Preparation of Carmustine-Loaded PLA Ultrasmall-Nanoparticles by Adjusting Micellar Behavior of Surfactants

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ABSTRACT: Carmustine-loaded poly(lactic acid) (PLA) nanoparticles were prepared with a spontaneous emulsification-solvent diffusion method by using Pluroinc F68 as emulsifying agent, and the influence of micellar behavior of surfactant in aqueous solution on the sizes of the nanoparticles was studied. The results revealed that F68 dissolved as unimers in water or sodium acetate-acetic acid buffer with pH value ranged from 5.0 to 5.5, resulting in larger PLA particles (226 nm) or clumps. In acidic media with pH of 4.0 and 4.5, however, F68 aggregated into micelles over the concentration of 3.7 and 4.8 mg/mL, as a result, ultrasmall nanoparticles with a size 50 and 80 nm could be obtained, respectively. The change in size had less effect on the drug content and entrapment efficiency, and the nanoparticles strongly enhanced the cytotoxic effect of the loaded drug *in vitro*, and this effect being more relevant for prolonged incubation times. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 110: 2446–2452, 2008

Key words: pluronic surfactant; critical micellization concentration (CMC); polylactic acid; nanoparticle; drug release

INTRODUCTION

Biodegradable poly(lactic acid) (PLA) nanoparticles are widely under investigation as delivery systems for anticarcinogen, protein, peptide, and gene drug[1–3] because of their good biocompatibility, biodegradability, and sustained release characteristics. For the clinical application, however, the desired therapeutic response with the nanoparticles delivery of biomolecules is influenced by various factors, and one of them is the size of nanoparticles. Particles with size above 100 nm tend to restrict their biodistribution, resulting from an increase in their capture by Kupffer's cells or other phagocytic cell populations within the MPS,[4] while particles with size below 100 nm, the hydrophilic surface exhibits a longer circulation in blood,[5] such systems should allow the control of the rate of drug administration that prolongs the duration of the therapeutic effect. In many cases, tumor tissues are supplied by a leaky neovasculature with an incomplete endothelial barrier and have a poor lymphatic drainage. This phenomenon is known as an enhanced permeability and retention effect.[6,7] These tumor characteristics provide an opportunity for the drug-loaded nanoparticles to reach their target site by diffusion (passive targeting).[8] It has been demonstrated that passive targeting is limited to carriers with a size of around 100 nm.[9,10] For the drug delivery targeting to brain tumor, the drug carriers must penetrate through blood-brain barrier (BBB) before getting to tumor tissue. For this reason, the size of drug-loaded nanoparticles should below 100 nm though BBB located at tumor was leaky.[11]

Three techniques have been usually used for the preparation of PLA nanoparticles based on preformed biodegradable polymers: (a) solvent-evaporation procedure[12]; (b) salting-out procedure[13]; (c) nanoprecipitation procedure.[14] Nevertheless, several difficulties have been found by using these techniques when working with toxic solvents (solvent-evaporation) and salts that are incompatible with bioactive compounds (salting-out). And these techniques are not useful to reduce the particle size and are impossible to produce nanoparticles with diameter less than 100 nm.[15]

The spontaneous emulsification-solvent diffusion method was recently developed by Niwa et al.[16] and followed by subsequent studies.[17–19] It involves the formation of a conventional oil-in-water emulsion within a partially water-soluble solvent. The subsequent addition of water to the system makes the sol-

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vent diffuse into the external phase, resulting in the formation of nanoparticles. Influence of process variables on the mean particle size of PLA nanoparticles has been extensively studied,[17–19] such as the type and concentration of the stabilizers, the stirring speed, the internal/external phase ratio, the PLA concentration in the organic phase, the pH and the viscosity of external phase. However, the use of this technique usually results in large particles (i.e., 100–450 nm).[20,21] Recently, It is reported that the PLA nanoparticles of size <100 nm were prepared,[22,23] and by adjusting PLA concentration, surfactant amount, aqueous to organic phase volume ratio, and adding of PLA nonsolvent (ethanol), the sizes of PLA nanoparticles could be controlled between 20 and 70 nm. But there is no clear explanation in mechanism in these reports. It is interesting to note, however, that both the two reports dealt with the incorporation of water-soluble drug (procaine hydrochloride and a tyrphostin compound AG-1295, respectively) to PLA carriers.

