Vanillin cross-linked chitosan microspheres for controlled release of resveratrol

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ABSTRACT

In this work, resveratrol (Res) was incorporated into chitosan microspheres for controlled release and stabilisation. The microspheres were prepared by emulsion chemical cross-linking method, and vanillin was used as the novel cross-linker. The microspheres showed a smooth surface with irregular small particles and internal voids with a size distribution between 53 and 311 μm. Interpenetrating network cross-linking mechanisms might account for the Schiff base reaction between chitosan and vanillin. The encapsulation efficiency of Res within microspheres was up to 93.68%. Res contained within microspheres was protected from light and heat compared with the free Res. In addition, release behaviours were governed by two distinct stages and dependent on pH of release media. Diffusion, swelling and erosion mechanisms might coexist for the full controlled release and Higuchi was the most suitable model for the whole release procedure. Thus, controlled release and stabilization of Res were achieved through incorporation of Res into cross-linked chitosan microspheres by vanillin.

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1. Introduction

Resveratrol (Res) is a naturally occurring polyphenolic phytoalexin, synthesised by a wide variety of plant species such as grapes, berries, peanuts, and a variety of food sources in response to injury or fungal attack (Fremont, 2000). Recently, Res has drawn great attentions because of its beneficial effects against many diseases such as cancer (Signorelli & Ghidoni, 2005), cardiovascular disease (Vella, Bowen, & Fenning, 2008), inflammatory disease (Kang et al., 2009), and platelet aggregation (Pace-Asciak, Hahn, Diamandis, Soleas, & Goldberg, 1995). However, Res has some drawbacks of unstabilization (Signorelli & Ghidoni, 2005), poorly water soluble (Lu, Cheng, Hu, Zhang, & Zou, 2009), and short biological half time (Juan, Buenafuente, Casals, & Planas, 2002), all of which limit the utilisation of Res in food and pharmaceutical industries. In order to overcome such drawbacks, incorporation of Res into a polymer matrix is one of the useful methods available.

In recent years, incorporation of active agents into polymer matrices for extending their shelf life, protecting against oxidation and increasing biological half time have been growing rapidly (Jameela, Kumary, Lal, & Jayakrishnan, 1998). Chitosan is a natural polysaccharide derived from chitin by alkaline deacetylation, and it is the second most abundant biopolymer after cellulose (Ravi Kumar, 2000). Due to its good properties of non-toxicity, good biocompatibility, mechanical film forming ability and antimicrobial

activity, chitosan has been widely used in food industry such as metal chelation in wastewater, purification of water, clarification and deacidification of fruit juice, formation of edible films and preservation of foods against microbial deterioration (Jeon, Kamil, & Shahidi, 2002; Shahidi & Abuzaytoun, 2005; Shahidi, Arachchi, & Jeon, 1999; Shahidi & Synowiecki, 1991). The potential of applying chitosan in food industries has recently been shown for controlled delivery system of active agents in the format of microspheres or nanospheres. To increase the time frame and controlled release property of active agents, chitosan microspheres usually need to be cross-linked. Several chemical cross-linkers have been used such as glutaraldehyde, formaldehyde, glyoxal and other reactive cross-linking agents (Ajit, Namdev, Sangamesh, & Tejraj, 2007; Gupta & Jabrail, 2006). However, these agents, classified as toxic substances and residual cross-linkers in microspheres, would give rise to health concerns and cause undesirable side effects (Fürst & Banerjee, 2005; Rathna, 2008). To avoid the disadvantages of chemical cross-linking, some physical cross-linkers have been used. However, physical cross-linkers have shown poor mechanical strength and the resulted microspheres have exhibited inferior release properties due to their weak electrostatic interactions between anions and chitosan (Gupta & Jabrail, 2006; Mi et al., 1999). Therefore, it is very necessary and crucial to search for novel cross-linkers for chitosan microspheres.

Vanillin, obtained from the bean or pod of the tropical *Vanilla* orchid (principally *Vanilla planifolia* Andrews, syn. *V. fragrans*) (Karathanos, Mourtzinos, Yannakopoulou, & Andrikopoulos, 2006), is often used as flavouring agent and food preservative,

and is generally regarded as safe (GRAS) substance (Mourtzinos, Konteles, Kalogeropoulos, & Karathanos, 2009). Apart from its flavouring properties, vanillin also exhibits several bioactive properties (Sinha et al., 2008). Moreover, vanillin is also used for other purposes such as constituent in cosmetic and drug preparations. The aldehyde groups in vanillin and the amino groups in chitosan may undergo Schiff base reaction and form a network structure which will favour the stabilization and controlled release of the Res.

