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Preparation and characterization of *N*-acetylchitosan, *N*-propionylchitosan and *N*-butyrylchitosan microspheres for controlled release of 6-mercaptourine

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Abstract

In this paper, biodegradable *N*-acylchitosan microspheres were prepared by a water-in-oil (w/o) interfacial *N*-acylation method for controlled release of 6-mercaptourine (6-MP). Acetic, propionic and *n*-butyric anhydrides were used as reagents for the interfacial *N*-acylation reaction to prepare *N*-acetylchitosan, *N*-propionylchitosan and *N*-butyrylchitosan microspheres. The onset time for the gelation of *N*-acylchitosan microspheres increased with increasing the molecular weight of used anhydride. While, the particle sizes of *N*-acetylchitosan microspheres (>50 μm), *N*-propionylchitosan microspheres (20–50 μm) and *N*-butyrylchitosan microspheres (~ 5 μm) decreased with the increase of substituted acyl chain length. The introduction of long acyl chains onto chitosan not only increased hydrophobicity, but also interfered crystallization of the polymer chains, leading to affecting the swelling and drug release properties of prepared *N*-acylchitosan microspheres. Drug release rates of the *N*-acylchitosan microspheres decreased in the sequence of *N*-propionylchitosan ($t_{50\%} \sim 19$ h) > *N*-butyrylchitosan ($t_{50\%} \sim 36$ h) > *N*-acetylchitosan ($t_{50\%} > 100$ h), and were fitted with a two-phase percolation model. The enhancement of drug release for *N*-propionylchitosan and *N*-butyrylchitosan microspheres under lysozyme degradation were less significant than their *N*-acetylchitosan counterpart. The results suggested that the *N*-acylchitosan microspheres excipient as interesting candidates for drug delivery application.

Keywords: *N*-acylchitosan; Microsphere; Drug release; Water-in-oil (w/o)

1. Introduction

Carbohydrate polymers are usually environment friendly and biological compatible. Commercialized carbohydrate polymers such as alginate, dextran, cellulose and chitosan have received much attention in chemical, biomedical and pharmaceutical application. A special interest in developing these carbohydrate polymer-based materials is oriented to prepare microspheres and nanoparticles for drug delivery. Notable studies have been investigated to deliver drugs, therapeutic peptide, protein, antigen, oligonucleotide and gene (Genta, Costantini, Asti, Conti, & Montanari, 1998; Gombotz & Wee, 1998; Kim et al., 2003; Liu & Yao, 2002; Mi, Shyu, Chen, & Schoung, 1999; Tomer, Podczeck, & Newton, 2001; Vandamme, Lenourry, Charrueau, & Chaumeil, 2002; Wang, Nation, Evans, & Cox, 2004)

Chitosan [poly(1,4-β-D-glucopyranosamine)] is a naturally occurring polysaccharide which possesses valuable properties as a biomaterial (Muzzarelli, 1993). Owing to its good biocompatibility, chitosan has received much interest in the pharmaceutical industry for the last few years (Akbuga & Bergisadi, 1996; Hejazi & Amiji, 2003; Janes, Fresneau, Marazuela, Fabra, & Alonso, 2001; Liu et al., 2004; Mi, Sung, & Shyu, 2002; Sivakumar, Manjubala, & Panduranga Rao, 2002; Thacharodi & Panduranga, 1993; Thanoo et al., 2000; Win, Shin, Hong, & Kajiuchi, 2003). It has been reported that chitosan can be digested by lysozyme. Therefore, chitosan can be recognized as

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a biodegradable polymer. In contrast to cellulose that is insoluble in mild aqueous solution, chitosan can be dissolved in dilute acid. This solubility is typically reduced with the introduction of hydrophobic groups.

Chitosan has both reactive amino and hydroxyl groups, and can be used for modification of its physicochemcal properties. It has been modified by cross-linking, carboxy methylation, galactosylation, etc. for drug delivery and biomedical applications (Chen, Wu, Mi, Lin, & Sung, 2004; Jameela, Kumary, Lai, & Jayakrishnan, 1998; Muzzarelli, Ramos, Stanic, Bruno, & Mattioli-Belmonte, 1998; Park et al., 2003; Sashiwa et al., 2000; Shantha & Harding, 2002; Tokura, Nishi, Tsutsumi, & Somorin, 1983; Tokura & Tamura, 2001). Among the chitosan derivatives, *N*-acylchitosans are important because of their biodegradable and blood compatible properties (Hirano & Noishiki, 1985; Hirano, Tsuchida, & Nagao, 1989; Hirano & Usutani, 1997; Lee, Ha, & Park, 1995). Novel fibers of *N*-acylchitosans and their cellulose composites have been developed for textile application (Hirano & Midorikawa, 1998; Hirano, Usutani, & Midorikawa, 1997).

