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Research paper

Layer-by-layer assembly of poly(L-glutamic acid)/chitosan microcapsules for high loading and sustained release of 5-fluorouracil

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ABSTRACT

Hollow polyelectrolyte microcapsules based on poly(L-glutamic acid) (PLGA) and chitosan (CS) with opposite charges were fabricated by layer-by-layer (LbL) assembly technique using melamine formaldehyde (MF) microparticles as sacrificial templates. The LbL assembly of polyelectrolytes and the resultant PLGA/CS microcapsules were characterized. A hydrophilic anticancer drug, 5-fluorouracil (5-FU), was chosen to investigate the loading and release properties of the microcapsules. The PLGA/CS microcapsules show high loading capacity of 5-FU under conditions of high drug concentration and salt adding. The high loading can be ascribed to spontaneous deposition of 5-FU induced by hydrogen bonding between 5-FU and PLGA/CS microcapsules. The PLGA/CS microcapsules show sustained release behavior. The release rate of 5-FU drastically slows down after loading in PLGA/CS microcapsules. The 5-FU release from PLGA/CS microcapsules can be best described using Ritger–Peppas or Baker–Londale models, indicating the diffusion mechanism of 5-FU release from the PLGA/CS microcapsules. In vitro cytotoxicity evaluation by the MTT assay shows good cell viability over the entire concentration range of PLGA/CS microcapsules. Therefore, the novel PLGA/CS microcapsules are expected to find application in drug delivery systems because of the properties of biodegradability, high loading, sustained release and cell compatibility.

1. Introduction

5-Fluorouracil (5-FU), a water-soluble fluorinated pyrimidine analog, is an antineoplastic drug, extensively used in clinical chemotherapy for the treatment of metastatic carcinomas of breast, gastrointestinal tract, pancreas, head, neck, and ovary [1–3]. 5-FU is rapidly absorbed through the blood capillaries into systemic circulation. This results in relatively low levels of drug near the site of action with the subsequent loss of efficacy and increased risk of systemic toxicity [3,4]. So, 5-FU needs long-term and sustained release in clinical application.

By using polymer-based drug delivery systems (DDSs) [5] of 5-FU the incidence of side effects may be reduced and therapeutic effects increased. Various polymer-based DDSs of 5-FU, including several groups such as polymer–drug conjugates [6], block or graft copolymer vesicles and micelles [7,8], submicrometer-size particles [9,10], etc., have been researched. But these conventional techniques proposed show the unsatisfied 5-FU loading and release behavior, which may be attribute to the limitation of the designed drug delivery vehicles, the poor compatibility, and lack of strong interaction between 5-FU and polymer matrix. So, a new DDS of 5-FU should be developed to improve these situation.

Hollow microcapsules, fabricated via the layer-by-layer (LbL) self-assembly of oppositely charged polyelectrolytes, have attracted particular interest. The properties, such as the shape, particle size, composition, surface features, wall permeability, can be tailed on a nanometer-scale range.

The controllable wall permeability, which is the most important feature of the LbL polyelectrolyte microcapsules, makes them promising as drug delivery vehicles [11,12]. Utilizing this property, various drugs have been loaded into the hollow microcapsules by simply adjusting the permeability of the capsule wall at different pH value or salt concentration [13]. However, the applicability of this method is largely restricted to the capsule compositions or/and the encapsulated drugs, and the loadings achieved are typically low, as the maximum concentration of drug inside the capsules is often limited to the concentration in the solution [13,14]. Some water-soluble substances have been reported to spontaneously deposit in the interior of polyelectrolyte microcapsules with the driving force of electrostatic attraction with a negatively charged complex of MF residues in the capsules [15,16]. The phenomenon of spontaneous deposition provides a facile pathway to highly load various water-soluble drugs.

The polymer materials employed for the LbL assembly of microcapsules mainly focus on non-degradable synthetic

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polyelectrolytes, such as poly(diallyldimethylammonium chloride) (PDADMAC) [12,17], poly(allylamine hydrochloride) (PAH) [18–20], polyethyleneimine (PEI) [21], poly(styrenesulfonate) (PSS) [18,19], and poly(acrylic acid) (PAA) [20]. But for practical and biomedical applications, the use of nature polyelectrolytes seems an attractive alternative. Nature charged polysaccharides, such as chitosan [22] and alginate sodium [22,23], have already been researched. However, the disadvantage is that the properties of nature polymers vary greatly with natural sources and batches, and it is difficult to control the chemical structure, molecular weight, and degradation behavior. Biodegradable synthetic polyelectrolytes have the advantages of controllable chemical structure, molecular weight, and various performances. Synthetic charged polypeptides, as an important class of biodegradable polyelectrolytes, have received interest because they possess a more regular arrangement and a smaller diversity of amino acid residues than those derived from natural proteins. Examples of such synthetic charged polypeptides include poly(L-ornithine), poly (aspartic acid), poly(L-lysine), poly(L-glutamic acid), and so on. Thus, a hybrid polymeric microcapsules combining natural and synthetic polymers are expected to make up for the deficiencies of nature polymers.

The polyelectrolytes selected for this study, poly(L-glutamic acid) and chitosan, make a pair of a synthetic polypeptide and a nature polysaccharide with opposite charges. Poly(L-glutamic acid) (PLGA), a synthetic polypeptide, is unique in that it is composed of naturally occurring L-glutamic acid linked together through amide bonds [24]. It is biodegradable and non-toxic, and the pendent-free carboxyl groups in each repeating unit provide functionality for drug attachment. These features make PLGA a promising candidate as a carrier in drug delivery system [24]. Chitosan (CS), α(1–4)2-amino 2-deoxy β-D glucan, is a partially deacetylated form of chitin, an abundant polysaccharide present in crustacean shells [25]. In the field of biopolymers, chitosan is well known for its biological properties such as biodegradability and biocompatibility allowing its use in drug delivery systems [26]. For reference, the chemical structures of PLGA, CS, and 5-FU are shown in Fig. 1.

In our previous study, PLGA and poly(ethylene glycol)-b-poly(L-glutamic acid) (mPEGGA) diblock copolymer was synthesized [27]. Polyelectrolyte complexation between PLGA (or mPEGGA) and chitosan (CS) in the form of films and nanoparticles has been scrutinized [27–29]. Here, we intent to develop a new vehicle for 5-FU delivery system: the microcapsules fabricated through LbL self-assembly of PLGA and CS. The LbL assembly process of PLGA/CS microcapsules, the 5-FU loading, and release properties was investigated. The interaction between 5-FU and PLGA/CS microcapsules was discussed. The PLGA/CS microcapsules show high loading and sustained release of 5-FU, demonstrating a good prospect of application in controlled drug release.

