International Journal of Pharmaceutics 427 (2012) 443–451

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Nanoporous multilayer poly(L-glutamic acid)/chitosan microcapsules for drug delivery

Shifeng Yan[a], Shuiqin Rao[a], Jie Zhu[a], Zhichun Wang[a], Ying Zhang[a], Yourong Duan[b,***], Xuesi Chen[c,**], Jingbo Yin[a,*]

[a] Department of Polymer Materials, Shanghai University, Shanghai 201800, China
[b] Cancer Institute, Renji Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200032, China
[c] Key Laboratory of Polymer Ecomaterials, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, China

ARTICLE INFO

Article history:
Received 30 November 2011
Received in revised form 5 January 2012
Accepted 13 January 2012
Available online 24 January 2012

Keywords:
Nanoporous
Layer-by-layer assembly
Poly(L-glutamic acid)
Chitosan
Drug delivery systems

ABSTRACT

Nanoporous poly(L-glutamic acid)/chitosan (PLGA/CS) multilayer microcapsules were fabricated by layer-by-layer (LbL) assembly using the porous silica particles as sacrificial templates. The LbL assembled nanoporous PLGA/CS microcapsules were characterized by Zeta-potential analyzer, FTIR, TGA, SEM, TEM and CLSM. 5-Fluorouracil (5-FU) was chosen as model drug. The drug loading content of PLGA/CS microcapsules depends on loading time, loading temperature, pH value and NaCl concentration. High loading capacity of microcapsules can be achieved by simply adjusting pH value and salt concentration. Moreover, 5-Fu loaded microcapsules take on a sustained release behavior, especially in an acid solution, in contrast to burst release of bare 5-Fu. The kinetics of 5-Fu release from PLGA/CS microcapsules conforms to Korsmeyer-Peppas and Baker-Lonsdale models, the mechanism of which can be ascribed to priority of drug diffusion and subordination of polymer degradation. The MTT cytotoxicity assay in vitro reveals the satisfactory anticancer activity of the drug-loaded PLGA/CS microcapsules. Therefore, the novel nanoporous PLGA/CS microcapsules is expected to find application in drug delivery systems.

1. Introduction

The polyelectrolyte microcapsules prepared by layer-by-layer (LbL) assembly technique have attracted particular interest because of their tailored properties, such as size, composition, surface functionality and so on (Donath et al., 1998; Caruso et al., 2001), and find potential applications in medicine, drug delivery, micro-reactor, catalysis, etc. (Antipov and Sukhorukov, 2004; Ye et al., 2005; Liang et al., 2005; He et al., 2005).

With the LbL technique, various spherical templates can be used to fabricate polyelectrolyte multilayer hollow microcapsules with controlled wall permeability. Different drugs have been loaded into the hollow microcapsules by simply adjusting the permeability of the capsule wall at different pH value or salt concentration (Johnston et al., 2006). However, in the application of drug delivery system, hollow microcapsules still have several shortcomings as follows: (1) Hollow microcapsules are ready to rupture or collapse due to lack of the internal support, so more adsorption steps of polyelectrolytes during LbL assembly process are needed to get the thicker mutilayer wall. (2) The drug loadings achieved are typically low, as the maximum concentration of drug inside the capsules is often limited to the concentration in the solution (Johnston et al., 2006; Mao et al., 2005). (3) The polyelectrolytes employed for the LbL assembly mainly focus on non-degradable synthetic polyelectrolytes or nature polyelectrolytes with uncontrollable structure and properties because of different natural sources and batches.

In order to improve the drug loading amount, a technique combining porous particles and LbL assembly has been developed. The drug is absorbed within a porous particle, such as silica (Yu et al., 2005) and calcium carbonate (Wang et al., 2006) that can be subsequently LbL-coated. However, the activity and loading content of the drug may be affected in the process of porous templates removal. Wang et al. have reported the nanoporous polyelectrolyte microcapsules fabricated by LbL absorption of poly(acrylic acid) (PAA) and poly(allylamine hydrochloride) (PAH) onto the pores of the porous silica spheres and removing the silica template (Wang et al., 2006). Comparing with hollow microcapsules, nanoporous microcapsules with internal support of polyelectrolyte network and high surface area ensure their properties of good structural

* Corresponding author at: Department of Polymer Materials, Shanghai University, 20 ChengZhong Road, Jiading District, Shanghai 201800, China.
Tel.: +86 21 69982432; fax: +86 21 69982840.
** Corresponding author. Tel.: +86 431 85262112; fax: +86 431 85685653.
*** Corresponding author. Tel.: +86 21 64178312; fax: +86 21 64178312.
E-mail addresses: yrduan@shsci.org (Y. Duan), xschen@ciac.jl.cn (X. Chen), jbyin@shu.edu.cn (J. Yin).

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doi:10.1016/j.ijpharm.2012.01.025

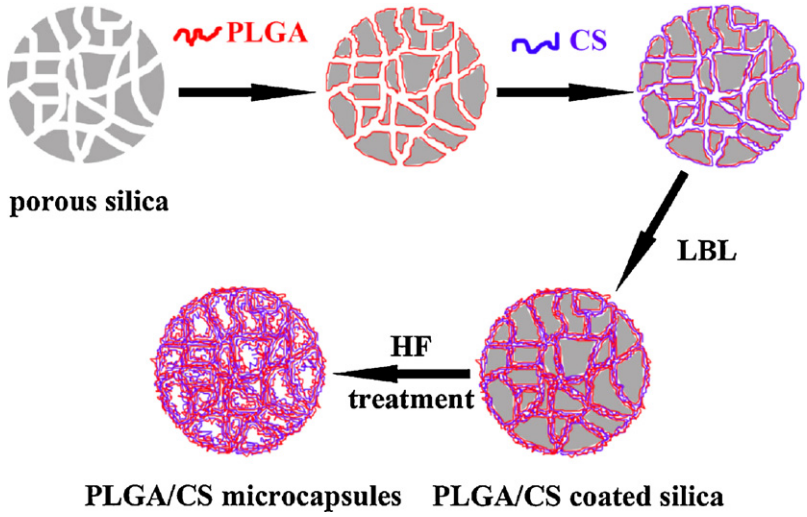

Fig. 1. Schematic illustration for the preparation of nanoporous PLGA/CS microcapsules.

stability and high drug loading capability. However, both PAA and PAH are non-degradable synthetic polyelectrolytes, which limits the biomedical applications. Moreover, the drug loading and release behavior is not detailed researched.

As for the wall substance of polyelectrolyte microcapsules, biodegradable synthetic polyelectrolytes have the advantages of controllable chemical structure, molecular weight, and various performances, which are superior to non-degradable synthetic polyelectrolytes or nature source-related polyelectrolytes. So the hybrid polymeric microcapsules combining natural and biodegradable synthetic polyelectrolytes are expected to make up for the deficiencies of nature polymers.