Based on the finding of aforementioned studies, we wish to examine the possibility of preparing *N*-acylchitosan microspheres for controlled drug release. In this study, biodegradable *N*-acylchitosan microspheres were prepared by a water-in-oil (w/o) interfacial *N*-acylation method. Aliphatic anhydrides such as acetic, propionic and *n*-butyric anhydride are used as reagents to examine the effect of hydrophobicity of an anhydride on the reactivity of the interfacial *N*-acylation reaction. An anticancer drug—6-mercaptourine (6-MP) was incorporated into the *N*-acylchitosan (*N*-acetyl-, *N*-propyl-, *N*-butyrylchitosan) microspheres for the examination of drug release. The work aims to evaluate the effect of *N*-acylation substitution on the physicochemical properties (hydrophilicity and crystalline property) of the prepared *N*-acylchitosan microspheres. Based on the analysis of hydrophilicity and crystalline property, the effects of different *N*-acylation substitution on the releases of 6-MP from *N*-acylchitosan microspheres are experimentally investigated. Additionally, the enhancements of drug releases from various *N*-acylchitosan microspheres by lysozyme hydrolysis were also studied. It was expected that such *N*-acylation derivatization would dominate the physicochemical properties and enzymatic hydrolysis of prepared *N*-acylchitosan microspheres and could play a role in controlling drug release.

2. Experimental

2.1. Materials

Chitosan (molecular weight 4×10^5) were purchased from Fluka Chemical Co. (Switzerland). The degree of deacetylation of chitosan was approximately 85%. 6-Mercapoturine purchased from Sigma Chemical Co. (USA) was used as a model drug. Hen egg-white lysozyme (Sigma, USA), 48,000 U/mg, was a commerical product from Sigma Chemical Co. (USA) and used without further purification. Acetic, propionic and *n*-butyric anhydride were all purchased from Merck, Germany. All other reagents and solvents used were of reagent grade.

2.2. Preparation of the chitosan microspheres

Chitosan powder (0.3 g) was dispersed in 20 ml of water containing 1.0 wt% acetic acid. The polymer solution was prepared by magnetic stirring for 3 h to dissolve chitosan powder. After dissolution, 6-mercaptourine was added and homogeneously mixed with the polymer solution. The final solution was added into 150 ml of soybean oil in a 250 ml beaker and was stirred at 500 rpm for 60 min to form the water-in-oil (w/o) dispersion. Acetic, propionic and *n*-butyric anhydride were, respectively, added by drop into the medium, and the stirring was, respectively, continued for 40 min–90 h. The hardened *N*-acylchitosan microspheres were collected by centrifugation at $3000\times g$ for 5 min using high-speed centrifuge (HERMLE, ZK 365). After the upper layer was removed by decantation, the microspheres were rinsed with ethyl acetate twice, and dried in air for 24 h.

2.3. Structural analysis

The prepared *N*-acylchitosan microspheres were analyzed by using Perkin–Elmer 1600 spectrophotometer. The intensity of amide adsorption at 1560 and 1655 cm^{-1} was monitored to examine the *N*-acylation reaction of chitosan.

2.4. Measurement of degree of N-acylation

The degree of *N*-acylation, could be determined by ninhydrin assay, was defined as the percentage of unchanged amino groups remaining after *N*-acylation (Curotto & Aros, 1995). In the ninhydrin assay, solutions of the acylated chitosan (0.1 mg/ml) were prepared in CH_3COOH (3%, w/v) and HCl (1%, w/v) by continuous stirring at 20 °C for 24 h. Acetate buffer (0.5 ml, 4 M, pH 5.5) was added to the resulting acylated chitosan solution. Subsequently, ninhydrin reagent was added and the solution was heated for 20 min. After heating with ninhydrin, the optical absorbance of the solution at 570 nm was recorded with a spectrophometer (Model UV-150-02; Shimadzu Corp., Kyoto, Japan) using D-glucosamine at various known concentrations as standard. It is known that the amount of free amino groups in the test sample, after heating with ninhydrin, is proportional to the optical absorbance of the solution.

2.5. X-ray diffraction study

The diffraction patterns of native and acylated chitosan samples (powder diffraction) were recorded using a Shimadzu XD-5 diffractometer.

2.6. Swelling studies

The swelling capacities of *N*-acylchitosan microspheres were determined by immersing the microspheres in deionized water at room temperature. *N*-acylchitosan microspheres (200 mg) were placed in the media for a required period of time. The swollen *N*-acylchitosan microspheres were collected by centrifugation using high-speed centrifuge (HERMLE, ZK 365). The wet weight of microspheres were determined by first blotting the *N*-acylchitosan microspheres with a filter paper to remove the adsorbed water on the surface, then weighed immediately on an electronic balance. The percentage swelling of *N*-acylchitosan microspheres in the media were then calculated by the formula

$$E_{sw} = [(W_e - W_0)/W_0] \times 100$$

where E_{sw} is the swelling percentage of *N*-acylchitosan microspheres at equilibrium. W_e denotes the weight of *N*-acylchitosan microspheres at equilibrium swelling and W_0 is the initial weight of *N*-acylchitosan microspheres. Each swelling experiment was repeated three times and the average value was taken as the percentage swelling value.

