Development of photo and pH dual crosslinked coumarin-containing chitosan nanoparticles for controlled drug release

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A B S T R A C T
A new strategy has been developed to the fabrication of chitosan nanoparticles as anticancer drug nanocarriers with ultraviolet-responsive coumarin derivatives and pH-responsive imine groups. For this purpose 8-formyl-7-hydroxy-4-methylcoumarin (8-FHMC) was initially synthesized as novel and dual crosslinking agent in order to produce coumarin-containing chitosan nanoparticles via oil-in-water nanoemulsion system. The structure of the resultant compounds and nanoparticles were confirmed by means of 1H NMR, FT-IR, UV–vis spectroscopy and XRD. The morphology and size distribution of the coumarin-containing chitosan nanoparticles was also characterized using SEM, AFM and DLS. The drug-loaded coumarin-containing chitosan nanoparticles were stable at physiological conditions, and can also be dissociated by the cleavage of imine linkages in the crosslinking segments under acidic condition. Compared to non-photo-crosslinked chitosan nanoparticles, photo-crosslinked chitosan nanoparticles displayed controllable and slower release. Thus, we have showed that chitosan nanoparticles crosslinked by coumarin with photo- and pH-responsive properties is a promising and novel drug carrier for designing intelligent drug delivery systems.

1. Introduction
Chitosan (CS) is a natural hydrophilic polymer generally derived from chitin by the alkaline or enzymatic deacetylation. It is the second most widespread biopolymer present on the earth after cellulose found in the exoskeleton of crustaceans such as crabs, lobsters, shrimps and krill (Kumar, 2000). Chitosan displays many desirable characteristics such as biodegradability, good biocompatibility, non-toxicity, bioadhesion and antimicrobial activity. Therefore, considerable attention has been focused on chitosan which have great potential for a variety of applications including in food industries, agriculture, cosmetics, biotechnology and pharmaceutics (Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Kumaraswamy et al., 2018; Saharan et al., 2015; Shahidi, Arachchi, & Jeon, 1999). Chitosan has been widely reported for controlled delivery of various active compounds, such as drugs, proteins, vitamins and genes (Ajun, Yan, Li, & Huili, 2009; Felt, Buri, & Gurny, 1998; Gan & Wang, 2007; Malhotra et al., 2009). It has been also used in drug delivery systems in a wide variety of shapes and formats including nanoparticles, microparticles, films, composites and hydrogels (Banerjee, Mitra, Singh, Sharma, & Maitra, 2002; Lee et al., 2002; Wu, Yang, Wang, Hu, & Fu, 2005; Xu & Du, 2003). To enhance the temporal control and release property of bioactive agents, chitosan nanoparticles usually need to be crosslinked. In this regard, various chemical crosslinkers have been employed for preparation of chitosan particles such as diisocyanates, diepoxy compounds, dialdehydes and other active crosslinking agents (Rinaudo, 2010; Rokhade, Shelke, Patil, & Aminabhavi, 2007). Among them, glutaraldehyde is the most common chemical crosslinking agent known to make chitosan nano/micro particles. However, it has been recently shown that crosslinking agents in general and glutaraldehyde in particular exhibit significant toxicity to the human body and cause adverse effects (Fürst & Banerjee, 2005).

To overcome these potential side effects, natural and biocompatible crosslinking agents with therapeutic properties has recently attracted much attention from scientific community to form chitosan nanoparticles. On the other hand, the fabrication of chitosan nanoparticles with natural and non-toxic monoaldehydes along with biomedical applications is a new and interesting challenge in chitosan chemistry (Iftime, Morariu, & Marin, 2017). So far, several monoaldehydes such as salicyaldehyde, 2-formylphenyl boronic acid, bornetaldehyde, vanillin and cinnamaldehyde have been used as crosslinking agent for preparation of chitosan particles (Allincui et al., 2016; Babu & Kannan, 2012; Fu et al., 2017; Iftime et al., 2017; Marin et al., 2015; Peng et al., 2010).

