International Journal of Pharmaceutics 372 (2009) 125–131

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

9-NC-loaded folate-conjugated polymer micelles as tumor targeted drug delivery system: Preparation and evaluation in vitro

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ARTICLE INFO

Article history:
Received 23 August 2008
Received in revised form 12 December 2008
Accepted 29 December 2008
Available online 4 January 2009

Keywords:
Folate-conjugated polymer micelles
9-Nitro-camptothecin
In vitro
Anti-tumor
Avoid the macrophages
Folate receptor mediated

ABSTRACT

In this study, folate-conjugated polymer micelles were synthesized by mixing folate-poly(ethylene glycol)-distearoylphosphatidylethanolamine (FA-PEG-DSPE) and methoxy-poly(ethylene glycol)-distearoylphosphatidylethanolamine (MPEG-DSPE) to encapsulate anticancer agent 9-nitro-camptothecin (9-NC). Formulations were characterized by critical micellization concentration (CMC) values of copolymers, micelle particle size, zeta-potential, encapsulation efficiency and drug loading efficiency. The molar ratio of FA-PEG-DSPE and MPEG-DSPE was chosen to avoid the macrophages and at the same time express highly active targeting ability. The targeting ability of folate-conjugated polymer micelles was investigated against three kinds of tumor cell lines (HeLa, SGC7901 and BXPC3). The drug efficacy in vitro of folate-conjugated polymer micelles was evaluated by using the methylthiazolete-trazolium (MTT) method. The results showed that the CMC values of MPEG-DSPE and FA-PEG-DSPE were 0.97×10^{-5} M and 1.0×10^{-5} M, respectively. The average size of folate-conjugated micelle was about 21–24 nm and the micelle size distribution of both empty and drug-loaded micelles were rather narrow. The encapsulation efficiency and drug loading efficiency were 97.6% and 4.64%, respectively. The drug-loaded micelles were stable during storage at 4 °C for 4 weeks. Micelles maintain the similar size and did not show 9-NC leakage. The best molar ratio of FA-PEG-DSPE and MPEG-DSPE in folate-conjugated micelles was 1:100 which can effectively solubilize 9-NC, avoid the macrophages in vitro and has a higher anti-tumor activity than both drug-loaded MPEG-DSPE micelles and free anticancer agents. The folate-conjugated polymer micelle which can avoid the macrophages is a kind of promising carrier for poorly soluble anticancer agents via folate receptor (FR) that mediated endocytosis to target tumor cells.

1. Introduction

9-Nitro-camptothecin (9-NC) is a potent topoisomerase-I inhibitor, and it has been applied for clinical trials in cancer treatment. Pharmacological studies disclosed that the anti-tumor activity of 9-NC was superior to that of camptothecine (CPT) in human tumors xenografted in nude mice (Gao et al., 2008). The anti-tumor activity of 9-NC closely depended on its structure: the lactone form of 9-NC was important to its anti-tumor activity. However, the applications of 9-NC were limited due to its poor solubility, instability and low oral bioavailability (You et al., 2008; Zerrin et al., 2007; Ferrec et al., 2001). To address these concerns several novel delivery systems have been tried. Liposomes and polymeric micelles have been reported previously and have shown encouraging efficacy. Though liposomes as carriers are effective in cellular internalization of drugs, the size and stability of liposomes are limitations in applications (Gao et al., 2008; Kumaresh et al., 2001; Unezaki et al., 1995). Normally, the size of a liposome is more than 100 nm, which would mean that only a small fraction of the liposomes could aggregate in a solid tumor with enhanced permeability and retention (EPR) effect (Gao et al., 2008; Unezaki et al., 1995). The stability of liposomes is not suitable for long-time drug release; it would lead to burst release if the liposomes are broken. Besides liposomes, there are rare other carriers reported for 9-NC delivery (Sha and Fang, 2004). Recently, much attention has been focused on polymeric micelles.

Polymeric micelles such as biodegradable block copolymers with poly(ethylene glycol) (PEG) and aliphatic polyesters have been used as a potential carrier for a wide variety of drugs, due to their low toxicity, long circulation, solubilization, targeting and nano-size (You et al., 2008; Torchillin et al., 2003). In an aqueous phase, the amphiphilic block copolymers formed micelles self-assembly,

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doi:10.1016/j.ijpharm.2008.12.035

which have a hydrophobic core and a hydrophilic shell (Zerrin et al., 2007). The hydrophobic core serves as a reservoir for poorly soluble anticancer drugs and transports to the tumor cells. The biocompatible and hydrophilic PEG shells could help the polymeric micelles escaping from the reticuloendothelial system (RES), evading scavenging by the mononuclear phagocyte system (MPS) and protecting the polymeric micelles from phagocytizing by the macrophages, in order to achieve long circulation in blood (Maeda, 2000).