2.7. Scanning electron microscopy (SEM)

The *N*-acylchitosan microspheres were sprinkled onto double-sided tape, sputter-coated with gold to about 500×10^{-8} cm thickness using an Hitachi coating unit IB-2 coater under a high vaccum, 0.1 Torr, high voltage, 1.2 kV and 50 mA. Coated samples were examined using Hitachi S-2300 scanning electron microscopy.

2.8. In vitro release

The release of 6-mercaptourine from *N*-acylchitosan microspheres was measured by using the dissolution (Hanson research, Dissoette II) and auto-sampling (Hanson research, SR6) systems. The dissolution medium was 500 ml of PBS solution(pH 7.4). The medium was placed in a 1 l round flask fitted with a pump for auto-sampler to remove medium and stirred with a mechanical stirring at a rate of 100 rpm. The dissolution medium temperature was maintained at 37 °C. The *N*-acylchitosan microspheres (100 mg) were placed in the dissolution medium. After a predetermined period, 5 ml of the medium was removed and the amount of 6-mercapoturine was analyzed spectrophotomerically at 360 nm. In order to maintain the original volume, 5 ml of the medium was replaced with fresh water meanwhile.

2.9. Enzymatic degradation

The *N*-acylchitosan microspheres were placed in 500 ml, pH 7.4 and 37 °C of lysozyme solution (1000 U/ml). The release of 6-mercaptourine from the *N*-acylchitosan microspheres and the amount of released 6-mercaptourine were, respectively, performed and determined as aforementioned process.

3. Results and discussion

3.1. Preparation and properties of N-acylchitosan microspheres

The amino groups on chitosan could react with anhydrides to form *N*-acylchitosans. The reaction of chitosan with straight chain aliphatic anhydrides (acetic, propionic and *n*-butyric anhydrides) increased the hydrophobicity, and finally resulted in the formation of insoluble gels. The schematic *N*-acylation reactions for the preparation of *N*-acylchitosan microspheres are shown in Fig. 1. The *N*-acetylchitosan, *N*-propionylchitosan and *N*-butyrylchitosan microspheres were prepared by a water-in-oil (w/o) interfacial *N*-acylation method. Firstly, the chitosan solution (the dispersion phase) and the soybean oil (the continuous phase) were heterogeneously mixed to form water-in-oil (w/o) dispersion. Afterward, the anhydride was dropped into the w/o dispersion at a slow rate. While an anhydride was added to the w/o dispersion, the anhydride might be partitioned into the continuous phase (soybean oil phase)

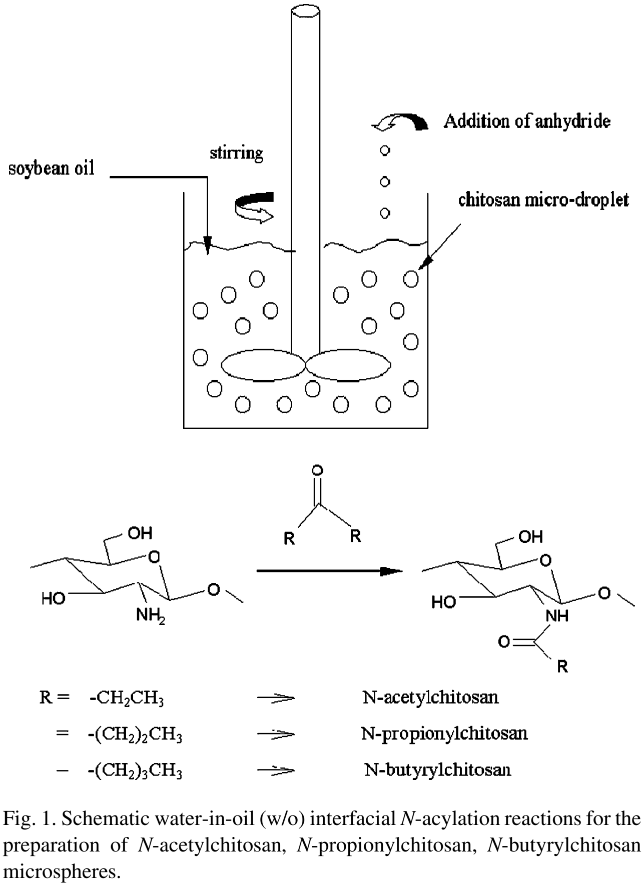

Fig. 1. Schematic water-in-oil (w/o) interfacial *N*-acylation reactions for the preparation of *N*-acetylchitosan, *N*-propionylchitosan, *N*-butyrylchitosan microspheres.

