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Effective loading and controlled release of camptothecin by *O*-carboxymethylchitosan aggregates

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Abstract

Increasing the solubility in aqueous environment while alleviating the toxicity of the delivery system at the same time are the vital challenges related to the administration of the antitumor drugs. In the present paper we describe a novel drug delivery system for the well known anticancer drug, camptothecin (CPT). This system is the aggregates of *O*-carboxymethylchitosan (OCMCS), which is a kind of biocompatible and amphiphilic chitosan derivative. The amount of drug loaded was examined by stead-state fluorescence. The release behaviors of CPT from this proposed controlled release system in PBS solution at 37 °C were studied by UV spectroscopy. The antiproliferative activity of cancer cell was evaluated using MTS assay. The results demonstrate that not only the aggregates but also the unimers of OCMCS can help to enhance the solubility of CPT. After CPT is loaded in OCMCS, the release of CPT is significantly sustained, which is caused by the interactions between OCMCS and lipophilic CPT. In vitro cancer antiproliferative activity test further confirms the slow release of CPT from OCMCS-drug system. The result of OCMCS unimers showing as good drug-loading and controlled release capability as its aggregates indicates that this novel release system can solve the commonly existing problem of unavailability of micelles assembled from amphiphilic copolymer in the significant dilution accompanying IV injection. These findings propose a new concept of a localized drug delivery.

Keywords: O-carboxymethylchitosan aggregates; Camptothecin; Delivery systems; Antiproliferative activity

1. Introduction

Recently, a number of novel delivery systems have been developed including the entrapment of drugs, proteins or genes in small vesicles or within polymeric matrices (Stolnik et al., 1995). Colloid delivery system is one of the most promising delivery systems because it may reduce the unwanted toxic side effect and improve the therapeutic effect (Kreuter et al., 1994). Colloid delivery systems include nanoparticles, liposomes, microemulsions, polymeric self-assemblies, and so on. Among them, core-shell structure of micelles and nanoparticles assembled from amphiphilic block copolymers (Bae et al., 2000; Discher and Eisenberg, 2002; Gan, Jim, Li, Yuer, Wang and Wu, 1999; Ge et al., 2002; Rosler et al., 2001) and self-aggregates of polymers have been widely applied to the field of biotechnology and pharmaceutics (Lee and Jo, 1998; Lee, Jo, Kwon, Kim, & Jeong, 1998; Lee, Kwon, Kim, Jo, Jeong, 1998; Ouchi and Nishizawa, 1998; Shantha and Harding, 2002). Though the micelles assembled from amphiphilic block copolymers have been widely studied as the drug delivery systems, the lack of stability to prevent degradation caused by significant dilution accompanying IV injection was the problems that still remained (Rapoport, 1999).

Chitosan is the second most plentiful biomass and is already known as a biocompatible, biodegradable, and non-toxic natural polymer (Dureja et al., 2001). It has been found a number of applications in drug delivery including that of adsorption enhancer of hydrophilic macromolecular drugs (Artursson et al., 1994; Kumar et al., 2004). When protonated (pH <6.5), it is able to increase the paracellular permeability of peptide drugs across mucosal epithelia

