Synthesis and evaluation of methionine and folate co-decorated chitosan self-assembly polymeric micelles as a potential hydrophobic drug-delivery system

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In this study, an amphiphilic copolymer folate-succinyl-methionine-chitosan-octyl (FSMCO) was successfully synthesized step by step for self-assembling polymeric micelles. The copolymers formed micelle-like nanoparticles by their amphiphilic characteristics and structures were examined by UV-Vis absorption and Fourier transform spectroscopy. The sizes of blank and ICG derivative-loaded micelles measured by dynamic light scattering were about 170 and 140 nm, respectively, which were spherical in shape with an average zeta potential of -10 mV. Further studies on the stability showed that the micellar solutions maintain their sizes at room temperature for 1 month without distinct aggregation or dissociation. ICG derivative was much better photostable after being entrapped by the new carrier. The prepared FSMCO micelles displayed a good drug loading content (11.7%), entrapment efficiency (66.5%) and sustained release rate for the model drug fluorescein. The copolymers demonstrated weeny cytotoxicity toward Bel-7402, L02 and A549 cells when incubated for 2 d. Ligands modified micelles endowed preferable cell targeting capability and beautiful cell inhibition of HCPT-FSMCO on Bel-7402 tumor cells. This kind of polymeric micelles may be a promising nanovehicle in delivering near-infrared dyes for tumors imaging and chemotherapeutic drugs for cancer therapeutics.

folate, methionine, chitosan, micelles, ICG derivative

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For a long time, numerous cytotoxic anticancer drugs display water-insoluble, poor tumor selectivity, toxic side effects [1] and the potential of resistance, and so on. The above limitations entail an urgent need for developing new drug formulation. Increasing interest has being paid to the design and development of drug delivery platforms for selective delivery (i.e. targeted delivery) to solid tumors, developing new non-invasive strategies for the diagnosis and treatment of cancer [2]. For instance, nanomicellar systems are promising carriers to figure out the above issues, which produced significant improvements in tumor accumulation of drugs and/or a reduction in systemic toxicity with en-

hanced permeation and retention (EPR) effect [3] and/or together with active targeting. Positively charged chitosan is an outstanding choice of semi-synthetic natural carrier materials can be modified to form micelles. Nowadays, folic acid (FA) has been proven to mediate favorable transcytosis of coupled nanocarriers through folate receptor (FR) [4] mediated pathway. The vast expression of FR in many cancers makes it a fascinating marker and target molecule for cancer diagnosis and therapy [5]. Some drugs conjugated with folate and imaging agents have arrived clinical test stage [6].

Methionine (Met) is sulfur containing essential amino acid for normal growth and development in mammals [7]. It plays many critical roles in mammalian metabolism such as

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methylation of DNA, protein synthesis, cell survival, and reproduction. Methionine can be transported by LAT1 and LAT2 receptors strongly expressed in many malignant cell membranes [8]. A characteristic feature had been found that many malignant cell lines need a large amount of exogenous methionine named methionine-dependence because of the metabolic defect of regenerating methionine, whereas non-cancerous cells are generally methionine independent [9,10]. Methionine restriction or even methionine-free diets was connected to cancer therapy [11]. However, methionine in this study was reacted to micelles severed as a targeting group.

With regards to the hydrophobicity of most therapeutic drugs and the ligands mentioned previously to enhance anti-cancer efficacy, we designed a new folate-succinyl-methionine-chitosan-octyl amphiphilic copolymer for the encapsulation of hydrophobic drugs in this study for the first time, in which FA and Met as the bi-model targeting moieties emerged an ingenious synergistic effect to amplify tumour targeting [12]. This dual receptors targeting would be a fresh tactic for delivering drugs effectively and specifically than single receptor targeting. Significant advances have been made in synthetic methodology, such that prepared the kind of micelles with highly controlled size, shape, surface charge and physicochemical characteristics, with FA and Met further decorated in their surface in order to improve biocompatibility and achieve active targeting. Fluorochromes, fluorescein and ICG derivative, were successfully encapsulated by FSMCO micelles. Identification, *in vitro* release and *in vitro* cytotoxicity of the polymeric micelles were also studied in detail. These results suggest that this novel FSMCO copolymer based on the polysaccharide is likely to be a promising nanocarrier for *in vivo* tumor imaging and anticancer drug delivery.