2. Materials and methods

2.1. Materials

Poly(L-glutamic acid) ($M_\eta = 6.0 \times 10^4$) was prepared from poly(γ-benzyl-L-glutamate) (PBLG) synthesized by the ring-opening polymerization of the *N*-carboxyanhydride (NCA) of γ-benzyl-L-glutamate in our laboratory. CS ($M_\eta = 4.0 \times 10^4$) was purchased from Jinan Haidebei Marine Bioengineering Corp. (Shangdong, China). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was purchased from Covalent Chemical Technology Co., Ltd. (Shanghai, China). Fluorouracil (5-FU) was purchased from Nantong Pharmacy Co., Ltd. (Jiangsu, China). 3-(4,5-Dimethylthiozol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) supplied by Sigma was used without further treatment. Other reagents were all analytical grade and used as received.

2.2. Preparation of hollow PLGA/CS multilayer microcapsules

Weekly crosslinked melamine formaldehyde (MF) colloidal microparticles with monodisperse size were used as template in this work. They were synthesized according to a previous method [30,31]; 1.4 g melamine was mixed with 3 g formaldehyde and 5 g de-ionized water under stirring of 200 rpm at 60 °C for 20 min to obtain a prepolymer. Then, the prepolymer was added into 45 mL poly(vinyl alcohol) (4.5 mg/mL) aqueous solution under stirring of 200–300 rpm. The solution pH was adjusted to 5.1 with acetic acid, and temperature was kept at 60 °C. Turbidity appeared within 10 min of stirring but the stirring was continued for another 10 min. The reaction was stopped by pouring the mixture into de-ionized water precooled. The produced MF microparticles were rinsed using the circle of centrifugation (3500 rpm, 5 min)/washing/redispersing in de-ionized water for three times. The dried MF microparticles were obtained by centrifugation (3500 rpm, 5 min) and subsequent freeze-drying (−80 °C, 48 h).

The assembly of stable polyelectrolyte PLGA/CS multilayers on the top of MF particles was performed by applying the LbL assembly technique, as shown in Fig. 2. The bare, positively charged MF particles (100 mg) were incubated with 10 mL PLGA solution containing 0.5 mol/L NaCl for 60 min, followed by three centrifugation (3500 rpm, 5 min)/washing cycles. Then, the MF particles were added into 10 mL CS solution containing 0.5 mol/L NaCl, 60 min was allowed for adsorption, and three centrifugation/washing cycles were performed (as above). The PLGA and CS adsorption steps were repeated to build multilayers on the MF particles. The alternative adsorption steps were continued until five pairs of PLGA/CS coating were reached. Hollow capsules were finally obtained by dissolving the MF core in 100 mL HCl solution (pH 1.1) for 30 min, followed by centrifugation (16,000 rpm, 5 min), washing with de-ionized water for three times and subsequent freeze-drying (−80 °C, 48 h) for further use.

2.3. 5-FU loading and release

One milligram PLGA/CS microcapsules were dispersed in 5 mL 5-FU aqueous solution. Various factors that affect the loading capacity of microcapsules were studied, including drug concentration, salt concentration, loading time and temperature. In a drug concentration-dependent experiment, the concentration of 5-FU solution varied from 0.2 to 1.0 mg/mL. In a loading time-dependent experiment, the microcapsules were incubated in 5-FU solution of

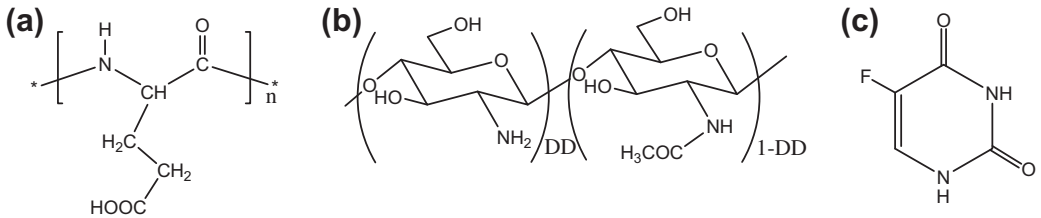

Fig. 1. Structures of (a) PLGA, (b) CS (DD refers to the degree of deacetylation), and (c) 5-FU.

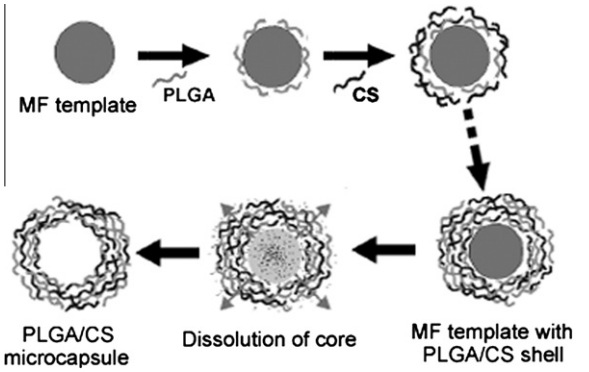

Fig. 2. Schematic illustration for the preparation of hollow PLGA/CS microcapsules.

1.0 mg/mL for 1–8 h. As for NaCl concentration-dependent experiment, the microcapsules were incubated in 5-FU solution of 1.0 mg/mL with NaCl concentrations ranging from 0.0005 to 0.5 M. In order to research on temperature effect, microcapsules were incubated at 25, 37, and 60 °C for 1 and 4 h, respectively. After being incubated under various conditions, the 5-FU-loaded microcapsules were centrifuged (16,000 rpm, 5 min), washed two times with de-ionized water, and freeze-dried for further use in release experiments. The concentration of 5-FU remained in the supernatant after centrifugation was measured at 265 nm wavelength, which is the UV–vis spectroscopy characteristic absorption wavelength. The 5-FU loading inside the microcapsules was calculated from the change of 5-FU concentrations in the supernatant. All the data are averaged from three parallel experiments.

The release of 5-FU from the microcapsules was determined by the dialysis method. One milligram 5-FU-loaded PLGA/CS microcapsules were carefully enveloped in the dialysis bag and immersed in either 100 mL phosphate buffer solution (PBS) (pH 7.4) or hydrochloric acid buffer solution (pH 1.4), ensuring the removal of the air bubbles in the dialysis bag before sealing. During the drug release experiment, 5-mL aliquots of the release media was taken out with reconstitution of 5 mL fresh buffer solution at every predetermined time interval, and the concentration of the 5-FU released from this drug delivery system was monitored at 265 nm of UV absorbance.

For comparison, the drug release experiment of bare 5-FU was conduct. The release from crosslinked PLGA/CS microcapsules was also studied. For crosslinking the microcapsule shell, 1 mg PLGA/CS microcapsules were added into 2 mL EDC solution (60 mg/mL) and cured at room temperature for 2 h. Here, EDC treatment was used to form amide bonds between the —COOH groups in PLGA and —NH_2 moieties in CS. Then, the crosslinked microcapsules were washed two times with de-ionized water and freeze-dried.