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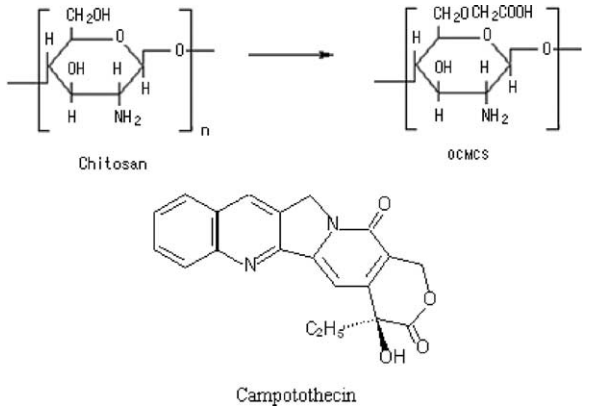
(Senel, Kremer, Kas, Wertz, Hincal and Squier, 2000). To overcome chitosan's limited solubility and effectiveness as absorption enhancer at neutral pH values such as those found in the intestinal tract, chitosan derivatives have been developed. For example, N-carboxymethylated chitosan was found to increase the permeation and adsorption of low molecular weight heparin, an anionic polysaccharide across intestinal epithelia. *O*-carboxymethylated chitosan (OCMCS) is another kind of water soluble chitosan derivative, with its *o*-hydroxyl group of each monomer substituted by a carboxymethylic group through the ether bond formation (Scheme 1), and has been proved to have biocompatibility (Cai et al., 2001; Zhu et al., 2004) as well as antibacterial activity (Chen et al., 1999). The presence of both carboxyl groups and amino groups in OCMCS macromolecules elicit special physicochemical and biophysical properties. For example, we recently have proved that OCMCS forms aggregates with swollen micro-gel morphology and its critical aggregation concentration (cac) is determined to be ~0.05 mg/ml (Zhu et al., 2005) and it can induce dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), the main component of cell membrane, vesicles fusion (Zhu et al., 2005). These novel properties of OCMCS motivate our great interests to further investigate the OCMCS aggregates as a matrix for drug delivery.

Camptothecin (CPT) (structure shown in Scheme 1) is an anticancer drug, which is a plant alkaloid and very effective in the treatment of gastric, rectum and bladder tumors (Brezova, Valko, Breza, Morris, Telser and Dvoranova, 2003). CPT displays its activity by inhibiting the enzyme topoisomerase I forming covalent enzyme-DNA complex, thereby resulting in a single-stand breaks (Bodley et al., 1995). CPT are usually administered either by continuous infusion or by multiple time-spaced injections, both resulting in a low patient comfort and compliance. In addition, the clinical use of CPT has some other practical disadvantages mainly due to (a) scarce water solubility and (b) a number of toxic effects. After prolonged administration CPT can indeed result in neutropenia, thrombocytopenia, anaemia and a number of non-haematological toxic effects such as alopecia, nausea, vomiting, diarrhea, fatigue and skin rash (De Oliveira and Chaimovich, 1993; Gabizon, 1989; Muggia et al., 1972). To overcome these drawbacks, it is important to develop the novel delivery systems for effective administration of CPT.



Scheme 1. The structures of chitosan, OCMCS and CPT.

In this study, the drug-loading as well as the controlled release behavior of OCMCS aggregates on CPT was investigated. The release kinetics was discussed after fitting the experimental data to a semiempirical equation based on Fickian diffusion model. Our results provide solid evidence that OCMCS aggregates are excellent candidates in drug delivery.

2. Experimental

2.1. Materials

Chitosan powder (average molecular weight: 6.7×10^5 g/mol; degree of deacetylation: 90%) was obtained from Lianyungang Biologicals Inc. (China). CPT ($C_{20}H_{16}N_2O_4$, $M_w=348.4$) were purchased from Sigma-Aldrich company. The other materials were of commercial grade and were used without further purification.

2.2. Synthesis of OCMCS

Two gram chitosan was immersed into 25 ml 50 wt% NaOH solution for 24 h in order to swell and alkalize the polymer. The alkalized chitosan was crushed into a filtration cake and then transferred into a flask. Monochloroacetic acid (5 g) was dissolved in isopropanol (25 ml), and added drop-by-drop into the alkalized chitosan inside the flask for 20 min. The reaction was carried for 8 h at room temperature. At the end of reaction, the mixture was filtered to remove the solvent. The filtrate was dissolved in 100 ml water, and then 2.5 M HCl was added to adjust the pH to 7. With centrifugation of the above solution for removal of the precipitate, and then addition of 150 ml anhydrous ethanol to the super-solution, the product precipitated from the solution. The solid was filtered and rinsed by anhydrous ethanol for three times and vacuum dried under room temperature. The mole fraction of carboxymethylated groups and amino groups within the OCMCS chain was found to be about 100 and 75%, respectively (Zhu et al., 2005).