1 Materials and methods

1.1 Materials

Chitosan, with deacetylation degrees of 91% and average molecular weight (MW) of 100 kD was purchased from Aoxing Biotechnology Co. Ltd. (Zhejiang, China). Folic acid (FA, folate, MW 441), 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC-HCl), *N,N'*-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS) and methyl thiazolyl tetrazolium (MTT) were all purchased from Sigma-Aldrich (St. Louis, USA). Indocyanine Green (ICG) derivative (MW 689) was prepared by our research group. *L*-Methionine, hydroxycamptothecin (HCPT) and fluorescein were purchased from Aladdin reagent (Shanghai, China). RPMI 1640 medium, trypsin, penicillin, calf serum, streptomycin and ethylenediaminetetraacetic acid (EDTA) were all purchased from Invitrogen-Life Technologies (Carlsbad, CA, USA). Many other reagents used in this study were all commercially obtained from Shanghai Chemical Reagent

Company (Shanghai, China) with analytical reagent grade.

Human hepatoma cell lines (Bel-7402), human lung carcinoma cell lines (A549) and immortal hepatic cell lines (L02) were all purchased from American Type Culture Collection (ATCC, Manassas, VA, USA).

1.2 Synthesis of folate and methionine co-modified chitosan micelles

(i) Preparation of *N'*-methionine-chitosan-*N*-octyl (MCO). In brief, 0.51 g octyl aldehyde was dropwise added to 0.5 g chitosan dissolved in 40 mL 1% acetic acid, followed by constant stirring for 4 h at room temperature. Then, the resultant solution pH was adjusted to 4.5. After that, 1.6 mL NaBH₄ solution (10 mg/mL) was rather slowly added drop by drop and followed by continuous stirring for 12 h. After conjugating octyl to the reactive amino groups of the chitosan, the product solution (chitosan-octyl, CO) was filtered and neutralized with gradual addition of 5% (w/v) NaOH solution, then purified by dialyzing against distilled water (molecular weight cut off (MWCO) 10 kD).

Then, diluted methionine was slowly dropwise added to the mixed solution (acidified to pH 5.0) of chitosan-octyl and excess EDC/NHS (molar ratio of Met:EDC:NHS=1:5:1), constant stirring for 8 h at room temperature to completely react to the remainder amino groups of chitosan. Still, a dialysis was done to purify the reaction product (Met-chitosan-octyl, MCO).

(ii) Synthesis of succinic anhydride modified MCO (SMCO). In order to obtain negatively charged nanoparticles, succinic anhydride dissolved in acetone (3 g/17 mL) was reacted with MCO (2 g) for 36 h. After that, the nanoparticles were washed several times with deionized water (DI water).

(iii) Preparation of folate-SMCO (FSMCO) micelles. γ -COOH group of FA (25 mg) dissolved in anhydrous dimethyl sulfoxide (DMSO, 2.5 mL) was activated for 4 h in the presence of DCC and NHS (molar ratio of FA:DCC:NHS=1:1.2:2) [13], followed by centrifuging and filtering to remove the side product dicyclohexylurea (DCU) precipitate, then continuous stirring in the dark for 12 h at room temperature to crosslink FA-NHS to a balanced amount of amino groups of SMCO, dialyzed, frozen and lyophilized.

1.3 Characterization of FSMCO micelles

To identify FSMCO, UV-Vis absorption spectra were used to analyze the synthesized products using UV-Vis spectrophotometer (JH 754PC, China). Successful preparations of FSMCO micelles were also confirmed by Fourier transform infrared (FTIR) spectra (Nicolet ECO 2000, USA).

1.4 Preparation of model drug-loaded FSMCO micelles

To figure out the maximal drug loading content, the optimum

weight ratios (Model drugs: FSMCO=2:1, 1:1, 1:2, 1:4, 1:5, 1:6) were investigated firstly. Model drugs with different hydrophobicities, fluorescein and ICG derivative, were dissolved in DMSO and dropwise added to FSMCO solution respectively with magnetic stirring at room temperature. Then, the resultant mixture was dialysed in dialysis tube (MWCO 10 kD) against DI water for 2 d, followed by centrifuging at 5000 r/min for 10 min to remove unloaded model drugs, the resultant supernatant was lyophilized and dissolved in dimethyl formamide (DMF), the absorbance of the resultant solutions at the absorption peaks of the two model drugs were analyzed, respectively.

