Sensitive Response of Chitosan Encapsulated Ibuprofen Microspheres to the narrow pH range for Drug Delivery System

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ABSTRACT

Controlled drug-delivery and release systems have received increasing attention for biomedical applications. Chitosan encapsulated ibuprofen (IBU@CS) microspheres were prepared according to oil-in-water micro emulsion polymerization method for an excellent narrow pH sensitive response targeted drug delivery system. The morphology and chemical composition of IBU@CS microspheres with different formulations were characterized. The cytotoxicity test was studied by MTT assay. Results showed that the IBU@CS microspheres were in a spherical structure with a diameter in the range of 50 nm-300 nm. The IBU@CS microspheres had no toxic effect on cells. The in vitro IBU release experiments in PBS solutions of pH 6.8 and 7.4 showed that the encapsulation of CS to IBU could not only reduce the release rate of IBU, but also make the microspheres have narrow pH sensitivity which can release IBU under pH of the inflammatory tissues (pH 6.8) more easily than that of normal tissues. This IBU@CS pH-responsive release system can provide a promising control-release manner to achieve a good therapeutic effect for localized drug delivery.

KEY WORDS: *Chitosan, Ibuprofen, Drug delivery system, Narrow pH response, Cytotoxicity test*

INTRODUCTION

Controlled drug-delivery and release systems are becoming more and more important in modern medication. For disease therapy, drug controlled delivery system can control the release rate of drugs and significantly reduce excessive release of drugs on the human body harm. The pH-responsive drug delivery systems have received increasing attention because of

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the different pH values in some special parts in human bodies. [1, 2] It has been known that pH of stomach is in the range of 1.0-2.5, small intestine pH is from 5.5 to 6.5 and colon pH is around 6.5 [3], while the normal body tissues pH is about 7.4 and the pH value at inflammatory tissue is approximately 6.8 [4]. Many present works on pH-responsive drug delivery systems focused on the conditions of stomach strong acid (pH=1.0~2.5), and few studies focused on slight pH differences between inflammatory and normal tissues. Therefore, it is necessary to establish a narrow pH-responsive drug delivery system which can release drugs under pH of the inflammatory tissues (pH 6.8) more easily than that of normal tissues (pH 7.4).

Ibuprofen (IBU) is the most commonly used nonsteroidal anti-inflammatory drug, which is often used for helping with fever and relieving mild-to-moderate pain after bone implantation surgery. It can inhibit prostaglandin synthesis and the physiologic signaling of pain by inhibiting the activity of cyclooxygenase [5]. However, recent studies show that the burst release phenomenon of IBU in initial stage is still a problem [6-10].

Chitosan is a partially deacetylated polymer of acetyl glucosamine obtained after alkaline deacetylation of chitin. In the 21st century, chitosan and its derivative have prospective applications in many fields because of their interesting properties such as non-toxic, biodegradable, biocompatible and antibacterial [11,12]. Chitosan-based polyelectrolyte complexes can be formed spontaneously by mixing oppositely charged polyelectrolytes in solution without any chemical crosslinker [13]. Recently, due to its physicochemical and biological properties, the study of chitosan as a carrier for drugs has become an interesting research area. In addition, there are a large number of amino groups on chains of chitosan (CS), whose ionization provides CS molecules with the pH-sensitive characteristics. Many studies [14-20] have reported the application of CS in pH responsive drug delivery systems. Yao et al.[21] reported that the amounts of cimetidine delivered from the pH-responsive chitosan/gelatin hybrid polymer microspheres in pH 7.8 solutions were less than that in the case of pH 1.0. Jayakrishnan et al. [22] prepared pH-sensitive progesterone-loaded chitosan microspheres in the size range of 45-300 μm, which dissolved rapidly in pH 5.0 and kept stable in pH 7.4. Li et al. [23] prepared chitosan-tripolyphosphate hydrogel bead with irregular spherical shape with pH sensitive in the range of pH 1.5-6.8. Considering that the acid dissociation constant (pKa) of CS is around 6.3-7.0, CS has different dispersing states in the near neutral pH range from 6.8 to 7.4 [24]. Moreover, ionized ibuprofen species are surface active molecules and able to adsorb onto polymers through hydrophobic and electrostatic bonds with their aromatic ring and hydrophilic carboxylic groups respectively [25-27] which may induce the controlled release process of the drug. Herein, taking advantage of chitosan's unique polymeric cationic character, pH-sensitivity microspheres prepared by chitosan and its derivatives have shown a good application prospect in the aspect of intelligent controlled release systems. The encapsulation IBU into CS may be able to solve the problem of burst release of IBU in initial stage, and make the drug delivery system have a narrow pH sensitive response.