The objective of this work was to explore the possibility of using vanillin to cross-link chitosan microspheres for controlled release and stabilization of Res, as well as to study the physicochemical properties of the microspheres.

2. Materials and methods

2.1. Materials

Chitosan (molecular weight 1×10^6 Da, deacetylation degree ⩾85%) was supplied by Sankang Health Products Co., Ltd. (Jiangxi, China). Res was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Vanillin, liquid paraffin, span 80 and tween 80 were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All other chemical agents used were of analytical grade.

2.2. Preparation of microspheres

Chitosan microspheres containing Res were prepared via emulsion chemical cross-linking method. Chitosan (100 mg) was dissolved in aqueous acetic acid solution (10 ml, 2%, v/v) and filtered through a nylon cloth to remove insolubles. Then, Res was dispersed directly into the chitosan solution to a final concentration of 10% (w/w of chitosan), which was used as the water phase. Liquid paraffin (50 ml) containing 2% (v/v) span 80 and tween 80 (1:1, v/v) emulsifier was used as an oil phase. Then, the water phase (10 ml) was added dropwise into the oil phase (50 ml) by an injector for emulsification over a 30 min period to form W/O emulsion with stirring at room temperature. Thereafter, vanillin dissolved in acetone (25 mg ml^{-1}) as the cross-linker was slowly and dropwise introduced into the W/O emulsion to solidify the chitosan droplets for 3 h with agitation. Finally, the microspheres were collected and washed three times with petroleum ether, acetone and ethanol, respectively. The product was dried in a vacuum oven at 45 °C for 12 h and kept in a desiccator for further analysis.

2.3. Scanning electron microscopy (SEM)

The morphological features were examined by scanning electron microscopy (Quanta 200F, FEI, Hillsboro, OR, USA). The samples were sprinkled onto a double-sided tape and sputter-coated with a 5 nm-thick gold layer. The inner structures of microspheres were also observed after fracturing microspheres by a razor blade.

2.4. Fourier transform-infrared spectroscopy (FT-IR)

FT-IR spectra were recorded by using a Spectrophotometer (Nicolet 5700, Thermo Electron Corporation, Waltham, MA, USA). Chitosan, vanillin and microsphere samples were prepared by processing compressed KBr disks.

2.5. Determination of encapsulation efficiency

The encapsulation efficiency (EE) of the microspheres was expressed as the percentage of the actual Res loading of the microspheres and the initially Res used. Microspheres (25 mg) were extracted exhaustively with ethanol (50 ml) for 12 h at room temperature under dark condition, centrifuged at 1200g for 30 min and filtered with a syringe filter (0.45 μm pore size). The filtrate was assayed by HPLC (1100 Series, Agilent Technologies Co., Ltd., Palo Alto, CA, USA) for Res content in microspheres at the wavelength of 306 nm. The analytical column was a C$_{18}$ (5 μm, 4.6 mm × 150 mm, Waters Company, Milford, MA, USA). The mobile phase was a mixture of methanol/acetonitrile/water/acetic acid (75:22.5:2.4:0.1, v/v/v/v) at a flow rate of 1.0 ml min^{-1} and the volume of injection was 20 μl. EE was determined using the following equation:

$$EE(\%) = (\text{Amount of Res in microspheres}/\text{amount of initial Res used}) \times 100.$$

2.6. Stability

Microsphere (25 mg) and free Res powders (reference samples) (25 mg) were added into a flask and stored under two conditions: (1) irradiated using a UV lamp (16 W at about 20 cm distance) for 60 min, and (2) exposed to two different temperatures (60 and 70 °C) under dark condition for 15 days. Aliquots of samples were removed, extracted with ethanol, and filtered. The test extracted solution was then subjected to HPLC as described above. The effect of encapsulation on the stability of Res was evaluated by the retention percentage, namely the ratio of the content of Res retained to the original one in the samples.