However, insufficient uptake at tumor sites will decrease the therapeutic benefit of the administered drug dose, and non-specific association with healthy tissues can lead to toxic side effects, limiting the maximum dosage that can be safely applied. This limitation prevents drug-loaded micelles from achieving the potential therapeutic effects they might otherwise attain. One strategy to achieve cancer-targeted drug delivery is the utilization of unique molecular markers that are specifically overexpressed within the cancerous tissues. It is well know that many malignant tissues, especially the ovary, nasopharyngeal, cervical and chorion carcinomas, consistently express high levels of folate receptors (FR), folate or folic acid has been widely used as a selective targeting moiety of various anticancer agents to specifically combine with the FRs (Zhao et al., 2008). Folic acid is a water soluble B vitamin, which is essential for de novo nucleotide synthesis and one-electron transfer reactions. A lot of studies have been reported on folate-mediated targeting of anticancer agents or genes by conjugating folic acid onto polymeric micelles, macromolecules, nanoparticles and liposomes. The most recent trend of folate targeting in the literature focuses on attaching folic acid to polymer micelles (Jones and Leroux, 1999; Torchilin, 2001, 2002; Torchillin et al., 2003; Lee et al., 2003).

In this study, the poorly soluble anticancer drug 9-NC was encapsulated in polymeric micelles (FA-M-9-NC), a mixture of folate-poly(ethylene glycol)-distearoylphosphatidylethanolamine (FA-PEG_{3350}-DSPE) and methoxy-poly(ethylene glycol)-distearoylphosphatidylethanolamine ($MPEG_{2300}$-DSPE) with different molar ratio. And the best molar ratio (1:100) was selected by avoiding the macrophages experiments and anti-tumor cell experiments. The targeting ability of FA-M-9-NC was investigated against three kinds of tumor cell lines: HeLa, SGC7901 and BXPC3. The drug efficacy in vitro of FA-M-9-NC was investigated by comparing with the 9-NC-loaded folate-free micelles (M-9-NC) and free 9-NC.

2. Materials and methods

2.1. Materials

For the synthesis of polymeric micelles, poly(ethylene glycol)-bis-amine (PEG-bis-amine, Mw: 3350), methoxy-poly(ethylene glycol) (MPEG-amine, Mw: 2300), Succinic anhydride (SUC), dicyclohexylcarbodiimide (DCC) and 4-dimethylamino pyridine (DMAP) were purchased from Sigma–Aldrich. Folate was purchased from Shanghai Shisheng Biotechnology Co., Ltd. All the other chemicals and components for buffer solutions were HPLC grade preparations.

For cell culture experiments, RPMI 1640 medium, folate-free RPMI 1640 medium, DMEM medium and Tripsin–EDTA were purchased from Gibco Co., USA. Fetal bovine serum (FBS) and bovine calf serum (BCS) were purchased from Hangzhou Si Jiqing Biological Engineering Materials Co., Ltd. The human pancreatic cancer cell line (BXPC3) was kindly provided by Huashan Hospital; the human uterine cervix cancer cell line (HeLa) was purchased from the cell bank of Chinese Academy of Science and the human gastric cancer cell line (SGC7901) was provided by Shanghai Cancer Institute.

2.2. Synthesis of FA-PEG-DSPE and MPEG-DSPE

The synthesis of FA-PEG-DSPE followed a three-step reaction: first, to synthesize FA-PEG-amine, FA, NH_2-PEG-NH_2, SUC, DCC and DMAP were dissolved in anhydrous dimethyl sulfoxide (DMSO), respectively, and then added sequentially. The reaction mixture was stirred for about 24 h at room temperature in the dark, and then added in deionized water (DH_2O) to terminate the reaction. The mixture was dialysed to remove DMSO and further purified by a DEAE-sepharose anion-exchange column (26 mm × 10 cm, Biosciences, Uppsala, Sweden) on a ÄKTA explorer 100 system (Amersham Biosciences, Uppsala, Sweden). The purity of FA-PEG-amine was detected by HPLC analysis (Agilent 1100 Series, Palo Alto, CA, USA). Second, FA-PEG-amine, DCC, SUC and DMAP were dissolved in dimethylformamide (DMF). The mixture was stirred for about 5 h at room temperature in the dark. The mixture was purified through Gel-filtration Chromatography (GFC) (Biosciences, Uppsala, Sweden) to remove the excess SUC. The purity of FA-PEG-SUC was also detected by HPLC analysis. Finally, to synthesize FA-PEG-DSPE, FA-PEG-SUC and DSPE reacted in $CHCl_3$ for about 24 h at 55 °C, dried on a rotary evaporation in a round bottom flask and then placed under vacuum over night to remove any traces of remaining solvent. The dried mixture was dissolved in absolute ethanol and centrifuged to remove excess DSPE. The final product FA-PEG-DSPE was a yellow dry powder and the purity quotient was detected by HPLC analysis.

The synthesis of MPEG-DSPE followed a two-step reaction as step two and step three described above.

