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Size-controlled self-aggregated N-acyl chitosan nanoparticles as a vitamin C carrier

Youngjin Cho[a], Jun Tae Kim[b], Hyun Jin Park[a,*]

[a] College of Life Sciences & Biotechnology, Korea University, Anam-dong, Seongbuk-gu, Seoul 136-701, Republic of Korea
[b] Department of Food Science and Technology, Keimyung University, Daegue 704-701, Republic of Korea

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ABSTRACT

N-acyl chitosans with various acyl chain lengths were synthesized to improve their hydrophobicity and stability in delivery system. N-acyl chitosan nanoparticles were fabricated by self-aggregation method and vitamin C was loaded into the particles. Particle sizes were ranged from 444 nm to 487 nm with various acyl chain lengths reduced to particle size 216–288 nm with vitamin C loading. Vitamin C in the N-acyl chitosan nanoparticles may cross-link and pull the particle wall resulting in reducing the particle size. N-acyl chitosan nanoparticles were characterized using FTIR, zeta potential, size analyzer, scanning electron microscope. The loading efficiency of vitamin C on N-acyl chitosan nanoparticles ranged 55–67% and increased the loading efficiency of vitamin C too, with N-acyl chain length. Vitamin C in N-acyl chitosan nanoparticles showed the controlled release properties at pH 1.3 and pH 7.4. Release rate of vitamin C load is reduced with increasing the length of acyl side chain.

1. Introduction

In the past decades, incorporation of bioactive compounds such as vitamins, probiotics, antioxidants and bioactive peptides into food systems has been essential to develop novel functional foods which provide many physiological benefits beyond their nutritional value (Chen & Subirade, 2006; Mermelstein, 2002; Pennington, 2002). It has been well reported that bioactive compounds reduced the risk of cancer, hypertension, stroke and cardiovascular disease (Hollman, Hertog, & Katan, 1996; Kris-Etherton et al., 2002). Among bioactive compounds, vitamin C, known as ascorbic acid, is one of the essential nutrients for the human body. As an important antioxidant, vitamin C has reduced the risk of cancer by neutralizing reactive oxygen or other free radicals that can damage DNA (Cathcart, 1985). Vitamin C has also been shown to decrease low density lipoprotein (LDL) cholesterol in the plasma of hyperlipidemia patients (McRae, 2008). However, it can be easily destroyed or oxidized when it is cooked, processed or exposed to the ambient conditions such as air, moisture, heat, light, and base resulting in lost its original functions (Shimoni, 2004; Zieliński, Kozłowska, & Lewczuk, 2001). To prevent the damage or oxidation of vitamin C and deliver it to the specific location, encapsulation and controlled release techniques have been developed in food and pharmaceutical areas (Champagne & Fustier, 2007; Cho, Shim, & Park, 2006; Kim, Hwang, Park, & Park, 2002).

Encapsulation is a process of packaging solid particles, liquids or gaseous materials which are wholly contained within the capsule wall material. This technique is used to trap active components and release them in controlled rates under specific conditions (Kissel, Maretschek, Packhäuser, Schnieders, & Seidel, 2006). In the food and pharmaceutical industries, biologically active food ingredients are encapsulated for variety of reasons including protection from volatilization during storage, protection from undesirable interactions with other food components, minimizations of flavor interactions or light induced deteriorative reactions, controlled release applications and protection against atmospheric conditions.

Chitin is a second mostly abundant biopolymer consisting of β-(1,4)-linked N-acetyl-D-glucosamine. Chitosan is obtained by deacetylation of chitin and composed of mostly β-(1,4)-linked D-glucosamine unit (Muzzarelli et al., 2012). The use of chitosan in oral delivery systems has been steadily increased with its biocompatible, biodegradable, mucoadhesive, and non-toxic natures (Hassan, Parish, & Gallo, 1992; Ohya, Takei, Kobayashi, & Ouchi, 1993; Tozaki et al., 1997). However, chitosan has a limitation in the application for controlled release of core materials due to its hydrophilic feature and high solubility in the acidic condition. Plain chitosan would be dissolved completely in acidic condition.