2.3. Loading capacity of OCMCS aggregates

OCMCS solutions were prepared in a series of concentrations from 0.03 to 3 mg/ml and subsequently used to dissolve the drug, CPT. Dry powder of the CPT was added in 10 ml of OCMCS solution and stirred for a week to make sure of saturation state of CPT in the OCMCS solutions. The undissolved CPT was removed by centrifugation. The amount of CPT dissolved in each OCMCS

aqueous solution was evaluated using steady-state fluorescence spectroscopy (SSF). SSF spectra were recorded on a Shimadzu RF 5301 PC spectrofluorimeter equipped with a jacketed cuvette holder. All measurements were made at the front-surfaces of the samples at 45° position with an excitation wavelength of 370 nm and the slit widths for excitation and emission were both kept at 1.5 mm. The emission intensities at 440 nm were recorded for all the solutions measured.

2.4. In vitro release study

The vitro release study was conducted in the way reported by Xiong et al. (Xiong et al., 2005), briefly: 5 ml of CPT/OCMCS and CPT aqueous saturated solution were put in the dialysis membrane (molecular weight (cut-off 12,000–14,000 Da). The sealed dialysis membrane placed in PBS (PBS, pH 7.4, 0.01 M) aqueous solution (100 ml). The whole solution was then placed in a shaking water bath at 37 °C for the drug release study. At predetermined time intervals released CPT solution (3 ml) outside the dialysis membrane was withdraw and measured at wavelength of 369.6 nm by UV–VIS-NIR Scanning spectrophotometer (UV-3101 PC) to determine the concentration of CPT. Fresh PBS solution (3 ml) was added to replenish the sample that was removed in order to maintain a constant volume. The error in the concentration profile attributed to this method of dilution is around 3%. The drug release experiments were performed in triplicate.

2.5. In vitro cancer cell activity

MCF-7(caspase-3 positive) cells were grown in DMEM (Dulbecco's modified Eagle's medium) containing 10% fetal bovine serum supplemented with 100 units/ml penicillin and 100 mg/ml streptomycin at 37 °C under a 5% CO_2 containing atmosphere. Cells were seeded into 96-well plate at 5000/well and grown to 50% confluency. CPT/drug solutions with 7.4 μg/ml concentration and saturated CPT solution (3.7 μg/ml) were diluted ten times with OPTI-MEM I (serum-reduced medium, GIBCO). The growth medium was removed from the 96-well plate and 100 μl of above diluted solution was added and incubated at 37 °C under a 5% CO_2 atmosphere for 4 h. Then 100 μl of growth medium containing 20% FBS was added to each well, and incubated at 37 °C under a 5% CO_2 atmosphere. After 24 h incubation, 20 μl combined MTS ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)/PMS (phenazine methosulfate) solution (Promega) was added to each well. After incubating the plate for 2 h at 37 °C in a humidified, 5% CO_2 atmosphere, the absorbance of each well at 490 nm was recorded by using a 96-well plate reader (Bio-Rad Labs). Six samples were used, and this in vitro experiment was performed in twice independently.

3. Results and discussion

3.1. Drug loaded in OCMCS aggregates

OCMCS forms aggregates in aqueous solution mainly driven by the intermolecular hydrogen bond, with a cac of ~0.05 mg/ml determined by surface tension, steady-state fluorescence spectroscopy and rheology techniques (Zhu et al., 2005). The glucose backbones of OCMCS form the hydrophobic domains, and the dissociated carboxylic groups as well as the hydrophilic groups around the backbone constitute the hydrophilic ones (Zhu et al., 2005). This structure of OCMCS aggregate provides an ideal system to load the hydrophobic antitumor drug.