The aim of this study is to prepare encapsulated IBU into CS microspheres using oil-in-water micro emulsion polymerization method. The IBU@CS microspheres were characterized in terms of morphology, particle size, chemical composition and cytotoxicity. The in vitro release experiments in PBS solution under pH 6.8 and 7.4 were studied. The release mechanism of IBU from the IBU@CS microspheres was investigated to provide some meaningful information for applications in biomedical areas.

EXPERIMENTAL

Materials

Chitosan (degree of deacetylation=95%, Mw=200000 g/mol) and IBU were obtained from Aladdin Bio-Chem Technology Co., LTD (Shanghai, China). Soybean lecithin with purity>98% was purchased from Macklin Biochemical Co., LTD (Shanghai, China). MTT (methylthiazolyldiphenyltetrazolium bromide) were provided by Sigma-Aldrich Co., LTD (Shanghai, China). The PBS solution was placed in a refrigerator at 4 ºC after high pressure steam sterilization. All chemicals were of analytical grade.

Preparation of IBU@CS microspheres

IBU@CS microspheres in the present study were prepared according to oil-in-water micro emulsion polymerization method (Table 1). Oil phase: a certain mass of lecithin (the molar ratio of lecithin: IBU was controlled as 1:1) as emulsifiers was dissolved in 400 μl ethanol, subsequently different amounts of IBU (the molar ratio of IBU to CS was controlled as 16:1, 32:1 and 64:1) was added to it and stirred until the system was homogenized. Water phase: 10 mg CS powder was dissolved in 20 ml acetic acid solution (1% v/v). Then oil phase was dispersed in the water phase with the mechanical stirrer at the speed of 1000 rpm for 2 h, and IBU@CS microspheres were obtained. After the loading process, the samples were installed in the dialysis bags (MD25 8000-14000D) and dialyzed in PBS with the pH value of 8.0 for 10 h in order to remove the unencapsulated IBU. The IBU@CS microspheres with different formulations were marked as IBU@CS-A, IBU@CS-B and IBU@CS-C, respectively.

TABLE 1. Formulations of IBU@CS microspheres.

Samples	Oil phase		Water phase
	Ibuprofen	Lecithin	Chitosan
A	32	7.6	10
B	64	12	10
C	128	24	10

Characterization

The morphology of the microspheres was observed using a HT7700 transmission electron microscopy (TEM, Hitachi, Japan). The particle size distribution of the microspheres and the zeta potential were evacuated by a 90Plus PALS laser particle size analyzer (Brookhaven Instrument Corporation, USA). The obtained homogenous suspensions were used to determine the mean diameter and diameter range. Fourier transform infrared spectrometry (FT-IR) analysis was carried out by a Thermo Scientific Nicolet iS50 FT-IR Spectrometer (America). The samples were prepared by processing compress KBr disks and scanned from 400-4000 cm^{-1} at a resolution of 4 cm^{-1}. Ultraviolet-visible (UV-vis) absorption spectra were performed on a U-3900H Spectrophotometer (Hitachi, Japan) using a pair of quartz cuvettes. The pH value was measured on a PHS-3E pH meter with a precision of 0.01 (Shanghai Yoke Instrument Co., LTD, China).

Calculation of encapsulation efficiency

The percentage of IBU within the prepared IBU@CS microspheres was calculated by weighting method and Ultraviolet-visible (UV-vis) spectroscopy. Encapsulation efficiency (EE) of IBU@CS microspheres was calculated with Eq. (1):

$$EE\% = \frac{m_0 - m_1}{m_0} \times 100\% \quad (1)$$

where m_0 and m_1 meant the weights of IBU before and after loading. m_1 was measured by UV-vis

spectrophotometer. The absorbance (ABS) of IBU at 264 nm was applied (Fig. 1). According to the ABS value and standard curve equation, the content of IBU in the solution after loading could be calculated. The result presented here was an average of five tests.