Therefore, chitosan was chemically modified with anhydrides or halides to N-acyl chitosan which is more hydrophobic and can

* Corresponding author. Tel.: +82 2 3290 4149; fax: +82 2 953 5892.
E-mail address: hjpark@korea.ac.kr (H.J. Park).

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reduce the solubility in the acid medium. In the present study, N-acyl chitosan nanoparticles were prepared by self-aggregation method without cross-linking agent and vitamin C was loaded in N-acyl chitosan nanoparticles. Vitamin C-loaded N-acyl chitosan nanoparticles were characterized and their loading efficiency and *in vitro* release test were performed.

2. Materials and methods

2.1. Materials

Vitamin C (L-ascorbic acid, 99+%, A.C.S. reagent), chitosan (molecular weight of 5.23×10^5 and degree of deacetylation (DD) of 84.9%) were purchased from Sigma–Aldrich Chemie (Steinheim, Germany). Propionic anhydride, hexanoic anhydride, nonanoyl chloride, lauroyl chloride, pentadecanoyl chloride and stearoyl chloride were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) and Sigma–Aldrich Chemie (Steinheim, Germany). All other chemicals were of analytical grade and used as received. Standard cellulose dialysis membrane (Spectra/Por® 6 dialysis membrane, MWCO = 1000 Da, 18 mm flat width, wet in 0.05% sodium azide) was purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, USA).

2.2. Synthesis of N-acyl chitosans

Chitosans were modified with anhydrides or halides to produce the N-acyl chitosans. For synthesis of each N-acyl chitosans, 2.0 g chitosan was dissolved in 200 ml mixing solution of 0.6% (w/v) acetic acid solution and methanol. A molar equivalent (1.2) of propionic anhydride, hexanoic anhydride, nonanoyl chloride, lauroyl chloride, pentadecanoyl chloride or stearoyl chloride was added slowly to the chitosan solution with magnetic stirring for 5 h, respectively. The mixture was poured into the same volume of methanol and ammonia solution in volume ratio of 7 to 3. The precipitates were filtered and rinsed with distilled water, methanol, and ether. Then, they were dried in a vacuum at 50 °C overnight. Various N-acyl chitosan derivatives have been obtained in propionyl chitosan, hexanoyl chitosan, nonanoyl chitosan, lauroyl chitosan, pentadecanoyl chitosan and stearoyl chitosan.

2.3. Preparation of vitamin C-loaded N-acyl chitosan nanoparticles

To prepare vitamin C-loaded N-acyl chitosan nanoparticles, N-acyl chitosans (propionyl chitosan to stearoyl chitosan, 500 mg) were suspended in 50 ml of phosphate buffer saline (PBS) solution (pH 7.4, 1% w/v) and incubated at 40 °C for 48 h. The vitamin C (20 mg) dissolved in 2 ml of citric acid (1% w/v) was added into the N-acyl chitosan suspension and sonicated using a probe type sonifier (Ultrasonic Homogenizer UH-600) at 20 W for 2 min. The sonication was repeated three times to get an optically clear solution using a pulse function (pulse on: 10 s and pulse off: 2 s). The resulting nanoparticles suspension was centrifuged at 20,000 rpm and 4 °C for 30 min to remove free vitamin C. After freeze-drying the precipitate, the resulting nanoparticles of a white powder around 450–490 mg were stored at 4 °C. Various vitamin C-loaded N-acyl chitosan nanoparticles obtained from propionyl chitosan, hexanoyl chitosan, nonanoyl chitosan, lauroyl chitosan, pentadecanoyl chitosan and stearoyl chitosan were coded as C3, C6, C9, C12, C15 and C18, respectively.