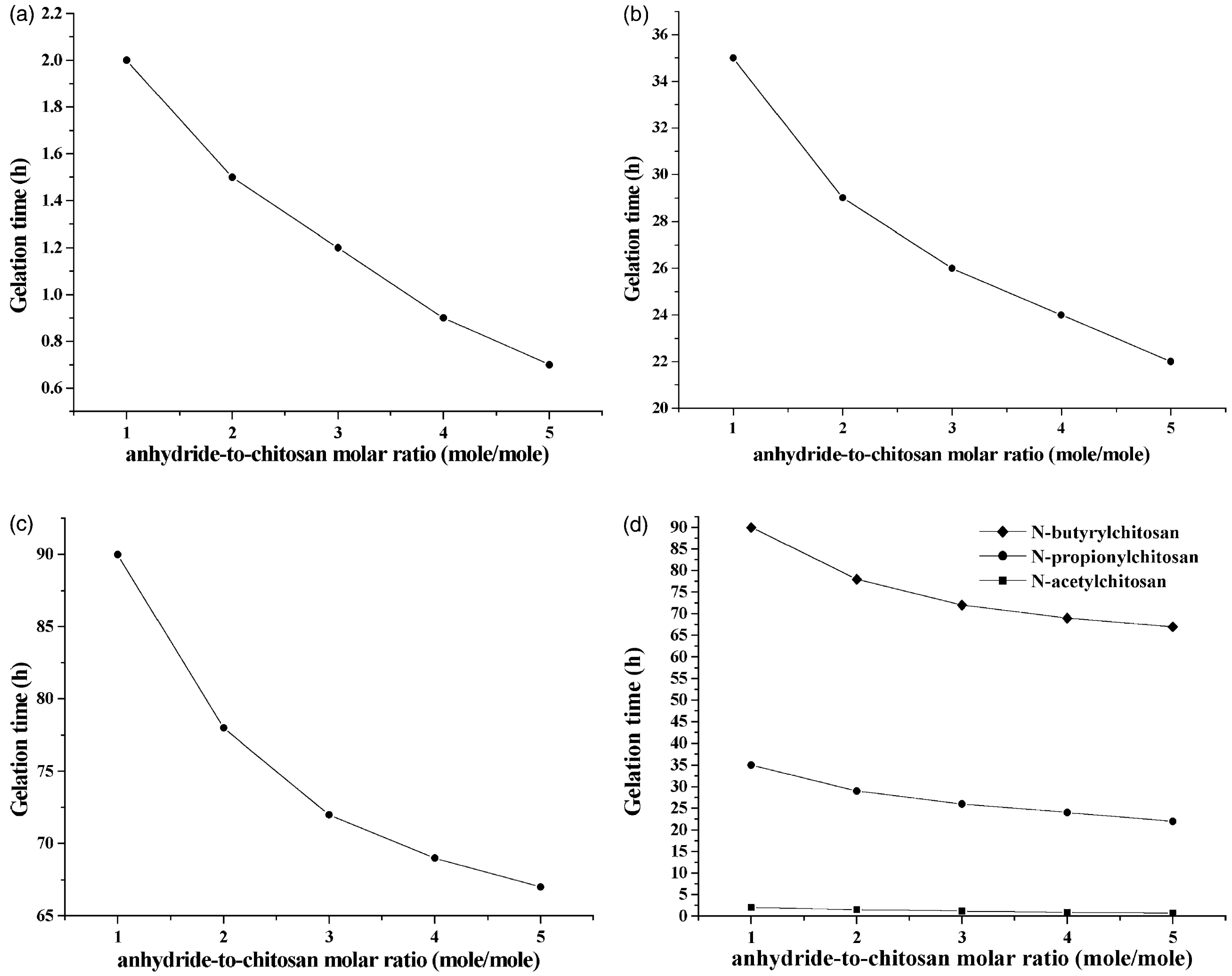

Fig. 2. The time to onset of gelation of *N*-acylchitosan microdroplets: (a) *N*-acetylchitosan; (b) *N*-propionylchitosan; (c) *N*-butyrylchitosan; (d) combination of *N*-acetylchitosan, *N*-propionylchitosan and *N*-butyrylchitosan.

and the water phase (chitosan microdrops). The partition ratio of an anhydride (water-to-oil phase) was dependent on the hydrophobicity of the aliphatic anhydride. The less hydrophobicity of the anhydride the more partitioned extent in the water phase (chitosan microdrops), which could result in a quicker gelation of the *N*-acylchitosan microspheres.

The time to onset of gelation increased with increasing the molecular weight of the anhydride. It took about 40–120 min and 24–35 h to gel the *N*-acetylchitosan and *N*-propionylchitosan microspheres, respectively. However, a much longer time (72–90 h) was needed to gel the *N*-butyrylchitosan microspheres (Fig. 2). This indicated that the time to onset of gelation would depend on the molecular weight of anhydrides. This dependence on the molecular weight of the anhydrides might be caused by increasing stereo hindrance at the reactive sites, leading to the reduction in the fraction of the effective intermolecular collisions. Furthermore, the remarkable decrease of gelation time for the preparation of *N*-acetylchitosan microspheres could be attributed to the less hydrophobicity of acetic anhydride.