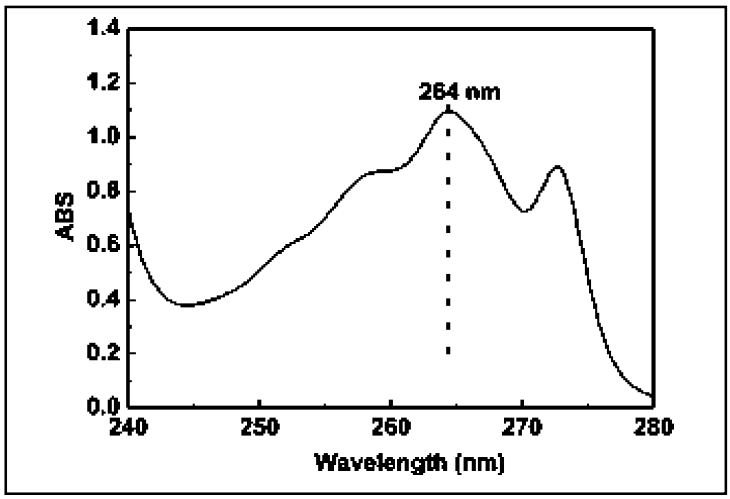

Fig. 1. UV-vis spectrum of IBU from IBU@CS microspheres during the loading process.

In vitro release experiments

All in vitro release of IBU was implemented by soaking equal weight samples in phosphate buffer saline (PBS). IBU@CS microspheres of the same volume of 15 ml were installed in the dialysis bags and soaked in PBS with the pH value of 6.8 and 7.4. The release temperature was kept at 37 °C. Then the IBU-release medium of 3 ml was extracted for UV-vis analysis at given time intervals and replaced with the same volume of fresh PBS solution to maintain a constant value. These samples were analyzed through UV/vis spectrophotometry at the wavelength of 264 nm and the concentration of IBU was calculated using the calibration curves. The amount of released IBU at time *t* was calculated through Eq. (2):

$$IBU\ release\% = \frac{C_n V_0 + V_e \sum_{i=1}^{n-1} C_i}{W} \quad (2)$$

being V_0 the total volume of PBS buffer (100 ml), V_e the removed volume from the release medium at each t interval (3 ml), C_n and C_i the IBU release concentration (mg/ml) and W the weight of IBU@CS microspheres (mg).

The release data were analyzed by using the Korsmeyer-Peppas equation, as shown in Eq. (3), which is often used to describe the drug release behavior from polymeric systems [28-30].

$$\mathrm{Log}(M_t / M) = \log k + n \log t \quad (3)$$

where M_t/M was the drug released fraction at time h, n was indicated the release exponent, indicative of the drug release mechanism, and k was a constant characteristic of the drug-polymer interaction (%/h). From the slope and intercept of log (M_t/M) versus log t, kinetic parameters n and k were calculated. The in vitro release studies were performed in triplicate for each of the samples.

Cytotoxicity test

The MTT assay is a popular tool in estimating the metabolic activity of living cells [31]. In this work, MTT assay was utilized to investigate the cytotoxicity of IBU@CS microspheres. Firstly, to prepare extract

samples: the IBU@CS microspheres were incubated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) under physiological conditions for 72 h and then supernatant fluid was filtered by 0.22 μm filter. Secondly, murine calvarial preosteoblasts (MC3T3-E1) were seeded at a density of 5×10^3 cell/well in a 96-well plate and incubated for 24 h. Then the cells were exposed to the extract of the samples for a period of 24 h and 48 h, respectively. Afterwards, cell culture medium was removed and 10 μl of a 5 mg/ml MTT solution in PBS was added to each well, and followed by incubation for another 4 h at 37 °C. The excess MTT solution was then removed from each well, and formazan crystals generated during the incubation period were dissolved by adding 100 μl of dimethyl sulfoxide (DMSO). After the crystals were fully dissolved, the optical density of each well was measured at a wavelength of 490 nm. Culture medium was employed as the control group, and wells containing culture media without cells were used as blanks.

3. RESULTS AND DISCUSSION

EE% of IBU@CS microspheres

The variation of the encapsulation efficiency with different formulations was displayed in Fig. 2. The measurement of the average EE% for IBU@CS-A, IBU@CS-B and IBU@CS-C was 32.5%±2.8, 81.7%±2.1 and 72.7%±3.6, respectively. With the increase of the molar ratio of IBU to CS, the EE% of IBU gradually increased. When the molar ratio of IBU to CS was adjusted to 32:1, the EE% was up to its relative maximum. With the molar ratio of IBU to CS continued to increase to 64:1, the EE% for IBU@CS microspheres was no longer increased, and was even slightly reduce. The results showed that the molar ratio of IBU to CS had a significantly influence on the EE%. The concentration of IBU should be high enough to ensure encapsulation efficiency. However, excessive doses would make the agglomeration of the IBU molecules, thus affecting the encapsulation.