The amount of CPT loaded in OCMCS aggregates was investigated by SSF measurement. Based on this linear relationship (the calibration curve of the SSF emission intensity as a function of CPT concentration dissolved in DMSO), the amount of the CPT loaded in OCMCS aggregates can be easily measured. Fig. 1 shows the SSF spectra of CPT as a function of OCMCS concentrations. As can be seen from Fig. 1, the SSF intensity does not increase monotonically with increasing OCMCS concentration, which indicates that the solubility of CPT does not increase linearly with OCMCS concentration. The solubility of CPT as a function of OCMCS concentration was plotted in Fig. 2. From Fig. 2, it can be seen clearly that the solubility of CPT is significantly enhanced in OCMCS solution compared to that in water. Up to a concentration of 0.0625 mg/ml, the drug's solubility increases monotonically with OCMCS concentration. After that CPT's solubility decreases and drops to a minimum at OCMCS concentration of 0.1 mg/ml. At higher OCMCS concentrations from 0.1 to 0.3 mg/ml, the solubility of the drug increases again but in a much more moderate way compared to that of OCMCS concentrations form 0 to 0.0625 mg/ml. The results show that the most

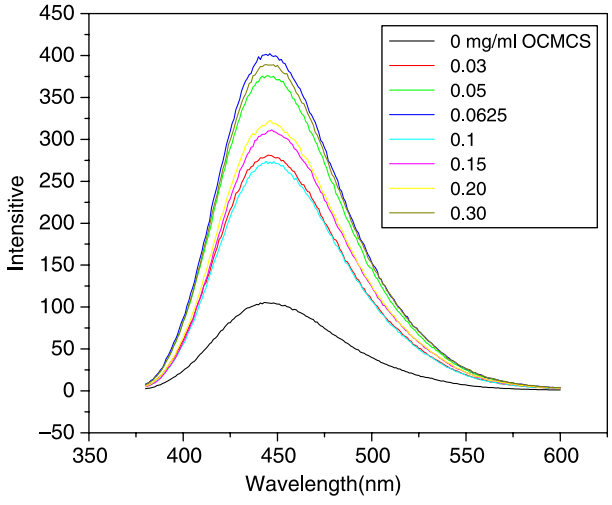


Fig. 1. Effect of OCMCS concentration on the fluorescence emission spectra of camptothecin in aqueous solution of OCMCS.

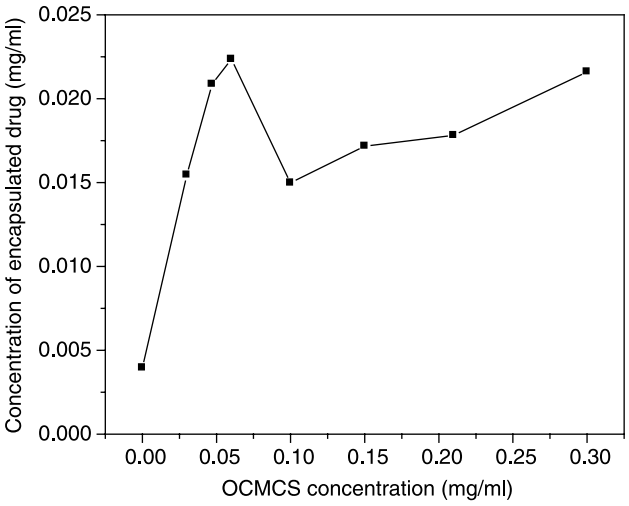
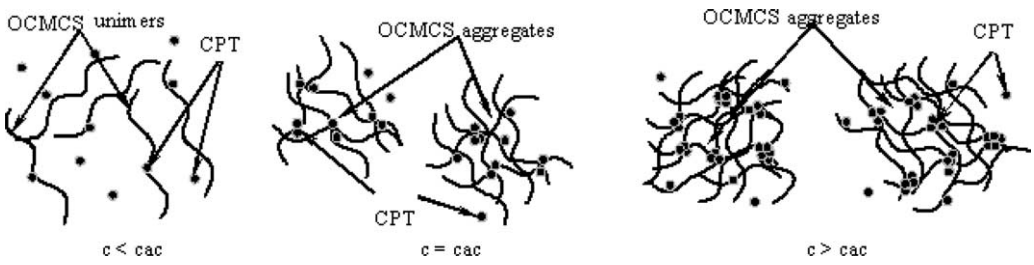