The drug encapsulation efficiency and drug loading content were measured based on the following equations [14]: encapsulation efficiency (EE)=(mass of drug encapsulated in micelles/mass of drug in feed)×100%; drug loading content=(mass of drug encapsulated in micelles/mass of drug encapsulated micelles)×100%.

1.5 Particle size and zeta potential measurement

Particle size is increasingly recognized as an important parameter in the effective delivery of therapeutic agents by particulate vehicles, as well as size distribution can have a profound effect on the drug release profile, organ targeting and the efficiency in penetrating tissues and cells [15]. In this study, the average sizes and polydispersity of blank and ICG derivative-loaded FSMCO micelles were characterized by Particle Size Analyzers (Brookhaven Instruments Corporation, Austin, Texas, USA) in phosphate-buffered saline (PBS, pH 7.4). Meanwhile, the zeta potential, a measure of the surface charge on the micelles, was determined by Zeta Potential Analyzers (Brookhaven Instruments Corporation, Austin, Texas, USA) in PBS (pH 7.4) solution.

1.6 Micelle stability

To study the storage stability, blank micelles and ICG derivative-loaded FSMCO micelles were suspended in PBS (1.0 mg/mL) and stored in dark place at room temperature for a month. Time-dependent change in micellar size was monitored by Particle Size Analyzers to evaluate the stability of the micelles.

1.7 Transmission electron microscopy (TEM) of micelles

The morphological evaluation of diluted blank FSMCO micelles and ICG derivative-loaded FSMCO micelles were performed using transmission electron microscope (TEM, Philips FEI Tecnai G2 20s-TWIN, the Netherlands) with accelerated voltage of 200 kV. Samples were prepared by dropping diluted micelles solutions on carbon-coated copper grids and allowed them to dry at room temperature. The specimens on the copper grids were avoided to be stained.

1.8 Status study of model drug in micelles

Fluorescein and ICG derivative were selected with the excitation and emission wavelengths 488/525 nm and 765/808 nm, respectively. The former is suitable for *in vitro* cellular study under confocal microscopy and the latter is available *in vivo* targeting evaluation under near infrared imaging system.

Optical properties about absorption and fluorescence of free and incorporated ICG derivative were measured by UV-Vis spectrophotometer and S2000 spectrometer (Ocean Optics, USA), respectively. In addition, relative fluorescence intensity was measured under successive exposure to the laser (765 nm) for 1 h to evaluate the photostability of free and incorporated ICG derivative, and data was gathered every 5 min.

1.9 *In vitro* drug release study

Fluorescein-loaded micelles and ICG derivative-loaded micelles were prepared to investigate the release profiles. *In vitro* drug release studies were carried out by a dialysis (MWCO 10 kD) using PBS (pH 7.4). Fluorescein and ICG derivative-loaded FSMCO solution was transferred into dialysis bags and dialyzed against 30.0 mL PBS in dark environment with constant agitation at 95 ± 5 r/min and 37°C, respectively. At selected time intervals, 3 mL dialysate was withdrawn and replaced with 3 mL fresh PBS to make the volume fixed at every time point during the assay. The concentration of the released model drugs was determined by UV-Vis spectrophotometer and calculated according to a previously obtained calibration curve. The release experiments were conducted in triplicate.

1.10 *In vitro* cell viability studies

The cytotoxicity of FSMCO was determined by employing MTT assay [16] against A549, L02 and MCF-7 cells. Cells were seeded into 96-well plate and cultivated at 37°C for 24 h. After that, cells were exposed to 200 μ L a series of concentrations of FSMCO solutions (0.0625, 0.125, 0.25, 0.5, 1, 2 mg/mL) for another 48 h. After incubation, FSMCO solutions were replaced with 180 μ L fresh RPMI 1640 medium and 20 μ L MTT solutions (5 mg/mL). Culture medium was removed after 4 h, and 200 μ L DMSO was added into each well with continuously shake for 10 min to dissolve the formazan crystals. The absorbance at 490 nm was measured using a Microplate Reader (Model 500; BIORAD, USA). Thus the metabolic activities of the cells were defined [17] by the following equation:

$$\text{Cell viability (\%)} = \text{OD}_a / \text{OD}_b \times 100\%,$$

where OD_a was the optical density of the cells incubated with FSMCO micelles, and OD_b was the optical density of the cells treated in the absence of FSMCO micelles only

with RPMI 1640 medium.

