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Self-aggregated nanoparticles from linoleic acid modified carboxymethyl chitosan: Synthesis, characterization and application *in vitro*

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ABSTRACT

The purpose of the present research work was to study the formation of linoleic acid (LA) modified carboxymethyl chitosan (LCC). Another objective was to evaluate effect of linoleic acid degree of substitution on loading capacity (LC), ADR loading efficiency (LE) and in vitro release profile of LCC nanoparticles. The hydrogel nanoparticles can be prepared using linoleic acid modified carboxymethyl chitosan (LACMCS) after the sonication. The critical aggregation concentration (CAC) of the self-aggregate of LA modified CMCS (LCC) was determined by measuring the fluorescence intensity of the pyrene as a fluorescent probe. The CAC values were in the range of 0.061–0.081 mg/mL. Self-aggregated nanoparticles exhibited an increased LC and LE, decreased sustained release with an increasing ratio of the hydrophobic LA to hydrophilic CMCS. LCC nanoparticles loaded with ADR exerted in vitro anticancer activity against Hela cells that was comparable to the activity of free (non-entrapped in nanoparticles) ADR.

1. Introduction

Chemotherapy is a major therapeutic approach for the treatment of cancer. As a chemotherapy agent, although adriamycin is widely used in cancer thermotherapy, its therapeutic effects are always limited by severe adverse effects, such as cytotoxicity in normal tissue and inherent multidrug resistance effect etc. [1]. To overcome these disadvantages and improve chemotherapeutic activity, researchers have focused on the development of nano-sized drug carriers [2]. Among these carriers, self-assembled nanoparticles comprised of hydrophobically modified polysaccharides have been extensively studied because of their excellent biocompatibility and unique structure [3–5]. Hydrophobically modified polysaccharides can self-aggregate due to their intra- and/or intermolecular hydrophobic interaction in aqueous media to form nanoparticles with hydrophobic core and hydrophilic shell. This kind of structure is suitable for trapping hydrophobic anticancer drugs such as adriamycin and taxol etc. [6,7].

Chitosan is a naturally occurring biopolymer made up of β-(1,4)-linked glucosamine units. Preparation of hydrogel nanoparticles based on hydrophobically modified chitosan (HMC) has attracted research interest, because chitosan has unique characteristics such as positive charge, bioadhesivity. Self-assembled nanoparticles from HMC can be used as DNA delivery system and anticancer drug delivery carriers [8,9]. But, due to insoluble nature of chitosan in neutral or basic aqueous phase, it is difficult for HMC to form stable self-assembled nanoparticles solution. Therefore, clinic trials using self-assembled nanoparticles are not available [10]. Recently, people pay more attention to various chitosan derivatives with improved hydrophilicity, e.g. *N*-trimethyl chitosan chloride (TMC), carboxymethyl chitosan (CMCS) [11], glycol chitosan [12–18], methoxy poly(ethylene glycol)-grafted-chitosan etc. [19]. Nanoparticles based on water soluble chitosan derivatives are emerging as novel carriers of drugs because of their stability in biological solution (pH 7.4) and biocompatibility *in vivo*. In our previous study, we reported preparation of the self-assembled nanoparticles based on linoleic acid (LA) modified carboxymethyl chitosan (LACMCS) and adriamycin was also successfully loaded within the nanoparticles [20]. The formation, characteristics and *in vitro* evaluation of nanoparticles as adriamycin carriers still need further study.

In this study, the fluorescence emission spectra of pyrene at various concentrations of LACMCS in an aqueous phase were used to determine critical aggregation concentration of self-aggregates. The usefulness of nanoparticles as carriers of ADR was evaluated by measuring their loaded efficiency, drug release profile and cytostatic properties of adriamycin loading nanoparticles.

2. Materials and methods

2.1. Materials

Chitosan (low-molecular-weight, Aldrich), pyrene, linoleic acids, 1-ethyl-3-(3-dimethylaminopropyyl) carbodiimide (EDC) were

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purchased from Sigma Chemicals. The molecular weight of chitosan was further determined by viscometric method using Mark-Houwink equation [21]: $[\eta] = K_m M^{\alpha}$, where $K_m = 1.81 \times 10^{-3}$, $\alpha = 0.93$. The average molecular weight (M_v) of the chitosan was 3.46×10^6. The %N-deacylation of chitosan was determined using the following relationship [21] which was found to be 81%: %N-deacylation= $(1 - A_{1655}/A_{3340} \times 1/1.33) \times 100$, where A is the logarithmic ratio of the absorbance and transmittance at the given wave number. Pyrene, as fluorescence probe, was purified by double recrystallization from absolute ethanol. Adriamycin (ADR) was purchased from Hairun Biochemical Company (Zhejiang, China). Acetic acid, isopropanol, monocloroacetic acid, methanol, ethanol, the filter, ammonia and ether were obtained from Chinese Medical Company of Shanghai Chimical Reagent (Shanghai, China). Phosphate-buffered saline (PBS) was purchased from Experimental Chemical Plant of Tianjin University (Tianjin, China). All chemicals were of analytical purity and were used as purchased.