Coumarin (2H-chromen-2-one or 2H-1-benzopyran-2-one) is a biological chemical compound, which was first reported and isolated in 1822 by Vogel (Iwakoshi, Lee, & Glimcher, 2003). It is a secondary
metabolite is found in many plants, like the tonka bean, sweet clover, cinnamon, vanilla grass and strawberries. Molecules possessing the coumarin nucleus exhibit various biological activities including anti-oxidants (Al-Majedy et al., 2016), antimutagenic (Bhattacharya, 2011), antiinflammatory (Bansal, Sethi, & Bansal, 2013; Chougala et al., 2017; Huang et al., 2012), antibacterial (Chougala et al., 2017), antifungal (Zhang et al., 2016), antimicrobial (Abd-El-Aziz et al., 2016), antithrombotic (Amin, Gawad, Rahman, & El Ashry, 2014), anticancer activity (Basanagouda, Jambagi, Barigidad, Laxmeshwar, & Devaru, 2014; Emami & Dadashpour, 2015; Luo et al., 2011; Thakur, Singh, & Jaitak, 2015) and antiinociceptive (De Almeida Barros et al., 2010). Several natural and synthetic drugs containing coumarin (Emami & Dadashpour, 2015) (Fig. S1) have been reported as antipsammodics agent (Hymecromone), antiaggessive agent (Batoprazine), vitamin K antagonists (Warfarin and Coundametraly), antibiotics (Albamycin), and anticoagulants (Methyl coumarin) as a novel light-sensitive crosslinking agent to investigate the controllable release pro- toxicity against HeLa cell lines.

Among the various crosslinking procedures, photo-crosslinking method is a good choice to tune the stability of polymeric nanoparticles, which demonstrates very interesting advantages, such as no need to chemical reagents and no unwanted byproducts are created during the procedure (Jiang, Qi, Lepage, & Zhao, 2007). Coumarin is an interesting candidate of photo-triggered crosslinking groups. One of the attractive features of using of coumarin moieties is photo-crosslinking reaction by the photodimerization of coumarin moieties, which has been well known from the beginning of the 20th century. Upon UV light at λ > 310 nm, the coumarin moieties are photodimerized via [2π + 2π] photocycloaddition reaction and leading to the formation of cyclobutane rings. Recall that the crosslinked bonds will be cleaved if irradiation occurs below 260 nm (Zheng et al., 2002). However, the short wavelength UV light was hard to penetrate into living body, because it’s extremely harmful to normal tissues. Therefore, the combined pH- and photo-responsive agents are necessary in ideal drug delivery systems.

To the best of our knowledge, no result of using of coumarin groups as crosslinking agent of the chitosan polymer is found in the literature. This together with the documented biological importance of coumarin-containing compounds prompted to synthesize the 8-formyl-7-hydroxy-4-methyl coumarin (8-FHMC) via the Duf formulation of 7-hydroxy-4-methyl coumarin (7-HMC) as a novel light-sensitive crosslinking agent in order to access the novel drug-loaded chitosan–coumarin nanoparticles by oil-in-water (O/W) nanoemulsion system. In addition, the chitosan–coumarin nanoparticles were also used to encapsulate quercetin to investigate the controllable release profile and potent cytotoxicity against HeLa cell lines.

2. Experimental

2.1. Materials

Low molecular weight chitosan (CS) (MW = 50,000–190,000 Da, degree of deacetylation: 85%), 3-(4,5-dimethylazolyl)-2,5-diphenyl tetrazolium bromide (MTT) and Dulbecco’s Modified Eagle’s medium (DMEM) were purchased from Sigma-Aldrich. Quercetin was obtained from Fluka Chemical Co. Resorcinol, ethyl acetocetate (EAA), glacial acetic acid (CH₃COOH, 100%), hexamethylenetetramine (HMTA), Tween 60 (polyoxyethylene sorbitan monostearate, HLB = 14.9), di-chloromethane (CH₂Cl₂, 99%), ethanol (EtOH, 99%) and other commercially available chemicals were purchased from Merck Chemical Company and used without further purification.