1.11 Targeting ability investigation of FSMCO at cellular level

In order to find out the target properties of the micelles at cellular level, Bel-7402 and A549 cells with a density of 5×10^5 cells/well in confocal laser scanning microscopy (CLSM) culture dishes were incubated with fluorescein loaded nanoparticles. 2 mL culture medium containing 0.1 mg fluorescein loaded succinyl-octyl-chitosan micelles [13] (SOC, pre-synthesized by our laboratory) or FSMCO was added to each well, medium was detached after 6 h incubation time and the cells were washed thrice with PBS. The fluorescence of the cells was obtained using CLSM (BX60, Olympus, Japan) under 488 nm excitation. Additionally, Bel-7402 cells were exposed to 2 mL culture medium containing free FA or Met or both as well as fluorescein-loaded nanoparticles for competition assay. Subsequent procedure was acted as the method described above.

1.12 Inhibition effect of drug-loaded FSMCO micelles on Bel-7402 cells

HCPT was encapsulated into FSMCO micelles to evaluate the suppression on Bel-7402 cells, cells were seeded in 96-well culture dishes and cultured for 24 h. HCPT, HCPT-SOC, HCPT-FSMCO at various concentrations (from 3.125 to 100 $\mu\text{g/mL}$) suspended in PBS were added to cell culture dishes and incubated for 24 h. MTT assay was also carried out to detect the livability of Bel-7402 cells.

2 Results and discussion

2.1 Synthesis and characterization of FSMCO

FA, Met, CO, MCO and FSMCO were studied by UV-Vis detection, and the results (Figure 1) showed that FSMCO had all three maximum absorption of Met (210 nm), CO (228 nm) and characteristic absorption peaks of FA (280 and 362 nm), which confirmed the successful acquisition of FSMCO.

Compared to chitosan, 1555 cm^{-1} appeared in the FTIR spectrum of MCO and FSMCO, which usually caused by secondary amines, illustrating part of amino groups of chitosan were substituted by Met and/or octyl (Figure 2). The new peaks at 2925 and 2855 cm^{-1} were attributed to aliphatic C-H stretching band of methyl groups of the alkyl chain ($-(\text{CH}_2)_7-\text{CH}_3$) [18], implied that octaldehyde was reacted to chitosan. The peak at 1645 cm^{-1} was C=O stretching band of acetyl group [19], confirmed that Met was linked to chitosan. Furthermore, the peak presented at 1700 cm^{-1} was the C=O stretching vibration of carboxyl group, declared that succinic anhydride was reacted to part of the amino groups of MCO. The appearance of the peak at 1600 cm^{-1} in the FTIR spectrum of FSMCO was related to

Figure 1 Identification of FSMCO with UV-Vis: absorbance of Met, FA, CO, MCO, and FSMCO.

Figure 2 Identification of FSMCO with FTIR: the FTIR spectra of chitosan (a), MCO (b) and FSMCO (c).

benzene ring of the folic acid. Both the above characterizations showed that the desired copolymers were synthesized successfully.

2.2 Size and morphology of micelles

Studies have shown that the perfect particle size for cancer treatment in the range of 70 to 200 nm, resulting in efficient accumulation in solid tumor tissues [20,21]. The hydrodynamic diameters of blank micelles and ICG derivative-loaded FSMCO micelles in PBS determined by dynamic light scattering (DLS) were shown in Figure 3. Protonation was avoided in PBS with pH 7.4 [22], and the size of the blank micelles was about 170 nm, and the size of the ICG derivative-loaded micelles was about 140 nm, which suggested that the micelle nanoparticles had satisfactory dimensions.