2.4. Instruments

Zeta (ζ) potentials were determined with Malvern Zetasizer 3000HS equipped with MPT-1 titrator (Malvern, Worcestershire, UK). Electrophoretic mobilities were converted to ζ-potentials using Smoluchowski's equation.

The FTIR spectra were recorded using a FTIR spectrophotometer (AVATAR 370, Nicolet, USA) in the region of 4000–500 cm^{-1}.

X-ray diffraction patterns were analyzed using a diffactometer (D/MAX2550, Rigaku, with Cu Kα radiation at a voltage of 40 kV and 30 mA. The samples were scanned between $2\theta = 5–40°$ with a scanning speed of 5°/min. Prior to testing, the samples were dried and stored in a desiccator.

Scanning electron microscopy (SEM, JEOL, JSM-6700F) and transmission electron microscopy (TEM, JEOL JEM-200CX, operated at 120 kV) were used to examine the morphologies of the samples.

UV–vis spectra were recorded on an Agilent 8453 UV–vis spectrophotometer.

2.5. Cell cytotoxicity assay

Cell cytotoxicity of PLGA/CS microcapsules was assayed using MTT assay. All samples subjected to cytotoxicity assay were previously sterilized by exposure to saturated steam of ethylene oxide. After sterilization, PLGA/CS microcapsules were respectively put into DMEM (Dulbecco's modified eagle medium, Gibco) supplemented with 10% FBS (fetal bovine serum, from Gibco) for 48 h at 37 °C to get their extract liquid with the concentration of 100, 50, 25, 12.5, 6.25, 3.125, and 1.5625 mg/mL, respectively. The common mouse fibroblast L929 cells were cultured onto a 96-well plate at a density of 12,000 cells per well and medium changed after 24 h incubation. Then, various concentrations of extract liquid were then added to the wells. After incubating for 24 h, 20 μL MTT stock solution in PBS (5 mg/mL) was added into each well with a final concentration of 0.5 mg/mL MTT. After 4 h of incubation at 37 °C, the supernatant was removed, and 200 μL DMSO was added to dissolve precipitated formazan crystals. The absorbance was measured using a microplate reader (Multiskan MK3, Thermo USA) at a test wavelength of 492 nm. Untreated cells were taken as control with 100% viability. The relative cell viability (%) was evaluated using the following equation:

$$\text{Cell viability} = A_{\text{sample}}/A_{\text{control}} \times 100,$$

where A_{sample} and A_{control} refer to the absorbances of the treated cells and untreated cells, respectively [32].

3. Results and discussion

3.1. PLGA/CS multilayer microcapsules fabricated through LbL assembly

The LbL assembly of PLGA and CS on colloidal MF microparticles is followed by microelectrophoresis (Fig. 3), as alternating ζ-potentials have been shown to be an excellent indicator of multilayer film growth on particles. With the assembly of PLGA/CS multilayers on the MF particles, the ζ-potentials alternate from ca. −15 to −40 mV for particles with outermost PLGA layers to ca. +25 to +40 mV for those having outer CS layers. The alternating ζ-potentials observed with each coating step suggest that multilayers are formed on the colloidal particles with the main driving force of electrostatic adsorption [21,33]. The ζ-potential increases with polyelectrolyte concentration, which can be attributed to the increasing charge density of outmost polyelectrolytes on the particles.

Fig. 4 presents the FTIR spectra of weakly crosslinked MF particles, PLGA, CS, and PLGA/CS microcapsules. For MF particles, the absorption band at 3377 cm^{-1} is ascribed to the superposition of stretching vibrations of —NH_2 and O—H groups, the peak at 1572 cm^{-1} is attributed to C—N and N—H stretching vibration, and the absorbances at 1500, 1012, and 814 cm^{-1} are assigned to the stretching vibrations of —NH_2, C—O—C, and C—N—C groups, respectively [34]. The absorption peaks of PLGA, i.e., those of the O—H, C=O, amide I and II groups, are located at 3346, 1734, 1628, and 1545 cm^{-1}, respectively [28,29]. With respect to CS, the characteristic absorption band at 3429 is attributed to the stretching vibration of the N—H group bonded to the O—H group, and the peaks at 1657 and 1597 cm^{-1} are ascribed to amide I and II bands [28,29]. For PLGA/CS microcapsules, the original char-

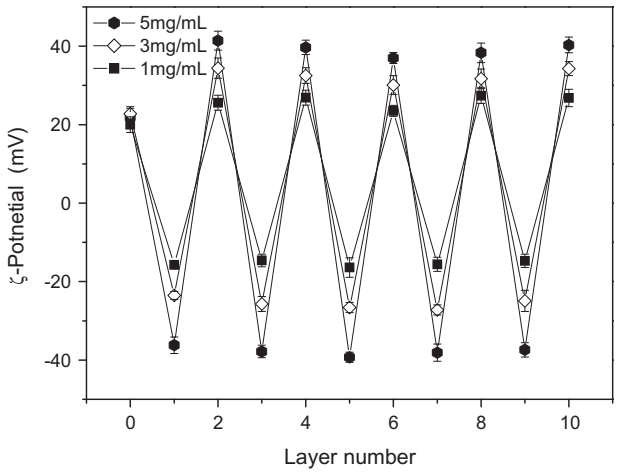

Fig. 3. ζ-Potential versus layer number for the LbL self-assembly of PLGA and CS on MF particles. The odd layer numbers correspond to PLGA deposition and the even layer numbers to CS adsorption.

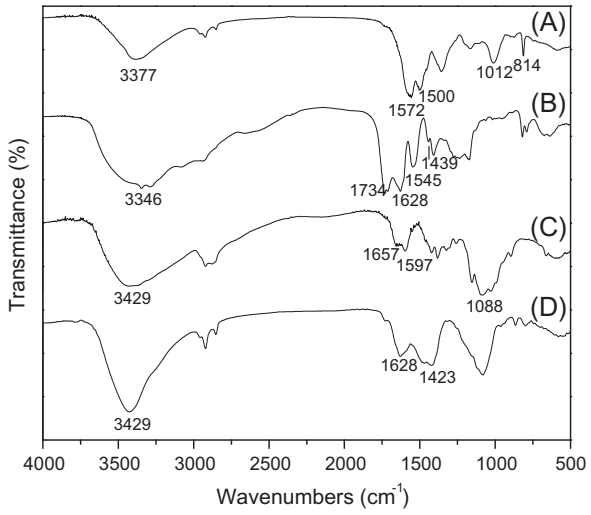

Fig. 4. FTIR spectra of (A) weakly crosslinked MF particles, (B) PLGA, (C) CS, and (D) PLGA/CS microcapsules.

acteristic absorptions of C═O group at 1734 cm^{-1} and amide II group at 1545 cm^{-1} for PLGA almost disappear, also do those of amide I and II bands for CS. New absorption bands that appear at 1628 and 1423 cm^{-1} are the characteristic bands of $—NH_3^+$ and $—COO^-$, respectively, revealing the strong electrostatic interaction between negatively charged carboxylic acid salts ($—COO^-$) on PLGA and the positively charged amino groups ($—NH_3^+$) on CS, and the acid–base complexation in PLGA/CS microcapsules [29,35].