2.4. Characterizations

FT-IR spectra of pure vitamin C, N-acyl chitosan and vitamin C-loaded N-acyl chitosan nanoparticles were obtained using a FTIR spectrometer-430 (Jasco, Japan). Each specimen was ground and mixed with potassium bromide (KBr). The KBr pellets were prepared by compressing the powders under the hydraulic press of 5 ton. All FT-IR spectra were obtained by 32 scans at a resolution of 2 cm^{-1} in the range of 3800–400 cm^{-1}.

To measure the zeta potentials and particle size distribution of N-acyl chitosan nanoparticles, 0.3% (w/v) of N-acyl chitosan nanoparticles dispersions were prepared in PBS solution at pH 7.4. The zeta potentials, average particle size, and size distribution of N-acyl chitosan nanoparticles were recorded using a quasielastic laser light scattering with Malvern Zetasizer (Malvern Instruments Limited, UK). Each specimen was measured three times to obtain the average values.

The surface morphology of vitamin C-loaded N-acyl chitosan nanoparticles was examined by a SEM (Hitachi S-4300, Japan). N-acyl chitosan nanoparticles were loaded on a brass stub using a double-sided adhesive tape and the specimens were coated with a thin layer of platinum (approximately 3–5 nm) at 30 W for 100 s. The SEM images were scanned at an excitation voltage of 15 kV and a magnification of 3–18k×.

2.5. Loading efficiency and in vitro release studies

25 mg of vitamin C-loaded chitosan nanoparticles were dissolved in 100 ml of 0.1 N HCl. The solution was passed through 0.2 μm filter (Millipore, USA) and then vitamin C content was assayed by measuring the absorbance of the diluted solutions at 244 nm (λ_{max} of vitamin C in 0.1 N HCl) using UV spectrophotometer (Shimadzu 1601PC, Japan). Experiments were performed in triplicate ($n = 3$) and loading efficiencies were calculated using Eq. (1).

$$\text{Loading efficiency } (\%) = \frac{C_c}{C_t} \times 100 \quad (1)$$

where C_c is the calculated vitamin C concentration and C_t is the theoretical vitamin C concentration.

To investigate the vitamin C release at acidic and neutral environment, 10 mg of vitamin C-loaded N-acyl chitosan nanoparticles (C3, C6, C9, C12, C15, and C18) were dispersed in 5 ml of PBS (pH 7.4) or diluted HCl (pH 1.3) buffers. Each dispersion was put into a dialysis bag (MWCO: 1000 Da) previously soaked in the medium for 3 h. Then the dialysis bag was introduced into a vial containing 50 ml of PBS or diluted HCl buffers. The systems were immersed in a thermostatic bath at 37 °C and kept at stirring speed of 100 rpm. At pre-determined time intervals (0, 0.5, 1, 2, 3, 4, 5, 6, 12, 24, 96 and 168 h), 1 ml samples were withdrawn from the vials and assayed for vitamin C release by measuring a absorbance at 265 nm (λ_{max} of vitamin C) using an UV spectrophotometer and replaced by 1 ml of fresh PBS or diluted HCl buffers. *In vitro* release tests were repeated three times by the identical manner.

3. Results and discussion

3.1. Characterization of N-acyl chitosans

The chemical structures of N-acyl-modified chitosans and vitamin C were depicted in Fig. 1. Various N-acyl chitosans were synthesized with alkyl anhydrides or acyl halides. N-acyl chitosans with propionyl, hexanoyl, nonanoyl, lauroyl, pentadecanoyl, and stearoyl side chains are indicated when the numbers of repeat unit (n) are 1, 4, 7, 10, 13, and 16, respectively in Fig. 1. In our previous study, the substitution of N-acyl chitosans was confirmed using FTIR and ^{1}H NMR spectroscopy (Cho, Kim, & Park, 2012). It was also reported that critical aggregation concentration (CAC) of N-acyl chitosan nanoparticles was 0.01 mg/ml (Cho et al., 2012).