2.2. Characterization

The 1H NMR measurement was recorded on a Bruker DRX-500-AVANCE spectrometer at 500 MHz using D₂O, CDCl₃ and DMSO-d₄ as the solvent at 25°C. Melting points were performed with an Electrothermal model 9100 apparatus and are uncorrected. Mass spectra of the organic products were obtained with an HP (Agilent technologies) 5937 Mass Selective Detector. Fourier transform infrared (FT-IR) spectroscopy of the products were performed in KBr pellets by using FT-IR spectrophotometer (IR Affinity, Shimadzu, Japan) in the range of 500–4000 cm⁻¹. The average particle size, polydispersity index (PDI) and zeta potential of chitosan particles were determined by dynamic light scattering (DLS, Brookhaven instrument, USA) at 25°C in triplicate. All samples were diluted 10 times with deionized water and the analysis was performed at a fixed scattering angle of 90°. Absorption spectrum and the drug release were determined with a Varian Cary 100 Bio UV/vis spectrophotometer using a quartz cuvette with 1 cm beam path length. The shape and morphological features of chitosan particles were determined using scanning electron microscopy (SEM) (HITACHI S-4160, Japan). The lyophilized samples were mounted on an aluminium stub by a double adhesive carbon tape and then introduced into an automated sputter coater and coated with gold before observations. XRD patterns of pure CS, blank crosslinked CS-NPs and quercetin-loaded crosslinked CS-NPs were performed by an X-ray diffractometer (XRD, Rigaku, Japan) with CuKα radiation at a voltage of 40 kV and 40 mA current to recognize the physical state (crystalline or amorphous) of the sample. The samples were scanned in 20 angle range from 5 to 40° at a scanning speed of 4° min⁻¹. The topography of the drug-loaded crosslinked CS-NPs was characterized by atomic force microscopy (AFM, ENTEGRA AFMNT-MDT, China) on a freshly cleaved mica substrate.

2.3. Synthesis and characterization of 7-HMC

Concentrated sulfuric acid (27 mL) was added to a 100 mL RB flask and cooled to 0–5°C in an ice bath. A solution of resorcinol (25 mmol) in ethyl acetocetate (30 mmol) was added to H₂SO₄ under constant stirring at 0–5°C. After stirring the reaction at room temperature overnight, the mixture was poured into the crushed ice with vigorous stirring. The precipitate was then filtered and recrystallization of the solid from EtOH finally afforded the product 1 (yield 75%; mp: 181–183°C); 1H NMR (500 MHz, CDCl₃): δ 2.4 (s, 3H), 6.15 (s, 1H), 6.77 (s, 1H), 6.86 (d, 1H), 7.52 (d, 1H); ESI-MS m/z: 176.0 [M+H]+ (Fig. S2).

2.4. Synthesis and characterization of 8-FHMC

To a solution of 7-HMC (15 mmol) in glacial acetic acid (20 mL) was added HMTA (45 mmol). The reaction mixture was heated at reflux in a water bath for 6 h at 80–85°C. After completion as indicated by TLC, 37 mL of 20% HCl was added and the reaction kept for 40 min. After cooling to the room temperature, the crude was extracted with diethyl ether twice (20 mL × 2) and washed with saturated NaHCO₃ and brine solutions, respectively. The organic layer was then dried over anhydrous MgSO₄ and after evaporation of the solvent under reduced pressure, the crude product was purified by column chromatography on silica gel using a mixture of Hex- AcOEt as eluent afforded 8-FHMC (yield 18%; mp: 175–177°C; FT-IR (KBr), ν, cm⁻¹: 1739 (C=O), 1638 (HC≡O), 1595 (C=C–); 1H NMR (DMSO-d₄, 500 MHz): δ 2.4 (s, 1H), 6.29 (s, 1H), 6.96 (d, 1H), 7.92 (d, 1H), 10.43 (s, 1H); 13C NMR (CDCl₃, 500 MHz): δ 19.31 (CH₃), 108.69, 110.87, 113.42, 114.70, 136.01, 143.37, 156.76 (C=O of lactone), 159.10, 165.50 (C=O, 192.93 (C=O of formyl); ESI-MS m/z: 204.0 [M+H]+ (Fig. S3). All these results displayed that 8-FHMC was synthesized successfully.
2.5. General procedure for preparation of chitosan nanoparticles