Morphology observation was conducted to follow the assembly of the PLGA/CS multilayers on the weakly crosslinked MF particles and examine the morphology of the particles before and after core removal. SEM and TEM micrographs of MF particles, MF particles coated with PLGA/CS multilayers, and PLGA/CS microcapsules are shown in Fig. 5. The spherical, uncoated MF particles have a smooth surface with an average diameter of about 1 μm. Deposition of the PLGA/CS multilayers on the MF particles causes a noticeable change in the particle morphology with a coarse surface and slight conglutination. This is owing to the irregular stack of oppositely charged PLGA and CS chains on the particle surface [36]. The diameter of the particles increases after PLGA/CS multilayers deposition. Similar phenomenon is reported for assembly of poly(4-vinyl-*N*-n-butylpyridinium bromide)/sodium poly(styrene sulfonate) (C4PVP/NaPSS) on PS latexes [36]. After removal of MF cores in HCl solution, the resultant PLGA/CS microcapsules maintain spherical morphology with relatively smooth surface. TEM images of PLGA/CS microcapsules show reduced electron density of intact spheres with somewhat overlap, a dark ring of PLGA/CS microcapsules is detected in the inset with high magnification, indicating the hollow nature. No broken capsules are observed from SEM and TEM images, and the integrity of PLGA/CS macrocapsules from the LbL technique is necessary for the following drug loading and release studies.

3.2. Interaction between 5-FU and PLGA/CS microcapsules

In order to elucidate the interaction between 5-FU and PLGA/CS microcapsules, the FTIR spectra and XRD of PLGA/CS microcapsules before and after 5-FU loading were studied. The FTIR spectra of 5-FU-loaded PLGA/CS microcapsules and blank PLGA/CS microcapsules are shown in Fig. 6. For comparison, the FTIR spectrum of 5-FU is also presented. In the spectrum of 5-FU, the characteristic peaks at 1724, 1248, and 1180 cm^{-1} are attributed to stretching vibration of C═O, C—N, and C—F groups, respectively [37]. They shift to 1736, 1275, and 1169 cm^{-1} in the spectrum of 5-FU-loaded PLGA/CS microcapsules. Also, the bands at 1430 and 642 cm^{-1} correspond to bending vibrations of ═C—H groups shift to 1383 and 623 cm^{-1} after 5-FU loading in PLGA/CS microcapsules. The above evidences indicate some interaction between 5-FU and PLGA/CS microcapsules and substantially encapsulation of 5-FU in PLGA/CS microcapsules [38].

Fig. 7 shows the X-ray diffraction patterns of pure 5-FU, PLGA/CS microcapsules before and after 5-FU loading. The pure 5-FU exhibits a strong characteristic peak at $2\theta = 28.6°$, indicating its crystalline structure [7]. For PLGA/CS microcapsules, only a broad peak centered at 21.6° is observed, corresponding to the amorphous nature. For 5-FU-loaded PLGA/CS microcapsules, the X-ray diffraction pattern shows the presence of the typical peaks for both 5-FU and PLGA/CS microcapsule at 28.4 and 22.7°, respectively, which indicates that the crystal state of 5-FU in PLGA/CS microcapsule remains the same. While the position of these two peaks shifts, and the intensity of these peaks is evidently reduced, revealing a certain degree of interaction between 5-FU and PLGA/CS microcapsules. It is also one of the evidence that 5-FU has been successfully loaded into the PLGA/CS microcapsules.

The interaction between 5-FU and PLGA/CS microcapsules and the main driving force for 5-FU loading can be ascribed to intermolecular hydrogen bonding [38,39]. Hydrogen bonding is formed based on the complexes between the strong electronegative atoms (O, N, F) in 5-FU and H atoms of —O—H groups in PLGA/CS, or between H atoms of —NH— groups in 5-FU and electronegative atoms (O, N) in PLGA/CS. The most stable hydrogen-bonded complexes form between 5-FU and PLGA, involving amide groups and carboxyl groups [40], as displayed in Fig. 8, which is in favor of 5-FU loading, considering the innermost PLGA layers in the microcapsules.

3.3. 5-FU loading capacity

Fig. 9 shows the relationship between the 5-FU loading capacity and loading time in the 5-FU aqueous solution (1 mg/mL) under fixed microcapsule concentration. The loading capacity increases rapidly and reaches the maximal value of 33.36% at 4 h, and then it slowly decreases and reaches an equilibrated value of about 27%. The 5-FU loading may be attributed to concentration difference and hydrogen bonding between 5-FU and component polymers of microcapsules.