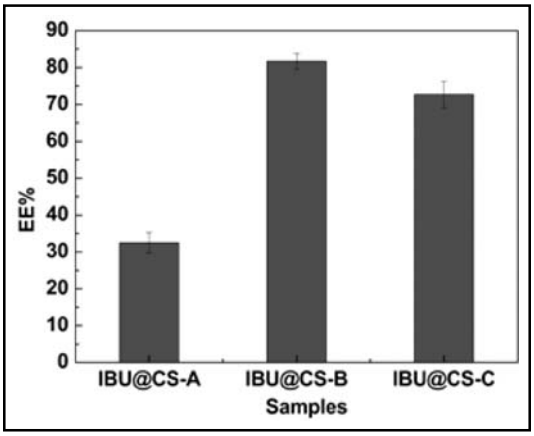

Fig. 2. The change of encapsulation efficiency for IBU@CS microspheres with different formulations.

Morphology observation

The morphologies of the IBU@CS microspheres were shown in Fig. 3. It could be seen that the IBU@CS microspheres displayed a regular spherical structure, with the diameter in the range of 50 nm-300 nm. Particle size distribution of IBU@CS microspheres was studied with dynamic light scattering (DLS). All microspheres were approximately normal distribution. The particle size of IBU@CS-A was in a range of 100-350 nm, with an average of 220 nm. While the particle size of IBU@CS-B and IBU@CS-C showed a gradual decrease to 150 nm and 100 nm, being significantly smaller than that of IBU@CS-A.

The polydispersity index (PDI) of IBU@CS microspheres was 0.56±0.02, 0.32±0.04 and 0.23±0.05, respectively. The PDI value of IBU@CS-C was smaller than 0.3, indicating the narrow distribution of particle size, as shown in Fig. 4. The zeta potentials of IBU@CS

microspheres were 43.01±4.87 mV, 37.02±2.29 mV and 20.75±1.15 mV. As we known, CS is positively charged, and IBU is negatively charged. Thus, these positive values of zeta potential indicated the encapsulation of CS to IBU. Moreover, the higher the absolute value of the zeta potential, the greater the electrostatic repulsion between particles, and the better the physical stability. Thus, the results showed that the IBU@CS-A microspheres were the largest with the best stability, while the IBU@CS-C

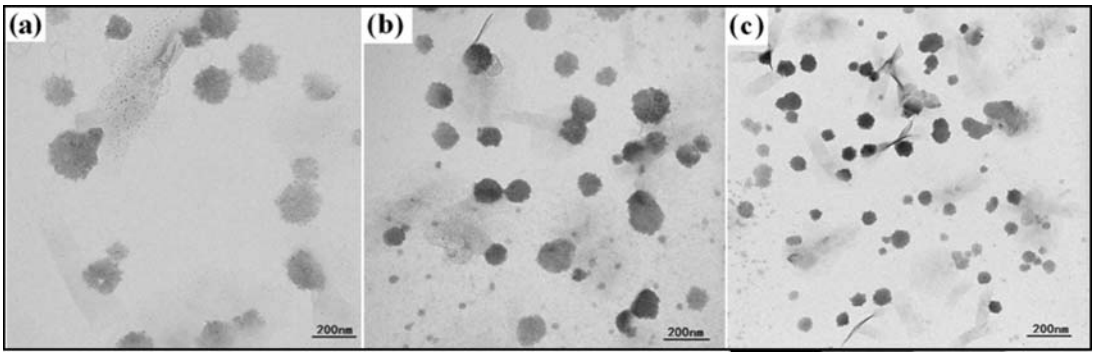

Fig. 3. TEM images of IBU@CS microspheres with different formulations: (a) IBU@CS-A; (b) IBU@CS-B; (c) IBU@CS-C.