Fig. 3 shows the FT-IR spectra of the prepared *N*-acylchitosan microspheres and native chitosan. The absorption peaks at 1560 cm^{-1} could be assigned to the N–H bending vibration of amide II band, and at 1655 cm^{-1} to the carbonyl stretching of amide I band. The native chitosan demonstrated characteristic bands of primary amines at 1570 cm^{-1} and secondary amides at 1655 cm^{-1}. After *N*-acylation, the band corresponding to primary amino groups at 1570 cm^{-1} disappeared, while the intensity of characteristic band of amide groups at 1560 and 1655 cm^{-1} increased, showing the evidence of *N*-acylation reaction to chitosan. The *N*-acylation degrees of the prepared *N*-acylchitosan microspheres (prepared at the condition of anhydride-to-chitosan molar ratio is 1:1) being calculated from ninhydrin assay are listed in Table 1.

The SEM of various *N*-acylchitosan microspheres prepared by the dispersion *N*-acylation reaction is shown in Fig. 4. It could be found that the shapes of all *N*-acylchitosan microspheres were in good sphericity. The diameters of *N*-acetylchitosan microspheres were almost larger than 50 μm. With increasing substituted acryl chain length, the diameters of prepared *N*-acylchiton

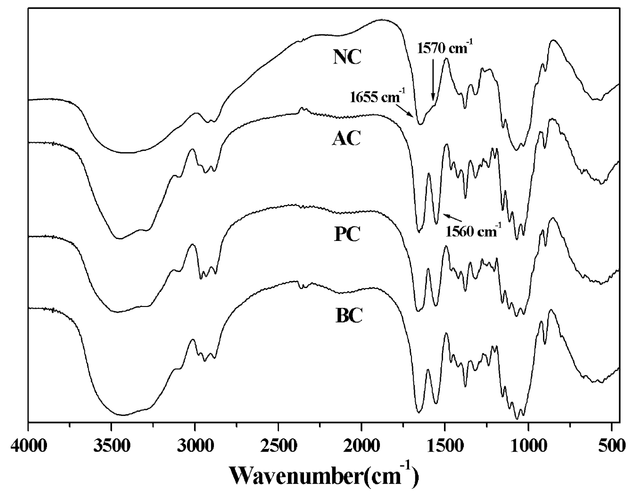

Fig. 3. FT-IR spectra of native chitosan (NC), *N*-acetylchitosan (AC), *N*-propionylchitosan (PC) and *N*-butyrylchitosan (BC) microspheres.

microspheres decreased. The diameters of *N*-propionylchitosan microspheres were mostly distributed in the range of 20–50 μm, which was much smaller than that of *N*-acetylchitosan microspheres. The particle size of *N*-butyrylchitosan microspheres was more uniform and even smaller (~5 μm) than those of *N*-acetylchitosan and *N*-propionylchitosan microspheres.

From the SEM analysis, it was assumed that, in the aforementioned w/o dispersion, the interfacial tension was reduced with the increase of substituted acryl chain length of chitosan, leading to the formation of microspheres with smaller particle size.

3.2. Crystallinity of N-acylchitosan microspheres

Fig. 5 shows the X-ray diffraction patterns of *N*-acylchitosan microspheres and native chitosan. The crystalline form of chitosan was gradually altered with the increasing acyl chain length. The X-ray diffractogram of *N*-acetylchitosan showed sharp and well-defined peaks at the same 2θ position, suggesting the formation of a more structured chitosan derivative. In the case of *N*-propionylchitosan microspheres, the diffractogram showed broad peak located at around $2\theta=20°$. It can be concluded that crystalline forms of this chitosan derivate is transferred from crystalline structure of native chitosan to less structured *N*-propionylchitosan. The low crystallinity of *N*-propionylchitosan could be related to the pendant chain length, long enough to interfere the formation of hydrogen bonding. It was worth noting that new sharp peaks appeared at $2\theta=8°$ for *N*-butyrylchitosan microspheres. This result on chitosan modification suggested important changes in crystalline structure induced by the hydrophobic side chains. It seems that the hydrophobic interactions can enhance the arrangement of macromolecular chains of *N*-butyrylchitosan in order to form higher crystalline of *N*-acylchitosan derivative, as compared with *N*-propionylchitosan.