2.3. Cytotoxicity of materials assay

The cytotoxicity of FA-PEG-DSPE and MPEG-DSPE was evaluated by using the methylthiazoletetrazolium (MTT) method. Briefly, HeLa cells were plated at 7.5×10^3 cells per well density in 96-well plates. After 24 h incubation at 37 °C, 5% CO_2, the medium was replaced with 200 μl medium containing empty FA-PEG-DSPE and MPEG-DSPE micelles, respectively, at equal molar concentration ranging from 0.2 μM to 20 μM. After additional 24 h incubation at 37 °C, 5% CO_2, each well was added with 20 μl MTT solution (the concentration of MTT solution was 5 mg/ml). After 4 h incubation at 37 °C, 5% CO_2, each well was replaced with 200 μl DMSO. The cell viability was determined by measuring the absorbance at 490 nm using an ELISA reader (PowerWave XS, Bio-Tek, USA).

2.4. Preparation of drug loading micelle

The drug-loaded folate-conjugated micelles were successfully prepared by film formation method. To obtain 9-NC-loaded micelles, 0.5 mg 9-NC dissolved in acetonitrile was added to 10 mg FA-PEG-DSPE/MPEG-DSPE (molar ratio = 1:100) solution in 5 ml chloroform. The organic solvents were removed by the rotary evaporation to form a thin film of drug/micelle material mixture. This film was further dried under high vacuum overnight to remove any traces of remaining solvent. The dried film was hydrated in 5 ml 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES)-buffered saline (HBS) (pH 5.5). The mixture was incubated in water bath at 37 °C for 20 min. Non-incorporated 9-NC was separated by filtration through a 220-nm membrane (Fig. 1).

2.5. Characterization of micelles

2.5.1. Micelle size determination

Micelle size was measured by dynamic light scattering (DLS) using NICOMP 380 ZLS Zeta-potential/Particle System (PSS. Nicomp, Santa Barbara, CA, USA). The stability of the micelles was monitored by the changes in particle size in the samples of 9-

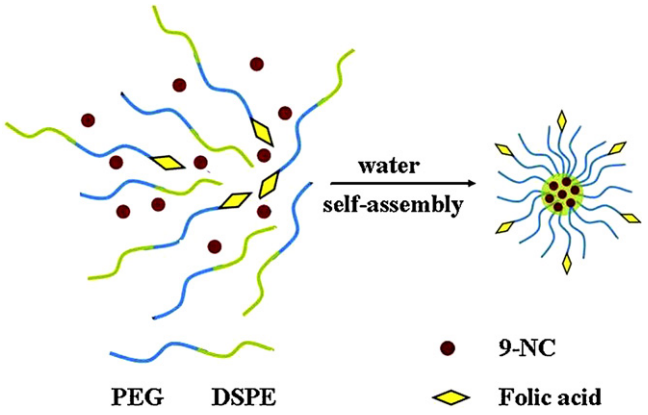

Fig. 1. Schematic film formation method of 9-NC-loaded folate-conjugated micelles.

NC-loaded micelles during the storage period. To test the storage stability, 9-NC-loaded micelles were stored in the dark at 4 °C for 1 month. The samples were diluted in HBS buffer (pH 5.5) by 1000-fold and analyzed for the presence of micelles and their size by the DLS.

The morphological examination of micelles was performed using a transmission electron microscope (TEM, Philips CM120, Netherlands). Briefly, a drop of micellar solution was placed on a copper grid and observed at 80 kV in the electron microscope.

2.5.2. Zeta-potential measurement

Zeta-potential of micelle formulation was measured by Zeta Phase Analysis Light scattering (PALS) using NICOMP 380 ZLS Zeta-potential/Particle System. For each sample, zeta-potential measurement was repeated 5 times.

2.5.3. Critical micelle concentration (CMC) determination

CMC was estimated by the Pyrene method (Zerrin et al., 2006). A series of MPEG-DSPE dissolved in chloroform (0.002–0.04 mg/ml) were added into clean, dry test tubes and each one of the tubes was added with 0.014 mg/ml pyrene. The mixtures were blown with nitrogen to remove most of the chloroform and then were further dried under high vacuum overnight to remove any traces of remaining solvent. The dried mixtures were hydrated in 1 ml HBS (10 mM, pH 7.4) and shaken in dark for 24 h at 25 °C. The undissolved pyrene was separated by filtering through 220 nm membrane filters. The concentration of solubilized pyrene in micellar phase was determined spectrofluorometrically at wavelengths of excitation 350 nm and emission 440 nm (LS55 luminescence spectrometer, PerkinElmer).

The CMC determination of FA-PEG-DSPE was similar as the method described above.