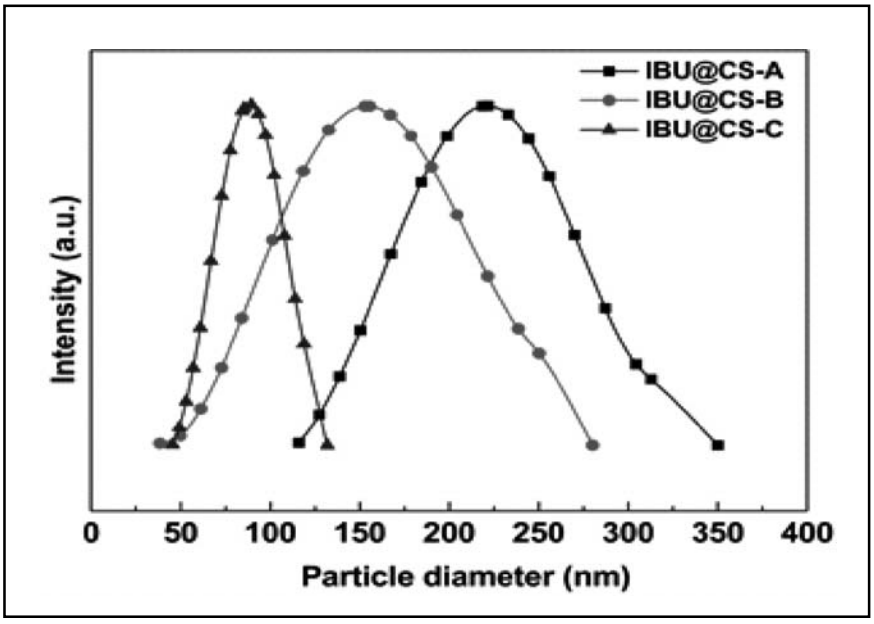

Fig. 4. Particle size distribution of IBU@CS microspheres with different formulations.

microspheres had the narrowest distribution of particle size.

FTIR study

In order to determine the encapsulation of CS to IBU, FTIR spectra of CS, IBU and IBU@CS microspheres were shown in Fig. 5. The peaks at 3356 cm^{-1} and 1032 cm^{-1} in all curves contributed to -OH and C-O groups stretching vibration, respectively. The characteristic adsorption bands of native CS was the acylamino in chitosan matrix which showed the N-H peak at 1595 cm^{-1} [32]. The absorption spectra of IBU at about 1710 cm^{-1}, 1515 cm^{-1} and 1412 cm^{-1} which were caused by the C=O stretching vibration, C=C vibration of the phenyl ring and the C-H bond vibration[33]. The absorption peaks at 2930 cm^{-1} and 2850 cm^{-1} were the stretching vibration of $-CH_3$ and $-CH_2$, respectively. FTIR spectrum indicated that the IBU@CS microspheres were successfully formed with the existence of the amine group

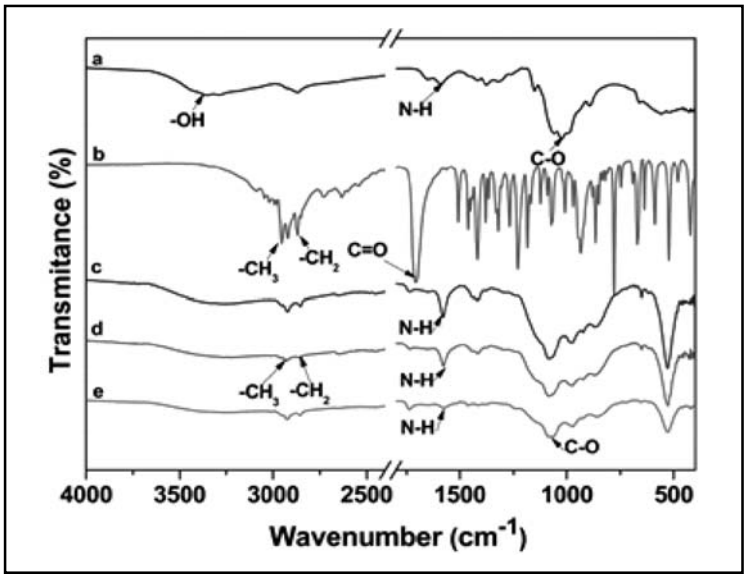

Fig. 5. FTIR spectrum of samples: a-CS; b-IBU; c-IBU@CS-A; d-IBU@CS-B; e-IBU@CS-C.

of CS and the methyl/methylene group of IBU. This result indicated the presence of IBU in prepared IBU@CS microspheres.