2.7. In vitro release

Microsphere samples (25 mg) were placed into dialysis bags and suspended in 250 ml SGF (simulated gastric fluid, pH 1.2), PBS (phosphate buffer saline, pH 7.0), or SIF (simulated intestinal fluid, pH 7.5) as the release media at 37 ± 0.1 °C and stirred at 100 rpm using the USP 23 paddle method. At predetermined time, samples (5 ml) were withdrawn with a syringe filter (0.45 μm pore size) from the release media and replaced with equal volume of the corresponding fresh media to maintain a constant volume. The test solution was analysed by HPLC and the amount of Res released from the microspheres was calculated as a percentage of the initial amount of Res incorporated in the microspheres prior to dissolution test.

2.8. Release mechanism

In order to gain insight into the release mechanism of Res, the release data were analysed according to the zero-order, first-order, Higuchi and Hixson–Crowell models, and the release exponent n of Peppas model was also calculated by Ritger and Peppas's method (Ritger & Peppas, 1987).

3. Results and discussion

3.1. Morphology of chitosan microspheres

SEM images of the chitosan microspheres are shown in Fig. 1. The microspheres had smooth surface but with some irregular small particles (Fig. 1A and B), which were attributed to the results of the mechanical stress during the stirring process or the movement of the moisture during the drying period. The microspheres are heterogeneous in size, ranging from 53 to 311 μm in diameter. The internal structure of microspheres showed a compacted and continuous network with many voids (Fig. 1C and D). The formation of voids may be related to the mechanisms of air bubbles or
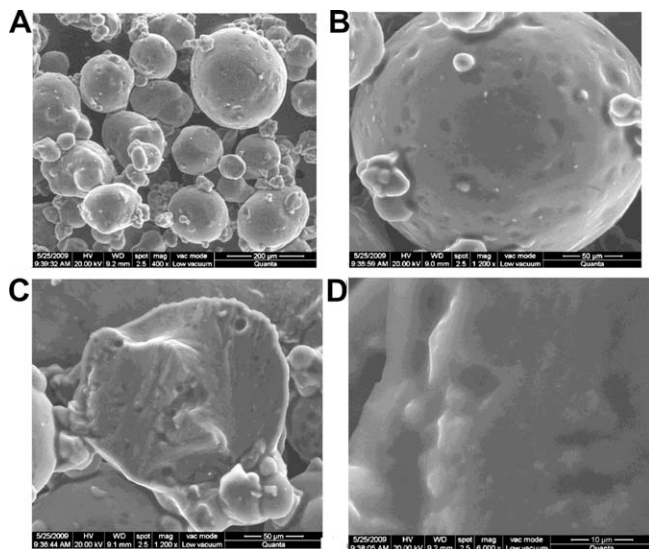
Fig. 1. SEM images of microspheres: Surface morphology (A and B) and internal structure (C and D).

entrapped fluid formed during the cross-linking and solidification process.