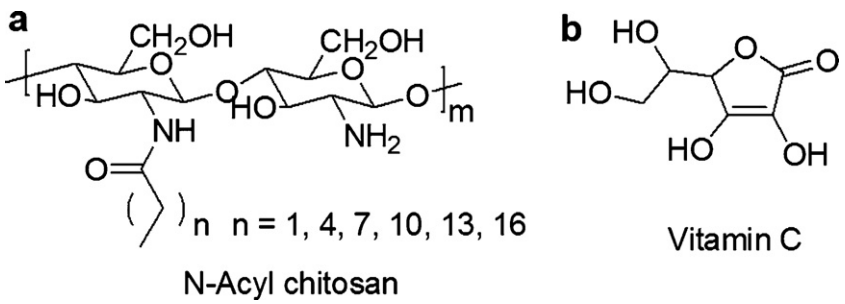

Fig. 1. The chemical structures of (a) N-acyl chitosans and (b) vitamin C.

3.2. Zeta potential and particle size

The ζ-potentials and particle sizes of N-acyl chitosan nanoparticles with and without vitamin C loading were summarized in Table 1. The ζ-potentials of N-acyl chitosan nanoparticles were positive (+) charged in ranges of 10.2–28.9 mV. Due to the positive charges of N-acyl chitosan nanoparticles, N-acyl chitosan nanoparticles can be interact to the negative charged cell wall and have a great potential to increase the absorbance and penetration of the core vitamin C. The ζ-potentials of N-acyl chitosan nanoparticles decreased significantly after vitamin C loading. Carbonyl and hydroxyl groups of vitamin C can interact with the positive charged amine groups of N-acyl chitosan by hydrogen bond and electrostatic interaction. The interactions between vitamin C and N-acyl chitosan could reduce the surface charge resulting in decrease with the ζ-potentials ranged from 5.9 mV to 18.4 mV. Although it is core material in N-acyl chitosan nanoparticles, some vitamin C can also act as a cross-linker and increase the inter- and intra-molecular interactions of N-acyl chitosan nanoparticles.

Fig. 2 shows the particle size distributions of the lauroyl chitosan nanoparticles and vitamin C-loaded lauroyl chitosan nanoparticles. The particle size distribution of the lauroyl chitosan nanoparticles ranged from 221 nm to 1434 nm and its mean diameter was 463.3 nm as shown in Table 1. By vitamin C loading, the particle size distribution was narrowed significantly with ranging from 116 nm to 501 nm. The particle size of N-acyl chitosan nanoparticles decreased after the vitamin C loading. Generally, the particle size of chitosan nanoparticles increased with the length of side chain of the chitosan, molecular weight of chitosan, and the amount of loaded core materials. The mean particle size of N-acyl chitosan nanoparticles increased with acyl chain length from 444.2 nm to 486.6 nm. However, the particle sizes of N-acyl chitosan nanoparticles decreased about 50% by vitamin C loading. This is one of the evidence that vitamin C could crosslink the N-acyl chitosan and pull the N-acyl chitosan nanoparticles matrix.

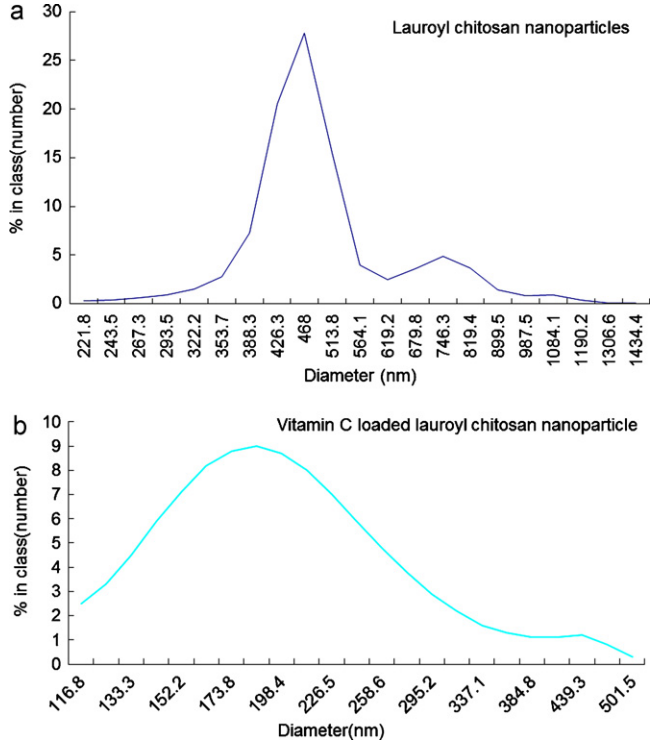

Fig. 2. The size distribution of the lauroyl chitosan nanoparticles (a) and vitamin C-loaded lauroyl chitosan nanoparticles (b) in particle number.