Here, in order to improve the forementioned dimerits of hollow microcapsules as drug delivery vehicle, nanoporous poly(L-glutamic acid)/chitosan (PLGA/CS) multilayer microcapsules were developed for drug delivery system using porous silica as templates. 5-Fluorouracil (5-Fu), an antineoplastic drug, was used as model water-soluble drug to research the loading and release properties of the microcapsules. 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. PLGA, as a synthetic polypeptide, is unique in that it is composed of naturally occurring L-glutamic acid linked together through amide bonds (Li, 2002). It possesses controlled molecule weight, designed structure and excellent biocompatibility. CS is a partially deacetylated form of chitin, an abundant polysaccharide present in crustacean shells (Janes et al., 2001). CS is well-known for its biological properties such as biodegradability and biocompatibility allowing its use in drug delivery systems.

The LbL assembly process of PLGA/CS microcapsules, the 5-FU loading and release properties were investigated. The nanoporous PLGA/CS microcapsules show stable structure of the walls, 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) ($M_\eta = 2.0 \times 10^4$) was prepared 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). Other reagents were all analytical grade and used as received.

2.2. Preparation of nanoporous PLGA/CS microcapsules

Porous silica microspheres were used as template in this work. They were prepared through controlled hydrolysis of sodium metasilicate using cetyltrimethylammonium bromide as a structure directing agent, as reported previously (Schulz-Ekloff et al., 1999). The surface of nanoporous silica microspheres was functionalized using γ-aminopropyltriethoxysilane. Amine ($-NH_2$) surface functional groups were introduced and the positive charged silica microspheres were achieved.

The absorption of polyelectrolyte PLGA/CS multilayers on the surface of nanoporous silica microspheres was performed using LbL assembly technique, as shown in Fig. 1. The positively charged porous silica microspheres (10 mg) were incubated with 4 mL aqueous PLGA solution (5 mg/mL, containing 0.5 M NaCl, pH = 4.4). 2 h was allowed for adsorption at 25 °C. NaCl was used to facilitate the infiltration of polyelectrolytes in the pores (Wang and Caruso, 2006). And the electrostatic bonding of $-COOH$ and $-NH_2$ is strongest at pH = 4–5 (Dai et al., 2007). Excess PLGA was removed by two cycles of centrifugation (13,000 rpm, 3 min) and washing with NaCl solution (0.5 M, pH = 4.4). Cross-linking of absorbed polyelectrolyte layer was performed by immersing the sample in 0.5 mL EDC (60 mg/mL, pH = 5.5) for 2 h. The following CS layer was deposited with 4 mL CS solution (5 mg/mL, containing 0.5 M NaCl, pH = 4.4, and the processes of centrifugation/washing/crosslinking were performed as above. The PLGA and CS adsorption steps were repeated until two pairs of PLGA/CS coating was reached.

The porous microcapsules were obtained by exposing the PLGA/CS coated silica template to 2 mL HF (5 M) aqueous solution for 30 min. After several cycles of centrifugation (26,000 rpm, 3 min) and washing with deionized water, nanoporous PLGA/CS microcapsules were lyophilized for further use.

2.3. 5-Fu loading and release

1 mg PLGA/CS microcapsules were dispersed in 5 mL 5-Fu aqueous solution (1 mg/mL) for absorption. Various factors that affect the loading content of microcapsules were studied, including loading time, temperature, pH value and salt concentration. After being incubated under various conditions, the 5-FU-loaded

microcapsules were centrifuged (16,000 rpm, 5 min), washed 2 times with de-ionized water, centrifuged 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 inside the microcapsules was calculated through the difference between the input amount and the amount in the supernatants produced during the washing process. According the following formula to reckon the 5-Fu loading content into microcapsules:

$$\text{Loading content} = (m_d/m_t) \times 100\%$$

where m_d is the mass of 5-Fu into microcapsules and m_t the total mass of 5-Fu-loaded microcapsules.

For the examination of 5-Fu release, 10 mg 5-Fu loading microcapsules were put into dialysis bag and exposed to either 100 mL phosphate buffer solution (PBS) (pH 7.4) or hydrochloric acid buffer solution (pH 1.0). 5 mL buffer solution was fetched from release system at each interval and measured at 265 nm by UV-VIS spectrophotometer. 5-Fu cumulative curve can be plotted according to 5-Fu PBS (pH = 7.4) release calibration curve and cumulative release formula:

$$Q = 100 \times C_t + \sum_{i=1}^{t-1} 5 \times C_i$$

$$Q\% = (Q/m_d) \times 100\%$$

where Q is total quantity of 5-Fu released from microcapsules in μg, C_t is 5-Fu concentration in buffer solution at time t and m_d the mass of 5-Fu in microcapsules.

For comparison, the drug release experiment of bare 5-FU and 5-FU loaded porous silica was conduct.

2.4. In vitro cytotoxicity measurement

The antitumor activity of 5-Fu-loaded PLGA/CS microcapsules was evaluated by MTT method. LM3 cells (human hepatocellular carcinoma cells) were cultured on a 96-well tissue culture plates (100 μL/well; Cellstar) at 1×10^5 cells/mL in DMEM (Dulbecco's modified Eagle's medium; Sigma–Aldrich, USA) containing 10% fetal bovine serum (FBS, Biochrom Ag, Germany), and then incubated at 37 °C in 5% CO_2 for 12 h. Then, the culture medium was replaced by 100 mL of medium containing different concentrations of 5-Fu-loaded PLGA/CS microcapsules. The cell viability cultured with DMEM medium was used as control. 25 μL of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide, 5 mg/mL) was added into each well followed by 2 h incubation at 37 °C. Dimethylsulfoxide (100 μL) was added into each well and incubated for 15 min. The reaction was optically monitored at 570 nm (A_{570}) using a 96-well microtiter plate reader (Bio-Rad Model 550, USA). All experiments were carried out in triplicate. The inhibitory rate of LM3 cells was calculated according to following equation (Huang et al., 2007):

$$\text{Inhibitory rate} = \frac{A_{570}(\text{control}) - A_{570}(\text{drug})}{A_{570}(\text{control})}$$

where A_{570}(control) was the absorbance in the control groups, and A_{570}(drug) was the absorbance in the 5-Fu-loaded PLGA/CS microcapsules groups.

2.5. Characterization

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.

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. Samples for SEM observation were prepared by depositing suspensions of porous silica or PLGA/CS microcapsules on Si slides.

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

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

TGA experiments were conducted using Simultaneous Thermal Analysis (STA409PC). The samples were heated from 25 to 950 °C with a heating rate of 10 °C/min in air.