3.2. Fourier transform-infrared spectroscopy (FT-IR)

FT-IR spectra are shown in Fig. 2 for vanillin, chitosan, and chitosan microspheres, respectively. In the FT-IR spectrum of vanillin (Fig. 2A), the stretching vibration absorption of -OH was at $3183 \, cm^{-1}$. The absorption of C–H stretching of methyl group is at $2867 \, cm^{-1}$. The peak at $1668 \, cm^{-1}$ corresponds to stretching vibrations of C=O of aldehyde group, and the characteristic stretching vibration absorption of benzene ring corresponds to three peaks at 1591, 1509 and $812 \, cm^{-1}$, respectively. The peak at $1266 \, cm^{-1}$ corresponds to bending vibrations of phenolic hydroxyl group.
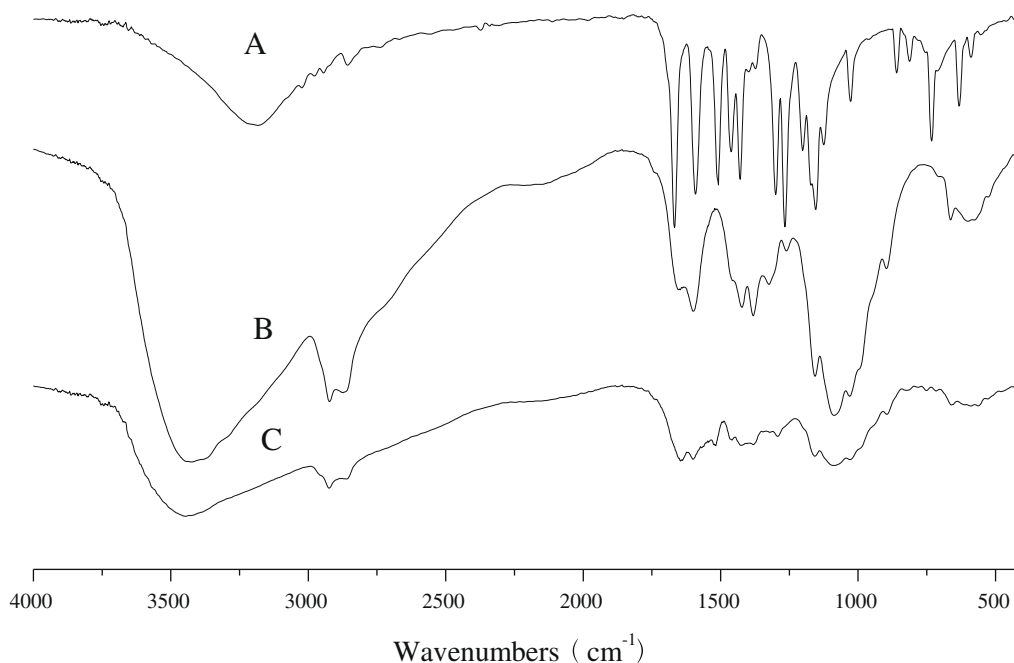
Fig. 2B shows the FT-IR spectrum of chitosan, the presence of –OH and N–H are confirmed due to stretching vibration at $3424 \, cm^{-1}$, in which the –OH stretching vibration are overlapped by N–H stretching. The absorption of C–H stretching of methyl or methylene group of chitosan is at $2923 \, cm^{-1}$, and the peak at $1656 \, cm^{-1}$ corresponds to the amide bands. The peak at $1560 \, cm^{-1}$ corresponds to the symmetrical stretching vibration absorption of amino group. The peak at $1381 \, cm^{-1}$ corresponds to stretching vibrations of C–N bond. At $1086 \, cm^{-1}$ the stretch vibrations of C–O are found.

The FT-IR spectrum of the chitosan microspheres (Fig. 2C) shows the peak at $1646 \, cm^{-1}$ corresponding to characteristic stretching vibration of C=N, which can be attributed to the Schiff base reaction between the aldehyde group of vanillin and amino group of chitosan. The peak of –OH/N–H of chitosan and phenolic hydroxyl of vanillin shifts from 3424 to $3447 \, cm^{-1}$ and from 1266 to $1292 \, cm^{-1}$, respectively, and their intensity has been reduced significantly after cross-linking, which may be due to the hydrogen bond interaction between chitosan and vanillin. From these results it is assumed that chitosan was cross-linked by vanillin successfully through Schiff base reaction and hydrogen bond interaction.

3.3. Encapsulation efficiency (EE)

The EE dependence on the preparation techniques has been reported in previous works. For example, the EE of Res within liposomes (Caddeo, Teskac, Sinico, & Kristl, 2008), microencapsules (Shi et al., 2008) and nanoparticles (Shao et al., 2009) was 76.00%, 18.75% and 91.00%, respectively. The EE of Res within the chitosan microspheres was increased up to 93.68%. Vanillin was used as the cross-linker for chitosan microspheres showing a comparable and improved EE.

3.4. Stability

Res is known to be a highly photosensitive compound and easily degraded by light and heat, which limit its application

Fig. 2. FT-IR spectrum of vanillin (A), chitosan (B), and microspheres (C).

for commercialisation. The protective effect of the microspheres under the light stress of Res was investigated. As shown in Fig. 3, Res in all samples were gradually degraded under UV irradiation, although the degrees of which were different. After 60 min irradiation, the Res retention rate of reference samples and chitosan microspheres were 61.65% and 78.04%, respectively. It is obvious that the chitosan microspheres using vanillin as cross-linker could largely prevented the degradation of Res.

Thermal stability experiments were also carried out at different temperature (60 and 70 °C) (Fig. 4). The retention percentage of Res within reference samples at about levels of 71.59% and 48.08% at 60 and 70 °C after 15 days storage, respectively. In contrast, incorporation of Res into chitosan microspheres using vanillin as the cross-linker protected the Res from heat effects and the thermostability increased considerably, about 83.81% and 74.88% of Res remained after heat at 60 and 70 °C for 15 days. The high stability against heat may arise from two factors. One factor may be the hydrogen bond formation between the hydroxyl groups of Res and chitosans, which might increase the melting point of Res and stabilize Res against heat effects. The other factor may be the physical incorporation into the sphere matrix and the resulted wall showing the protective effect to the core of Res.

