in Scheme 1, triethylenetetramine was incorporated into the FA *via* the reaction between amine and the carboxyl groups. However, since there are two carboxyls (α and γ) in FA, the feed ratio of triethylenetetramine to FA should be controlled around 1:1 in order to get single substituted FA as the predominant product.



Scheme 1 The synthetic route of CFA

Figure 1 shows the IR spectra of FA and CFA. The amide characteristic peaks at 1690 cm^{-1} and 1610 cm^{-1} and the amine peak at 3400 cm^{-1} were both observed on the IR spectra of FA and CFA. However, the amine peak of CFA became much broader compared with that of FA. The broadening of the peak resulted from the formation of large number of hydrogen bonds due to the incorporation of triethylenetetramine, and this implied the successful introduction of triethylenetetramine into FA molecules.

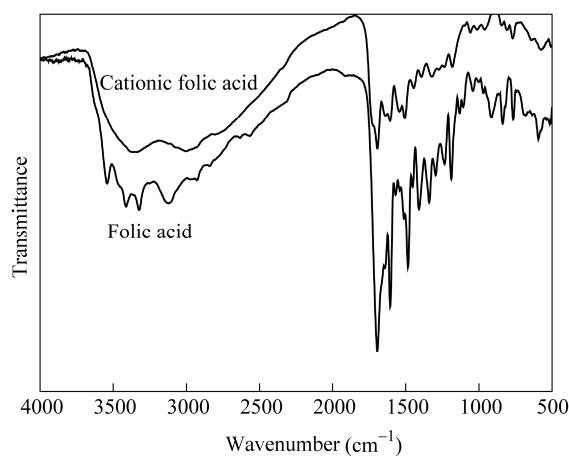


Fig. 1 The IR spectra of CFA

The NMR spectra of FA and CFA are available in Fig. 2. Compared with FA, some resonance peaks corresponding to triethylenetetramine including methylene group at $\delta = 2.5\text{--}3.5$, primary and secondary amine groups at $\delta = 4.0\text{--}6.0$ and the amide groups at $\delta = 8.0\text{--}9.0$ occurred, which indicated the incorporation of triethylenetetramine. Furthermore, two methylene peaks (g and h) adjacent to carboxyl in FA split from one to two peaks after reaction. It was obvious because both carboxyls were partially substituted by triethylenetetramine. And the conversion ratio of these two carboxyls can be calculated by integral ratio of their split peaks. It was found the conversion of α -carboxyl (g) is around 30% and that of γ -carboxyl (h) is about 59%. The conversion of γ -carboxyl is much higher than that of α -carboxyl, and this result is consistent with the report of literature that the reactivity of γ -carboxyl is higher than the one of α -carboxyl. In addition, FA carboxyl conversion was calculated as 89% by sum of two carboxyl conversion, and it means averagely approximate one carboxyl was substituted in one FA molecule. On the other hand, FA conversion can also be calculated according the changing of integral ratio of carboxyl hydrogen (peak j), and it was 91% almost as same as the result calculated from peaks g and h. Thus, it can be concluded that the predominant product of the reaction is single substituted CFA.