Confocal micrographs of PLGA/CS microcapsule samples were taken with an LSM700 confocal laser scanning microscopy (CLSM) (Carl Zeiss Inc.). CS was conjugated using rhodamine B isothiocynate, which is a fluorescent dye, with excitation at 510–550 nm and fluorescence emission of red light. The rhodamine B-labeled CS and was used to prepare PLGA/CS microcapsules as described in Section 2.2.

3. Results and discussion

3.1. Preparation of nanoporous PLGA/CS microcapsules by LbL assembly

As mentioned above, in order to increase the stability of microcapsules fabricated by LbL assembly, nanoporous silica microspheres were chosen as templates and crosslinking of polyelectrolytes was performed to form a network sustainment. So the porous microcapsules can be obtained by depositing less polyelectrolyte layers. Fig. 2 shows the characterization of LbL assembly of polyelectrolytes and the resultant PLGA/CS microcapsules.

The LbL assembly of PLGA and CS on porous silica templates is followed by microelectrophoresis. The surface of modified porous silica possesses a large number of $-NH_2$ groups, which turns to be $-NH_3^+$ with positive charge after ionized in aqueous solution. In a similar way, PLGA with $-COO^-$ possess negative charge while CS with $-NH_3^+$ is positive charged. As shown in Fig. 2a, the silica particles with outermost layers of PLGA and CS yield alternating ζ-potentials of −16 to −38 mV and +26 to +42 mV. The alternating ζ-potentials observed with each coating step suggest that multilayers are formed on porous silica templates with the main driving force of electrostatic adsorption (Schüler and Caruso, 2001; Tao et al., 2006). The ζ-potential increases with polyelectrolyte concentration, which can be attributed to the increasing charge density of outmost polyelectrolytes on the particles.

Morphology observation of silica microspheres and PLGA/CS microcapsules was conducted using SEM and TEM, as shown in Fig. 2b. The silica templates have a rough surface with an average diameter of about 2 μm, as shown in Fig. 2b-A and b-E. Upon high magnification, nanoporous structure is clearly seen in Fig. 2b-C with pore size of 10–20 nm. Compared with silica templates, PLGA/CS microcapsules retain the original shape of silica templates with no sign of rupture or collapse, but the size of which is much smaller than that of bare silica template, as shown in Fig. 2b-B and b-F. Wang et al. also found the shrinkage of polymer-based spheres after dissolve the templates (Wang et al., 2006). High magnification of the SEM observation also shows the nanoporous structure of PLGA/CS microcapsules. The integrity and nanoporous structure of PLGA/CS microcapsules indicates their high potential in drug delivery system. TGA curve of PLGA/CS microcapsules shows very little remaining weight (about 2.3 wt%) after heated to 950 °C in air, as shown in Fig. 2b-G, revealing substantial remove of silica

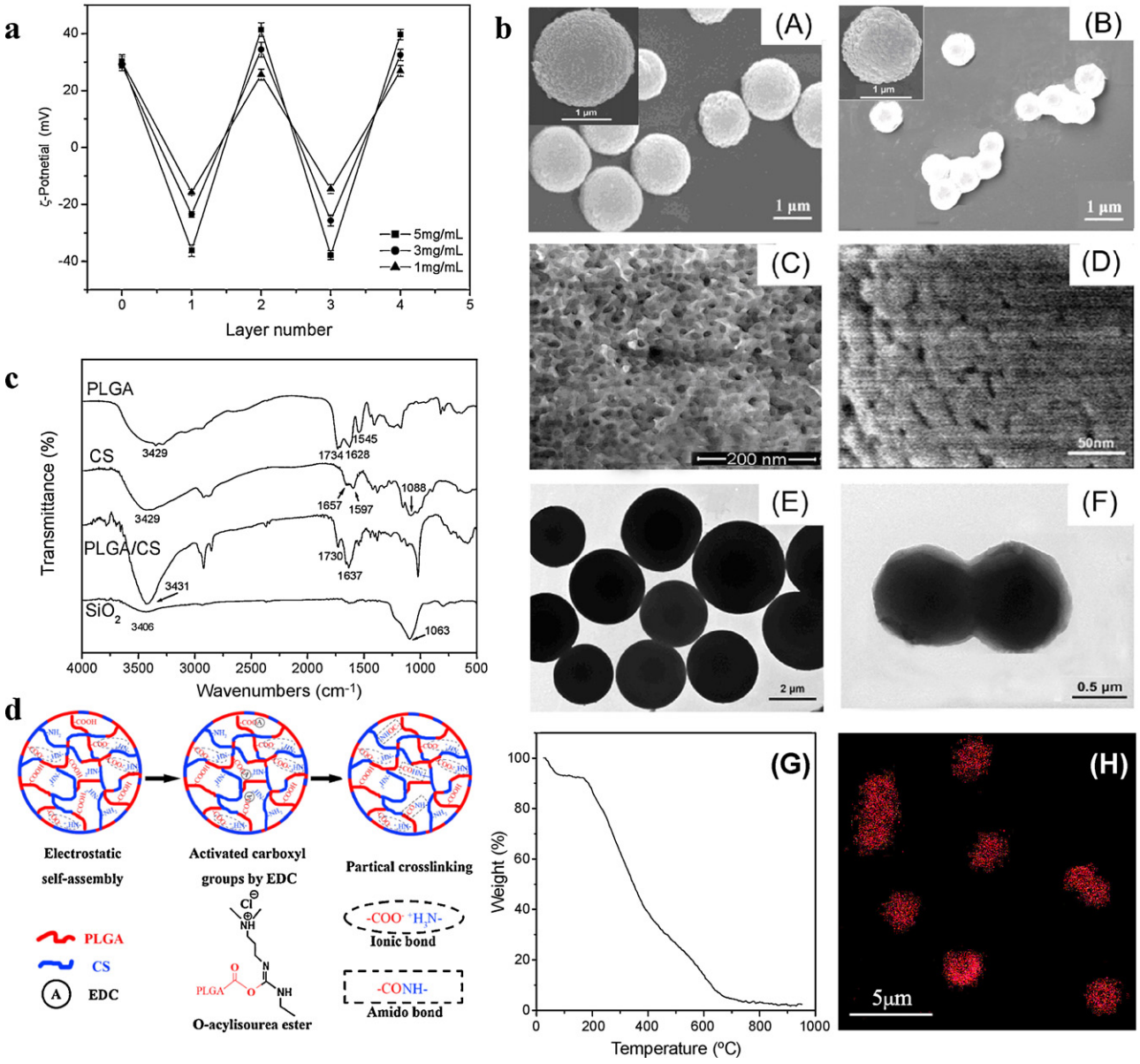

Fig. 2. Fabrication of nanoporous PLGA/CS microcapsules by LbL assembly. (a) The ζ-potential versus layer number for the LbL self-assembly of PLGA and CS on porous SiO_2 particles. The odd layer numbers correspond to PLGA deposition and the even layer numbers to CS adsorption. (b) SEM (A–D) and TEM (E and F) images of porous SiO_2 particles (A, C and E) and PLGA/CS microcapsules (B, D and F), TGA curve of PLGA/CS microcapsules (G), and CLSM image of PLGA/CS microcapsules (CS was labeled using rhodamine B isothiocynate). (c) The FTIR spectra of PLGA, CS, PLGA/CS microcapsules and SiO_2 templates. (d) Schematic illustration for the interaction between component polymers in PLGA/CS microcapsules.