3.3. Surface morphology

The particle shape and surface morphology of N-acyl chitosan nanoparticles and vitamin C-loaded N-acyl chitosan nanoparticles were investigated using a SEM. Fig. 3 shows the SEM photomicrographs of lauroyl chitosan nanoparticles with magnifications of 10k× and 100k×. Lauroyl chitosan nanoparticles (C12) were not complete spherical shape and wrinkle surface could be observed as shown in Fig. 3. However, vitamin C-loaded lauroyl chitosan nanoparticles showed more spherical shape and smaller particles as shown in Fig. 4 due to sufficient interactions between vitamin C and lauroyl chitosan.

3.4. FTIR analysis

The typical IR peaks of vitamin C was observed at 1027, 1120, 1141, 1321, 1673, 1755, 3031, 3218, 3316, 3411, and 3527 cm^{-1} as shown in Fig. 5. The peaks at 1141, 1321, 1673 and 1755 cm^{-1} are assigned to the stretching and bending vibrations of C=O and C—O hydroxyl groups present in the vitamin C. The spectrum of the vitamin C-loaded N-acyl chitosan nanoparticles also showed these characteristic peaks confirming the stability of the vitamin C loading. These peaks showed at 1074, 1400, 1604, 2944 and 3417 cm^{-1}. On the other hand, placebo N-acyl chitosan nanoparticles showed comparatively broader peaks at 1082, 1383, 1554, 1654, 2895 and 3419 cm^{-1} (Cho et al., 2012) which resulted in appearance of superimposed peaks of the vitamin C and N-acyl chitosan nanoparticles at that region in the vitamin C-loaded N-acyl chitosan nanoparticles spectrum. In special, after loading of vitamin C, the peak of stretching vibration of C=O shift the lower wavenumber region (1602, 1610, 1604, 1596, 1635, and 1604 cm^{-1} from vitamin C-loaded propionyl chitosan nanoparticles to stearoyl chitosan nanoparticles, respectively). This result demonstrated that vitamin C was strongly interacted with N-acyl chitosan by hydrogen bonding in nanoparticles.

Table 1
The characterizations of N-acyl chitosan nanoparticles and vitamin C loaded N-acyl chitosan nanoparticles.

N-acyl chitosans	Nanoparticles	ζ-Potential[a]	ζ-Potential[b]	Mean diameter[c]	Mean diameter[d]
Propionyl chitosan	C3	26.0 ± 4.6	18.4 ± 2.7	444.2 ± 12.2	215.6 ± 18.1
Hexanoyl chitosan	C6	10.2 ± 3.2	5.9 ± 2.0	469.3 ± 15.6	233.9 ± 13.5
Nonanoyl chitosan	C9	12.5 ± 3.8	6.3 ± 1.9	481.2 ± 13.8	239.3 ± 11.4
Lauroyl chitosan	C12	14.6 ± 2.4	6.9 ± 3.1	463.3 ± 10.4	245.4 ± 14.5
Pentadecanoyl chitosan	C15	22.0 ± 5.5	13.2 ± 3.3	469.8 ± 9.6	275.6 ± 12.8
Stearoyl chitosan	C18	28.9 ± 7.4	18.1 ± 2.5	486.6 ± 20.1	288.2 ± 10.2

Values were measured three times and the standard deviations were obtained.
[a] ζ-Potential (mV) of N-acyl chitosan nanoparticles.
[b] ζ-potential (mV) of vitamin C loaded N-acyl chitosan nanoparticles.
[c] Mean diameter (nm) of N-acyl chitosan nanoparticles.
[d] Mean diameter (nm) of vitamin C loaded N-acyl chitosan nanoparticles.