It is well known that the addition of inorganic salt influences the micellar properties of nonionic surfactant especially for Pluronic family.[24] The purpose of the present study was to investigate the relationship between micellar behavior of surfactant F68 and the sizes of PLA nanoparticles. In view of the side effect of inorganic salts on biomolecules, however, the micellar properties of F68 were altered through adjusting pH level of aqueous phase by changing the quantity of sodium acetate and acetic acid buffer added in. This operation may complicate the micellar behavior of surfactant F68, but it is very similar to the biological operation, making the technique developed here suit to adapt to biomacromolecules. Carmustine (BCNU) was chosen as a model drug because of the fact that such drugs are effective to treat brain tumor, and it is known prone to break down and its stability is sensitive to pH of medium. Therefore, the effect of pH on the loading of biomolecules could be simultaneously studied. Finally, inhibition of BCNU-loaded PLA nanoparticles to glioma cell was evaluated *in vitro*.

EXPERIMENTS

Materials

Poly(D,L-lactic acid)(PLA) with weight-average molecular weight of 30 kD was purchased from Shandong Parmacy and Mechanism C.(China) Carmustine original drug was supplied by Tianjin Pharmaceuticals Group Corporation. Pluronic F68 (product of Nanjing WELL Chemical Corporation, Ltd., w (EO) = 80%, w (PO) = 20%, weight-average molecular weight of 8370) had molecular formula $(EO)_x$-$(PO)_y$-$(EO)_x$, x and y was determined by ^{1}H-NMR as 76 and 29, respectively.

Preparation of PLA nanoparticles

The PLA nanoparticles were prepared by using the spontaneous emulsification-solvent diffusion method. In a typical experiment, 200 mg of PLA was dissolved in an organic mixture consisting of 19.5 mL acetone and 0.5 mL dichloromethane. The polymer solution obtained was then added dropwise into 50 mL of aqueous F68 solution under the moderate magnetic stirring at 25°C. The pH values of F68 solution were controlled by altering the ratio of sodium acetate to acetic acid (Table I). The organic solvent was evaporated under atmospheric pressure, and the temperature of emulsion was controlled at 25°C with a water bath. The prepared nanoparticles were separated by centrifugation, followed by washed with deionized water under sonication and centrifugation again. This procedure was repeated for three times to remove unincorporated F68, and then the suspension was lyophilized. To formulate nanoparticles loaded with BCNU, the drug 80 mg was added in PLA solution in dark, followed by the same sequence as mentioned above. The basic recipe for the preparation of PLA nanoparticles is shown in Table I.

Characterizations of nanoparticles

Morphology of nanoparticles was observed with a transmission electron microscope (TEM, JEOL JEM-

TABLE I
Parameters for the Preparation of BCNU-Loaded PLA Nanoparticles[c]

	C_{HAc} (mol/L)	C_{NaAc} (mol/L)	pH	Mean particle size[a] (nm)	Poladispersity[a]
1	0.202	0.036	4.0	54	0.110
2	0.202	0.112	1.5	83	0.129
3	0.202	0.363	5.0	Coalescence	–[b]
4	0.202	1.124	5.5	Coalescence	–[b]
5	0	0	7.2	226	0.113

[a] The mean size and the size polydispersity of the nanoparticles were obtained by laser light scatter size-analysis.
[b] Referred to no data.
[c] Concentration of F68 was 0.45%.

100CXII). The average particle diameter and distribution of nanoparticles were assessed by light scattering method (90Plus/BI-MAS, USA).