2.2. Preparation of CMCS

Chitosan (CS, 5 g), sodium hydroxide (6.75 g), and 1% (w/v) aqueous acetic acid solution (100 mL) were added into a flask (500 mL) to swell and alkalize at a given temperature for 1 h. The temperature was maintained in a water bath. The monochloroacetic acid (7.5 g) was dissolved in isopropanol (10 mL), and added into the reaction mixture drop-wise over about 30 min and reacted for 4 h at the same temperature. The solid was filtered and rinsed in 70–90% methyl alcohol to desalt and dewater, and dried at room temperature under vacuum. The products were sodium salt CMCS.

Sodium salt CMCS (1 g) was suspended in 80% ethyl alcohol aqueous solution (100 mL), hydrochloric acid (10 mL, 37%) was added and stirred for 30 min. The solid was filtered and rinsed in 70–90% ethyl alcohol to neutrality and dried by vacuum. The products were the H-form of CMCS.

2.3. Preparation of LACMCS

LA can be coupled to CMCS by the formation of amide linkages through the EDC-mediated reaction as described in previous study [20]. Briefly, CMCS (1 g) was dissolved in 1% (w/v) aqueous acetic acid solution (100 mL) and diluted with 85 mL methanol. LA was added to the CMCS solution at 0.54 mol/mol glucosamine residue of CMCS followed by drop-wise addition of 15 mL EDC methanol solution (0.07 g/L) while stirring at room temperature. A 1:1 mol ratio of EDC to LA was used in this study. After 24 h, the reaction mixture was poured into 200 mL of methanol/ammonia solution (7/3, v/v) with stirring. The precipitated material was filtered, washed with distilled water, methanol, and ether and then dried under vacuum for 24 h at room temperature.

2.4. Preparation of LCC nanoparticles

The modified chitosan was suspended in PBS (pH 7.4) at 37 °C for 24 h and sonicated by using a probe type sonifier (Ultrasonic Homogenizer UH-600, Sonics & Materials, Inc. USA) at 20 W for 3 min. The sonication was repeated twice to get an optically clear solution by using the pulse function (pulse on, 10.0 s; pulse off, 2.0 s). The clear solution of nanoparticles was filtered through to remove dust. Solutions of different concentrations were obtained by diluting the 1% (w/w) stock solution with PBS buffer.

2.5. Measurement of fluorescence spectroscopy

Fluorescence measurements were used to determine the critical aggregation concentration as described by Liu et al. [22]. Pyrene, used as a hydrophobic probe, was purified by repeated recrystallization from ethanol and vacuum-dried at 20 °C. Purified pyrene was dissolved in pure ethanol at a concentration of 0.04 mg/mL. About 20 μl of the resulting solution was added into a 20 mL test tube and the ethanol was evacuated under purging by nitrogen gas. Four milliliters of LCC nanoparticles solution was subsequently added to the test tube, resulting in a final pyrene concentration of 1.0×10^{-6} M. The concentration of nanoparticle in the solution ranged from 1×10^{-2} mg/mL to 2 mg/mL. The mixture was incubated for 3 h in a water bath at 65 °C and shaken in a SHA-B shaking water bath (GuoHua Company, Hebei, China) overnight at 20 °C. Pyrene emission spectra were obtained using a Shimadzu RF-5301PC fluorescence spectrophotometer (Shimadzu Co., Kyoto, Japan). For measurements of intensity ratios for the first and the third peaks (I_{372}/I_{383}) in the emission spectra for pyrene, the slit openings for excitation and emission were set at 15 and 1.5 nm, respectively. The excitation (λ_{ex}) and emission (λ_{em}) wavelengths were 343 and 390 nm, respectively. The spectra were accumulated with an integration time of 3 s/nm.