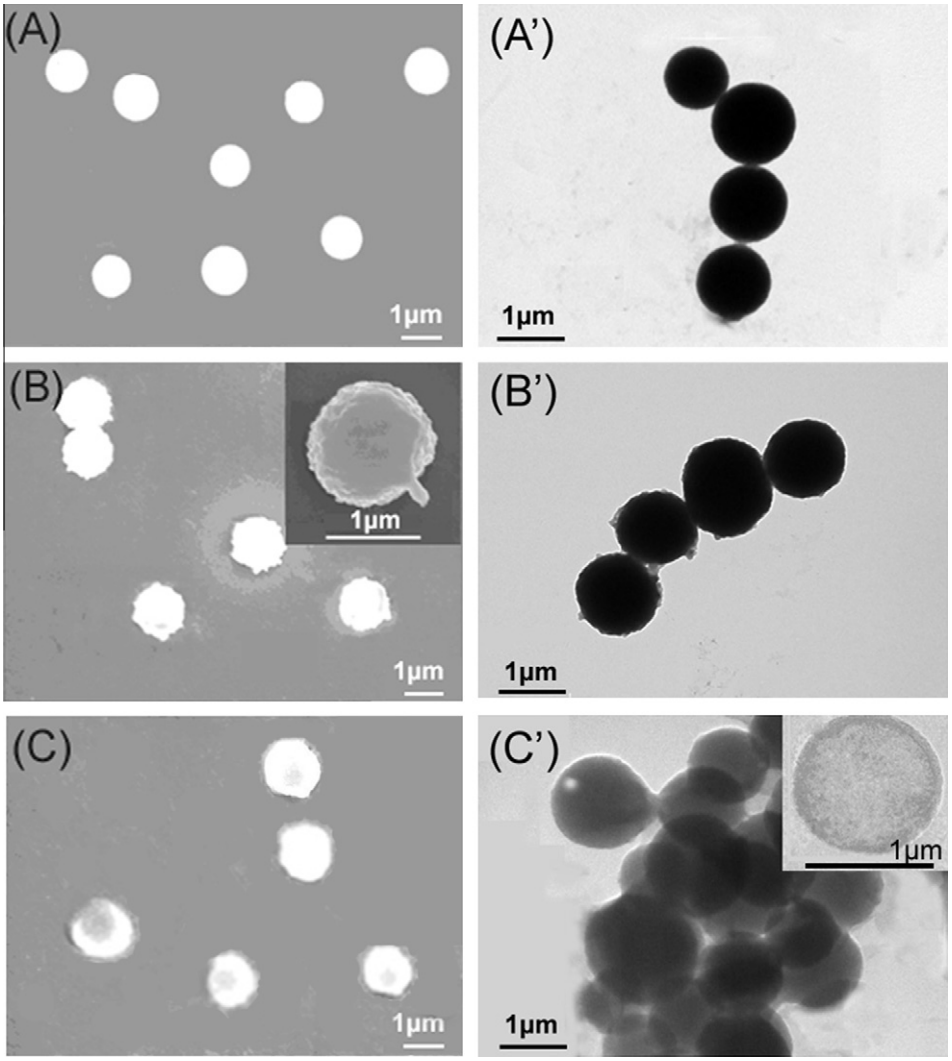

Fig. 5. SEM (A–C) and TEM (A′–C′) images of (A and A′) weakly crosslinked MF particles, (B and B′) MF particles coated with PLGA/CS multilayers, and (C and C′) PLGA/CS microcapsules.

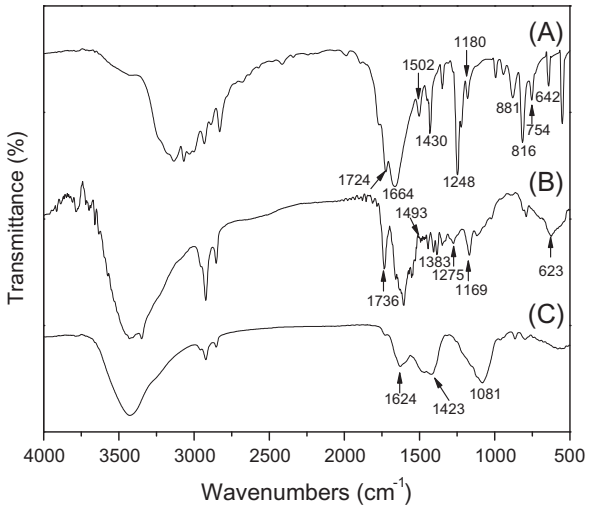

Fig. 6. FTIR spectra of (A) 5-FU, (B) 5-FU-loaded PLGA/CS microcapsules, and (C) PLGA/CS microcapsules.

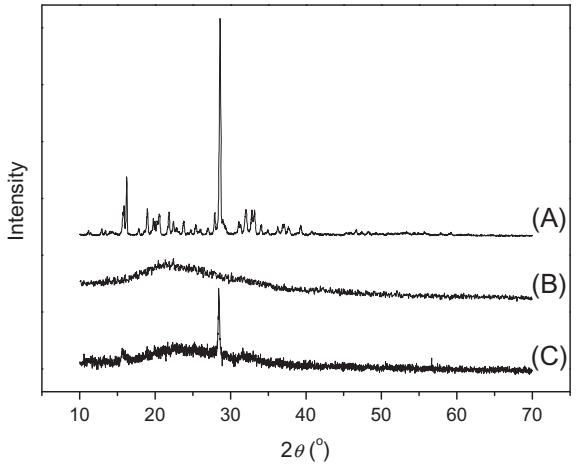

Fig. 7. X-ray patterns of (A) 5-FU, (B) PLGA/CS microcapsules, and (C) 5-FU-loaded PLGA/CS microcapsules.

In order to research the influence of 5-FU concentration on the drug loading capacity, the PLGA/CS microcapsules are mixed with 5-FU solution with different concentrations. As shown in Fig. 10, when 5-FU concentration increases from 0.2 to 1.0 mg/mL, the drug content increases nonlinearly from 16.56% to 33.36% after 4 h loading at 25 °C. The high loading and nonlinear increase of drug content with concentration can be attributed to the spontane-

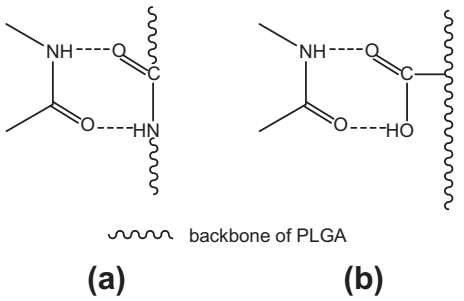

Fig. 8. Schematic representation of the most stable structures of the hydrogen-bonded complexes between 5-FU and PLGA.

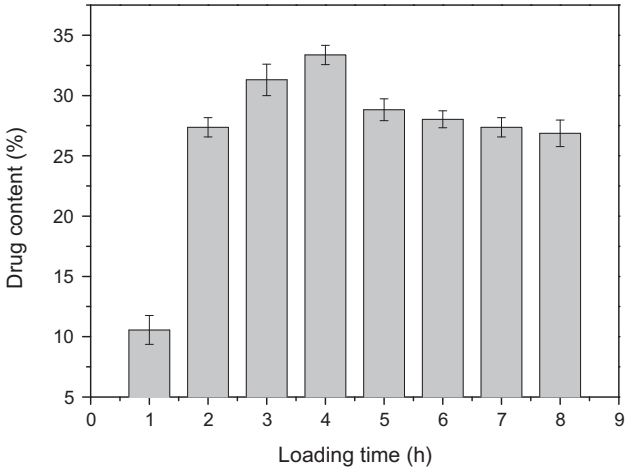

Fig. 9. The loading capacity of PLGA/CS microcapsules as a function of loading time.