Coumarin-containing chitosan nanoparticles were prepared by the O/W nanoemulsion method. Briefly, a solution of chitosan (1–5%, w/v) was prepared in a glacial acetic acid solution (0.7%, v/v) at room temperature overnight under vigorous magnetic stirring (1000 rpm). Thereafter, Tween 60 (1%, w/v) was added as a surfactant into the solution followed by sonication for 10 min in an ultrasonic probe to obtain a homogeneous mixture as water phase (W). Quercetin was dissolved in EtOH-CH₂Cl₂ (1:3, v/v) to make a final concentration of 10% (w/w of chitosan) and stirred for 10 min to obtain a homogeneous oil phase mixture (O). Now the oil phase was added dropwise into the aqueous phase and sonicated for 5 min to form O/W emulsion. The volume ratio of O/W was fixed at 1:5. Next, a solution of 8-FHMC (0.2–1.2%, w/v) gently and dropwise poured into the O/W emulsion to form the crosslinked chitosan-coumarin nanoparticles suspension. Then half of the solution was exposed to an ultraviolet lamp and irradiated at 365 nm for 12 min to photo-cross-link the chitosan nanoparticles. Afterwards, both photo- and non-photo-crosslinked chitosan nanoparticles solutions were centrifuged at 14,000 rpm for 30 min at room temperature. Finally prepared nanoparticles were washed several times with DI water and then freeze-dried for 12 h. The blank nanoparticles prepared similarly without adding of quercetin.

2.6. Characterization of crosslinking property of the photo-crosslinked chitosan nanoparticles

To study the crosslinking kinetic of the photo-crosslinked CS-NPs, 5 mL of sample solution was irradiated at 365 nm using UV-light. At predetermined intervals, 500 μL of the above solution was taken and its absorbance at 321 nm measured. The photo-dimerization degree (PD) of coumarin groups was calculated from UV–vis absorption spectra using the following equation:

$$\text{PD} = \frac{[A_0 - A_t]}{A_0} \times 100$$  \hspace{1cm} (1)

where $A_0$ and $A_t$ are the initial absorbance and the absorbance after an irradiation time $t$ at 321 nm via alternating light of 365 nm, respectively.

2.7. Determination of the drug loading and encapsulation efficiency

The encapsulation efficiency (EE) and loading capacity (LC) of quercetin loaded in CS-COU nanoparticles was expressed by separation of the unentrapped quercetin from nanoparticles by centrifugation at 14,000 rpm for 30 min. Finally prepared nanoparticles were washed several times with DI water and then freeze-dried for 12 h. The obtained quercetin was dissolved in ethanol and this solution was considered as supernatant. The clear supernatant was analyzed by UV–vis absorption spectra at 373 nm to determine the quercetin content. The encapsulation efficiency and loading capacity were estimated as follows:

$$\text{EE} = \frac{Q_{Ut} - Q_{Ut}}{Q_{Ut}} \times 100$$  \hspace{1cm} (2)

$$\text{LC} = \frac{Q_{Ut} - Q_{Ut}}{(\text{Weight of nanoparticles})} \times 100$$  \hspace{1cm} (3)

where $Q_{Ut}$ and $Q_{Ut}$ are the total amount of quercetin used in the fabrication of nanoparticles and $Q_{Ut}$ is the free quercetin present in the supernatant.

2.8. In vitro drug release study

The drug-loaded photo- and non-photo-crosslinked CS-NPs were dispersed in phosphate saline (PBS, pH 7.4) and acetate (pH 5.8) buffers at a concentration of 1 mg/mL. These systems were then transferred into a cellophane membrane dialysis bag (MWCO = 12,000) followed by suspending into a container with 10 mL of buffer solution at the same pH value. The outer phase of the release media was kept at $37 \pm 0.5 ^\circ C$ and stirred gently at a speed of 50 rpm. At regular intervals, 5 mL of aliquots was withdrawn from the release media for UV–vis analysis and replaced with an equal volume of fresh buffer solution to maintain a constant volume. The cumulative amount of quercetin released from crosslinked CS nanoparticles in each buffer was determined by its absorbance at 373 nm. The relative percentage of quercetin release was calculated using Eq. (4):

$$\text{Drug release} = \frac{M_t}{M_\infty} \times 100$$  \hspace{1cm} (4)

where $M_t$ is the amount of quercetin released from the nanoparticles at time $t$ and $M_\infty$ is the amount of quercetin loaded in the nanoparticles.