Drug delivery applications

In vitro release behaviors of IBU from IBU@CS microspheres in PBS buffer solutions of pH 6.8 and pH 7.4 (37 °C) for different time intervals were shown in Fig. 6. It can be seen from Fig. 6 that the IBU release took place very fast during the entire experiment. 8-15 wt.% of IBU released from IBU@CS microspheres within 30 min, which suggested that the drug was included inside of CS shell. If it was a case of IBU release from the CS surface, most of the adsorbed drug would be a burst release

in the early stage of dissolution [34]. For pure IBU, it is clear that the IBU release was dependent on the pH. The amount of released IBU in pH 7.4 was slightly higher than that in pH 6.8, which was due to the carboxylic acid group and hydrophobic property of IBU molecule (i.e. a-methyl-4(2-methylpropyl) benzeneacetic acid) [35]. IBU is a poorly soluble drug molecule and difficult to diffuse in aqueous medium. As discussed in Sánchez-Sánchez's and Sun's study [36,37], the molecules of IBU were in different states, either protonated at low pH value (pH=2.0) or deprotonated (in anionic form) at pH=7.4. At pH 7.4, the deprotonated form of IBU was predominant and the -COO^- groups of IBU were dissociated. It would be beneficial for the diffusion of ionized IBU, leading to a higher release rate. While with a decreasing pH value, because of the H^+ in the release system, the ionized IBU would change into molecular IBU, which was with poor solubility. IBU was in the protonated form due to the hydrogen bonding among the -COOH groups and protons, leading to a decrease

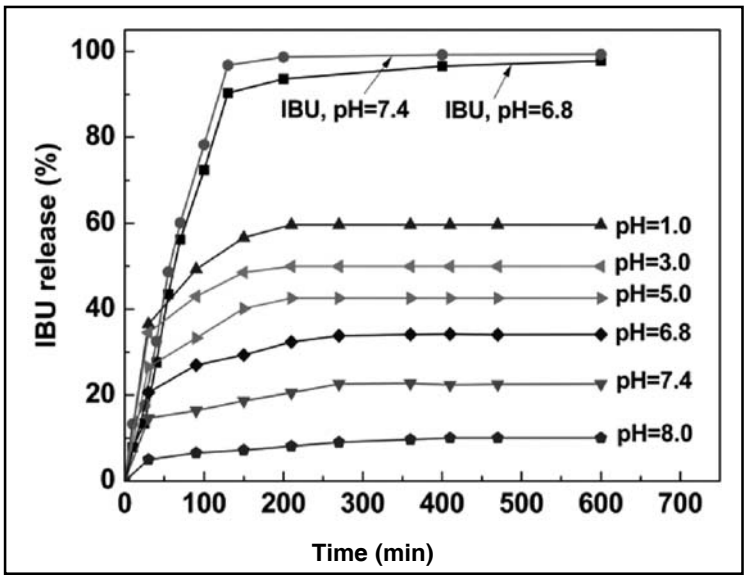

Fig. 6. In vitro IBU-release profiles of IBU from unencapsulated IBU and IBU@CS microspheres in PBS solution of different pH values.

release of IBU. Hence, the release of pure IBU showed a little increasing tendency with the increase of pH value from 6.8 to 7.4.

However, after the encapsulation of CS to IBU, the amount of released IBU from IBU@CS microspheres changed dramatically with pH values. The amount of released IBU from IBU@CS microspheres in pH 7.4 solutions was about 12% for 100 min and 32% for 600 min, whereas the amount of released IBU from IBU@CS microspheres in pH 6.8 solution was around 25% for 100 min and 44% for 600 min.

Moreover, according to the release curve in the same pH medium, because of the encapsulation of CS to IBU, IBU was released more slowly from IBU@CS microspheres than that of the unencapsulated for the same release time period, in which more than 90% IBU was released within 200 min. Thus, in vitro released experiments showed that IBU was successfully loaded into the IBU@CS microspheres.

The encapsulation of CS to IBU could not only reduce the release rate of IBU and improve the burst release of IBU in initial stage, but also make the IBU@CS microspheres have narrow pH sensitive response. The released IBU from the IBU@CS microspheres at pH of 6.8 was easier than that of pH 7.4. The narrow pH response of IBU@CS microspheres was because CS owned different dispersing states in pH 6.8 and pH 7.4 aqueous solutions. The morphologies of their chains were in slightly entangled semirigid state, heavily entangled flexible state and orderly aggregated state, respectively [25]. When CS was in a solution of pH 7.4, the hydrogen bonding was strong and the amide groups did not carry any charge, so there was no electrostatic repulsion between CS molecules, and CS were in orderly aggregated state. In this case, the chains crystallize in an orthorhombic unit cell with dimensions a=8.95(4), b=16.97(6), c(fiber axis)=10.34(4) Å [38]. This crystal structure could efficiently hinder the release of IBU which was approximately 1.0285 nm and diameter was around 0.5237 nm [39]. When CS was in a pH 6.8 release medium, the hydrogen bonding was weakened and CS molecules existed in the gel network state, which was less efficiently influence the release of IBU.