templates. Fig. 2b-H shows the CLSM image of PLGA/CS microcapsules. Uniform fluorescence can be observed across the particle cross-sections due to the homogeneous distribution of rhodamine B isothiocynate-labeled CS throughout the PLGA/CS microcapsules, reflecting the non-hollow structure and the porous nature of the PLGA/CS microcapsules, which is in contrast to ring fluorescence for traditional hollow microcapsules (Gao et al., 2001; Tao et al., 2006).

Fig. 2c presents the FTIR spectra of silica templates, PLGA, CS and PLGA/CS microcapsules. For PLGA, the absorption bands located at 3429, 1734, 1628 and 1545 cm^{-1} originate from O—H, C=O, amide I and II, respectively. With respect to CS, the characteristic absorption band at 3429 cm^{-1} 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 (Dai et al., 2007; Song et al., 2009). The silica template shows the characteristic band at about 1063 cm^{-1}, which is ascribed to the stretching vibration of Si—O—Si band (Yan et al., 2004). This characteristic absorption of silica template can hardly be detected in the spectrum of PLGA/CS microcapsules, indicating the substantial removal of silica templates. For PLGA/CS microcapsules, the original characteristic 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 peaks of 1730 and 1637 cm^{-1}, which means strong interaction between PLGA and CS. As mentioned above, there exists strong electrostatic interaction between negatively charged carboxylic acid ions ($-COO^-$) on PLGA and the positively charged amino groups ($-NH_3^+$) on CS, which facilitates the alternating deposition of PLGA and CS. What is more, in order to further improve the stability of microcapsules, EDC treatment was used to form amide bonds between the —COOH groups in PLGA and $-NH_2$

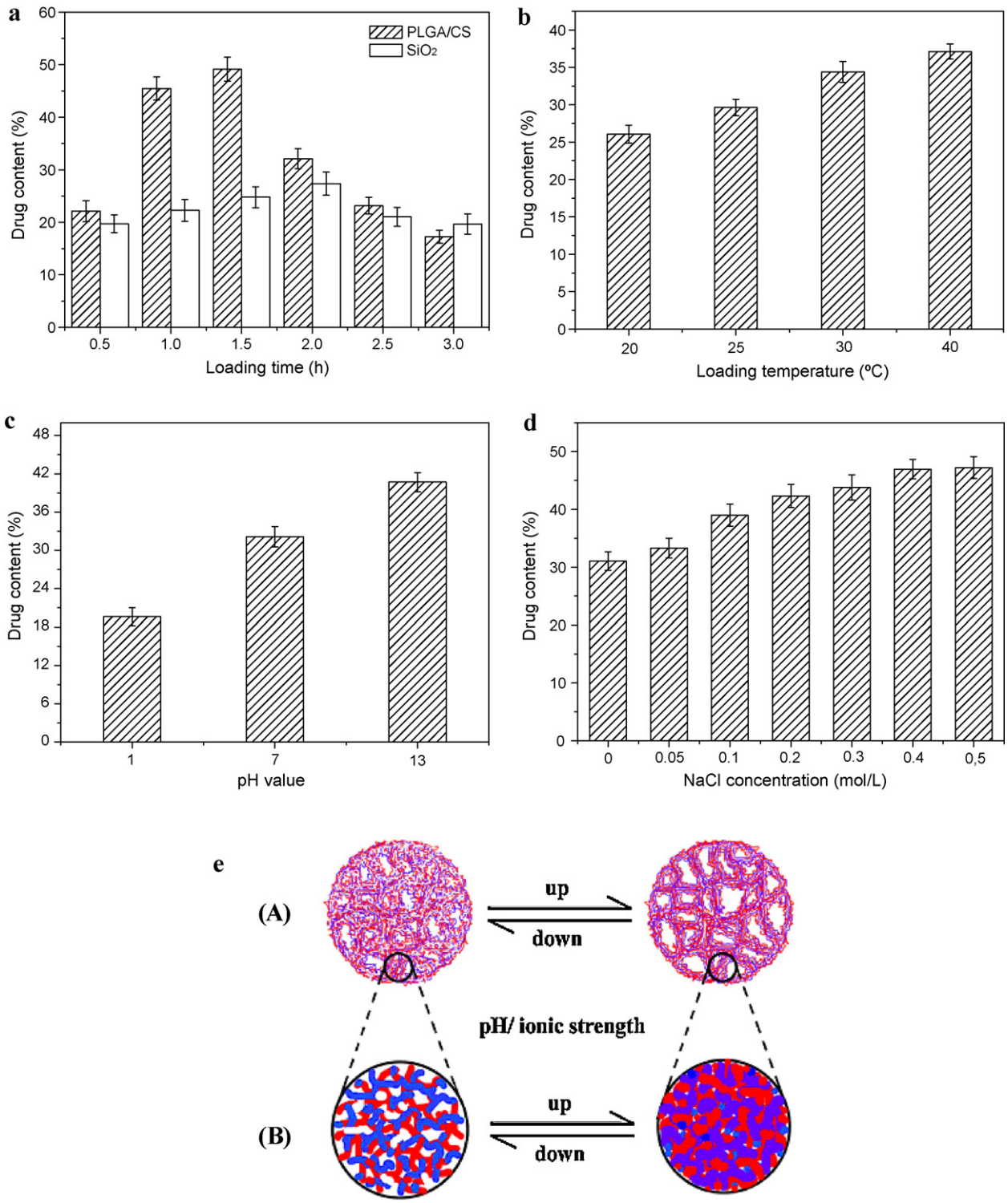

Fig. 3. 5-Fu loading in various conditions. (a) Drug content of PLGA/CS microcapsules and SiO_2 as a function of loading time. (b) 5-Fu content as a function of loading temperature. (c) 5-Fu content as a function of pH value of loading solution. (d) Drug content of microcapsules as a function of NaCl concentration. (e) Schematic diagram of (A) PLGA/CS microcapsule and (B) partial enlargement of polyelectrolyte wall in different pH values.

moieties in CS (Pouyani et al., 1992, 1994; Kuo et al., 1991). While absorption band at 3431 cm^{-1} represents the non-crosslinked —OH and —NH_2 groups of PLGA and CS. According to the result of FTIR analyzing, only partial crosslinking have occurred between carboxyl groups of PLGA and amino groups of CS. The presence of both ionic interaction and amide bone linkage provides tenacity and sensitivity to pH and salt. The interaction between PLGA and CS in the microcapsules is schematically depicted in Fig. 2d.