Determination of BCNU content

According to the previous study, Bratton-Marshall colorimetric method was used to determine the drug content, which was adopted by LOO and DION.[25] Ten mg BCNU-loaded PLA nanoparticles was dissolved in 0.5 mL dichloromethane and was then adjusted to 10 mL with ethanol to precipitate the polymer. The suspension was centrifuged at 15000 rpm for 20 min (4°C). 0.2 mL of the supernatant was added to 1 mL of 0.5% sulfanilamide in 2 *N* HCl and 1.8 mL of deionized water. The sample was placed in a water bath at 50°C for 45 min. After incubation, it was rapidly cooled with ice bath. Then 0.1 mL of N-(1-naphthl) ethylenediamine dihydrochloride aqueous solution(3 mg/mL) was added. After 10 min, the absorbance at 540 nm was measured.

The drug content and drug entrapment were respectively, defined by the following expressions:

$$\text{Drug Content (\%w/w)} = \frac{\text{mass of drug in nanoparticles} \times 100}{\text{mass of nanoparticles recovered}}$$

$$\text{Drug Entrapment (\%)} = \frac{\text{mass of drug in nanoparticles} \times 100}{\text{mass of drug used in formulation}}$$

Micellar behavior of F68 in different pH aqueous solution

Surface tension

The polymer solutions of various concentrations were prepared by dissolving of different amount of F68 in sodium acetate-acetic acid buffer of different pH (Table I) and were allowed to equilibrate for 2 days. The surface tension of polymer aqueous solution was measured by a dynamic Wilhelmy plate technique (DCAT21, Data physics, Germany) at 25°C. The critical micellar concentration (CMC) was determined from the intersection of two straight lines of surface tension γ versus logarithm bulk concentration (log C) of F68 above and below CMC.

Naphthalin solubilization

Excessive condensed nucleus aromatic hydrocarbon naphthalin was added into F68 solution with different concentration and pH. Solutions obtained were kept shaking for 24 h at 25 ± 0.2°C on a horizontal shaking water bath to make sure the solubilization of naphthalin in micelle reached equilibrium. At the end of incubation period, the solutions were centrifuged for 15 min in 1000 r/min to separate the undissolved portion of naphthalin. The amount of naphthalin in supernate was determined using ultraviolet spectrophotometric method at 276 nm (UV-9100 ultraviolet light spectrophotometer).

Inhabiting of glioma cell growth in vitro

The MTT assay was carried out to measure the cell proliferation using a C6 rat glioma cell line. The rat glioma cell line was gifts from NIH (USA). C6 glioma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells (1×10^5) were plated in 60 mm cell culture dishes and grown overnight at 37°C with 5% CO_2 and 95% air until they were 50–80% confluent. Then the cells were trypensized with 0.125% (w/v) trypsin/ethylene-diamine-tetra-acetic acid (pH 8.0) and plated into each well of a 96 well plates. 10 μg BCNU was added into wells as BCNU group, and BCNU-loaded PLA nanoparticles containing 10 μg BCNU were also include as BCNU-PLA group. Empty nanoparticles and PBS were also used as negative controls. On each day of consecutive 6 days, 20 μL MTT (5 mg/mL) was added to each well, and the cells were incubated at 37°C for additional 4 h, then the reaction was stopped by lysing the cell with 200 μL of DMSO for 5 min and quantification measurements (optical density) were obtained at wavelength of 570 nm, and expressed as normalized percentage.

RESULTS AND DISCUSSION

Effect of pH on the sizes of PLA nanoparticles

Formulation for the preparation of drug-free PLA nanoparticles is shown in Table I. Since BCNU was very stable in weak acid environment,[26] pH values of F68 solution were chosen ranging from 4 to 5.5, and F68 solution with pH 7.2 was used as control. Figure 1 shows the morphologies of nanoparticles observed with TEM, and the size and size distribution of the nanoparticles were measured by laser light scattering size-analysis. The average diameter of nanoparticles obtained at pH 7.2 is 226 nm. Whereas at the pH of 5.0 and 5.5, PLA was coalescent during solvent evaporation and no nanoparticles were obtained. With the pH of F68 solution decreasing to 4.0 and 4.5, however, the size of nanoparticles decreases sharply to about 50 ~ 80 nm. These variations should be ascribed to the alteration in the physical-chemical properties of surfactant, because all formulation variables were kept equal except for the pH of F68 aqueous solution.