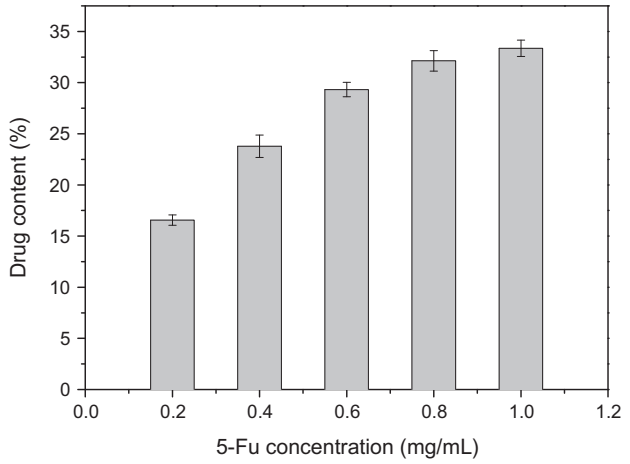

Fig. 10. The loading capacity of PLGA/CS microcapsules as a function of 5-FU concentration.

ous deposition of water-soluble 5-FU into the PLGA/CS microcapsules. Some positively charged substances, such as rhodamine, dextran, have been reported to spontaneously accumulate inside the PSS/PAH microcapsules, and the driving force is the electrostatic attraction between the incorporated substances and a negatively charged complex of PSS combining with MF residues in the capsules formed during the dissolving process [15,16]. Because 5-FU is a neutral weak acid [41], the driving force should be ascribe to hydrogen bonding between 5-FU and PLGA/CS microcapsules, as discussed above. The deposited material is in an aggregated or complexed form, rather than existing in its free state, which constrains the system so that the real concentration of the deposited substance within the interior of the capsule remains lower than in the bulk [15,16,31].

The influence of NaCl concentration on the 5-FU loading capacity of the PLGA/CS microcapsules is demonstrated in Fig. 11. The loading temperature and time are fixed at 25 °C and 4 h. The loading capacity is 37.41% in 5-FU aqueous solution without salt addition, and it increases with NaCl concentration and then levels off at NaCl concentration between 0.4 and 0.5 mol/L. The loading capacity reaches 52.32% at NaCl concentration of 0.5 mol/L, about 40% higher than that without NaCl addition. Ibarz et al. [42] have reported salt-induced wall changes of PSS/PAH capsules, and the minimum amount of salt required to induce polymer penetration is 0.02 mol/L. For PLGA/CS microcapsules, the salt addition can screen the repulsion between polyelctrolyte molecules; salt may also induce lateral shrinkage of the multilayers to enlarge the pores of the wall of PLGA/CS microcapsules [43]. Therefore, the salt addition can enhance the permeability of PLGA/CS microcapsules and contribute to the 5-FU loading.

The temperature effect on the loading capacity is depicted in Fig. 12. For 1 h loading, the loading capacity increases gradually from 10.51% to 19.02%, when the loading temperature increases from 25 to 60 °C. While for 4 h loading, the loading content decreases from 33.32% to 22.68%. Increasing temperature has two effects on the 5-FU loading: enhancing the penetrating movement of 5-FU through the PLGA/CS multilayer wall and accelerating the rearrangement of the polymer chains in the shell to form more compact structure [31]. For the microcapsules loading for 1 h, the structure change of shell in relatively short time has little effect on drug loading. The increasing temperature contributes to the movement of 5-FU through the multilayer wall of PLGA/CS microcapsules, so the drug loading increases. On the contrary, for the microcapsules loading for 4 h, the time is long enough for the rearrangement of the shell and formation of a more perfect multilayer film, the latter effect seems to be dominant, blocking the 5-FU from penetrating through the microcapsule shell at higher temperatures. It has also been reported that the loading capacity of small molecule fluorescein for PSS/PAH microcapsules decreases significantly at higher temperature [44].

Therefore, the 5-FU loading capacity of PLGA/CS microcapsules can be adjusted by loading procedure and solution condition, and it reaches the maximum value of 52.32%. To the authors' knowledge, such a high 5-FU loading capacity for drug delivery systems is first reported.

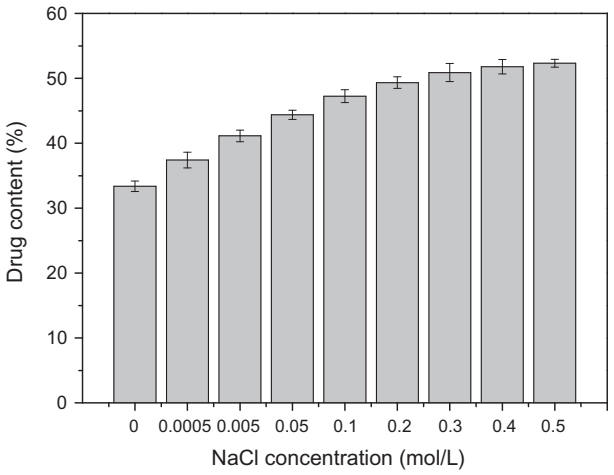

Fig. 11. The loading capacity of PLGA/CS microcapsules as a function of NaCl concentration.

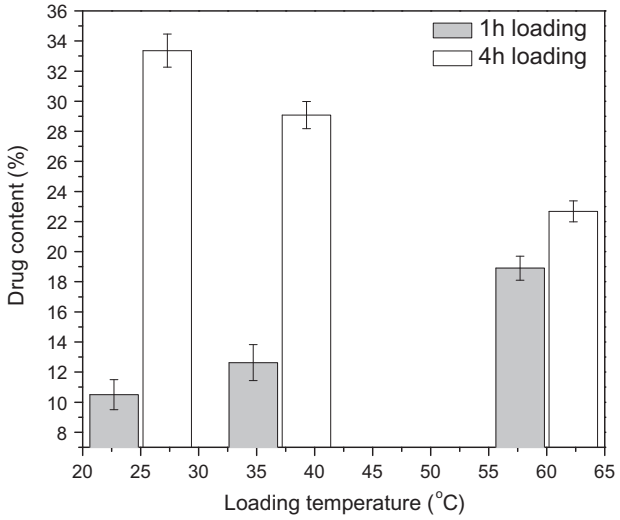

Fig. 12. The loading capacity of PLGA/CS microcapsules as a function of loading temperature.

3.4. Controlled release of 5-FU from PLGA/CS microcapsules

Fig. 13 depicts the release profiles of bare 5-FU, 5-FU-loaded capsules in pH 1.4 HCl buffer and pH 7.4 phosphate buffer, respectively. The bare 5-FU in both pH 1.4 and pH 7.4 buffers releases rapidly. The initial burst release is severe with complete drug release within 300 min. Compared with the release rates from the bare 5-FU, the release rates of 5-FU from PLGA/CS microcapsules in both pH 1.4 and pH 7.4 buffer are obviously delayed. High release rates are still observed in the initial 100 min, which may correspond to release of drug on the surface of microcapsules. Then, the drug release rates in both pH 1.4 and pH 7.4 buffer slow down and finally reach equilibrium, suggesting the entrapped 5-FU release from PLGA/CS microcapsules; 99.4% of the loaded 5-FU releases at 6000 min in pH 1.4 buffer and 99% at 7680 min in pH 7.4 buffer, respectively. Mao et al. investigated the release behavior from PSS/PAH microcapsules, they found the cumulative release of ciprofloxacin hydrochloride at equilibrium was 70% at 3d [14]. So, the PLGA/CS microcapsules show more preferable release behavior with longer equilibrium time and higher cumulative release.