3.2. 5-Fu loading in various conditions

5-Fluorouracil (5-FU), a water-soluble fluorinated pyrimidine analog, is an antineoplastic drug, yet its short half life, bad oral absorption and unneglectable side effect restrain its clinical application (Bonglev et al., 2003). Here, 5-FU was used as model drug to study the loading and release behavior of PLGA/CS microcapsules.

Fig. 3 shows 5-Fu loading content of PLGA/CS microcapsules in various conditions. A comparison study of 5-Fu loading capability between PLGA/CS microcapsules and porous silica templates with loading time is shown in Fig. 3a. The concentration of 5-FU is set at 1 mg/mL. As a whole, the loading capability of microcapsules is superior to that of silica templates. The initial higher 5-Fu concentration in aqueous solution makes it diffuse into microcapsules and templates quickly. The loading capacity increases rapidly and reaches the maximal value of 49% at 1.5 h. Then it slowly decreases and reaches equilibrium. The 5-FU content in microcapsules decreases more quickly than that in templates, which may be attributed to the rearrangement of polymer chains and formation of more compact structure, with desorption of some 5-Fu from the microcapsules.

The influence of temperature on 5-FU loading capacity is depicted in Fig. 3b. The loading capacity increases gradually from 26.1% to 37.1%, when the loading temperature increases from 20 to 60 °C. 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 (Ye et al., 2006). For the PLGA/CS microcapsules, the structure change of shell is relatively small because of both ionic interaction and amide bone linkage between component polyelectrolytes, as aforementioned. The first effect seems to be dominant, so the drug loading increases.

Fig. 3c demonstrates the influence of pH value on the 5-FU loading capacity. The loading content increases with the growth of pH value, from 19% of loading capacity in acid solution to 40% in alkali solution. The pH dependence of 5-FU loading can de ascribed to change of both the pore size of entire PLGA/CS microcapsules and permeability of multilayer walls.

The charge density and conformation of the weak polyelectrolyte in solution can be readily regulated by pH values of bulk solutions (Hiller and Rubner, 2003; Yang et al., 2003), changing pH value can initiate imbalance charge distribution between polyanions and polycations, leading to the formation and disappearance of little "holes" of capsule wall with the role of on-off switch (Mauser et al., 2004). For porous PLGA/CS microcapsules, the outermost CS layer plays a dominant role in pH-sensitivity. As pH values decreases, the charge density on the CS chains increases, the molecular chains on the surface assumes a linear conformation due to electrostatic repulsion. That is, its permeability of multilayer walls increases with decreasing pH values, as illustrated in Fig. 3d-B. However, there exist a lot of pores generated from the nanoporous templates, as characterized in Fig. 3b-D, providing more space for the expansion of the CS molecular chain (Ito et al., 1990). So the pores are squeezed, as shown in Fig. 3d-A. As the pores of microcapsule are much bigger than the "holes" resulting from permeability variation of multilayer walls, as a whole, the latter influence seems to be dominant, so the drug loading deceases with decreasing pH value.

The chemical stability of the porous PLGA/CS microcapsules is greatly improved because of amide bones formed between PLGA and CS after EDC treatment. The porous PLGA/CS microcapsules can not be degraded or dissociated during drug loading process, even in extreme pH (such as in strong acid or base) conditions.

The influence of ionic strength on drug loading is shown in Fig. 3d. With the growth of NaCl concentration in 5-Fu solution, the drug content increases and then levels off. The free salt ions can screen the charges hanging on the outermost CS chains to be non-charged, causing the macromolecules to adopt a more coiled, compact conformation (Wang et al., 2007), and leading to the increasing permeability of multilayer walls, as illustrated in Fig. 3d-B. However, the pores of nanoporous PLGA/CS microcapsules are enlarged, as shown in Fig. 3d-A. So the drug loading increases with ionic strength.

3.3. Sustained release of 5-Fu from PLGA/CS microcapsules and corresponding release models

Fig. 4a and b depicts the release profiles of bare 5-FU, 5-FU-loaded PLGA/CS microcapsules and 5-FU-loaded silica templates in pH 7.4 buffers at 37 °C, respectively. The burst release of the bare 5-FU is severe with complete drug release within 260 min. Compared with the release rates from the bare 5-FU, the release rates of 5-FU from PLGA/CS microcapsules and silica templates are obviously delayed. For porous silica templates, the initial release rate is rather rapid with 30% 5-Fu being released within 30 min, the cumulative release was only 56% at 2500 min. For PLGA/CS microcapsules, initial burst release is still observed, which may correspond to the release of drug on the surface of microcapsules. About 40% 5-FU has been released within the first 240 min. Then the drug release slows down and finally reaches equilibrium with a cumulative release amount of 93% at 3200 min. We think that the sensitivity of PLGA/CS microcapsules to Na^+ and PO_4^{3-} in PBS buffer may promote 5-FU release from microcapsule. Compared with porous silica templates, the PLGA/CS microcapsules show more preferable release behavior with longer equilibrium time and higher cumulative release.

However, when 5-FU-loaded PLGA/CS microcapsules are exposed in pH 1.0 HCl solution, the 5-Fu releases slowly and only 51% of loaded 5-Fu can be released within 53 h, as shown in Fig. 4c. The reduction of release rate and cumulative release amount can be ascribed to the decrease of pore size of microcapsules at low pH value and low ionic strength, as mentioned above.

In order to describe the kinetics of the drug release process from the controlled release formulations, various models and mathematical equations, including zero-order, first-order, Higuchi, Ritger-Peppas, Baker-Londale and Hixson-Crowell models (Korsmeyer et al., 1983; Schwartz et al., 1968; Higuchi, 1963; Dredán et al., 1996), are applied for describing 5-FU release process from silica templates and PLGA/CS microcapsules. The most suited being the one which best fits the experimental results. Simulated equations and correlation coefficients are calculated and compared in Table 1.