The TEM micrograph (Figure 4) clearly revealed that the blank micelles and ICG derivative-loaded micelles were spherical in shape, which had a homogeneous size distribution (Polydispersity <0.2) with an average size of 140 and 110 nm, respectively. When encapsulate with hydrophobic

Figure 3 Size distributions of blank FSMCO micelles (a), and ICG derivative-loaded FSMCO micelles (b) at 25°C in PBS (pH 7.4).

Figure 4 Transmission electron microscope (TEM) pictures of blank FSMCO (a), and ICG derivative-loaded FSMCO micelles (b).

dye ICG derivative, the hydrophobic interaction between ICG derivative and octyl would bring about the hydrophobic core much more intense, representing the decreased size compared to blank micelles; one ICG derivative molecule possesses two “-COO-”, repulsive force increased among micelles because of the negative charge of the entirety increased after encapsulating with ICG derivative, thus acquired better dispersibility tested by Particle Size Analyzers. The smaller sizes were observed by TEM as compared to that determined by DLS is most likely due to the evaporation of water around the micelles during the sample preparation of TEM, which caused the shrinkage of the hydrophilic shell [23].

The zeta potential gives an indication in the physical stability of the colloidal system *in vitro* and *in vivo*, and affects its interaction with proteins and cell membranes in blood vessels [24]. MCO was positively charged, and blank micelles and ICG derivative-loaded micelles were both negatively charged about -10 mV. It was proved that succinic anhydride was successfully reacted to MCO. Thereby, negatively charged colloidal system would barely adhere to cells in vessels when intravenous injection.

2.3 Stability of micelles

Diluted blank micelles solution and ICG derivative-loaded micelles solution were used in the stability study. The results (Figure 5) indicated that the micelles were almost

Figure 5 Particle size of blank FSMCO micelles, and ICG derivative-loaded FSMCO micelles at 25°C in PBS (pH 7.4) as a function of time.

steady during storage at room temperature (25°C) for a month. No change in the micelle size and size distribution in the first 10 d and no precipitation of ICG derivative were found during the month, moreover, ICG derivative-loaded micelles were more stable than blank micelles because of the similar polarity between octyl and ICG derivative which enhanced the hydrophobic association action.

2.4 Drug loading and *in vitro* drug release

We concluded the maximum drug loading content with the feed ratio 1:5. The optimal drug loading content was 11.74% for fluorescein (EE=66.52%), while the drug loading content was just 37.46% for ICG derivative (EE=6.97%). The drug loading content and EE for fluorescein were more than that of the more hydrophobic ICG derivative which may be due to the rapid precipitate of the intense hydrophobicity when at the dynamic drug loading process in aqueous solution. Consequently, precipitation resulted in lower entrapment into the inner hydrophobic core.

Figure 6 shows *in vitro* release profile of model drugs from FSMCO micelles at 37°C in PBS solution (pH 7.4). Obvious burst release was observed in the release profile of fluorescein and the cumulative percentage release reached about 70% during the initial 40 h. More than 90% of the loaded fluorescein was released in the following slow release stage for up to 96 h. In contrast to fluorescein, the ICG derivative cumulative percentage release by 240 h reached only 59% with a continuous slow-release started from about the 5th hour. The results were manifest and indicated that the release rate of model drug was closely related to the hydrophobicity, the hydrophobic binding force of fluorescein to the inner octyl of micelles was much weaker than that of ICG derivative because of its inferior hydrophobicity, which induced a fairly fast release rate for fluorescein from

Figure 6 Release kinetics of fluorescein-loaded FSMCO micelles and ICG derivative-loaded FSMCO micelles in PBS (pH 7.4, 37°C). Inset: The release kinetics of the initial 72 h. Data were given as mean $\pm$ SD ($n=3$).

FSMCO carrier.