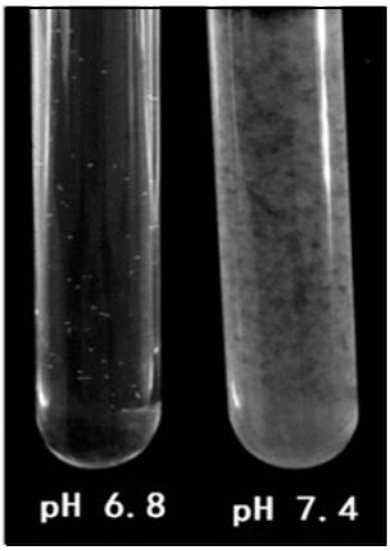

Fig. 7. Different states of CS under pH 6.8 and pH 7.4

In addition, the pH-responsive property of IBU@CS microspheres should be attributed to a different swelling behavior of CS under varying acidic environment. As seen in Fig. 6, when the pH value was lower than pH 7.4, the IBU release% increased with decreasing the pH value. That was because at low pH, the amide groups on the CS could become protonated, forming the hydrophilic NH_3^+ group. The resulting electrostatic repulsion between the protonated amino groups weakened the intermolecular and intramolecular hydrogen bonding interaction of chitosan molecules. In neutral conditions, no such protonation occurred and the swelling ratios of the microspheres were low [40].

The IBU@CS microspheres release behaviors corresponded well with the swelling pH sensitivity of CS. At pH 7.4, the microspheres were contracted since the charge density of the chitosan was low, which leads to a lower IBU release rate.

When the pH decreased to 1-2, dissociation of physical linkages and dissolution of the network may occur [41]. The loose state of CS in acid conditions results in a high release rate of IBU. The release mechanism of IBU from IBU@CS microspheres under pH 6.8 and pH 7.4 was shown in Fig. 8.

To understand the release behavior of IBU in PBS buffer solutions with different pH, the release kinetics of IBU@CS was studied. *n* was an empirical parameter characterizing the release mechanism. On the basis of diffusion exponent, the *n* value found to be around 0.5 indicated the drug release mechanism approached to a Fickian type of diffusion controlled release, whereas *n* equal to 1.0 showed the drug release mechanism approached to a zero-order profile. The *n* value from 0.5 to 1.0 was a time dependent mechanism and it was called non-Fickian type

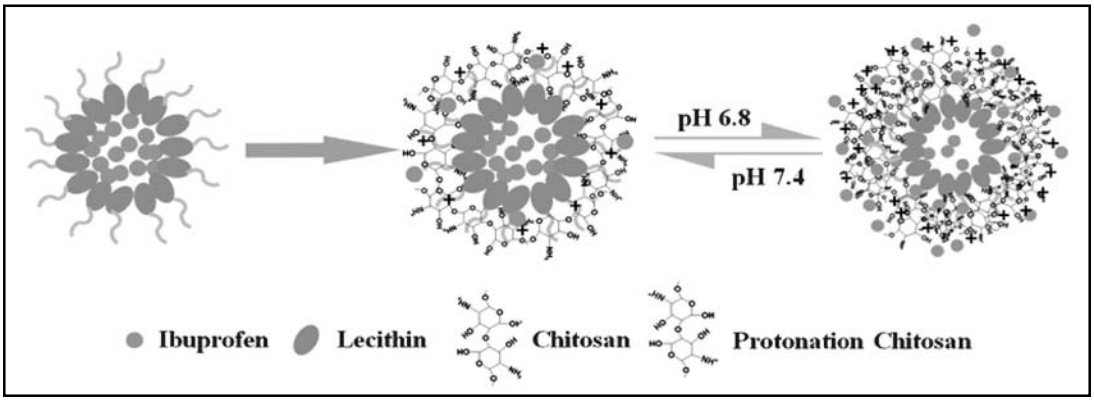

Fig. 8. Release mechanism of IBU from IBU@CS microspheres under pH 6.8 and pH 7.4.