Two possible mechanisms may involve in the drug-release process from matrix tablets, which may be evaluated from Korsmeyer-Peppas semi-empirical model.

$$Q/Q_\infty = kt^n$$

where Q/Q_∞ is the fraction of drug released at time t, k a constant comprising the structural and geometric characteristics of the tablet, and n the release exponent, is a parameter which depends on the release mechanism and is thus used to characterize it. For spherical tablet, it is reported that threshold of n value between Fickian and non-Fickian mechanism is 0.43. In particular, $n \leq 0.43$ corresponds to a Fickian diffusion release, whereas n between 0.43 and 0.85 indicates an anomalous non-Fickian transport (Vuebaa et al., 2004).

The n value given in Table 1 for silica templates in pH 7.4 buffer is 0.27084 ($n < 0.43$), indicating mechanism of pure 5-Fu diffusion. For PLGA/CS microcapsules in pH 7.4 buffer and pH 1.0 buffer, n was determined to be equal to 0.53123 and 0.53332 ($0.43 < n < 0.85$), respectively. The characteristic exponent suggests the drug release may be modulated by both the 5-Fu diffusion and own erosion of PLGA/CS microcapsules. In spite of this, mathematical studies were carried out with the diffusion based Baker-Lonsdale kinetic models. $3/2[1-(1-Q)^{2/3}]-Q$ (where Q is the release percentage) is plotted as a function of time, as shown in Fig. 4d, a linear relationship is found for PLGA/CS microcapsules with correlation coefficient above 0.99, indicating the drug release behavior is mainly governed by diffusion mechanism (Pardakhty et al., 2007), i.e. the mechanism

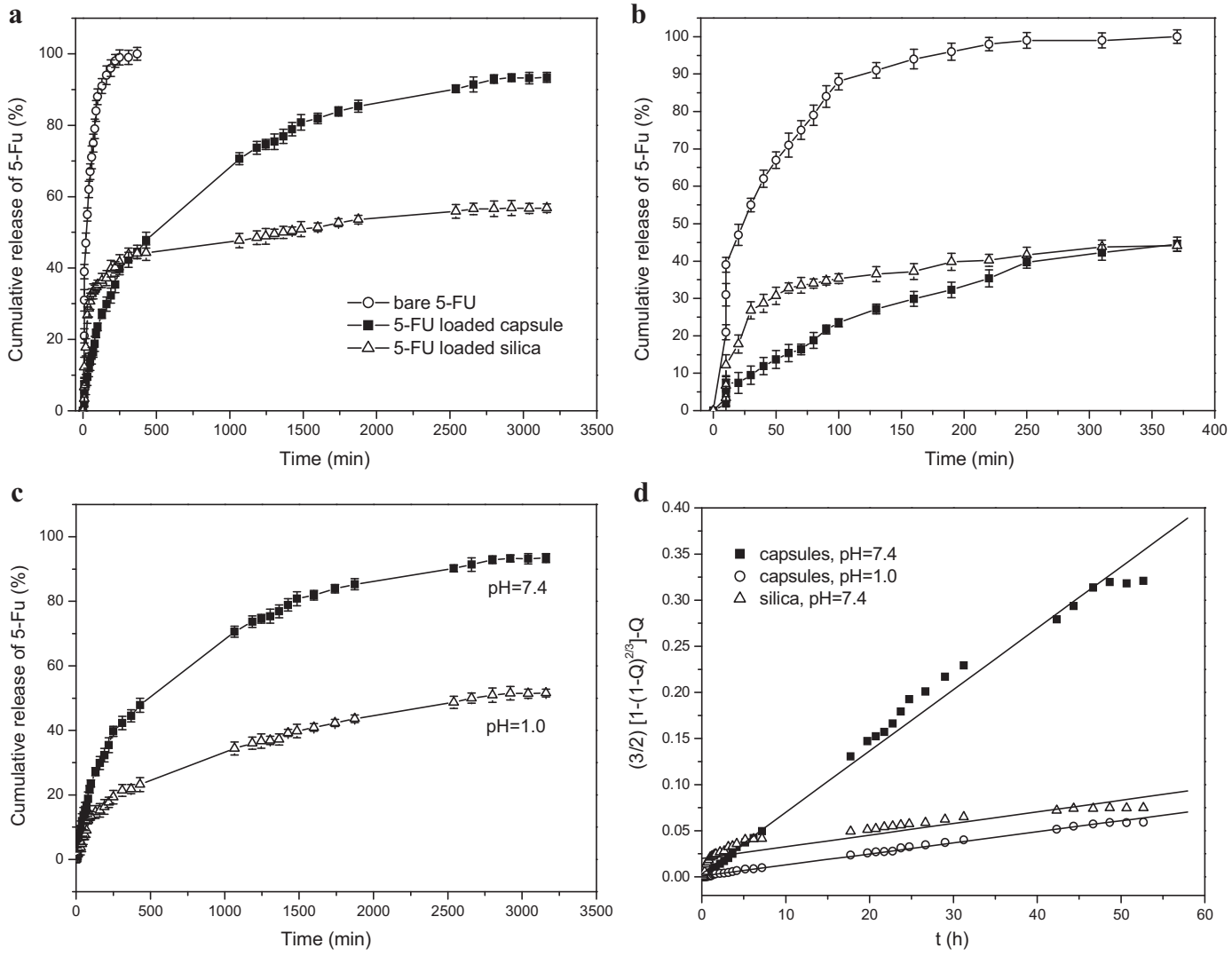

Fig. 4. 5-Fu release from PLGA/CS microcapsules. (a) Release profiles of bare 5-FU, 5-FU-loaded PLGA/CS microcapsules and 5-FU-loaded silica templates in pH 7.4 buffers at 37 °C. (b) The data of release curve (a) in the first 370 min. (c) The cumulative release curves of 5-Fu microcapsules at pH = 1.0 and pH = 7.4, 37 °C. (d) Baker-Lonsdale kinetics of PLGA/CS microcapsules at different pH values and silica spheres at pH = 7.4 (solid line: simulation results; dot: experimental results).

Table 1
Fitted equations of cumulative release curves.