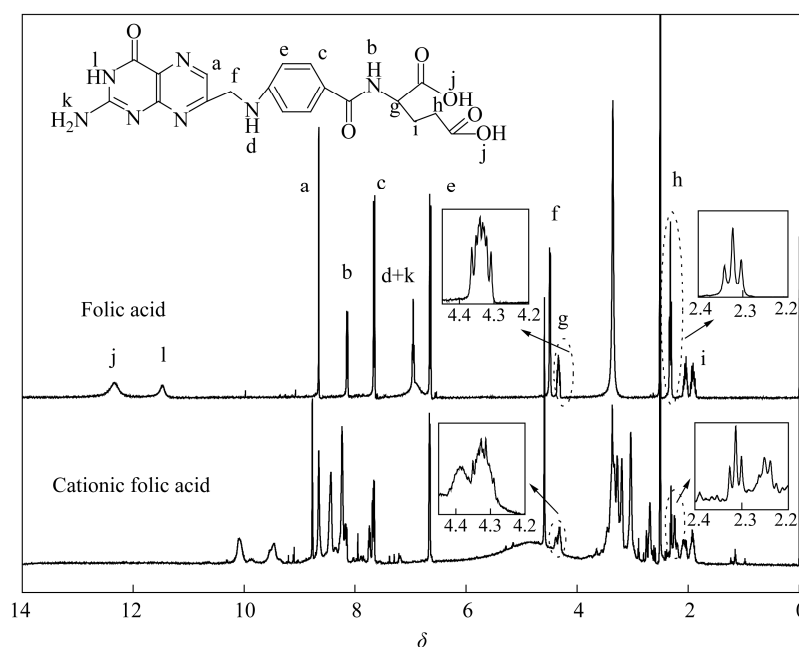


Fig. 2 NMR spectrum of CFA

The MS spectrum of CFA is depicted in Fig. 3. The above NMR results demonstrated that the predominant product is single substituted CFA and its molecular weight was calculated as 569.29 g/mol, whereas the molecular weight of double substituted CFA should be 697.31 g/mol. The molecular ion peak with MW = 570.2895 corresponding to M+1 peak of single substituted product was found in Fig. 3, while no peak corresponding to double substituted product can be found. Thus, this result further confirmed the predominant product of the reaction is single substituted CFA.

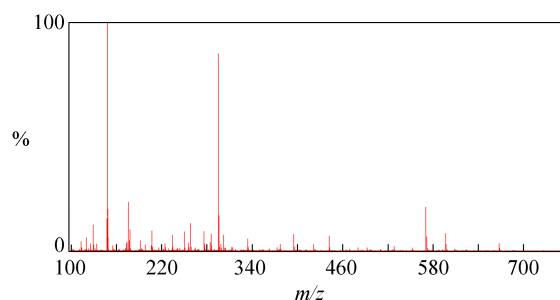


Fig. 3 The mass spectrum of CFA

The Preparation and Characterization of CFA Loaded Micelles

The PTX-loaded micelles can be prepared *via* dialysis according to our previous literature. Since the shell of PTX-loaded micelles was made from anionic heparin, it can be used to absorb various cationic substances. Thus, the CFA and cationic DOX·HCL were both absorbed on the shell *via* static interaction so as to prepare binary-drug loaded micelles with tumor targeting ability. As shown in Table. 2, three samples with different feed amounts of FA and DOX·HCL were prepared in this paper. The DLC of FA can be measured by HPLC and Fig. 4 was the standard curve of FA showing HPLC method is suitable for FA measurement. It was concluded from Tables 1 and 2 the loading amount of FA can be controlled by its feed amount while the content of PTX and DOX in the micelle were kept as constant. Thus, it is possible to evaluate the presence of tumor targeting ability by *in vitro* cytotoxic measurements of these three samples.

Table 2. The DLC and DLE of micelles

	DLC (%)			DLE (%)			Zeta potential (mV)
	PTX	DOX	FA	PTX	DOX	FA	
PTXC*	N/A	N/A	N/A	N/A	N/A	N/A	−35.1
BDC**	1.63	5.58	0	1.11	61.40	0	−9.1
BDC-FA1	1.61	5.43	0.16	1.07	59.75	34.9	−9.4
BDC-FA2	1.70	5.66	0.60	1.21	62.23	33.3	−10.7

* PTX-loaded drug carrier

** Binary drug loaded carrier

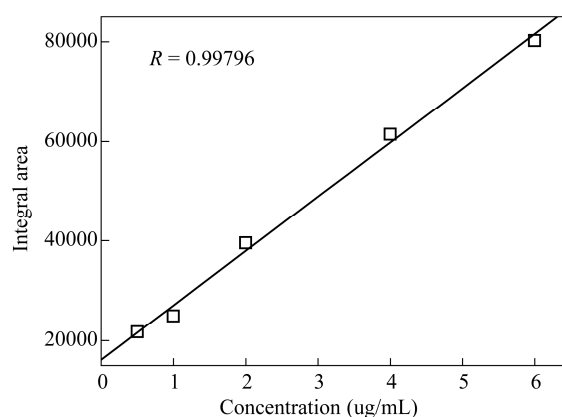


Fig. 4 The HPLC standard curve of CFA

In addition, the results of zeta potential measurements before and after DOX and CFA absorption are also listed in Table 2. Before absorption, the micelle's zeta potential was around −35.1 mV indicating the composition of its heparin shell. After absorption, all the zeta potential of three samples decreased to about −9 or −10 mV due to the neutralization effect of absorbed CFA and DOX. The micelle's TEM picture after absorption

is shown in Fig. 5. It was seen that the morphology of micelles appeared as slightly irregular spheres with the diameter ranging from 50 nm to 100 nm inferring the presence of EPR effect. The DLS result is shown in Fig. 6 which appears as a single peak with the diameter of around 120 nm. This result is well consistent with TEM observation. Thus, if the active tumor targeting ability can be proven in the following cytotoxicity measurement, the prepared micelle herein is expect to be able to assemble both active and passive tumor targeting ability simultaneously.