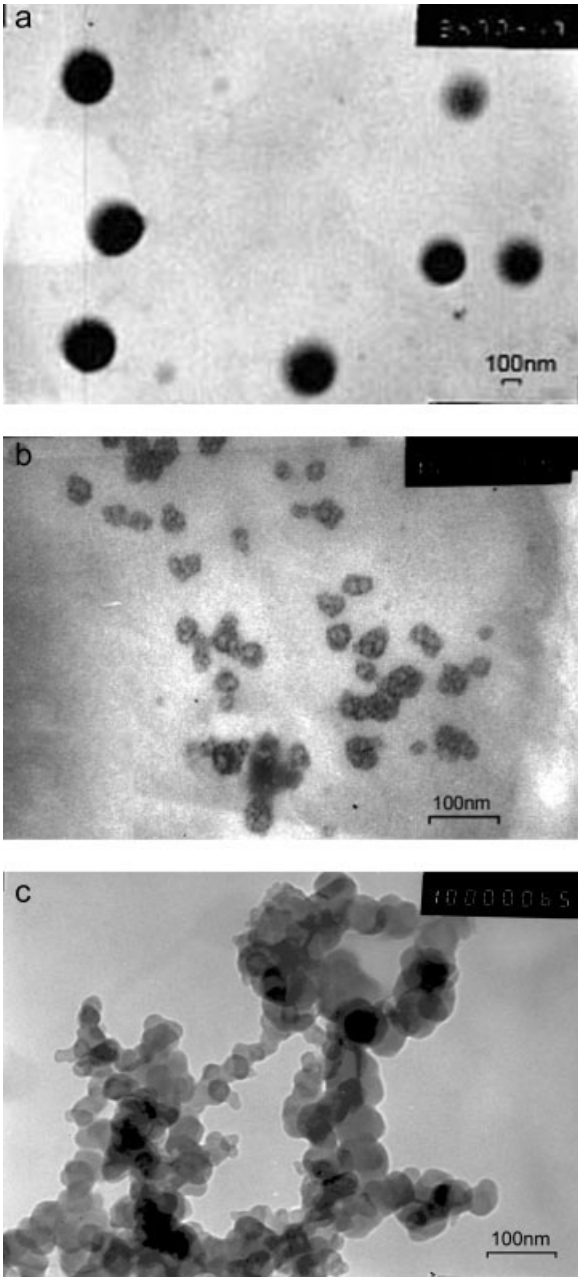

Figure 1 TEM micrograph of PLA nanoparticles prepared in F68 solution (pH = 7.2) free with HAC and NaAC (a), and in F68/HAc-NaAc buffer solution with pH 4.0 (b) and pH 4.5 (c).

Micellar behavior of F68 in different pH aqueous solution

To investigate the effect of physical–chemical properties of F68 on the size of PLA nanoparticles, F68 micellar behavior was measured using surface tension and naphthalin solubilization. Figure 2 shows the influence of pH on the surface tension of F68 solution. Inflection points on curves of F68 aqueous solutions at pH 4.0 and 4.5 appeared at 3.7 and 4.8 mg/mL, respectively. However, the surface tension for F68 solution with pH of 7.2 exhibits a complicated staircase shape, and no apparent inflection point was found on curve. This result was agreed with that reported by Svitova and Radke.[27] Similar phenomena could be seen in the case of F68 solution at pH 5.0 and 5.5.