Samples	Medium (pH)	Kinetic model	Equation	Correlation coefficient
Silica templates	Phosphate buffer (pH 7.4)	Zero-order	$Q = 0.30715 + 0.00637 \times t$	0.78332
		First-order	$\ln(1-Q) = -0.37660 - 0.01095 \times t$	0.85131
		Higuchi	$Q = 0.2361 + 0.05310 \times t^{1/2}$	0.87388
		Korsmeyer-Peppas	$\ln Q = -1.46053 + 0.27084 \times \ln t$	0.82981
		Baker-Londale	$3/2[1-(1-Q)^{2/3}] - Q = 0.02111 - 0.00123 \times t$	0.92167
		Hixson-Crowell	$(1-Q)^{1/3} = 0.88280 - 0.00303 \times t$	0.83000
Crosslinked PLGA/CS microcapsules	Phosphate buffer (pH 7.4)	Zero-order	$Q = 0.22366 + 0.01731 \times t$	0.92145
		First-order	$\ln(1-Q) = -0.18378 - 0.05257 \times t$	0.97630
		Higuchi	$Q = 0.0.05049 + 0.13813 \times t^{1/2}$	0.98402
		Korsmeyer-Peppas	$\ln Q = -1.93333 + 0.53123 \times \ln t$	0.97202
		Baker-Londale	$3/2[1-(1-Q)^{2/3}] - Q = 0.00357 - 0.00665 \times t$	0.99506
		Hixson-Crowell	$(1-Q)^{1/3} = 0.92704 - 0.01155 \times t$	0.99366
PLGA/CS microcapsules	HCl buffer (pH 1.0)	Zero-order	$Q = 0.10825 + 0.00938 \times t$	0.94800
		First-order	$\ln(1-Q) = -0.10982 - 0.01359 \times t$	0.97220
		Higuchi	$Q = 0.01895 + 0.07344 \times t^{1/2}$	0.99340
		Korsmeyer-Peppas	$\ln Q = -2.60369 + 0.53332 \times \ln t$	0.98003
		Baker-Londale	$3/2[1-(1-Q)^{2/3}] - Q = 0.00101 - 0.0012 \times t$	0.99772

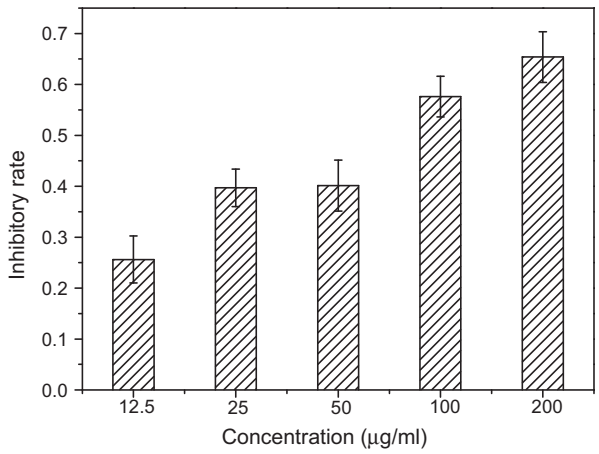

Fig. 5. Cytotoxicity assay of 5-Fu-loaded microcapsules against LM3 cells.

of 5-Fu release from PLGA/CS microcapsules is priority of drug diffusion and subordination of polymer degradation.

3.4. Anticancer effect assay of 5-Fu-loaded PLGA/CS microcapsules

The potential applications in the biomedical fields were assessed by investigating the cancer cell inhibition of 5-Fu-loaded PLGA/CS microcapsules using the MTT assay. As revealed by Fig. 5, 5-Fu-loaded PLGA/CS microcapsules exhibit obvious inhibition effect. The inhibitory rate can be seen to increase with loading levels of 5-Fu-loaded PLGA/CS microcapsules. Higher 5-Fu amounts are expected to release from the drug-loaded PLGA/CS microcapsules with initial drug concentrations over time, resulting in a cytotoxic effect on the cancer cells. The results also indicate the activity of 5-Fu is maintained during its loading into PLGA/CS microcapsules (Gao et al., 2007). So the PLGA/CS microcapsules are a very promising candidate for potential cancer drug delivery applications.

4. Conclusions

Nanoporous multilayer microcapsules were fabricated by layer-by-layer assembly of poly(L-glutamic acid) and chitosan using the porous silica microspheres as sacrificial templates. The electrostatic interaction and amide bone linkage between PLGA and CS were characterized by Zeta-potential analyzer, IR and XRD. The PLGA/CS microcapsules were characterized by SEM, TEM and CLSM. The microcapsules show high loading capacity of 5-Fu, and the loading content can be adjusted by pH value and salt concentration. 5-Fu release from microcapsules take on a sustained release, especially in an acid solution, and its release amount can reach 93% for about 53 h at physiological pH. Fitted equations of release curves correspond to Korsmeyer-Peppas and Baker-Lonsdale models. The MTT cytotoxicity assay in vitro reveals the satisfactory anticancer activity of the drug-loaded PLGA/CS microcapsules. These results show that PLGA/CS microcapsules can serve as a kind of efficient drug delivery carrier in the future.

Acknowledgments

The work was supported by the National Natural Science Foundation of China (Nos. 51003055, 50973060 and 51173101), the Science and Technology Commission of Shanghai Municipality (No. 11JC1404200), the Shanghai Leading Academic Discipline Project (No. s30107) and Innovation Program of Shanghai Municipal Education Commission (No. 11YZ06). Yuliang Chu and Bo Lu from Instrumental Analysis Research Centre (Shanghai University) are acknowledged for their help in SEM and X-ray diffraction measurement.