Table 1
Estimation of degree of substitution by ninhydrin assays

Samples	Degree of substitution (%)
N-Acetylchitosan	88.2 ± 6.3
N-Propionylchitosan	83.5 ± 5.1
N-Butyrylchitosan	79.9 ± 5.8

3.3. Swelling studies of N-acylchitosan microspheres

The effect of *N*-acylation on the swelling of microspheres is shown in Fig. 6. The swelling capability of a polymeric material is dependent on the hydrophilic groups and amorphous region of the polymer. The macromolecular chains in crystal region were tightly packed, therefore the hydration effect can be reduced. As shown in Fig. 6, the swelling ratios of *N*-acylchitosan microspheres prepared from different anhydrides increase in the sequence of *N*-acetylchitosan < *N*-butyrylchitosan < *N*-propionylchitosan. The swelling of the *N*-acylchitosan microspheres was not found to proportionally decrease with the increase of acyl chain length. The result was attributed to the reason that the introduction of a long acyl chain as a side group (especial for *N*-propionyl group) on chitosan would not only increase the hydrophobicity but also decrease the crystallinity. This explained why *N*-propionylchitosan and *N*-butyrylchitosan microspheres generated higher swelling ratio as compared with the more hydrophilic *N*-acetylchitosan microspheres.

3.4. In vitro drug release

Fig. 7 shows the drug release behavior of various *N*-acylchitosan microspheres. The drug release rate of the *N*-acylchitosan microspheres decreased in the sequence of *N*-propionylchitosan ($t_{50\%}\sim 19$ h) > *N*-butyrylchitosan ($t_{50\%}\sim 36$ h) > *N*-acetylchitosan ($t_{50\%}>100$ h). As pre-aforementioned, the introduction of an acyl chain as a side group results in the increase of hydrophoribicity, as well as the decrease of crystallinity of prepared *N*-acylchitosan microsphere. It was known that the increase of hydrophobicity and crystallinity of a polymer-based drug carrier would limit the access of water and decrease the dissolution of drug. The results of drug releases suggested that the effect of crystallinity on drug release is even more evident for *N*-acetylchitosan microspheres, as compared with the *N*-acylchitosan microspheres prepared from propionic and butyric anhydrides.

Fig. 8 shows the log–log polts of M_t/M_∞ (release percent) vs. t (release time) for drug release profiles of the *N*-acylchitosan microspheres. These results demonstrate non-linear release profiles, suggests that the release of 6-mercaptopurine from those *N*-acylchitosan microspheres can be classified to a two-phase percolation model of release behavior. Generally speaking, the gel structure can be

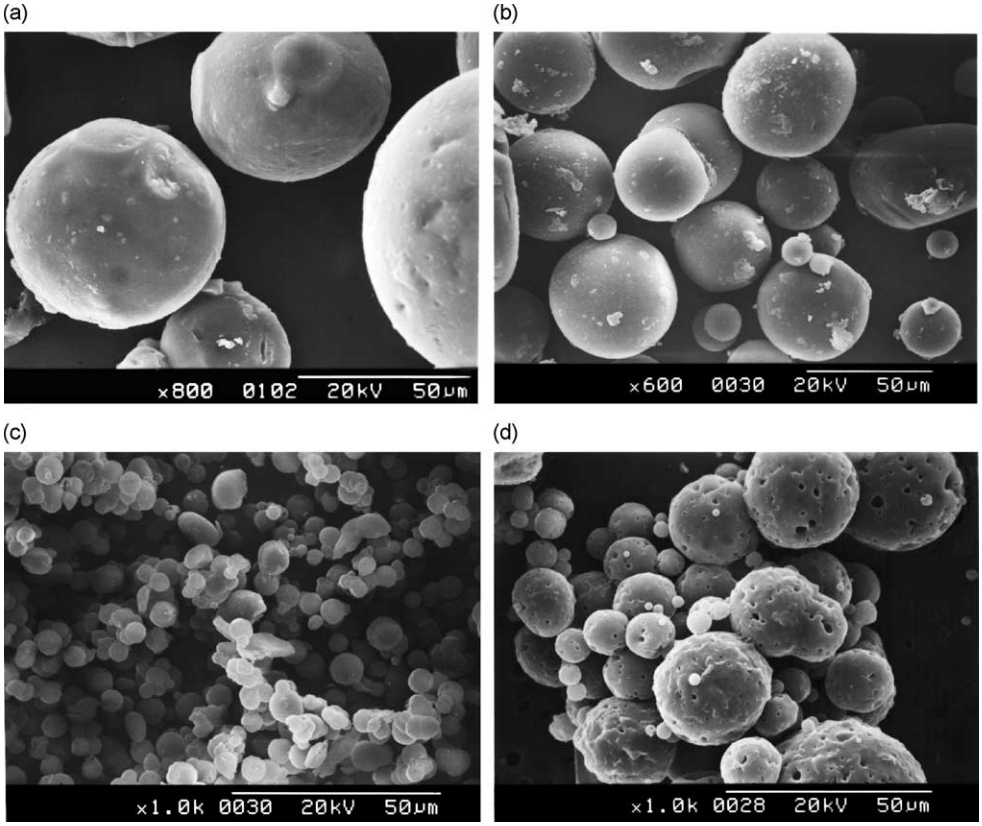

Fig. 4. SEM micrography of various *N*-acylchitosan microspheres: (a) *N*-acetylchitosan microspheres; (b) *N*-propionylchitosan microspheres; (c) *N*-butyrylchitosan microspheres prepared by the w/o interfacial *N*-acylation method; and (d) *N*-acetylchitosan microspheres prepared by a spray in-liquid *N*-acetylation method (Mi, Wong, Shyu, & Chang, 1999).