The PLGA/CS microcapsules show similar drug release behavior in pH 1.4 and pH 7.4 buffers, which is due to neutral nature of 5-FU in acidic or neutral solution. The release rate is slightly higher at pH 1.4. This may be attributed to increase of the pores and enhancement of the permeability of the wall of microcapsules under this condition. Similar result is obtained for 5-FU release from chitosan-reinforced alginate microparticles [45]. In contrast with the charged drug, such as insulin, with the isoelectric point of pH 5.5, the release rate from polyelectrolyte microcapsules templated on MF microparticles at pH 7.4 is found much faster than that at pH 1.4 because of charge reverse of drug induced by the pH change [31].

Fig. 14 shows the release profiles of 5-FU from PLGA/CS microcapsules before and after crosslinking using EDC. The release profiles indicate the reduction in the release rate and cumulative release amount after the crosslinking of PLGA and CS. This is because the crosslinking restricts the movement of molecular chain and enhances the obstruction to the 5-FU movement passing through the microcapsule shell, which is in accordance with the result previously reported [31].

3.5. Release model and kinetic mechanism

In order to describe the kinetics of the drug release process from the controlled release formulations, various models and mathematical equations are applied, which are listed in Table 1.

The kinetics of 5-FU release from PLGA/CS microcapsules are evaluated according to zero-order, first-order, Higuchi, Ritger–Peppas and Baker–Londale models [46–48]. For the mathematical evaluations, the drug release kinetics are obtained by fitting standard release equations to the experimental data. The most suited being the one which best fits the experimental results. Simulated equations and correlation coefficients are calculated and compared in Table 2. The correlation coefficients calculated from five models clearly indicate that 5-FU release from PLGA/CS microcapsules can be best described using Ritger–Peppas and Baker–Londale models where correlation coefficient is greater than 0.99 under all conditions.

The mathematical equation of Korsmeyer–Peppas model is given in Table 1, where n is the diffusional exponent characteristic of the release mechanism. For spheral tablet, it is reported that threshold of n value between Fickian and non-Fickian mechanism is 0.43 [49]. In particular, $n \leqslant 0.43$ corresponds to a Fickian diffusion release, whereas n between 0.43 and 0.85 indicates an anomalous non-Fickian transport [46]. The n values given in Table 2 for three set of PLGA/CS microcapsules are 0.3897, 0.3976, and 0.3893, respectively. These results suggest that the PLGA/CS microcapsules

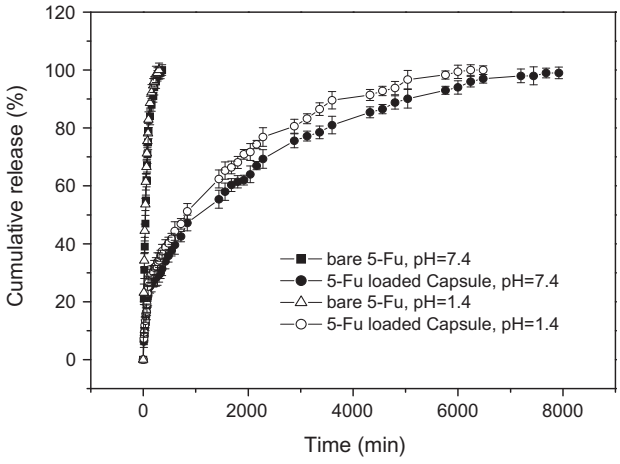

Fig. 13. Release profiles of bare 5-FU, 5-FU-loaded capsules in pH 1.4 and pH 7.4 buffers at 37 °C.

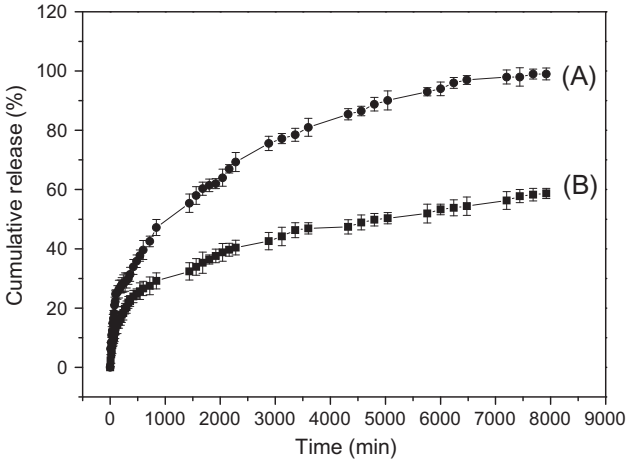

Fig. 14. The cumulative release curves of 5-FU from PLGA/CS microcapsules before (A) and after (B) crosslinking in pH 7.4 buffer at 37 °C.

Table 1
Various release kinetic models and corresponding mathematical equations.

Release kinetic model	Mathematical equation	Remark
Zero-order	$Q = Q_0 + k_0 t$	Q is the amount of drug released in time t
First-order	$Q = Q_\infty(1 + e^{-k_1 t})$	Q_0 the amount of drug in the solution at $t = 0$
Higuchi	$Q = k_H t^{1/2}$	Q_∞ the total amount of drug in the matrix
Korsmeyer–Peppas	$Q/Q_\infty = kt^n$	k_0 the zero-order kinetic constant k_1 the first-order kinetic constant k_H the Higuchi rate constant
Baker–Londale	$(3/2)[1 - (1 - Q)^{2/3}] - Q = kt$	n the release exponent k the release constant for Korsmeyer–Peppas or Baker–Londale model

Table 2
Fitting results of the experimental 5-FU release data to different kinetic equations.

Samples	Medium (pH)	Kinetic model	Equation	Correlation coefficient
PLGA/CS microcapsules	Phosphate buffer (7.4)	Zero-order	$Q = 0.2776 + 0.0068 \times t$	0.9362
		First-order	$\ln(1 - Q) = -0.1207 - 0.0298 \times t$	0.9878
		Higuchi	$Q = 0.1126 + 0.0844 \times t^{1/2}$	0.9914
		Korsmeyer–Peppas	$\ln Q = 0.3897 \ln t - 1.8247$	**0.9937**
		Baker–Londale	$(3/2)[1 - (1 - Q)^{2/3}] - Q = -0.0015 + 0.0034t$	**0.9987**
Crosslinked PLGA/CS microcapsules	Phosphate buffer (7.4)	Zero-order	$Q = 0.1737 + 0.0376 \times t$	0.9224
		First-order	$\ln(1 - Q) = -0.1871 - 0.0060 \times t$	0.9581
		Higuchi	$Q = 0.0806 + 0.0471 \times t^{1/2}$	0.9848
		Korsmeyer–Peppas	$\ln Q = 0.3976 \ln t - 2.3679$	**0.9905**
		Baker–Londale	$(3/2)[1 - (1 - Q)^{2/3}] - Q = 0.0049 + 0.0006t$	**0.9912**
PLGA/CS microcapsules	HCl buffer (1.4)	Zero-order	$Q = 0.2941 + 0.0083 \times t$	0.9288
		First-order	$\ln(1 - Q) = -0.1421 - 0.0376t$	0.9766
		Higuchi	$Q = 0.1253 + 0.0937 \times t^{1/2}$	0.9887
		Korsmeyer–Peppas	$\ln Q = 0.3893 \ln t - 1.7181$	**0.9923**
		Baker–Londale	$(3/2)[1 - (1 - Q)^{2/3}] - Q = -0.0021 + 0.0044t$	**0.9968**