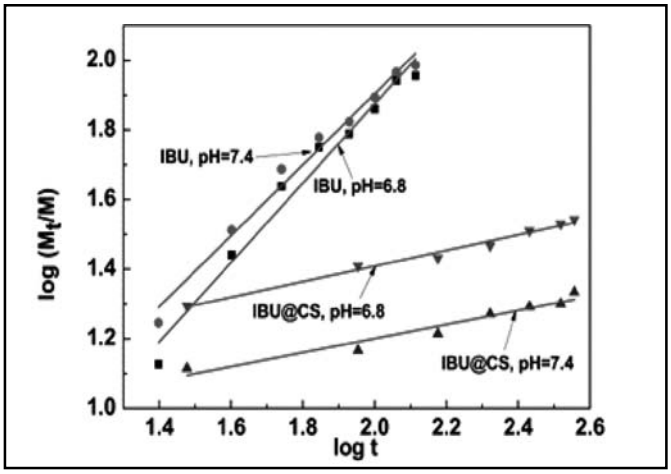

Fig. 9. Release kinetics of IBU from unencapsulated IBU and IBU@CS microspheres in PBS solution of pH 6.8 and pH 7.4.

TABLE 2. The value of *k*, *n* and correlative coefficients (R^2) following linear regression of release data.

Sample	pH=6.8			pH=7.4		
	n	*k*	R^2	*n*	*k*	R^2
IBU	1.040	0.667	0.973	1.019	0.873	0.982
IBU@CS	0.202	2.220	0.935	0.224	2.611	0.981

of diffusion or chain relaxation control release [42]. From the logarithmic plot of release data log(M_t/M) versus log t, the values of *n* and *k* were calculated as shown in Fig. 9.

Table 2 summarized the values for the IBU@CS microspheres in pH 6.8 and pH 7.4 medium. As illustrated in Table 2, the release data fit well with Korsmeyer-Peppas model as a correlation coefficient (R^2) greater than 0.90 was obtained in all cases. The *n* value of unencapsulated IBU was around 1 at pH 6.8 and pH 7.4 medium, which suggested that the IBU release was nearly zero-order release, while the *n* value of IBU@CS microspheres was in the range from 0.202 to 0.224 at pH 6.8 and pH 7.4 medium. It was believed that the IBU release mechanism of IBU@CS microspheres was non-Fickian diffusion. Metters and Kulkarni [43,44] pointed that the quantity of drug released from the samples was controlled by diffusion, swelling and the degradation process of the sample. Thus, the release of IBU from the IBU@CS microspheres involved two different mechanisms: diffusion through the swollen CS and release due to CS degradation. Due to the electrostatic forces (attractive force) between the positively charged CS and negatively charged IBU, the cumulative release of IBU from the IBU@CS microspheres could not reach 100%.

Cytotoxicity test

To evaluate cytotoxicity of IBU@CS microsphere, cells were cultured in regular medium as control and extraction medium of IBU@CS microspheres for 24 and 48 h. Cell viability of MC3T3-E1 were demonstrated in Fig. 10. Compared with that of control group, cell viability at 24 h was not significantly affected by the extract medium of IBU@CS

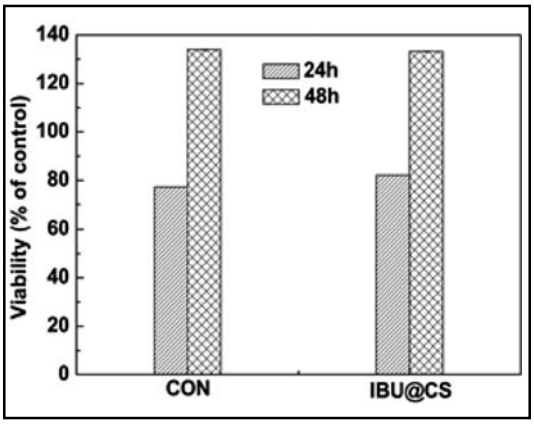

Fig. 10. Cell viability of MC3T3-E1 cultured in regular media (control) and extraction media of IBU@CS microspheres

microspheres. Similar patterns were found in cell culture results at 48 h. The data obtained from MTT experiments suggested that the IBU@CS microspheres prepared in this study were potentially non-toxic to cells and could be suitable for clinical use.

CONCLUSIONS

The narrow pH sensitive IBU@CS microspheres were prepared according to oil-in-water micro emulsion polymerization method for drug delivery system. The morphology of IBU@CS microspheres had a regular spherical structure with a diameter in the range of 50 nm-300 nm. FTIR spectrum indicated that the IBU@CS microspheres were successfully formed with the amine group of CS and the methyl/ methylene group of IBU. In vitro release experiments indicated that the encapsulation of IBU into CS could not only reduce the release rate of IBU, but also make the microspheres have narrow pH sensitive response. The release of IBU in IBU@CS microspheres was higher under pH of the inflammatory tissues (pH 6.8) than that of normal tissues (pH 7.4). The cytotoxicity test presented that the IBU@CS microspheres had no toxic effect on cells.

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