2.5 Optical characterization of entrapped ICG derivative

ICG derivative was easily packaged into FSMCO by hydrophobic association effect. The excitation and emission spectra for ICG derivative and ICG derivative-loaded FSMCO are shown in Figure 7(a) and (b). No change was observed

Figure 7 Optical characterization of free ICG derivative and ICG derivative-loaded FSMCO. (a) Absorbance spectra; (b) fluorescence spectra; (c) light stability.

in optical properties of ICG derivative-loaded micelles compared to free ICG derivative, including maximum absorption peak, excitation wavelength and emission wavelength. Furthermore, better resistance to fluorescent bleaching was found after ICG derivative being encapsulated into FSMCO micelles (Figure 7(c)), the relative intensity of fluorescent emitted from ICG derivative encapsulated by FSMCO was strengthened from 1000 to nearly 2200 in the beginning 20 min, slow decrease was found in the following time, but still much higher than the relative intensity of fluorescent emitted from free ICG derivative. It was suggested that ICG derivative together with FSMCO micelles can be used to trace the dynamic process of FSMCO in the mouse model evaluated by near-infrared imaging system [25].

2.6 Cytotoxicity assay of FSMCO micelles

The *in vitro* cytotoxicities of prepared FSMCO against Bel-7402, L02 and A549 cells are shown in Figure 8. The composite was proved to be weeny cytotoxicity even in 2 mg/mL by MTT assay. After the 48 h incubation, cell viabilities in the presence of micelles at different concentrations from 0.0625 to 2 mg/mL were more than 93%, even at high con-

Figure 8 Cytotoxicity study of FSMCO micelles against A549 cells, Bel-7402 cells, and L02 cells. Data represent mean $\pm$ SD ($n=5$).

centration. It was therefore presupposed that the micelles exhibited reasonable biocompatibility. The confirmation of little or nontoxicity of chitosan-based micelles was also reported in other places [26,27].

2.7 Cellular uptake of nanoparticles

The exceeded expression of LAT1, LAT2 and FA receptors of Bel-7402 cells was previously reported, while the expression levels of both receptors on A549 cells (used as control) were low [13,28]. In order to prove the increased uptake in tumor cells, results (Figure 9) were observed by fluorescence microscope. Fluorescence intensity of fluorescein in cells was decreased as the following order: FSMCO-fluorescein>FSMCO-fluorescein together with Met>FSMCO-fluorescein together with FA>FSMCO-fluorescein together with FA and Met>SOC-fluorescein. Extra added free FA and Met in medium reduced the endocytosis because of the competitive inhibition. By contrast, low fluorescence intensity can be seen in A549 cells visibly. These results indicated that both the presence of FA and Met modified on micelles can promote cellular uptake effectively by Bel-7402 cells.

2.8 Inhibition effect of HCPT loaded FSMCO micelles on cellular level

The results (Figure 10) of MTT assay confirmed that the anti-tumor efficacy was advanced as the concentration of drug increased. After 24 h incubation, therapeutic effects were shown to varying degrees: HCPT-FSMCO>HCPT>HCPT-SOC. HCPT-FSMCO put up greatest cell inhibition. Cell viability was down to 46% at the maximum concentration of HCPT-FSMCO due to the receptors-mediated endocytosis. Results here demonstrated that folic acid and methionine played indispensable roles in increasing the cytotoxic effect of HCPT.

3 Conclusions

The newly developed folate-succinyl-methionine-chitosan-octyl amphiphilic copolymer in this paper could self-assemble

Figure 9 The fluorescence microscopy images of Bel-7402 cells (a) and A549 (b) tumor cells after incubated with fluorescein loaded micelles (scales in images were 20 μ m).

Figure 10 Anti-tumor capacity assessment of HCPT-FSMCO, HCPT-SOC, HCPT in Bel-7402 tumor cells. Cell viability (%) at 24 h.

into core-shell micelles in aqueous medium by hydrophobic interactions, which proved to be an excellent water-insoluble drug reservoir due to the octyl as the hydrophobic core. FSMCO micelles may be used for drug delivery of hydrophobic anti-cancer drugs without any influence to drugs, which showed enhanced stability indeed. The obtained nanomicelles had spherical shape with uniform negatively charged particle size, more cyto-compatible with cancer cells or normal cells. Fluorescein encapsulation efficiency as high as 66.5% was achieved and drug loading reached to 11.7%. Dual-receptor mediated selective Bel-7402 cells targeting capability and preferable cell inhibition efficacy were verified. These particular properties of FSMCO conjugates demonstrated that it is a promising injectable nanosystem for drug delivery and imaging in cancer research.

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