Fig. 2. CPT loading as a function of OCMCS concentrations.

efficient loading of CPT occurs at a concentration of 0.0625 mg/ml, which is a little bit higher than cac of OCMCS. This result is different from previous report that the amount of hydrophobic substance *N*-phenyl-1-naphthylamine (PNA) uptaken was found sharply increased around CMC where the intermolecular aggregation of chitosan-*g*-PEG occurred (Shantha and Harding, 2002). Our explanation for the present result is that before cac, although the aggregates have not formed in the solution, the interactions such as H-bond and hydrophobic interactions should exist between OCMCS chains and lipophilic CPT, because OCMCS has amphiphilic property and there are lots of active groups in its molecules. These possible formed interactions facilitate CPT to dissolve in OCMCS unimer solution (Scheme 2 (a)). When the concentration is beyond cac, aggregates form in OCMCS solution, hydrophobic drugs can be solubilized by the hydrophobic core inside the aggregates. Therefore the comparatively high amount of CPT can be entrapped in the aggregates when the concentration is beyond cac of OCMCS (Scheme 2 (b)). The low extremum appeared at the concentration of 0.1 mg/ml is probably because much more compact aggregates form at this concentration (Scheme 2(c)). It has been reported that the compact aggregates can only uptake a smaller amount of drug in comparison with the loosen ones (Shantha and Harding, 2002). Present result suggests that the re-organization of aggregate structure may occur with increasing OCMCS concentration, and more stable aggregates form at higher concentrations. The current results demonstrate that not only aggregates but also unimers of OCMCS are able to enhance the solubility of hydrophobic CPT.

3.2. The release of CPT from OCMCS aggregates

The slow release kinetics of a drug from colloid release system is of scientific significance and applied importance. The release profiles of CPT from OCMCS solution are shown in Fig. 3, where an initial burst release was observed for all the cases, however, after 50 min, drug releases from OCMCS solution become significantly slower than that from saturated CPT aqueous solution. The model-independent parameters were obtained from individual experimental curves and summarized in Table 1 where $t_{50\%}$, t_{eq} and F_{eq} represent percentage release after the time at which 50% amount of the drug was released, time to achieve equilibrium and the fraction of CPT released at equilibrium, respectively. From Table 1, it can be seen that it takes only 100 min for CPT to diffuse out of the dialysis membrane sack filled with saturated CPT aqueous solution (Fig.3 (a)), while it needs more than 550 min for the CPT release from the sack filled with CPT/OCMCS solutions to achieve equilibrium. It is obvious that the release of CPT can be effectively controlled by the delivery matrix of unimers and aggregates of OCMCS. Because cac is determined to be ~0.05 mg/ml, OCMCS unimers exit in 0.03 mg/ml of OCMCS solution. The release behavior of CPT from OCMCS unimers (Fig. 3(b)) is quite similar to that of CPT from OCMCS aggregates (Fig. 3(c) and (d)). This result is interesting and meaningful. Though the micelles assembled from amphiphilic block copolymers have been widely studied as the drug delivery systems, the lack of stability to prevent unavailability caused by significant dilution accompanying IV injection is the problem that still remains (Rapoport, 1999). For example, the presence of pluronic unimers results in burst release of drug and enhanced cytotoxicity of the drug. Our present results provide a good example of slow release behavior of CPT from OCMCS unimers. OCMCS aggregates have potential as a novel drug delivery system, because they can prevent the above mentioned problems of the amphiphilic copolymeric colliod drug delivery systems when the concentration



Scheme 2. The Scheme of the interaction between CPT and OCMCS.