3.5. In vitro release

Release of the active agents from chitosan or derivatives microspheres involves three different mechanisms: (a) releasing from the surface of particles, (b) diffusion through the swollen rubbery matrix, and (c) releasing due to polymer erosion (Ding, Huang, Li, & Liu, 2007). The release behaviours and stages of Res from microspheres in SGF, PBS or SIF media are shown in Fig. 5. Controlled release of Res was dependent on pH value of media conditions (Fig. 5A), with slower release kinetics at higher pH conditions, which was attributed to amino groups of chitosan protonation resulting in a soluble, and positively charged polysaccharide leading to faster swelling in acidic medium. The Higuchi model was applied to describe the release of Res (Fig. 5B). The release data were presented as the fractional release and plotted against the square root of time. These plots revealed that there were two stages for the release of Res from the microspheres. The first stage of release was initially rapid (burst release), which may be result from the rapid diffusion of the Res onto the surface of microspheres from the initial swelling of the spheres. Later, the second stage of release was slow from microspheres (controlled release). The burst release
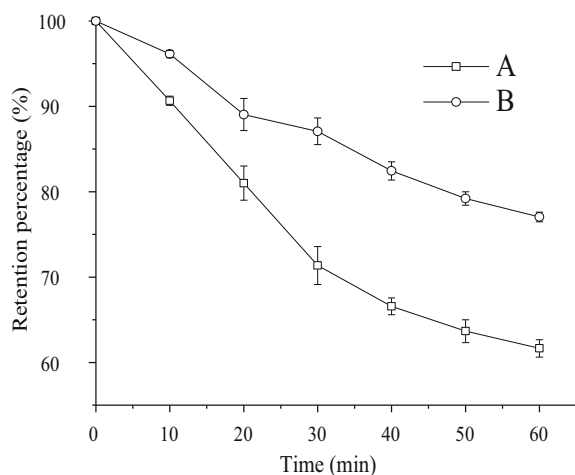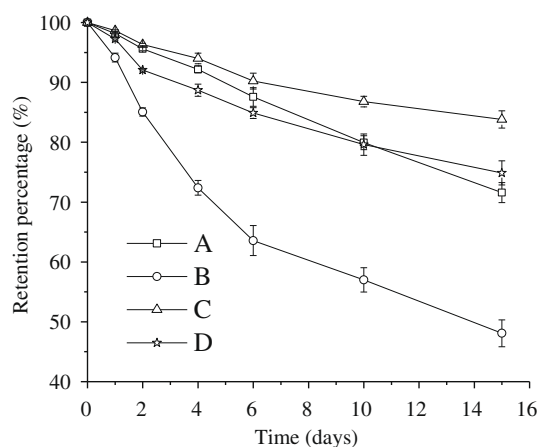

Fig. 4. The retention percentage of resveratrol in reference and microsphere samples at different temperature. (A) reference samples at 60 °C; (B) reference samples at 70 °C; (C) microsphere samples at 60 °C; (D) microsphere samples at 70 °C. Data represent the mean ± SD ($n = 3$).

may help to reach the effective concentration of Res rapidly in plasma, whereas the controlled release would maintain the effective concentration of Res in plasma for a long time. The results suggested that the poor bioavailability of Res because of the rapid metabolism and elimination could be supplemented by encapsulation method and thus prolonged its biological half time in *vivo*.

3.6. Release mechanism

Zero-order, first-order, Higuchi, Hixson–Crowell and Peppas models were used to investigate the probable mechanism of Res from microspheres at each release stage:

Zero-order model: $M_t/M_\infty = kt$

First-order model: $\ln(1 - M_t/M_\infty) = -kt$
Higuchi model: $M_t/M_\infty = kt^{1/2}$
Hixson–Crowell model: $(1 - M_t/M_\infty)^{1/3} = -kt$
Peppas model: $\ln M_t/M_\infty = n \ln t + \ln k$

where M_t/M_∞ is the fractional active agents release at time t, k is a constant incorporating the properties, n gives an indication of the release mechanism.