2.5.4. Encapsulation efficiency and drug loading

The amount of 9-NC in the micellar phase was measured by the reversed phase-HPLC. The micelle solution before filtration and after filtration through a 220-nm membrane was added with

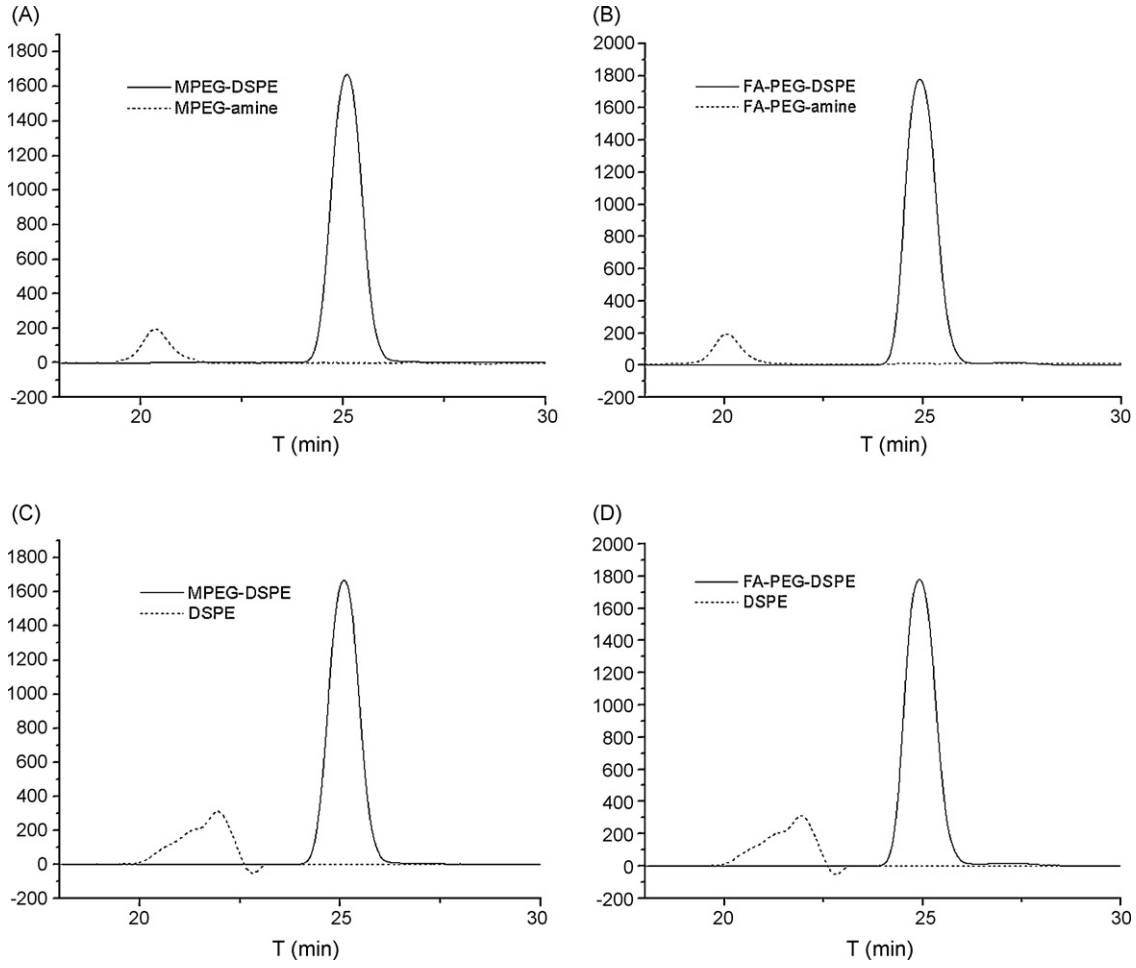

Fig. 2. HPLC analysis of FA-PEG-DSPE and MPEG-DSPE. Tosoh TSK-GEL G4000PWXL 300 mm × 7.8 mm, 300 Å, 10 μm; 20/80 acetonitrile/water containing 0.1 mol/l NaCl (A and B); Dikma RP C18 column 150 mm × 4.6 mm, 5 μm; 94/5/1 methanol/tetrahydrofuran/0.17 M ammonium acetate buffer solution (C and D).

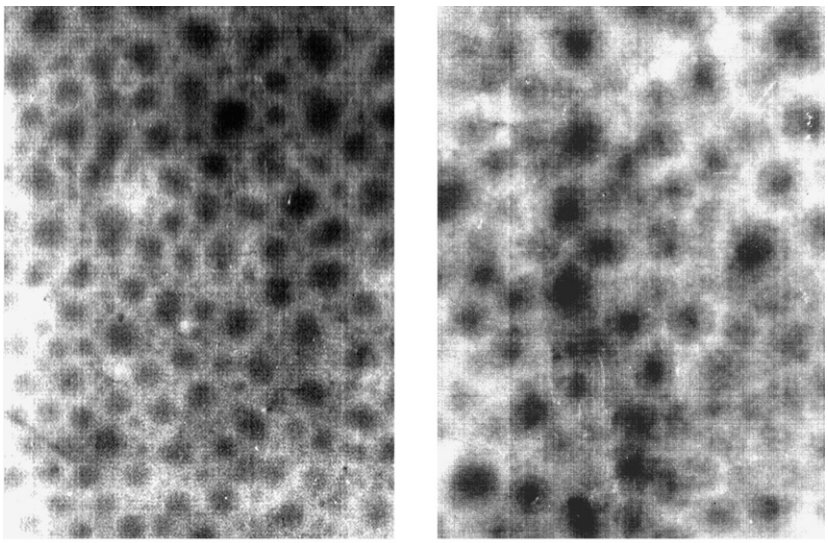

Fig. 3. Transmission electron microscope photograph of drug-loaded folate-conjugated micelles. (A) Empty micelles and (B) FA-M-9-NC.