have similar diffusion properties regardless of the crosslinking of microcapsules and pH value of buffer. Two possible mechanisms may involve in the release of 5-FU from PLGA/CS microcapsules, and the characteristic exponents for PLGA/CS microcapsules indicate the diffusion of 5-FU from PLGA/CS microcapsules rather than its own erosion modulate drug release. Since the total drug release time (132 h) in our experiments is considerable shorter than the degradation lifetime of PLGA/CS microcapsules, the bulk degradation of polymers is trivial and PLGA/CS microcapsules may retain their integrity without significant degradation/dissolution. As a result, drug release from the microcapsules can be attributed to the diffusion mechanism. The 5-FU release from PLGA/CS microcapsules may be the combination of diffusion through the polyelectrolyte matrix and partial diffusion through water-filled pores [50].

The drug release from polymeric systems might conform to two diffusion based models, i.e., Higuchi's matrix dissolution model or Baker–Lonsdale equation. It is reported that Higuchi model is originally derived for a planar matrix system but not for the spherical formulation. On the other hand, Baker–Lonsdale model is proposed for drug release from a spherical matrix [51]. In order to investigate whether drug release conforms to the Baker–Lonsdale model, $3/2[1 - (1 - Q)^{2/3}] - Q$ (where Q is the release percentage) is plotted as a function of time, as shown in Fig. 15, a linear relationship is found for each model, also indicating diffusion mechanism of drug release from PLGA/CS microcapsules [51,52].

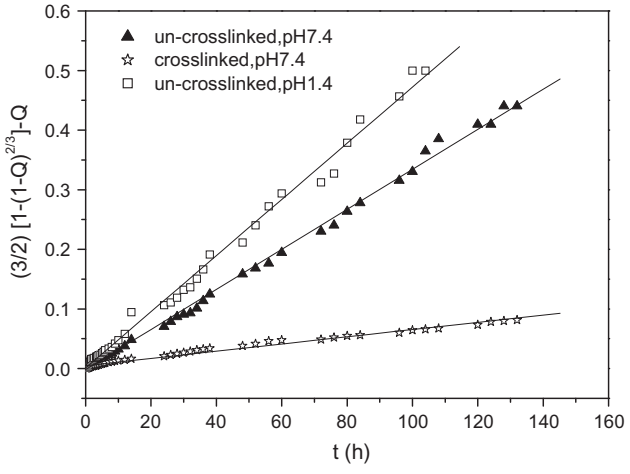

Fig. 15. Baker–Londale kinetics of PLGA/CS microcapsules before and after crosslinking at different pH values (solid line: simulation results; dot: experimental results).

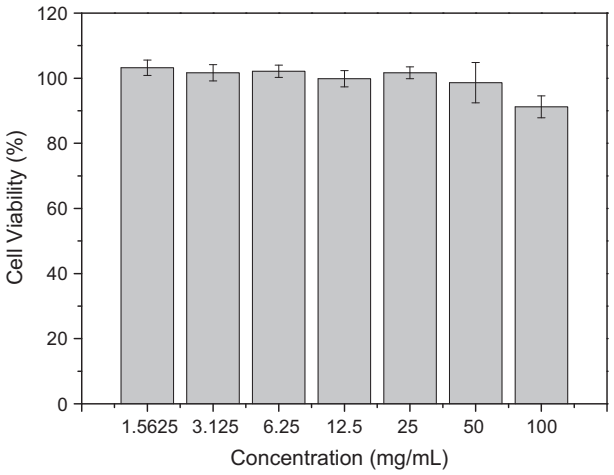

Fig. 16. Cytotoxicity evaluation by the MMT assay for the extract of PLGA/CS microcapsules.

3.6. In vitro cytotoxicity assay of PLGA/CS microcapsules

From the pharmaceutics standpoint, the toxicity of carrier is fatal for a drug delivery system [53]. In this study, a MTT assay is introduced to evaluate the possible cytotoxicity of the PLGA/CS microcapsules and the results are presented in histogram, as shown in Fig. 16. Because 5-FU would be harmful to cell growth, blank PLGA/CS microspheres are used. Cells are incubated in the extract solution of microspheres with the increasing concentration ranging from 1.5625 to 100 mg/mL. The cells treated with extracts of PLGA/CS microspheres show quite good cell viability over the entire concentration range, and above 90% viability is retained even when the concentration is up to 100 mg/mL. In our previous study, the PLGA/CS multilayer coating fabricated by LbL assembly could obviously promote C2C12 (muscle myoblast cell) attachment and growth [28]. These results suggest that LbL assembly multilayer of PLGA/CS could be developed into a safe carrier for drug delivery systems.

4. Conclusions

Weekly crosslinked MF colloidal microparticles were prepared and used as template for the LbL assembly of PLGA and CS. PLGA/CS microcapsules were obtained after removing of MF cores at low pH.

The PLGA/CS microcapsules show high loading capacity of hydrophilic neutral anticancer drug 5-FU by simply soaking in 5-FU solution, which can be ascribed to the spontaneous deposition of 5-FU into the PLGA/CS microcapsules with the driving force of hydrogen bonding between 5-FU and PLGA/CS microcapsules. The maximum loading of 5-FU was achieved under conditions of high drug concentration and salt adding. The loading capacity increases gradually with loading temperature for 1 h loading, while decreases for 4 h loading. The PLGA/CS microcapsules show sustained release behavior. Prolonged 5-FU release is achieved from PLGA/CS microcapsules, in contrast to burst release of bare 5-FU. The release rate and cumulative release amount are both reduced after crosslinking the capsule wall by treatment of EDC.

Different models are applied for the evaluation of the drug release data. The 5-FU release from PLGA/CS microcapsules is best fitted in Ritger–Peppas or Baker–Londale models, suggesting diffusion controlled release mechanism. MTT assay shows the good cell viability in the extract solution of microspheres, indicating the security of PLGA/CS microcapsules.

Because of the properties of biodegradability, high loading, sustained release and cell compatibility, the novel PLGA/CS microcapsules promise high potential for encapsulation used in drug delivery systems.

Acknowledgments

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