Naphthalin solubilization was carried out to verify the result of surface tension measurement. Naphthalin was hydrophobic and its solubilization in surfactant aqueous solution arose from the formation of hydrophobic environment provided by micelles formed by Pluronic copolymer above CMC. Therefore, the concentration corresponding to inflection point on solubilization curve was usually assigned to the CMC of Pluronic copolymer. Figure 3 shows the solubilization curves of naphthalin in F68 solution at pH 7.2 and pH 4.5, respectively. In F68 solution at pH 4.5, the solubilization of naphthalin is obvious and the CMC measured by solubilization method is very close to that by surface tension method. In F68 solution at pH 7.2, however, no evident enhancement in solubility of naphthalin can be found, indicating that no micelle was formed in the concentration range being measured. Our result was in agreement with the previous reports where F68 existed as monomer at room temperature and can not form micelles till temperature above 40°C.[28]

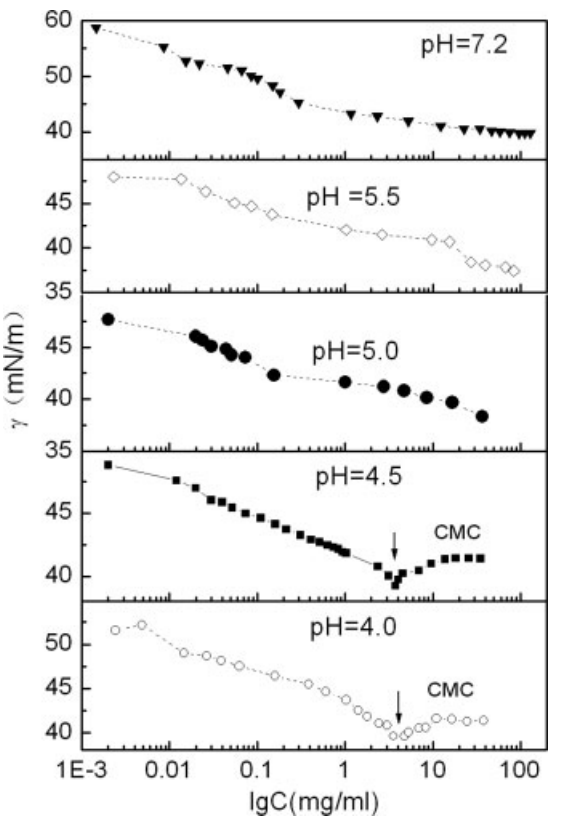

Figure 2 Surface tension for F68 solution with different pH values, plotted as the F68 concentration at 25°C.

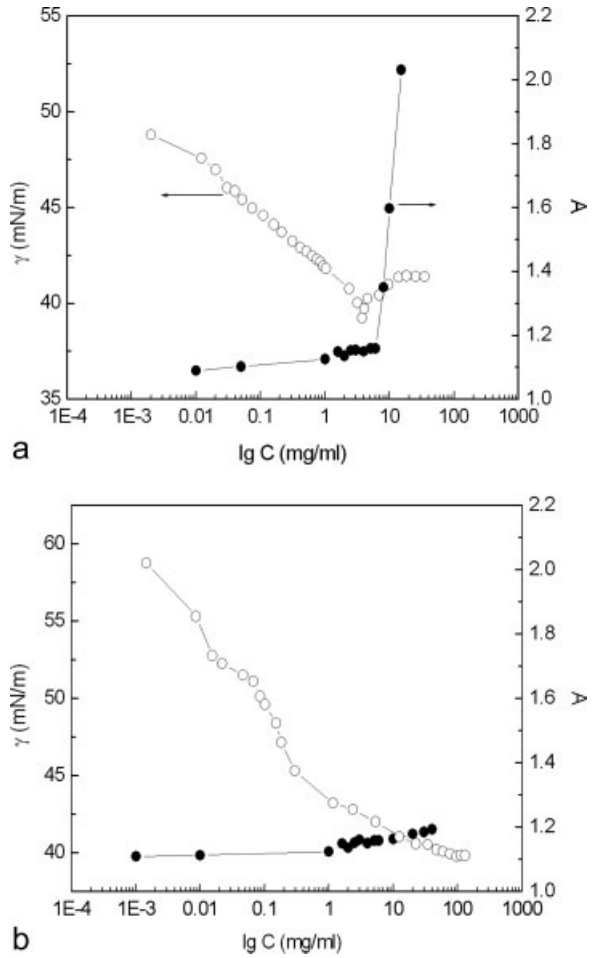

Figure 3 Solubilization of naphthalene in F68 solution with different pH values, and the contrast with the surface tension measurement. (a). pH = 4.5, (b). pH = 7.2.