mobile phase, respectively. Since the mobile phase contained acetonitrile, micelles were disrupted and free 9-NC was determined. The drug content of the micelle solution before filtration was the initially added drug amount and the one after filtration was the drug amount which incorporated into micelles. The HPLC system equipped with a UV detector and Dikma RP C18 column (4.6 mm × 150 mm) was used. The column was eluted with acetonitrile/water/fomic acid (45/55/0.169, v/v/v) at 1.0 ml/min. 9-NC was detected at 368 nm. All samples were analyzed in triplicate.

The encapsulation efficiency was evaluated by the percentage of the drug amount which incorporated into micelles with respect to the initially added drug amount. The drug loading efficiency was expressed as the percentage of the extracted drug amount from micelles with respect to the total amount of drug-loaded micelles.

To test the stability of the drug-loaded micelles which were stored for 1 month, encapsulation efficiency and drug loading efficiency were evaluated by the HPLC method described above.

2.6. Cell culture

BXPC3 cell line, SGC7901 cell line and HeLa cell line were maintained in RPMI1640 cell culture medium with 10% heat-inactivated BCS at 37 °C, 5% CO_2. Macrophages were maintained in DMEM cell culture medium with 10% heat-inactivated FBS at 37 °C, 5% CO_2.

2.7. Molar ratio of FA-PEG-DSPE and MPEG-DSPE selection

PEG could protect the polymeric micelles from phagocytizing by the macrophages and folate molecules have targeting ability. The targeting ability of folate-conjugated micelles relies on the specific binding of folate and FRs overexpressed on cancer cells (Zhao et al., 2008). If few folate molecules are on the surface of the micelles, it may not be sufficient for FR recognition. However, if too many folate ligands are present, the folate-conjugated micelles could be eliminated by the macrophages. So it is important to choose a suitable molar ratio of FA-PEG-DSPE and MPEG-DSPE to avoid the macrophages and at the same time express highly active targeting ability. We use flow cytometry (FCM) (FACSCalibur, Becton Dicknson, USA) on macrophages with Rhodamine-123 (Rh-123)-containing folate-conjugated micelles (FA-M-Rh-123) of different molar ratio of FA-PEG-DSPE and MPEG-DSPE (0:100, 1:100, 2:100, 5:100 and 10:100) to find out from which type of molar ratio, the Rh-123-containing folate-conjugated micelles cannot be phagocytized by the macrophages.

Meanwhile, 9-NC-loaded micelles and HeLa cell line were used to find out the relationship between the survival rate of the cancer cells and the molar ratio of FA-PEG-DSPE and MPEG-DSPE (0:100, 0.1:100, 0.2:100, 0.5:100, 1:100, 2:100 and 5:100 as experimental groups). We also used folate acid inhibition group (added 10^{-3} M folic acid to FA-M-9-NC micelles medium which the molar ratio of FA-PEG-DSPE and MPEG-DSPE was the same as the experimental groups). The concentration of 9-NC in each group was 0.9 μg/ml. The survival rate was evaluated by using the MTT method. Briefly, HeLa cells were plated at 7.5×10^3 cells per well density in 96-well plates. After 24 h incubation at 37 °C, 5% CO_2, the medium was replaced with folate-free RPMI1640 medium containing 10% FBS. After 24 h incubation at the same condition, each well was replaced with the experimental groups and control groups. After additional 24 h incubation, each well was added with 20 μl MTT solution and 4 h later each well was replaced with 200 μl DMSO. The cell viability was determined by measuring the absorbance at 490 nm using an ELISA reader.

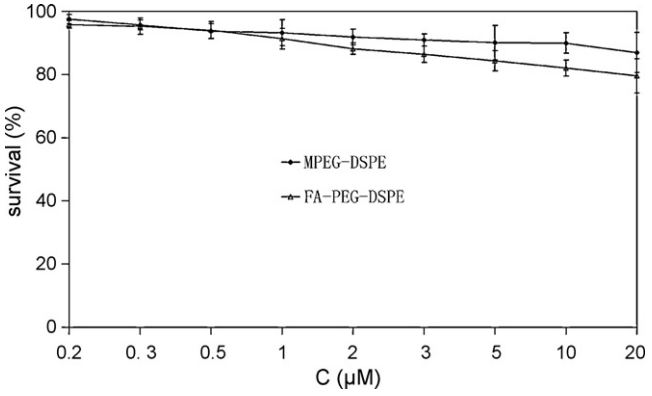

Fig. 4. 24 h cytotoxicity test results for MPEG-DSPE and FA-PEG-DSPE. Data were shown as mean ± S.D. ($n = 3$).