subdivided into two classes: rigid gels, which form a lattice that is also stable in the presence of swelling agent, and soft gels, which may undergo structure deformation in time (Adrover, Giona, & Grassi, 1996). The release behavior of *N*-acylchitosan gel matrix can be modeled by assuming the presence of two phases: a rigid phase in crystalline region and a sol phase in amorphous (or loose structured) region. The drug releasing from the amorphous region is faster than releasing from the crystalline region. The initial quick release and the continued slow release indicated that the *N*-acylchitosan microspheres contained rigid and sol phases. It is believed that the two-phase model was suitable for the explanation of drug releasing from *N*-acylchitosan microspheres.

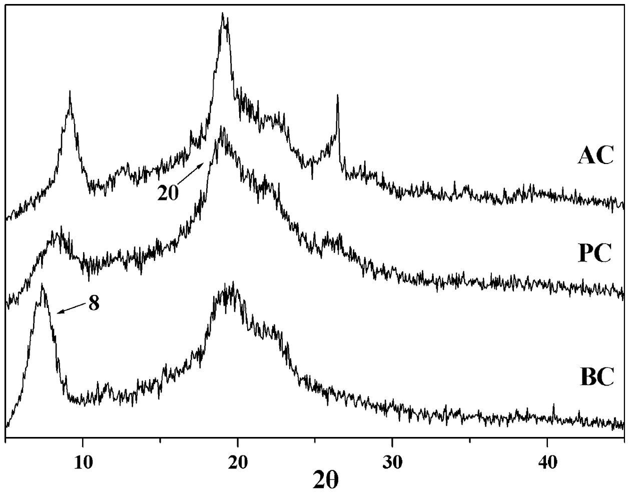

Fig. 5. X-ray diffraction of *N*-acetylchitosan (AC), *N*-propionylchitosan (PC) and *N*-butyrylchitosan (BC) microspheres.

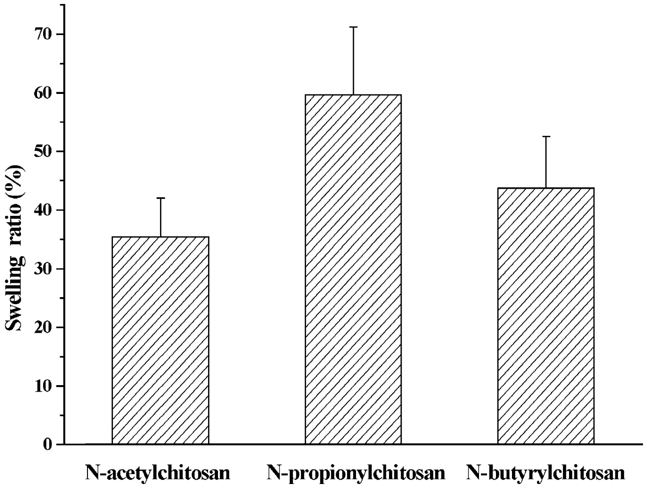

Fig. 6. Swelling degrees of *N*-acetylchitosan, *N*-propionylchitosan and *N*-butyrylchitosan microspheres (microspheres prepared at the condition of anhydride-to-chitosan molar ratio is 1:1).

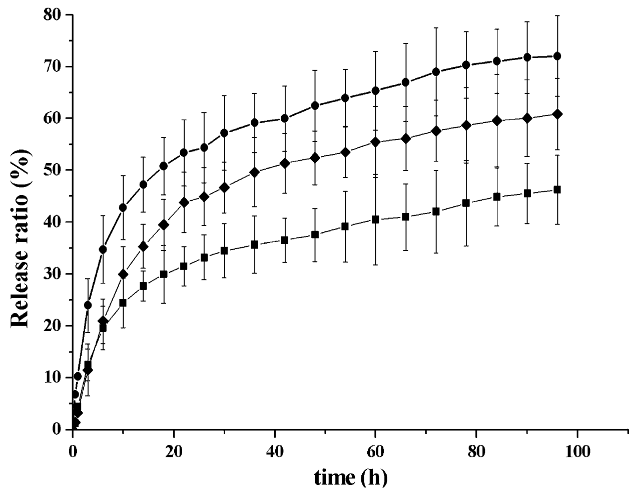

Fig. 7. Profiles of 6-MP releasing from *N*-acylchitosan microspheres (microspheres prepared at the condition of anhydride-to-chitosan molar ratio is 1:1): (■) acetylchitosan, (●) *N*-propionylchitosan, (♦) *N*-butyrylchitosan.