2.6. Preparation of ADR-loaded nanoparticles

Different amount of ADR was added to 0.1% solution of nanoparticles while stirring and stored in a dark place for 24 h. The excess ADR, which was not incorporated into the nanoparticles, was removed by ultrafiltration using a membrane filter with molecular weight cut-off at 1.0×10^4 (Amicon, USA). Ultrafiltration was repeated until the 490 nm intensity of the filtrate was zero. ADR-loaded nanoparticle solution was added to acetic acid at a final concentration of 1%, and the 490 nm intensity of ADR was measured.

The loading capacity (LC) of nanoparticles and ADR loading efficiency (LE) were calculated using following equation:

$$\mathrm{LC} = \frac{(A-B)}{C} \times 100; \quad \mathrm{LE} = \frac{(A-B)}{A} \times 100$$

A = total amount of ADR in added solution; B = total amount of ADR in supernatant after ultrafiltration; and C = weight of the nanoparticles measured after freeze-drying.

2.7. In vitro release studies

Release rate measurements in vitro were carried out as follows: 2 mL of ADR-loaded nanoparticles solution (1 g/L in a dialysis tube) was placed in 20 mL of PBS. The release medium was stirred at 100 rpm and 37 °C.

At predetermined sampling times, 2 mL medium was removed and replaced by 2 mL fresh PBS to maintain sink conditions. The amount of ADR in the solution was determined by UV spectrometry at 490 nm.

2.8. In vitro antitumor activity

HeLa cells (human epithelioid cervical cancer cell line) were routinely cultured in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C, 5% CO_2 and 95% humidity. Cell viability was determined using an MTT assay. ADR-loaded nanoparticles (LCC-1) were used to measure antitumor activity in vitro. HeLa cells were incubated in conditioned media containing different concentrations of ADR-loaded nanoparticles or free ADR solution, in 96-well microplates at 37 °C in a humidified atmosphere containing 5% CO_2. After predetermined incubation periods, MTT solution was added to each well. After 4 h of incubation at 37 °C, the medium was removed and any formazan crystals formed were solubilized with DMSO. After slow shaking for 5 min, the absorbance of each well was determined at

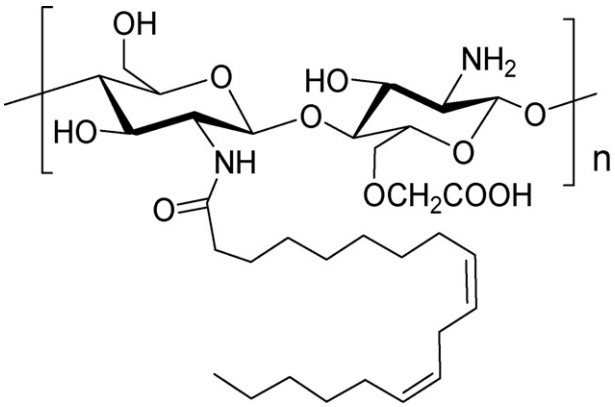

Fig. 1. The structure of LCC.

490 nm using a Microplate Reader. Cell viability was expressed as a percentage compared to a control (only test cells were added).

3. Result and discussion

3.1. Characterization of nanoparticles

LA was covalently coupled to an amino group of CMCS using EDC, a water soluble carbodiimide (Fig. 1). Conjugation was confirmed by infrared and ^{1}H NMR spectra. The average particle size (hydrodynamic diameter of 417.8 ± 17.8 nm) and the size distribution were determined by quasielastic laser light scattering with a mean [22]. TEM images of the nanoparticles showed that nanoparticles with structural integrity were formed after the sonication of LCC molecules (Fig. 2). The degree of substitution (DS), defined as the number of linolenic acid groups per anhydroglucose unit of CMCS, was determined by a titration method [10]. The data are shown in Table 1.

3.2. Critical aggregation concentration of self-aggregates

The aggregation behavior of LCC in aqueous media was monitored by fluorometry in the presence of pyrene as a fluorescence probe. Pyrene was chosen as the fluorescence probe because its condensed aromatic structure is sensitive to polarity, and it produces

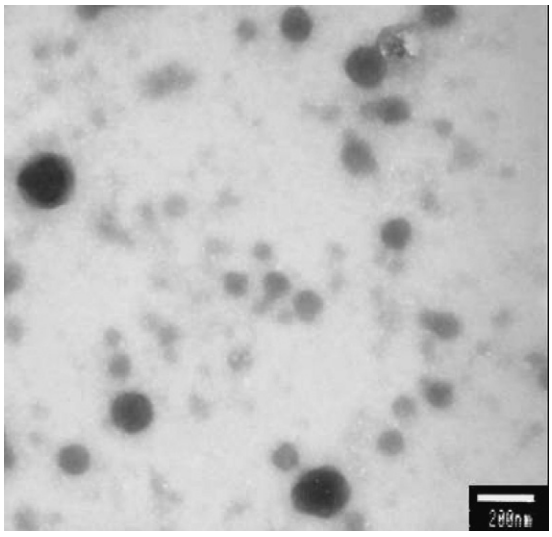

Fig. 2. Transmission electron micrograph of self-aggregates based on LCC.