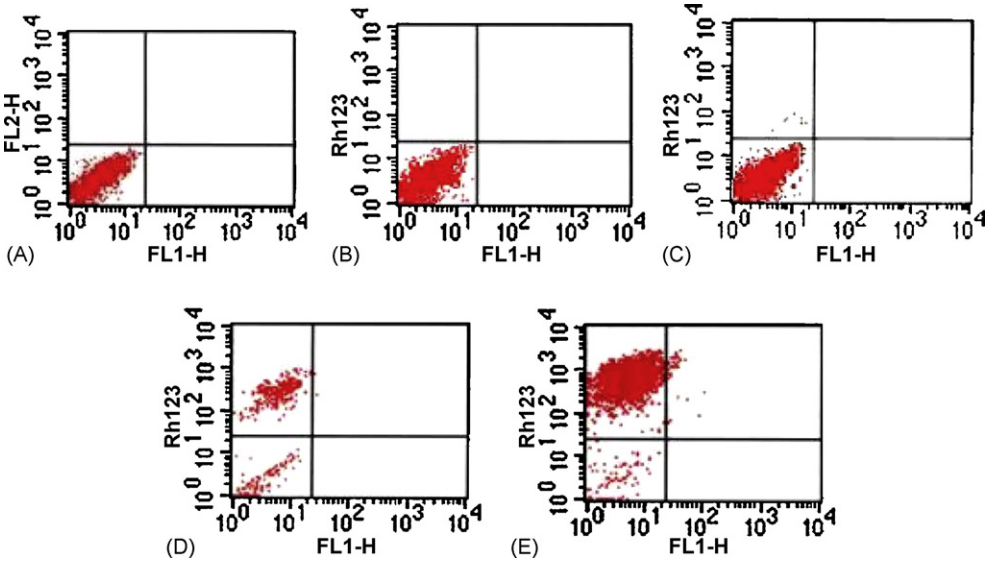

Fig. 5. FCM evaluation on macrophages with Rh-123-containing folate-conjugated micelles of different molar ratio of FA-PEG-DSPE and MPEG-DSPE: (A) only MPEG-DSPE; (B) FA-PEG-DSPE/MPEG-DSPE = 1:100; (C) FA-PEG-DSPE/MPEG-DSPE = 2:100; (D) FA-PEG-DSPE/MPEG-DSPE = 5:100; (E) FA-PEG-DSPE/MPEG-DSPE = 10:100.

2.8. Study in vitro of 9-NC-loaded polymeric micelles for tumor cells

In practice, BXPC3 cells, SGC7901 cells and HeLa cells were plated at 7.5×10^3 cells per well density in 96-well plates, respectively. After 24 h incubation, the medium was replaced with 200 μl medium containing FA-M-9-NC, M-9-NC and free 9-NC. After additional 24 h and 48 h incubation, the drug efficacy of 9-NC-loaded polymeric micelles was evaluated respectively by using the MTT method.

3. Results and discussion

3.1. Purity of FA-PEG-DSPE and MPEG-DSPE

FA-PEG-DSPE and MPEG-DSPE were synthesized by decarboxylation reaction as described in Section 2 and the purity of them were confirmed by HPLC analysis. As shown in Fig. 2, there was no MPEG-amine or free DSPE in MPEG-DSPE (as shown in Fig. 2A and C), and there was no FA-PEG-amine or free DSPE in FA-PEG-DSPE (as shown in Fig. 2B and D). The purity quotient of MPEG-DSPE and FA-PEG-DSPE was approximately 95.8% and 94.3%, respectively.

3.2. Characteristics of micelles

3.2.1. Micelle size and zeta-potential

The average size of folate-conjugated micelle was about 21–24 nm and the micelle size distribution of both empty and drug-loaded micelles were rather narrow. The morphology of the drug-loaded micelles was investigated by TEM. As shown in Fig. 3, all these micelles had a spherical shape. The particle surface was very smooth and no drug crystal was visible.

The drug-loaded micelles were stable during storage at 4 °C for 4 weeks. No precipitation of drug or micelle size/size distribution changes was noted during this period. The average size of folate-conjugated micelle was still about 25 nm and the micelle size distribution of both empty and drug-loaded micelles were still rather narrow. It permits to hope that these micelles will be sufficiently stable even diluted in HBS buffer (pH 5.5) by 1000-fold.

Both folate-free empty micelles and folate-conjugated empty micelles were negatively charged with zeta-potential of approximately −15.7 mV and −13.2 mV, respectively. Drug-loaded micelles slightly decreased the negativity of the micelles without significant deviation ($P > 0.05$).