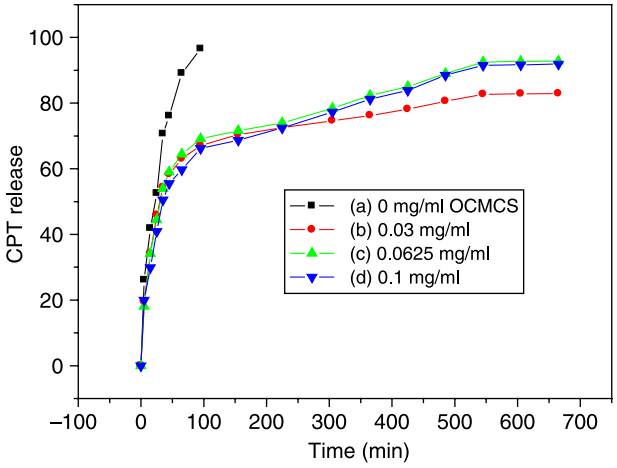


Fig. 3. Release behavior of CPT from OCMCS in PBS (pH 7.4, 0.01 M) solution at 37 °C.

is lower than CMC. The M_η of OCMCS is 670,000, which is much higher than that of amphiphilic block copolymers. Moreover, there are lots of active functional groups, such as amino and carboxylic groups, along the long chains of OCMCS, which is absent on the molecules of amphiphilic block copolymers. As a result, there are interactions between OCMCS chains and lipophilic CPT driven by H-bond and hydrophobic interactions. These interactions result in slow release of CPT from OCMCS unimers and low F_{eq} for CPT/0.03 mg/ml OCMCS.

3.3. Diffusion coefficients

Generally, the release kinetics of drug from polymeric micelles is affected by several factors such as particle size, block composition, molecular weight, concentration, and degradation rate (Kim et al., 2000). The release mechanism can be divided into diffusion controlled, degradation controlled or a mix of them (Siepmann and Peppas, 2001; Zuleger and Lippold, 2001). The release of drug from OCMCS aggregates is considered to take place by diffusion due to the lack of the enzyme to degrade chitosan based biomaterials on the present experimental conditions. Diffusion coefficient thus appears as a key parameter for designing OCMCS aggregates to release a drug at a pre-determined rate.

The following equation is generally used to describe a diffusion controlled release, where M_t and M_∞ are the absolute cumulative amounts of drug released at time t and infinite time, respectively; l is the thickness of the flat sample and D is the diffusion coefficient, which is assumed to be constant.

$$\frac{M_t}{M_\infty} = 1 - \frac{8}{\pi^2}\sum_{n=0}^{\infty}\frac{1}{(n+1)^2}\exp\left\{-\frac{(2n+1)^2 D\pi^2}{l^2}t\right\} \quad (1)$$

By taking only the first term in the summation (Σ) series and performing a logarithmic transformation, Eq. (1) can be simplified to Eq. (2):

$$\ln\left(1-\frac{M_t}{M_\infty}\right) = \ln\frac{8}{\pi^2} - \frac{D\pi^2}{l^2}t \quad (2)$$

By plotting $\ln(1-M_t/M_\infty)$ against t, D can be determined from the slope, $-D\pi^2/l^2$, of a linear regression of Eq. (2). The method has been extensively adopted to obtain diffusion coefficients from drug release data (Han et al., 2000; Liu et al., 2004; Yilmaz and Pekcan, 1998).

Applying Eq. (2), data in Fig. 3 was plotted in Fig. 4. From Fig. 4, it can be seen that the release of CPT in the presence of OCMCS normally divides into two stages, up to 100 min, the release behaviors are very similar to the one without OCMCS, but after 100 min, much slower release was clearly observed. Therefore it is necessary and reasonable to fit the two-stage release curves separately.

The release diffusion coefficients D_1 and D_2 values are obtained, respectively and are listed in Table 1. The D of CPT saturated aqueous solution is more than 2 times than D_1 and about 15 times than D_2 of CPT/OCMCS systems, respectively. The much lower diffusion coefficient (D_2) for CPT loaded in OCMCS unimers suggests that there are more interactions formed between OCMCS chains and CPT than that between OCMCS aggregates and CPT, which consists well with our previous conclusions (Scheme 2). The slow release behaviors of CPT from CPT/OCMCS systems propose that OCMCS as a potential candidate in effective administration of CPT.