Table 1
The characterization of LCC nanoparticles.

LCC	DS (%)	CAC (mg/mL)
LCC-1	1.8	0.061
LCC-2	2.1	0.072
LCC-3	2.6	0.081

distinctive excimer fluorescence under conditions of sufficiently high concentration and mobility. Fig. 3 shows the fluorescence emission spectra of pyrene at various concentrations of LCC-3 in an aqueous phase after sonication at 25 °C. The excitation spectra showed no significant changes in the total fluorescence intensity at low concentration ranges, indicating that LCC does not form aggregates in dilute solution. The total emission intensity increased with the increase in concentration of LCC. This result indicates that LCC start to form hydrophobic domains in aqueous media resulting from intermolecular hydrophobic interactions at a certain concentration, which was defined as the critical aggregation concentration (CAC), meaning the threshold concentration of self-aggregation of polymeric amphiphiles. Below the CAC, pyrene is solubilized in water,

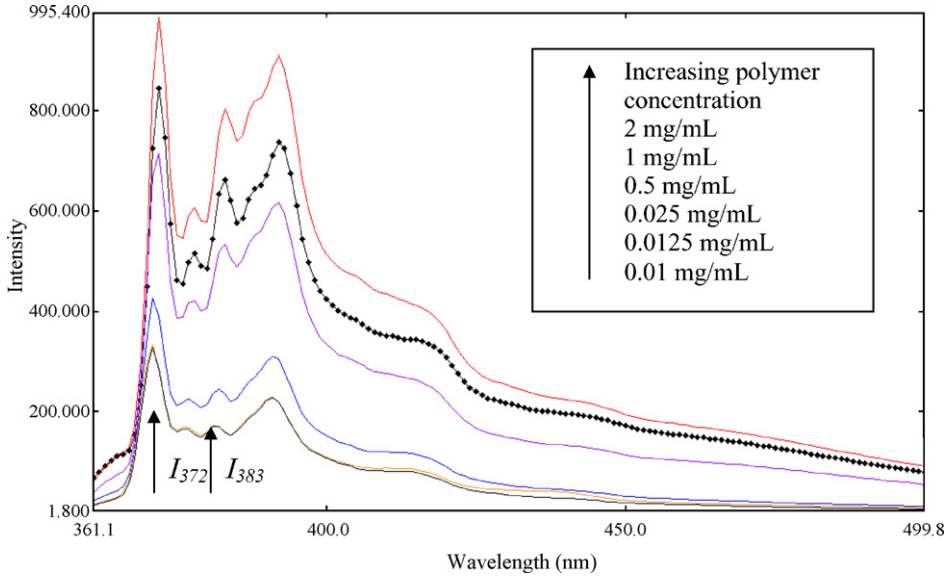

Fig. 3. Fluorescence emission spectra of pyrene in the presence of LCC nanoparticles.

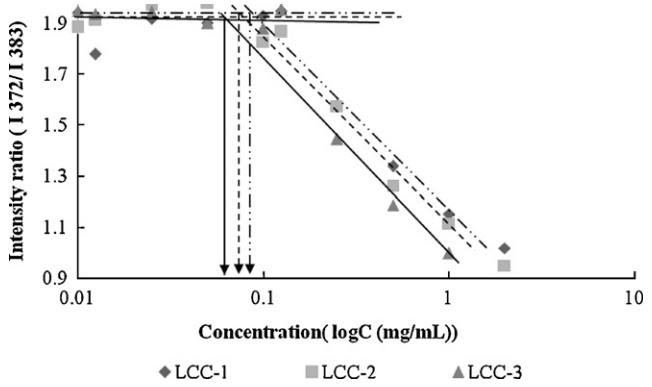

Fig. 4. Intensity ratios (I_{372}/I_{383}) from pyrene emission spectra as a function of logarithm of LCC conjugate concentration in distilled water.