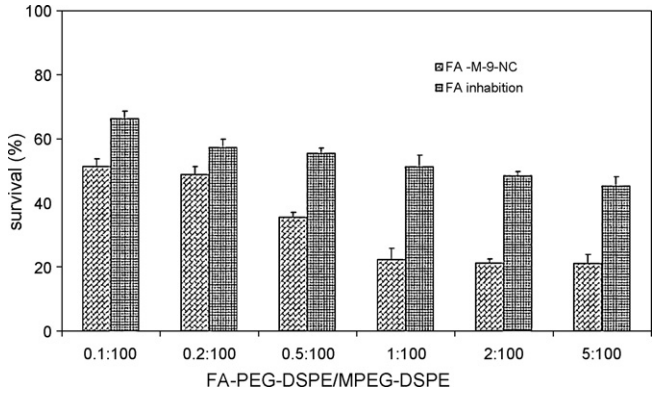

Fig. 6. The relationship between the survival rate of HeLa cells and the molar ratio of FA-PEG-DSPE and MPEG-DSPE (0.1:100, 0.2:100, 0.5:100, 1:100, 2:100 and 5:100) Mean ± S.D. ($n = 3$).

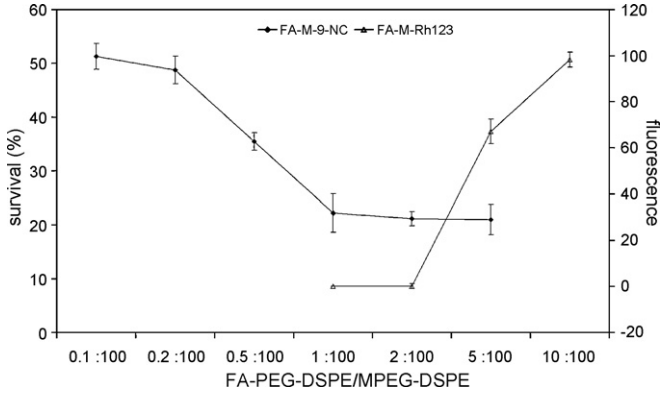

Fig. 7. The comparison between the fluorescence of FA-M-Rh-123 and the survival rate of HeLa cells of different molar ratio of FA-PEG-DSPE and MPEG-DSPE. Mean ± S.D. ($n = 3$).

Table 1
The IC_{50} values of the three kinds of tumor cells after exposing to three different formulations of 9-NC for 24 h and 48 h, respectively.

	Time								
	24 h			48 h			24 h/48 h		
	HeLa	SGC7901	BXPC3	HeLa	SGC7901	BXPC3	HeLa	SGC7901	BXPC3
$IC_{50(FA\text{-}M\text{-}9\text{-}NC)}$	0.52	1.50	8.31	0.005	0.02	7.10	104.00	75.00	1.17
$IC_{50(M\text{-}9\text{-}NC)}$	1.93	8.14	9.20	0.15	0.55	7.52	12.86	14.80	1.22
$IC_{50(9\text{-}NC)}$	8.85	11.25	14.00	1.52	3.15	9.61	5.82	3.57	1.46

3.2.2. Critical micelle concentration

The CMC values of MPEG-DSPE and FA-PEG-DSPE were shown to be as low as 0.97×10^{-5} M and 1.0×10^{-5} M, respectively. This result suggested that the polymeric micelles had high stability and they also had the ability to maintain the integrity even upon strong dilution in the body.

3.2.3. Encapsulation efficiency and drug loading

The encapsulation efficiency and drug loading efficiency of 9-NC incorporated into micelles were 97.6% and 4.64%, respectively. The drug-loaded micelles were stable during storage at 4 °C for 4 weeks. No obvious changes of drug content of the micelles were noted during this period. The encapsulation efficiency and drug loading efficiency in the fourth week were 97.4% and 4.63%, respectively.

3.3. Safety of materials

The concentration (0.2–20 μM) of materials we chose can provide a high drug concentration in the micelle suspension. The figure presented in Fig. 4, clearly showed low cytotoxicity of the materials we used to prepare micelles. The range of cell survival in the presence of various concentration of empty micelles was $96.21 \pm 7.261\%$ to $84.18 \pm 1.697\%$ for MPEG-DSPE and $93.47 \pm 3.402\%$ to $73.20 \pm 2.043\%$ for FA-PEG-DSPE. This result demonstrated that the polymeric micelles had good biocompatibility and produced minimal cytotoxicity. We inferred that, FA-PEG-DSPE was transferred into tumor cells more easily than MPEG-DSPE via folate receptor mediated endocytosis, and produced more cytotoxicity than MPEG-DSPE. So the cell survival of FA-PEG-DSPE group was lower than that of MPEG-DSPE group. However, the cytotoxicity of FA-PEG-DSPE can be ignored due to its low ratio (only 1%) in the mixed materials of the micelles.

3.4. Influence on the uptake of cells with different molar ratio of FA-PEG-DSPE and MPEG-DSPE

Flow cytometry evaluation on macrophages with FA-M-Rh-123 of different molar ratios of FA-PEG-DSPE and MPEG-DSPE was shown in Fig. 5. There was no fluorescence in folate-free micelles group (A) and in group 1:100 (B); the fluorescence rate was 0.11% in group 2:100 (C); 67.06% in group 5:100 (D); and 98.30% in group 10:100 (E).