For zero-order, first-order, Higuchi and Hixson–Crowell models, the analysis of correlation coefficient (r^2) of linear relationship between Res release and time was established for the evaluation of the release mechanism. From the results of Table 1, it can be observed that Higuchi model was the most suitable model for describing Res release kinetics from microspheres, which indicated that the diffusion through the matrices of spherical shape was the main factor in controlling Res release from such microspheres. The r^2 analyses (Table 1) also suggested that the "quality of fit" was better for the first stage than that for the second stage. Kockish, Rees, Tsibouklis, and Smart (2005) also reported that the "quality of fit" of the diffusion controlled processes was generally better for the initial stage of the release than that for the terminal phase.

For Peppas model, n gives an indication of the release mechanism. For example, $n = 0.43$ for Case I Fickian diffusion, $n = 0.85$ for Case II transport, $0.43 < n < 0.85$ for anomalous behaviour or non-Fickian transport, and $n > 0.85$ for Super Case II transport (Ritger & Peppas, 1987). The calculated n values of chitosan microspheres were listed in Table 1. It is shown that the exponent n values for the stage 1 were higher than 0.85, which suggested that Super Case II transport was the major release mechanism, whereas
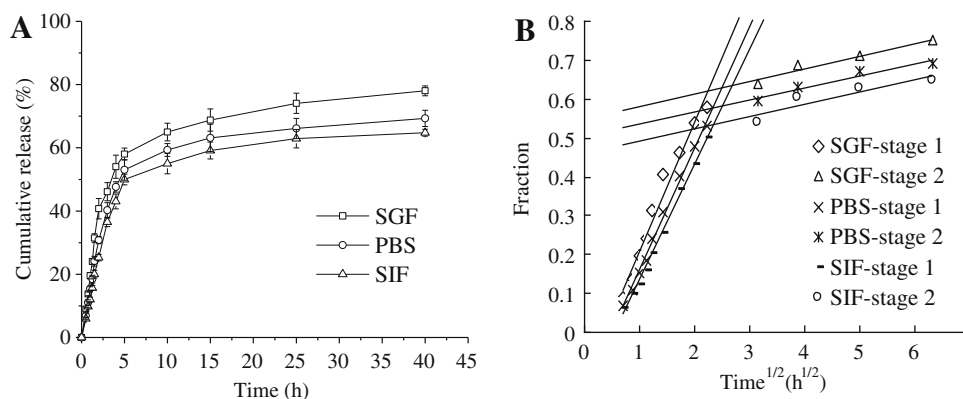

Fig. 3. The retention percentage of resveratrol in reference (A) and microsphere samples (B) exposed to UV lamp. Data represent the mean ± SD ($n = 3$).

Fig. 5. In *vitro* percentage cumulative release (A) and Higuchi model fractional release (B) of resveratrol from microsphere samples in different solution of SGF, PBS or SIF at 37 ± 0.1 °C. Data represent the mean ± SD (n = 3).

Table 1
Correlation coefficients (r^2) and release exponent (n) according to the different models used for describing resveratrol release mechanisms from the microspheres in SGF, PBS or SIF solution.

Model	SGF solution		PBS solution		SIF solution	
	Stage 1	Stage 2	Stage 1	Stage 2	Stage 1	Stage 2
Zero-order (r^2)	0.91	0.91	0.96	0.89	0.98	0.78
First-order (r^2)	0.96	0.94	0.98	0.92	0.99	0.81
Higuchi (r^2)	0.97	0.95	0.99	0.94	1.00	0.85
Hixson–Crowell (r^2)	0.94	0.93	0.98	0.91	0.99	0.80
Peppas (n)	0.85	0.11	0.88	0.11	0.92	0.12

the Fickian diffusion was the major release mechanism for stage 2 ($n < 0.43$). The diffusion, swelling and erosion release mechanisms may coexist for the full controlled release of Res from the microspheres.

4. Conclusions

In the current investigation, the chitosan microspheres containing Res were successfully prepared with vanillin as a natural and non-toxic cross-linker. The microspheres had smooth surfaces and continuous network. Cross-linking mechanism was via Schiff base reaction. The Res in microsphere samples had better stability than the free Res. Release of Res from the microspheres was initially rapid then followed by a plateau. The diffusion, swelling and erosion mechanisms might coexist for the full controlled release of Res, and Higuchi was the most suitable model for the whole release procedure. Thus, controlled release and stabilization of Res will provide a more effective and continuous supply of Res within the body.

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