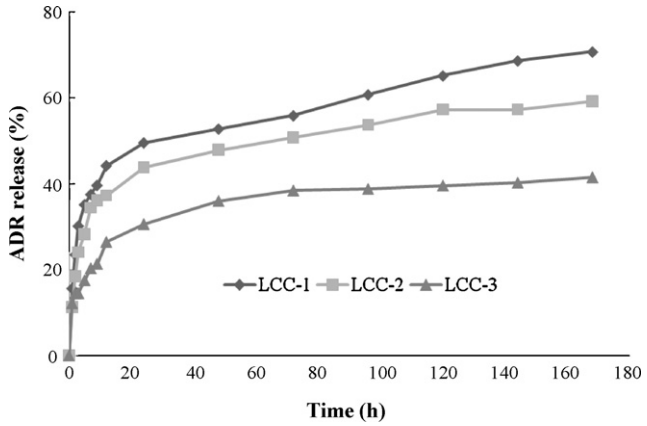

Fig. 5. In vitro release profiles of ADR from LCC nanoparticles.

a medium of high polarity. When aggregates are formed, pyrene partitions preferentially toward the hydrophobic domain afforded by the aggregate core and thus, experiences a non-polar environment [23,24]. The CAC can be determined by observing the change in the intensity ratio of the pyrene in the presence of polymeric amphiphiles.

Fig. 4 exhibits the intensity ratio (I_{372}/I_{383}) of the pyrene excitation spectra versus the logarithm of the LCC conjugates concentration. At low concentrations of polymeric amphiphiles, the I_{372}/I_{383} values are close to the value 1.9, which remains nearly unchanged. The intensity ratios begin to decrease with increasing concentration. The CAC is determined by the interception of two straight lines. The CAC values of the LCC are 0.061, 0.072 and 0.081 mg/mL (Table 1). The CAC values are lower than the critical micelle concentration (CMC) of low-molecular-weight surfactants, which may be one of the important characteristics of polymeric amphiphiles, indicating the stability of LCC self-aggregates in dilute condition. Furthermore, the CAC values of the LCC conjugates decreased with increase in the content of hydrophobic groups LA. The reason may be the increase of hydrophobicity [25,26].

3.3. Drug loading capacity and loading efficiency

The LC of nanoparticles and ADR LE were affected by the DS of LA. As seen in Table 2, when the DS was increased, the LC and LE in the nanoparticles was gradually increased from 58.65% to 65.84% and from 8.79% to 9.88%.

Amphiphilic polymers composed of a hydrophilic segment and a hydrophobic segment can self-assemble via intra- and/or intermolecular associations between hydrophobic moieties, primarily to minimize interfacial free energy. In such a way, the hydrophilic part is oriented outside which may induce steric repulsive forces and stabilize the particle interface, while the hydrophobic part is buried in the core that has been used to serve as a reservoir of hydrophobic drugs, such as paclitaxel and ADR [22,23]. We synthesized a LCC conjugate to form nanoparticles that solubilized and stabilized unprotonated and hydrophobic ADR molecules in aqueous solution. We obtained a relatively high LC and LE. The results showed that free ADR mostly was inserted into nanoparticles successfully. Furthermore, the LC and LE of ADR is affected by the degree of LA substitution, the higher the degree of substitution, the higher the LC and LE.

Table 2
Loading capacity and loading efficiency.

LCC	LC (%)	LE (%)
LCC-1	58.65 ± 0.67	8.79 ± 0.02
LCC-2	63.59 ± 0.78	9.54 ± 0.02
LCC-3	65.84 ± 0.39	9.88 ± 0.01

3.4. In vitro release studies

ADR is one of the most effective anticancer agents; however, high incidences of adverse reactions of the drug have limited its use. To suppress the adverse reactions of ADR, sustained release of ADR from polymeric nanoparticles has been proposed. Fig. 5 shows the in vitro release profiles of ADR of LCC nanoparticles. All of LCC nanoparticles showed an initial burst release phase and a sustained release phase: the entrapped ADR was released from 26.48% to 43.98% during 12 h and release was related to the degree of LA substitution; afterwards, the release rate was slowed down because drugs come out of the interior of nanoparticles. The ADR loaded into the inner core of the nanoparticles showed sustained release of about 70% (LCC-1), 60% (LCC-2) and 40% (LCC-3) over 7 days, respectively. And we found that the greater was the ratio of the hydrophobic LA to hydrophilic CMCS, the slower was sustained release. The initial burst release may be due to the ADR adsorbed on the shell of the nanoparticles. On the other hand, the mechanisms of slow release may involve the chitosan nanoparticles that had a rigid and hydrophobic core. This structure led to the migration of ADR which was strongly restricted, and slow release behavior from the core was observed [20,27]. Furthermore, this may be attributed to the strong hydrophobic interaction between LA segments and ADR. The higher the degree of substitution, the stronger the affiliation between nanoparticles and ADR, thus slower the sustained release [28]. These results indicate that LC, LE and release rate are adjustable by changing the linoleic acid degree of substitution on CMCS.