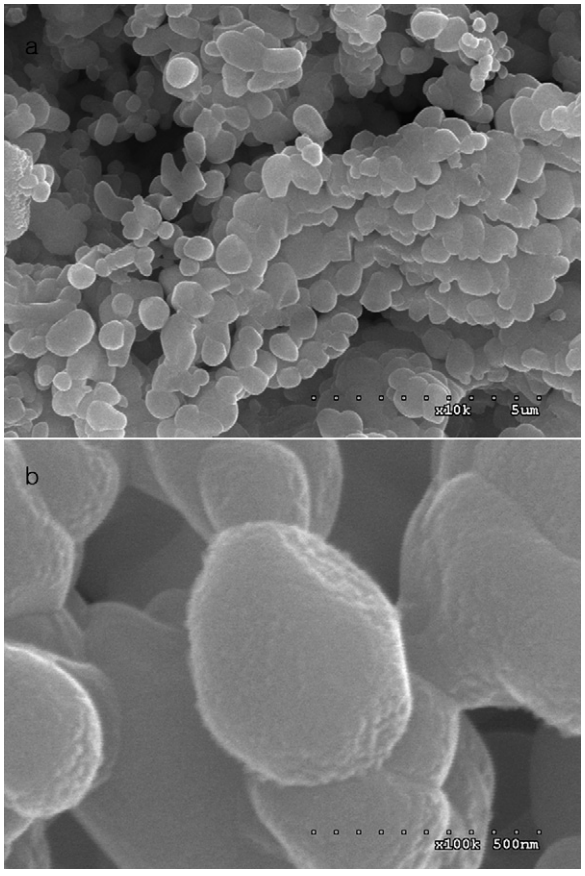

Fig. 3. SEM photomicrograph of lauroyl chitosan nanoparticles with magnification of (a) 10k× and (b) 100k×.

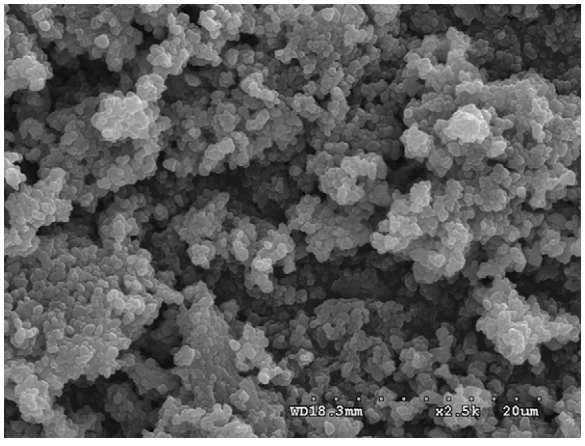

Fig. 4. SEM photomicrograph of vitamin C-loaded lauroyl chitosan nanoparticles (C12).

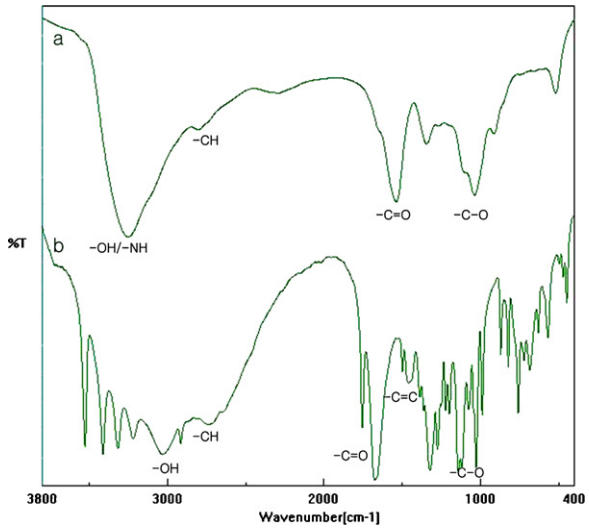

Fig. 5. FT-IR spectra of (a) vitamin C-loaded lauroyl chitosan nanoparticles (C12) and (b) vitamin C.