The relationship between the survival rate of HeLa cells and the molar ratio of FA-PEG-DSPE and MPEG-DSPE was shown in Fig. 6. In experimental groups, when the concentration of 9-NC was 0.9 μg/ml, the cell survival rates were respectively 52.19% (FA-PEG-DSPE/MPEG-DSPE = 0:100), 51.29% (0.1:100), 48.78% (0.2:100), 35.42% (0.5:100), 22.19% (1:100), 21.15% (2:100) and 21.0% (5:100). There was no significant difference between the last three groups ($P > 0.05$). In folic acid inhabition groups, the cell survival rates were respectively 66.19% (FA-PEG-DSPE/MPEG-DSPE = 0.1:100), 57.26% (0.2:100), 55.43% (0.5:100), 51.22% (1:100), 48.40% (2:100) and 45.21% (5:100). The survival rates of HeLa cells were higher than the corresponding ones in experimental groups, that is to say, the free folic acid reduced the cell uptake of FA-M-9-NC. Therefore, it suggested that FA-M-9-NC was transported into HeLa cells via FR mediated endocytosis.

The two important factors described above were compared in Fig. 7. Therefore, the suitable molar ratio of FA-PEG-DSPE and MPEG-DSPE was chosen to be 1:100.

3.5. Efficacy of 9-NC-loaded polymeric micelles for tumor cells in vitro

In vitro drug efficacy of 9-NC-loaded folate-conjugated micelles against HeLa, SGC7901 and BXPC3 cells was determined for 24 h and 48 h. The drug efficacy of different 9-NC formulations (FA-M-9-NC, M-9-NC and free 9-NC) was compared in Table 1. HeLa cells were reported to have overexpressed FRs on their surface and be sensitive to 9-NC. The IC_{50} value of M-9-NC group was lower than free 9-NC group significantly after 24 h exposure of HeLa cells to the three different formulations. However, the cellular uptake increased with folate conjugation. The killing ability of FA-M-9-NC increased about 3.7 and 17.0 times compared with M-9-NC and free 9-NC, respectively. Although the killing ability for SGC7901 cells was not as good as HeLa cells, higher internalization of folate-conjugated micelles was still observed in comparison with M-9-NC and free 9-NC (increased about 5.4 and 7.5 times, respectively). The cell survival had no significant deviation between M-9-NC group and 9-NC group ($P > 0.05$) because of the less sensitivity of SGC7901 cells to 9-NC than HeLa cells. Since BXPC3 cells expressed less FRs on cell surface and were less sensitivity to 9-NC, the killing ability of FA-M-9-NC and M-9-NC were almost the same, at most 1.7 times higher than 9-NC. It suggested that, if and only if the tumor cells overexpressed FRs on their surface, FA-M-9-NC can show a better targeting ability.

As shown in Table 1, after 48 h action on HeLa cells, the IC_{50} value of FA-M-9-NC decreased to 0.005 μg/ml, and the killing ability was 30 and 304 times higher than M-9-NC and free 9-NC, respectively. When the drug concentration was increased to 5 μg/ml, the cell survival of FA-M-9-NC group was close to zero. Similar situation was also observed on SGC7901 cells. For the IC_{50} value of FA-M-9-NC decreased to 0.02 μg/ml and the killing ability of FA-M-9-NC was still higher than M-9-NC and 9-NC (about 28 and 158 times). But for BXPC3 cells, the IC_{50} values of FA-M-9-NC, M-9-NC and 9-NC decreased not obviously, and there was no significant deviation between the three formulations ($P > 0.05$). Except for BXPC3 cells, the IC_{50} values of FA-M-9-NC for 48 h exposure decreased about 104 and 75 times for HeLa and SGC7901 cells, respectively, in comparison with those for 24 h. It suggested that, the targeting ability of FA-M-9-NC could be enhanced by extending the time of reaction properly with tumor cells with overexpressed FRs, thus emphasizing the significance of our effort to protecting the micelles from macrophages in order to achieve long retention in the body.

4. Conclusion

In summary, drug-loaded folate-conjugated micelles was prepared from FA-PEG-DSPE and MPEG-DSPE which was prepared by chemical synthesis method and had high purity and showed a

narrow size distribution (about 21–24 nm). The molar ratio of FA-PEG-DSPE and MPEG-DSPE 1:100, could avoid macrophages and express highly selective targeting ability. The folate-conjugated micelles showed a higher ability to actively target the tumor cells with overexpressed FRs on cell surface in comparison with folate-free micelles or free anticancer agents. The folate-conjugated polymeric micelle formulation described in the present study, which can avoid the macrophages, is a kind of promising carrier for poorly soluble anticancer agents via FR mediated endocytosis. The higher efficacy and lower toxicity research on primary tumors and lymphatic transfer tumors in vivo will be further studied.

Acknowledgements

We are grateful for the foundation support of the National High Technology Research and Development Program of China (863 Program) (No. 2006AA03Z325), Shanghai Key Technologies R&D Program (Nos. 0652nm011 and 074319117).

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