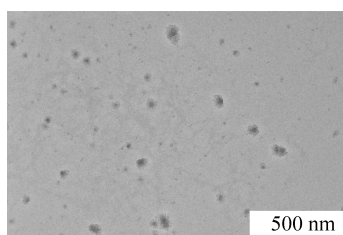


Fig. 5 The TEM picture of CFA loaded micelles

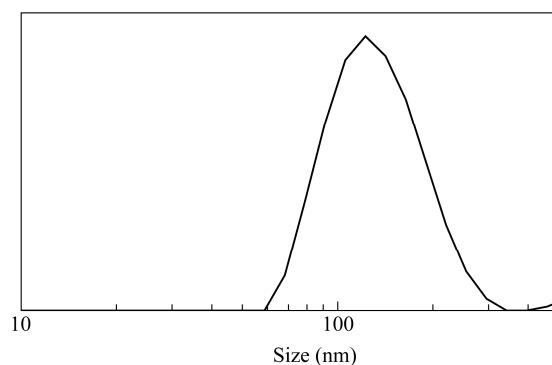


Fig. 6 The DLS results of CFA loaded micelles

The In Vitro Release Test of CFA

The CFA release profiles are shown in Fig. 7. It shows only very tiny release amount can be found under physiological circumstance ($\text{pH} = 7.4$) whereas large release amount is detected on acid circumstance. This release profile is similar with the profiles of DOX reported in our previous literature. It implied the static interaction between CFA and the shell is stable under physiological circumstance. Thus, it can be expected that the absorbed CFA will keep stable on the shell and is able to target the micelle onto the tumor site during the *in vivo* circulation.

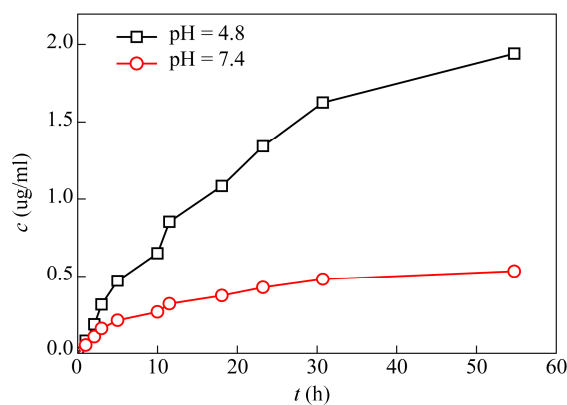


Fig. 7 The *in vitro* release profile of CFA

The In Vitro Cytotoxic Test

The *in vitro* cytotoxic test was measured with MDA-MB-321 as cancer cells and HUVEC as normal cells, and the results are illustrated in Figs. 8 and 9. Figure 8 was the cancer cell survival rate of three micelle samples under different micelle concentrations. It shows that when the content of PTX and DOX were almost same in three micelle samples, the two samples loaded with CFA presented the similar and highest cytotoxicity against tumor cells, the positive control (the combination of free PTX and DOX) showed medium cytotoxicity and the binary-drug loaded micelle without CFA possessed the lowest cytotoxicity. It is normal that the cytotoxicity of positive control is larger than that of the corresponding drug-loaded micelle because the release of drug from the micelle is restricted by the diffusion effect of the polymer. However, the phenomenon that CFA loaded micelle's cytotoxicity became larger than positive control clearly inferred the presence of active tumor targeting ability in CFA loaded micelle. On the other hand, the normal cell survival rates of the three samples are illustrated in Fig. 9. The cytotoxicity of binary-drug loaded micelle without CFA was still lower than that of the positive control, but the two CFA loaded micelles also showed lower cytotoxicity than the positive control. This indicated CFA loaded micelle didn't show any targeting ability to normal cells. In other word, it showed according to the results in Figs. 8 and 9 that CFA loaded micelles can selectively kill the tumor cells. Consequently, the active tumor targeting ability was successfully introduced by absorption strategy in this paper.