3.5. Enzymatic degradation

Fig. 9 shows the effect of enzymatic degradation on drug releases from the *N*-acylchitosan microspheres. The contribution of enzymatic degradation on drug release was significant for *N*-acetylchitosan microspheres as noted by the enhancement of drug release. On the contrary, enzymatic degradation did not obviously contribute to drug release from *N*-propionylchitosan and *N*-butyrylchitosan microspheres. These results can be attributed to the reason that *N*-propionylchitosan and *N*-butyrylchitosan microsphere are less susceptible to enzymatic degradation as compared with *N*-acetylchitosan microspheres. The degradation of chitosan and its derivatives by enzyme depends on the fraction of acetylated units, the substituted hydrophobic groups, and the crystalline properties (Muzzarelli, Francescangeli, Tosi, Bruno, & Muzzarelli, 2004; Muzzarelli, Xia, Tomasetti, & Ilari, 1995; Nordtveit, Vårum, & Smidsrød, 1994, 1996; Sashiwa, Saimmoto, Shigemasa, Ogawa, & Tokura, 1990). Hirano, Usutani, and Midorikawa (1997) have found that *N*-propionylchitosan fiber was about 11 times less hydrolyzable than *N*-acetylchitosan fiber, suggested that the introduction of *N*-propionyl group to chitosan made a higher stereo hindrance for the attachment of active site of enzyme. This limited the cleavage of glucosidic bonding in *N*-propionylchitosan. Accordingly, the *N*-propionylchitosan and *N*-butyrylchitosan microspheres appeared a slower degradation rates comparing to the *N*-acetylchitosan microsphere because of their enzyme–substrate specificity.

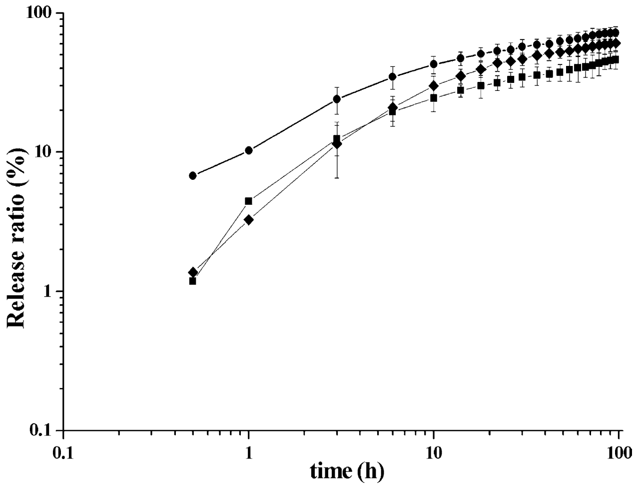

Fig. 8. Log–log plot of M_t/M_∞ vs. t of 6-MP releasing from *N*-acylchitosan microspheres (microspheres prepared at the condition of anhydride-to-chitosan molar ratio is 1:1): (■) *N*-acetylchitosan; (●) *N*-propionylchitosan; (♦) *N*-butyrylchitosan.

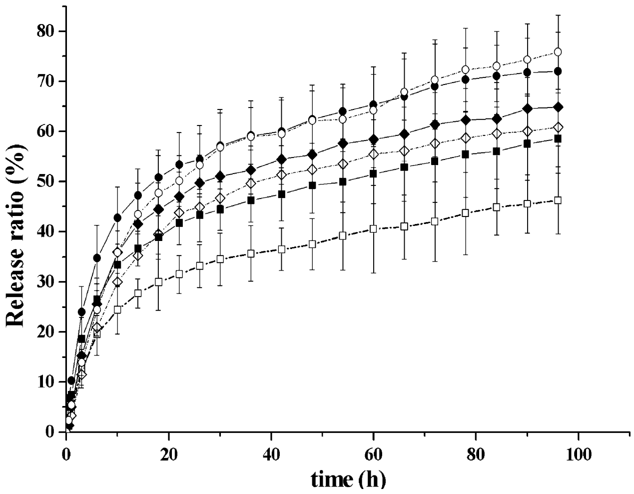

Fig. 9. Effect of enzymatic degradation on the release of 6-MP from *N*-acylchitosan microspheres (microspheres prepared at the condition of anhydride-to-chitosan molar ratio is 1:1): (■) acetylchitosan, (●) *N*-propionylchitosan, (♦) *N*-butyrylchitosan (without lysozyme degradation), (□) acetylchitosan, (○) *N*-propionylchitosan, (◇) *N*-butyrylchitosan (with lysozyme degradation).

4. Conclusion

In this work, we prepared novel chitosan-based microspheres by an interfacial *N*-acylation method. Pysicochemical (such as chemical reactivity, hydrophilicity, crystallinity) and drug release properties could be manipulated by the choice of different chain length of aliphatic anhydrides for the preparation of different *N*-acylchitosan (*N*-acetylchitosan, *N*-propionylchitosan and *N*-butyrylchitosan) microspheres. The introduction of acyl group to chitosan is of importance for the domination of swelling capability, enzymatic degradation and the control of drug release. Such *N*-acylchitosan microspheres might be potential for using as a drug delivery system.

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