3.5. Loading efficiency and in vitro release of vitamin C

The loading efficiencies of N-acyl chitosan nanoparticles ranged around 55–67% as shown in Fig. 6. Damaged N-acyl chitosan nanoparticles such as surface irregularities and fragmentations might cause the substantial loss of vitamin C loading amounts during the sonication process. The vitamin C loading efficiency was slightly increased from C3 to C12 and rarely changed with further increase of the acyl side chain length.

Fig. 7 shows *in vitro* release from vitamin C-loaded N-acyl chitosan nanoparticles with various N-acyl side chain lengths. In order to study the effect of acidic condition on vitamin C release, *in vitro* release test was carried out at pH 7.4 of PBS and at pH 1.3 of HCl solution at 37 °C. As expected, vitamin C was released

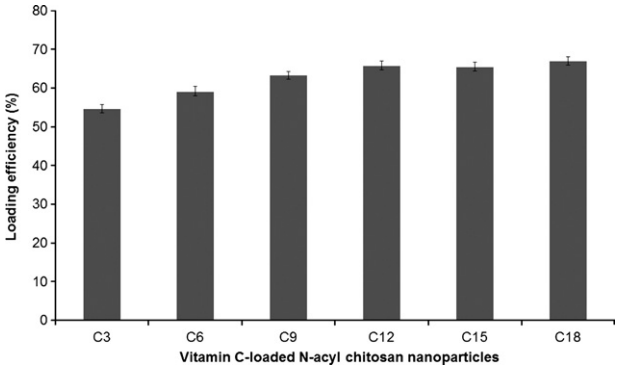

Fig. 6. Loading efficiency of vitamin C in N-acyl chitosan nanoparticles.