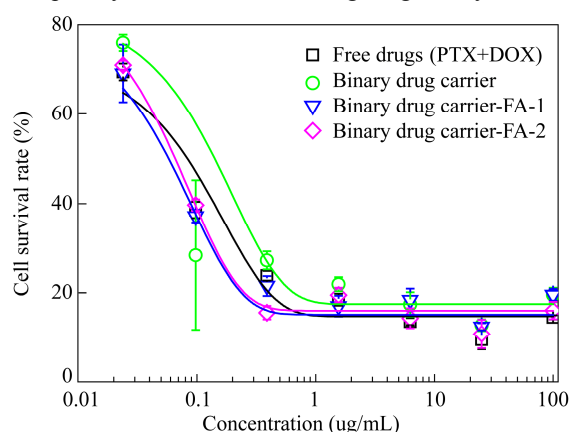


Fig. 8 The cytotoxicity against cancer cells

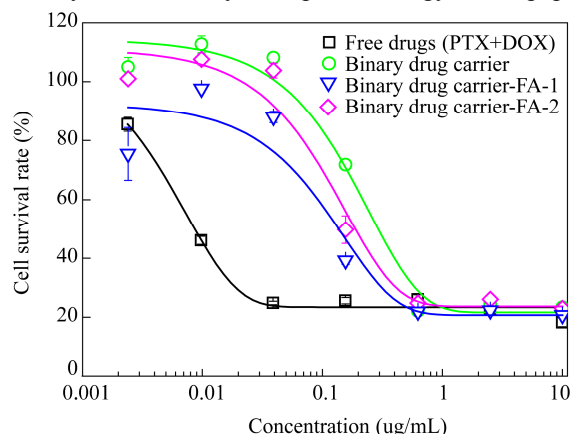


Fig. 9 The cytotoxicity against normal cells

In addition, although CFA loading amount was different in two CFA loaded micelles in Figs. 8 and 9, their cytotoxicity against both cancer and normal cells was almost the same. This implied the increasing of CFA amount didn't contribute to increasing the active tumor ability of the micelle within the concentration range used in this paper. And for PCL-heparin conjugate micelle, the largest absorption amount actually was restricted by the total amount of anionic charge of heparin shell. Thus, the lower the FA was loaded, the higher DOX might be loaded onto the micelle.

The Confocal Laser Scanning Microscopy (CLSM) Observation

The cellular uptake and intracellular distribution of CFA loaded micelles were observed by CLSM. Because the low fluorescence property of CFA as well as its low content, it's difficult to observe the CFA directly. PTX also cannot produce fluorescence for CLSM measurement, thus the fluorescence of DOX (red light) was used to indicate the intracellular distribution of CFA loaded micelles, and the CLSM images are shown in Fig. 10. It was seen that DOX mainly occurred in cytoplasm after 1 h incubation. Subsequently, DOX was found to be distributed in both cytoplasm and nucleus after 2 h incubation and more DOX was found to enter the nucleus after 24 h incubation. These results revealed CFA loaded micelle is able to target tumor cell, localize and release the drugs inside the tumor cell. According to previous pharmacodynamic study, both DOX and PTX take their effects in nucleus to kill tumor cells. The former interrupts DNA replication process in the nucleus, and the latter is able to prevent cell division by stabilizing cellular microtubules in the nucleus. Therefore, the localization of micelles inside the cell nucleus is highly beneficial for their pharmacodynamic effects.

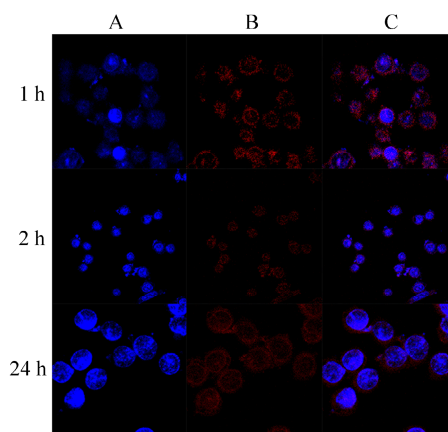


Fig. 10 The CLSM observation of CFA loaded micelles: (A) blue fluorescence from Hoechst 33342 stained nuclei, (B) red fluorescence from DOX and (C) merged channels of A and B

CONCLUSIONS

The cationic folic acid was prepared by incorporating triethylenetetramine into folic acid. The CFA can be absorbed onto the shell of PCL-heparin conjugate micelles *via* static interaction in aqueous solutions. The zeta potential measurement proved the successful incorporation of CFA *via* static interaction, and the stability of absorbed CFA was demonstrated by its *in vitro* release profile. When the content of PTX and DOX was the same, the CFA loaded micelles possessed higher cytotoxicity against cancer cell than the positive control and the micelles without CFA, while they showed lower cytotoxicity than positive control and similar cytotoxicity with the micelle without CFA against normal cells. This as well as CLSM observation implied the presence of active tumor targeting ability of CFA loaded micelle. The prepared CFA and binary drug loaded micelle has a promising potential as anti-tumor drug carrier since it not only can load two different anti-tumor drugs, possess pH sensitive release behavior but also assemble both active and passive tumor targeting ability in one micelle.

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