References

Antipov, A.A., Sukhorukov, G.B., 2004. Polyelectrolyte multilayer microcapsules as vehicles with tunable permeability. Adv. Colloid Interface Sci. 111, 49–61.
Bonglev, D.B., Harkin, D.P., Johnston, P.G., 2003. 5-Fluorouracil: mechanisms of action and clinical strategies. Nat. Rev. Cancer 5, 330–338.
Caruso, R.A., Susha, A., Caruso, F., 2001. Multilayered titania, silica, and laponite nanoparticle coatings on polystyrene colloidal templates and resulting inorganic hollow spheres. Chem. Mater. 13, 400–409.
Dai, Z.Z., Yin, J.B., Yan, S.F., Cao, T., Ma, J., Chen, X.S., 2007. Polyelectrolyte complexes based on chitosan and poly(L-glutamic acid). Polym. Int. 56, 1122–1127.
Donath, E., Sukhorukov, G.B., Caruso, F., Davis, S.A., Möhwald, H., 1998. Novel hollow polymer shells by colloid-templated assembly of polyelectrolytes. Angew. Chem. Int. Edit. 37, 2201–2205.
Dredán, J., Antal, I., Rácz, I., 1996. Evaluation of mathematical models describing drug release from lipophilic matrices. Int. J. Pharm. 145, 61–64.
Gao, C., Leporatti, S., Moya, S., Donath, E., Möhwald, H., 2001. Stability and mechanical properties of polyelectrolyte capsules obtained by stepwise assembly of poly(styrenesulfonate sodium salt) and poly(diallyldimethyl ammonium) chloride onto melamine resin particles. Langmuir 17, 3491–3495.
Gao, H., Gu, Y., Ping, Q., 2007. The implantable 5-fluorouracil-loaded poly(L-lactic acid) fibers prepared by wet-spinning from suspension. J. Control. Release 118, 325–332.
He, C., Liang, Z., Wang, C., Liu, X., Tong, Z., 2005. Preparation of nanoparticles loaded superoxide dismutase by layer-by-layer assembly and their bioactivity. Chem. J. Chin. Univ. 1, 88–92.
Higuchi, T., 1963. Mechanism of sustained action medication. Theoretical analysis of rate of release of solid drugs dispersed in solid matrices. J. Pharm. Sci. 52, 1145–1149.
Hiller, J., Rubner, M.F., 2003. Reversible molecular memory and pH-switchable swelling transitions in polyelectrolyte multilayers. Macromolecules 36, 4078–4083.
Huang, Z.X., Lei, X.Y., Zhong, M., Zhu, B.Y., Tang, S.S., Liao, D.F., 2007. Bcl-2 small interfering RNA sensitizes cisplatin-resistant human lung adenocarcinoma A549/DDP cell to cisplatin and diallyl disulfide. Acta Biochim. Biophys. Sin. 39, 835–843.
Ito, Y., Kotera, S., Inaba, M., Kono, K., Imanishi, Y., 1990. Control of pore size of polycarbonate membrane with straight pores by poly(acrylic acid) grafts. Polymer 31, 2157–2161.
Janes, K.A., Calvo, P., Alonso, M.J., 2001. Polysaccharide colloidal particles as delivery systems for macromolecules. Adv. Drug Delivery Rev. 47, 83–97.
Johnston, A.P., Cortez, C., Angelatos, A.S., Caruso, F., 2006. Layer-by-layer engineered capsules and their applications. Curr. Opin. Colloid Interface Sci. 11, 203–209.
Korsmeyer, R.W., Gurnya, R., Doelkera, E., Buria, P., Peppas, N.A., 1983. Mechanism of solute release from porous hydrophilic polymers. Int. J. Pharm. 15, 25–35.
Kuo, J.W., Swann, D.A., Prestwich, G.D., 1991. Chemical modification of hyaluronic acid by carbodiimides. Bioconjug. Chem. 2, 232–241.
Li, C., 2002. Poly(L-glutamic acid) – anticancer drug conjugates. Adv. Drug Delivery Rev. 54, 695–713.
Liang, Z., Wang, C., Tong, Z., Ye, W., Ye, S., 2005. Bio-catalytic nanoparticles with urease immobilized in multilayer assembled through layer-by-layer technique. React. Funct. Polym. 63, 85–94.
Mao, Z.W., Ma, L., Gao, C.Y., Shen, J.C., 2005. Preformed microcapsules for loading and sustained release of ciprofloxacin hydrochloride. J. Control. Release 104, 193–202.
Mauser, T., Déjugnat, C., Sukhorukov, G.B., 2004. Reversible pH-dependent properties of multilayer microcapsules made of weak polyelectrolytes. Macromol. Rapid Commun. 25, 1781–1785.
Pardakhty, A., Varshosaz, J., Rouholamini, A., 2007. In vitro study of polyoxyethylene alkyl ether niosomes for delivery of insulin. Int. J. Pharm. 328, 130–141.
Pouyani, T., Harbison, G.S., Prestwich, G.D., 1994. Novel hydrogels of hyaluronic acid: synthesis, surface morphology, and solid-state NMR. J. Am. Chem. Soc. 116, 7515–7522.
Pouyani, T., Kuo, J.W., Harbison, G.S., Prestwich, G.D., 1992. Solid-state NMR of N-acylureas derived from the reaction of hyaluronic acid with isotopically-labeled carbodiimides. J. Am. Chem. Soc. 114, 5972–5976.
Schüler, C., Caruso, F., 2001. Decomposable hollow biopolymer-based capsules. Biomacromolecules 2, 921–926.
Schulz-Ekloff, G., Rathoousky, J., Zukal, A., 1999. Mesporous silica with controlled porous structure and regular morphology. Int. J. Inorg. Mater. 1, 97–102.
Schwartz, J.B., Simonelli, A.P., Higuchi, W.I., 1968. Drug release from wax matrices. I. Analysis of data with first-order kinetics and with the diffusion-controlled model. J. Pharm. Sci. 57, 274–277.
Song, Z.J., Yin, J.B., Luo, K., Zheng, Y.Z., Yang, Y., Li, Q., Yan, S.F., Chen, X.S., 2009. Layer-by-layer buildup of poly(L-glutamic acid)/chitosan film for biologically active coating. Macromol. Biosci. 9, 268–278.
Tao, X., Sun, X.J., Su, J.M., Chen, J.F., Roa, W., 2006. Natural microshells of alginatee-chitosan: unexpected stability and permeability. Polymer 47, 6167–6171.
Vuebaa, M.L., Carvalhob, L.A., Veigaa, F., Sousaa, J.J., Pinaa, M.E., 2004. Influence of cellulose ether polymers on ketoprofen release from hydrophilic matrix tablets. Eur. J. Pharm. Biopharm. 58, 51–59.

Wang, C., He, C., Tong, Z., Liu, X., Ren, B., Zeng, F., 2006. Combination of adsorption by porous $CaCO_3$ microparticles and encapsulation by polyelectrolyte multilayer films for sustained drug delivery. Int. J. Pharm. 308, 160–167.

Wang, Y.J., Angelatos, A.S., Dunstan, D.E., Caruso, F., 2007. Infiltration of macromolecules into nanoporous silica particles. Macromolecules 40, 7594–7600.

Wang, Y.J., Caruso, F., 2006. Template synthesis of stimuli-responsive nanoporous polymer-based spheres via sequential assembly. Chem. Mater. 18, 4089–4100.

Yan, S.F., Ling, W., Zhou, E.L., 2004. Rapid synthesis of $Mn_{0.65}Zn_{0.35}Fe_2O_4/SiO_2$ homogeneous nanocomposites by novel modified sol–gel auto-combustion method. J. Cryst. Growth 17, 226–233.

Yang, S.Y., Mendelsohn, J.D., Rubner, M.F., 2003. New class of ultrathin, highly cell-adhesion-resistant polyelectrolyte multilayers with micropatterning capabilities. Biomacromolecules 4, 987–994.

Ye, S., Wang, C., Liu, X., Tong, Z., 2005. Deposition temperature effect on release rate of indomethacin microcrystals from microcapsules of layer-by-layer assembled chitosan and alginate multilayer films. J. Control. Release 106, 319–328.

Ye, S.Q., Wang, C.Y., Liu, X.X., Tong, Z., Ren, B.Y., Zeng, F., 2006. New loading process and release properties of insulin from polysaccharide microcapsules fabricated through layer-by-layer assembly. J. Control. Release 112, 79–87.

Yu, A., Wang, Y., Barlow, E., Caruso, F., 2005. Mesoporous silica particles as templates for preparing enzyme-loaded biocompatible microcapsules. Adv. Mater. 17, 1737–1741.