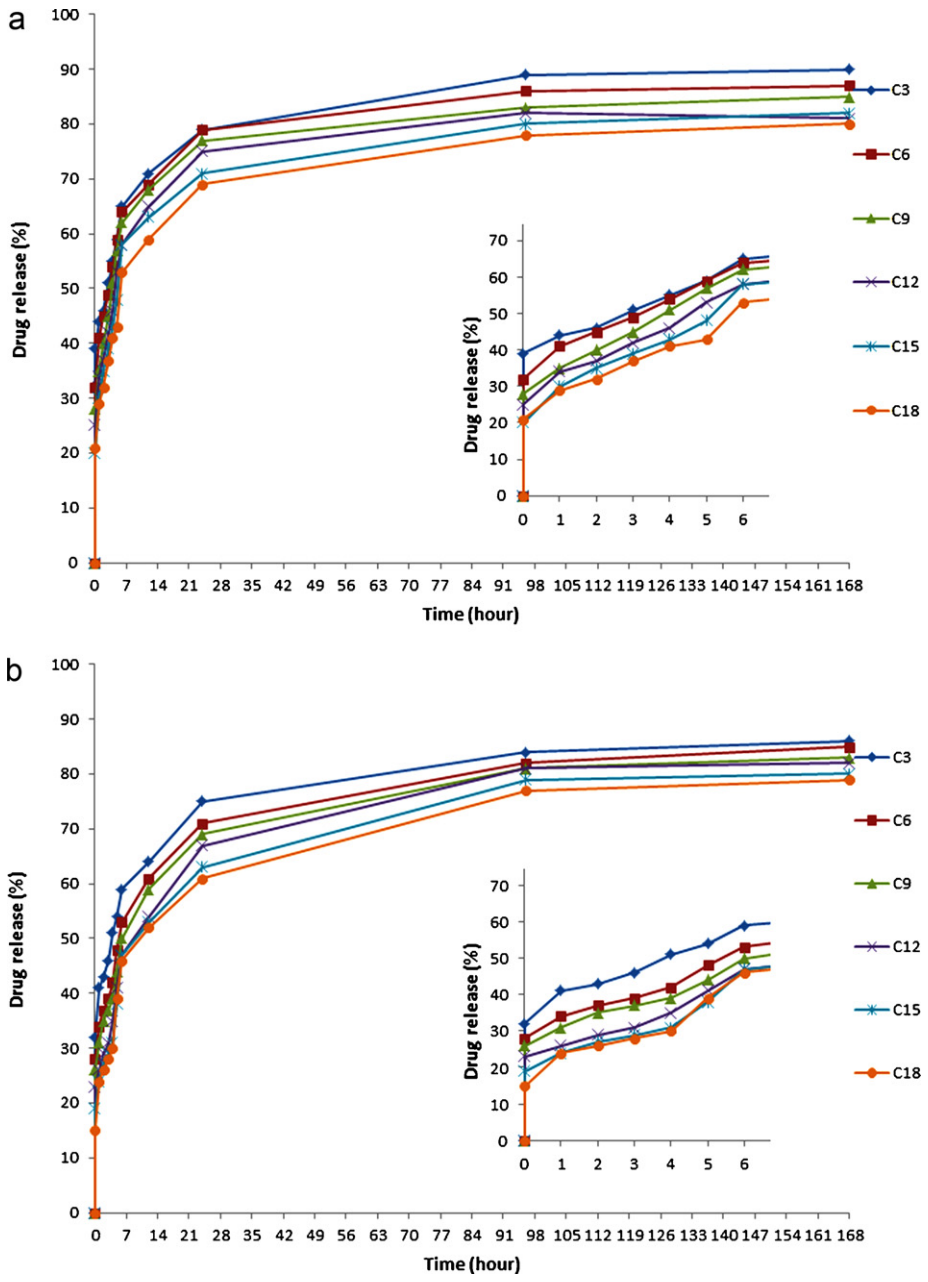

Fig. 7. *In vitro* release of vitamin C from N-acyl chitosan nanoparticles (a) at pH 1.3 and (b) at pH 7.4.

around 10–32% at pH 7.4 of PBS and 20–40% at pH 1.3 of HCl within 0.5 h as the initial burst and slowly released over the time. Since plain chitosan can be completely dissolved in acidic condition, its application in delivery system has been limited for the controlled release. However, N-acyl modified chitosan showed better resistance in acidic condition due to the hydrophobic N-acyl side chain. It was observed that vitamin C release rate of N-acyl chitosan nanoparticles is reduced with increasing the length of acyl side chain. This is due to the strengthening of the hydrophobic interaction in N-acyl chitosan nanoparticles with increasing the length of acyl side chain. Once chitosan has been modified with hydrophobic acyl groups, it could be swollen in aqueous condition but not dissolved completely even in acidic condition. Vitamin C release was slightly fast in acidic (pH 1.3) condition compared to the neutral (pH 7.4) condition as shown in Fig. 7. But N-acyl chitosan nanoparticles still showed the controlled release in acidic condition and showed similar trends with in neutral condition.

4. Conclusions

Chitosan was successfully modified with various N-acyl groups from propionyl to stearoyl to improve its hydrophobicity. Vitamin C was loaded efficiently in N-acyl chitosan nanoparticles by sonication method. The loading efficiency ranged 55–67% and improved with increasing the length of N-acyl side chain. The ζ-potentials of N-acyl chitosan nanoparticles showed positive charges and reduced significantly after vitamin C loading due to the interactions between the hydroxyl and carbonyl groups of vitamin C and the amine groups of chitosan. The mean particle sizes of N-acyl chitosan nanoparticles also reduced from 444–487 nm to 216–288 nm

after vitamin C loading. This is mainly due to the cross-linking effect of vitamin C in N-acyl chitosan nanoparticles. *In vitro* release test of vitamin C-loaded N-acyl chitosan nanoparticles verified that N-acyl modified chitosan nanoparticles was not dissolved in acidic condition and controlled released the core vitamin C over the releasing time.

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