3.4. Effect of OCMCS dilution

When pluronic micelles are used as drug carriers, there exists the fundamental difference between below or above the CMC. Below the CMC the intracellular uptake and cytotoxicity of the drug delivered were enhanced because of the presence of pluronic unimers (Rapoport, 1999), whereas above the CMC, the shielding properties of pluronic micelles are able to be used to prevent unwanted drug

Table 1
Release of CPT: experimental designation, release parameters and diffusion coefficients

Saturated CPT in	$t_{50\%}$ (min)	t_{eq} (min)	F_{eq} (%)	$D_1\times10^7$ (cm^2/s)	$D_2\times10^7$ (cm^2/s)
Water	22	96	99.5	21.75	/
0.03 mg/ml OCMCS	30	550	78.5	9.81	0.668
0.0625 mg/ml OCMCS	30	650	90.1	9.81	1.532
0.1 mg/ml (OCMCS)	35	650	88.9	9.81	1.702

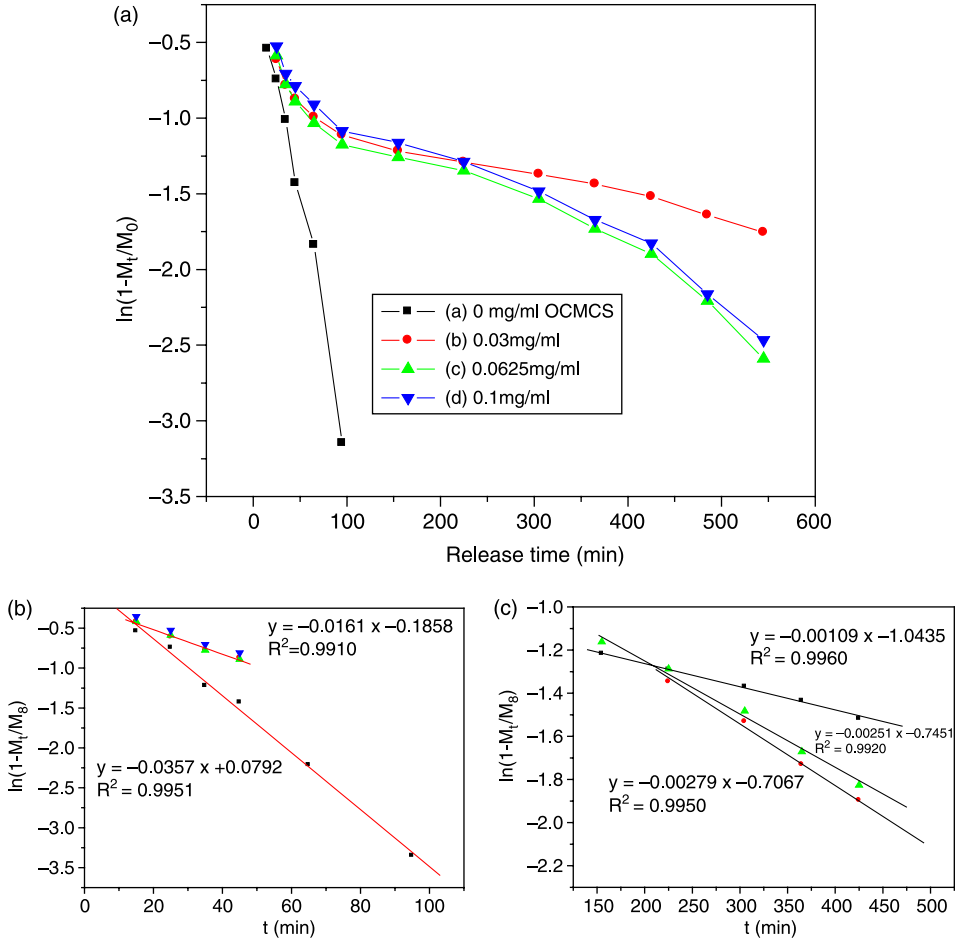


Fig. 4. Semi-logarithmic plots of the data in Fig. 3 as a function of time at different OCMCS concentration.

interactions with healthy cells. Due to the fact that the concentration of the polymeric drug carrier can drop to levels below the CMC upon IV injections, pluronic micelles have the stabilization problem on their efficiency of delivering drug.