The varieties of micellar behavior of F68 in different pH media were attributed to the salting out and salting in effect of inorganic electrolytes on F68 molecules. Na^+ enhanced the structure of water and competed with polyoxyethylene moiety of F68 for water of hydration, salting F68 out by dehydration and lowering the CMC of F68. In contrast, CH_3COO^- anion broke the structure of water, increasing the association of water molecules with ether groups of F68 via hydrogen bond, leading to the increase in CMC. At pH 4.0 to 4.5, the concentration of Na^+ was high enough to salt F68 out, as a result, the CMC of F68 decreased and the micelles could be formed at lower concentration. At pH 5.0 and 5.5, the concentration of CH_3COO^- increased, the salt in became the dominant effect, F68 tended to dissolve in solution and no micelles could be formed.

The physical–chemical properties of surfactant are crucial to the size of the nanoparticles obtained in emulsification-diffusion method. In this method, nano-order emulsion droplets form firstly due to the quick diffuses of acetone out from each emulsion droplet and nanoparticles are then obtained by the solidifying of the droplets due to the evaporation of dichloromethane.[29] The size of the nanoparticles obtained finally depends not only on the interfacial turbulence resulted from acetone diffuse but also on the stabilization of droplets and 'protonanoparticles' after diffusion process. Dimitrova et al.[30] suggested that the stability of the droplets could be affected by the continuing solute mass transfer. The droplets could collide and coalesce among themselves. Therefore, if the stabilizer remained at the liquid-liquid interface during the diffusion and evaporation process, and if its protective effect was adequate, then the size of the droplets formed at first step would be maintained and the nanoparticles with small sizes could be obtained. In present study, the decrease in CMC of F68 might increased their amount absorbed on the droplets surface at the lower F68 concentration, thereby improving packing of surfactant at the interface and reducing the size of the nanoparticles.

Drug release and inhibition of tumor growth *in vitro*

Table II summarizes the mean particle, encapsulation ratio and encapsulation yield of nanoparticles obtained at different pH environment. The drug-loading and entrapment efficiency of the nanoparticles prepared at pH 7.2 are 3.472% and 23.58% respectively, while the nanoparticles obtained at pH 4.0 possess a drug-loading and entrapment efficiency of 2.750% and 19.06%. This result indicates that drug encapsulation was only slightly affected by the size of nanoparticles. The possible reason is the stability of BCNU in weak acid media. BCNU were most stable in buffer solution of pH 4.0 with a half-life over 8 h,[21] in contrast, it tended to decompose quickly in pH 7.2 buffer and the half-life was less than 1.5 h. Therefore, it believes that at pH 4.0, the stability of BCNU leaded to more virginal drug was encapsulated in the particles.

In vitro release of BCNU from two types of nanoparticles, prepared according to Table II, in phosphate buffered saline is shown in Figure 4. The profiles are biphasic, with an initial burst of drug, followed by a phase of slower release as drug entrapped inside the particles diffuses out into the

TABLE II
Influence of the pH of F68 Solution on Drug Content and Encapsulation Yield

pH	Mean particle size (nm)	BCNU drug content (%)	Encapsulation yield (%)
7.2	226	3.472	23.58
4.0	54	2.750	19.06

release medium. For nanoparticles with size of 54 nm, 22% release of the drug occurs in 12 h, while nanoparticles with size of 226 nm had 15% release during the same time. By 262 h the total release is 27% for larger particles and 35% for smaller particles. Considering the drug content was close to each other, the size of the nanoparticles was a major factor to influence the release rate.

The cytotoxic activity of BCNU and BCNU-loaded PLA nanoparticles was evaluated by assessing cell viability by the MTT assay using the C6 glioma cell line. As shown in Figure 5, a marked reduction in cell viability is observed when C6 cells were incubated with 10 μg/mL BCNU for 24 h at 37°C. At this concentration, the cell growth was almost totally inhibited after 72 h of incubation. The cell viability, however, increases gradually for the other incubation times tested, demonstrating that the remaining free BCNU degraded completely, and the biological effect of single dose of BCNU cannot maintain for 1 week in C6 glioma cells *in vivo*.