3.5. In vitro antitumor activity

The antitumor activity of the LCC nanoparticles loaded with ADR against Hela cells was evaluated using the MTT method. Figs. 6 and 7 show the cell viability treated with the free ADR, the ADR-loaded nanoparticles (LCC-ADR) and the free nanoparticles at various ADR concentrations for various incubation times. The free nanoparticles show almost no effects on the cytotoxicity of cells. The activity of both free ADR and LCC-ADR increased with increasing ADR concentration and incubation time showing that cell killing with free ADR and LCC-ADR are both time and concentration dependent. LCC-ADR showed slightly decreased cytostatic activity relative to the free ADR over the same concentration range. LCC-ADR can only be internalized in the cells by the endocytosis process, rather than by the release of free drug in the cell culture medium which diffuses into

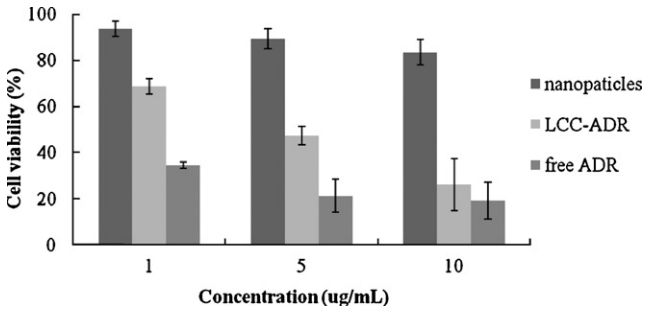

Fig. 6. Viability of Hela cells treated with the free ADR, the ADR-loaded nanoparticles and the free nanoparticles at various ADR concentrations for 3 days.

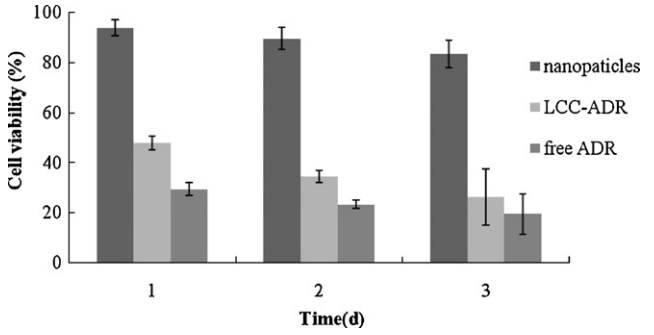

Fig. 7. Viability of Hela cells treated with the free ADR, the ADR-loaded nanoparticles and the free nanoparticles at ADR concentration of 10 μg/mL for various days.

the cells quickly as a small molecule. Furthermore, ADR was loaded in the cores of the nanospheres which might impede its release to the nucleus. When the drug content was 10 μg/mL after 3 days incubation, the cell viability for nanoparticles and free ADR was 26.22% and 19.45%, respectively. This implies that the ADR is released from the nanoparticles inside the cells without losing cytotoxicity once LCC-ADR is transported across the membrane by endocytosis. ADR was released in a continuous way which conformed to release characteristics (Fig. 5). A similar conclusion was reported in previous papers [29,30]. The results obtained from in vitro studies show that LCC nanoparticles may be used as a potential adriamycin delivery system.

4. Conclusions

Hydrogel nanoparticles can be prepared using LA modified carboxymethyl chitosan (CMCS) after the sonication. The formation of self-aggregates depends on the DS, and the CAC values decreased with increasing DS. Self-aggregated nanoparticles can be used as ADR carriers, which exhibited an increased LC and LE, decreased sustained release with increasing DS of LA. LCC nanoparticles loaded with ADR exerted in vitro anticancer activity against Hela cells that was comparable to the activity of free ADR. These studies suggest the potential applicability of nanoparticles to the pharmaceutical and biomedical fields, especially to the delivery of hydrophobic agents.

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