The dilution effect of CPT/OCMCS systems was investigated with the assay of in vitro antiproliferative activity of cancer cells. The results are shown in Fig. 5. The figure shows that the relative antiproliferative activity of cancer cells dependent on the OCMCS concentration. The highest activity appears in the system of 5 μg/ml of CPT in 0.05 mg/ml of OCMCS solution (64.9 ± 3.3%), while the lower activity shows in systems of both 5 μg/ml of CPT in 0.03 mg/ml of OCMCS solution (36.1 ± 2.4%) or 5 μg/ml of CPT in 0.3 mg/ml of OCMCS solution (26.3 ± 2.5%) after 24 h incubation in comparison with saturated CPT solution. The highest antiproliferative activity is found with the dilution of 0.05 mg/ml of OCMCS. This is reasonable because quite a few drug entrapped in the aggregates should burst release into the cell growth medium due to the disassociation of OCMCS aggregates. At low OCMCS concentration (0.03 mg/ml), the drugs could be loaded because of the interactions between CPT and OCMCS chain. The low antiproliferative activity is believed to be resulted from the incompletely drug release following 24 h incubation. At high OCMCS concentration, though it was ten times diluted by medium, its final concentration was 0.03 mg/ml, at which, still relative higher drug concentration can be incorporated (judged by Fig. 3), similarly, the drug could not release completely within 24 h incubation and resulting low free drug concentration in the medium.

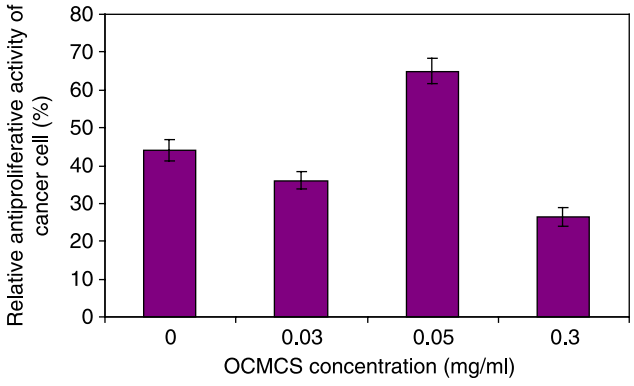


Fig. 5. The relative antiproliferative activity of cancer cell dependence on the OCMCS concentrations.

These results provide solid evidence that OCMCS can effectively control the release of CPT. Even the final concentration is less than cac of OCMCS, the drug does not show burst release behavior. These findings propose a new concept of a localized drug delivery.

4. Conclusions

In summary, a novel, biocompatible OCMCS aggregates were developed into a controlled drug delivery system. An antitumor alkaloid, CPT was used as the model drug. The amount of drug loaded in OCMCS is strongly dependent on its concentration. Not only the aggregates but also unimer of OCMCS can effectively load the hydrophobic CPT, the maximum amount of CTP loaded in OCMCS was found to be 6 times than that of camptothecin in water at a concentration of OCMCS of 0.0625 mg/ml, which is a little bit higher than its cac. The diffusion coefficient of CPT in the presence of OCMCS exhibits low, which is believed to be correlated with interactions between OCMCS and lipophilic CPT. In vitro measurements of antiproliferative activity of cancer cell further confirm the slow release behavior of CPT from OCMCS even when the final concentration of OCMCS is far lower than its cac in the significant dilution. The present work provides an excellent example of the great potential of OCMCS aggregates in application in the field of biotechnology and pharmaceutics.

Acknowledgements

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