BCNU loaded-nanoparticles exhibited a different behavior. For both the BCNU-loaded ultra-small nanoparticles and nanoparticles, increasing the time of incubation resulted in an enhancement of BCNU-PLA nanoparticles cytotoxicity. After 120 h of incubation with this formulation, a reduction of approximately 84% in cell viability was detected for all samples tested. No distinct difference in cell viability was observed between two types of BCNU-loaded nanoparticles.

Compared to the BCNU and BCNU-loaded nanoparticles groups, the empty polymer group demonstrated a little but sustained growth inhibition. The ultra-small empty nanoparticles groups, however, showed a obvious cytotoxic activity at 24 h, suggesting side-effect may arose from the sodium acetate – acetic acid buffer.

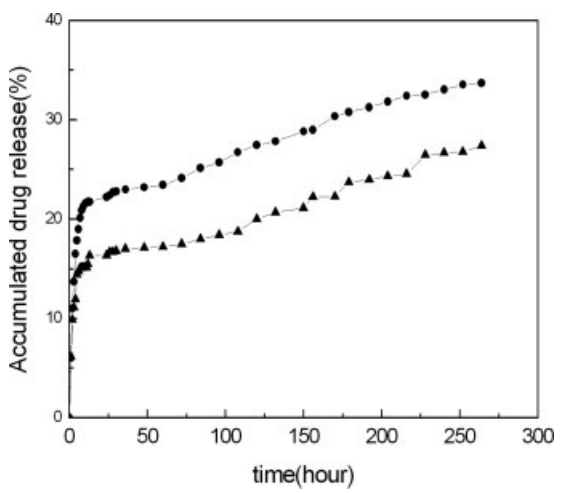

Figure 4 Release profiles of BCNU from PLA nanoparticles. ● BCNU-loaded PLA ultra-small nanoparticles; ▲ BCNU-loaded PLA nanoparticles.

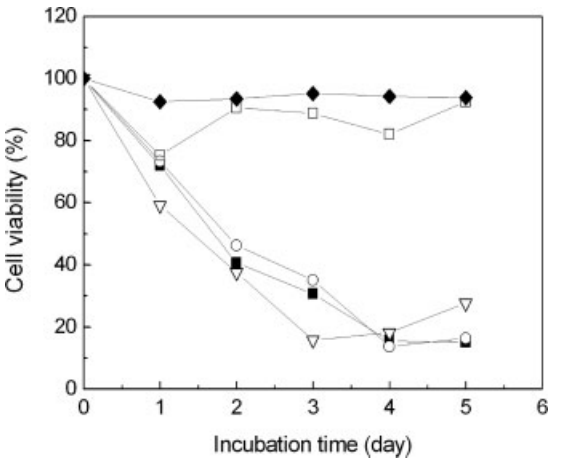

Figure 5 Inhibition of tumor growth by BCNU-loaded PLA nanoparticles *in vitro*. ■ BCNU-loaded PLA ultra-small nanoparticles; ○ BCNU-loaded PLA large nanoparticles; □ Empty PLA ultra-small nanoparticles; ◆ Empty PLA large nanoparticles; ▽ BCNU.

CONCLUSIONS

The micellar properties of F68 could be altered through adjusting pH of aqueous phase by changing the quantity of sodium acetate and acetic acid buffer added in it. In addition, the micellar properties of F68 were associated with its absorption ability on PLA globule surface, which could influence the stability of PLA globule and reduce the size of nanoparticles. When pH of F68 solution was adjusted to 4.0 and 4.5, ultra-small nanoparticles of about 50 ~ 80 nm could be obtained. And though the size decreased sharply, the drug content and entrapment rate kept at about 3 and 20% respectively. The ultra-small BCNU-loaded nanoparticles showed effective cell inhibition ratio, similar to the larger nanoparticles.

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