2.9. In vitro cytotoxicity

HeLa cells were chosen as model cells to evaluate the in vitro cytotoxicity of quercetin-loaded CS-COU nanoparticles and the free quercetin by an MTT viability assay. In the MTT assay, HeLa cell lines were seeded into 96-well plates at the density of $1.25 \times 10^4$ cells per well in 180 μL DMEM followed by incubating for 24 h at 37 °C in 5% CO₂ humidified incubator. Subsequently, the cells were incubated with pure quercetin, free 8-FHMC, crosslinked CS-NPs with and without UV irradiation at concentrations of 10–100 μg mL⁻¹. After incubated for 48 h, 20 μL of MTT solution (5 μg mL⁻¹ in PBS) was added and the cells were incubated for another 4 h. The medium containing MTT was then completely removed, and replaced with 150 μL of DMSO per well for 10 min at room temperature to dissolve the formazan products. Finally, the absorbance was measured at a wavelength of 570 nm using a microplate reader. Cell viability (%) was calculated using the following equation:

$$\text{Cell viability} = \frac{(\text{Abs (test cell)})/(\text{Abs (controlled cell)}) \times 100\%}{\text{Abs (controlled cell)}}$$  \hspace{1cm} (5)

3. Results and discussion

3.1. Preparation of 8-FHMC and CS-COU nanoparticles

8-Formyl-7-hydroxy-4-methyl coumarin was synthesized in two steps using the procedure reported previously (Al-Kawkabani et al., 2013) with a slight modification. As depicted in Scheme 1, 7-hydroxy-4-methyl coumarin (4-methylumbelliferone) that is a drug used in bile therapy and possessing an anticancer activity was initially synthesized using Pechmann condensation of resorcinol and EAA in the presence of Con. H₂SO₄. In the second step, 8-FHMC was prepared via the Duff formylation of 7-HMC in the presence of HMTA and acetate acid at 90–95 °C to afford PHMC. The Duff reaction presumably proceeds via
imine formation, protonation of the imine by acid thus increasing the electrophilicity of the imine bond, α-addition of the electrophilic iminium cation by 7-HMC nucleophilic attack, further protonation to another iminium ion and a series of hydride shifts tandem sequences. The finally generated conjugated system affords the formyl-derivative of the 7-HMC after hydrolysis by aqueous acid (Fig. S4).

The chemical structures of 7-HMC and 8-FHMC were confirmed using $^1$H NMR and $^{13}$C NMR spectrum (Fig. 1). As shown in Fig. 1A, peak b at 2.4 ppm was assigned to the methyl protons, peak a at 6.15 ppm, c at 7.52 ppm, d at 6.77 ppm and the peak e at 6.86 ppm were assigned to the methine protons of 7-HMC. In $^1$H NMR spectrum of 8-FHMC (Fig. 1B) peak b at 2.4 ppm was assigned to the methyl protons and the peaks a, c and d at 6.29, 6.96 and 7.92 ppm respectively, were assigned to the methine protons and the peak e at 10.43 ppm was assigned to the aldehyde proton of 8-FHMC. Moreover, in $^{13}$C NMR spectrum of 8-FHMC (Fig. S5) the aldehydic carbon (HC=O), the hydroxyl carbon (C-OH), the lactonic carbon (C=O of lactone) and the methyl carbon (CH$_3$) appeared at δ 192.93, 165.50, 156.76 and 19.31 ppm, respectively. All these results demonstrated that 8-FHMC was synthesized successfully.

Recall that 8-FHMC compound was selected as the desired cross-linking agent for the preparation of the chitosan nanoparticles based on the assumption that its structure can furnish either one chemical crosslinking via the formation of imine bond between the chitosan chain amine and aldehyde groups in 8-FHMC structure together with another crosslinking through the formation of hydrogen bond in order to increase the physicochemical properties of iminocoumarin-chitosan network. In addition, because of the position of ortho-7-hydroxycoumarin, further imine bond stability is possible via intramolecular hydrogen bond between the 8-FHMC hydroxyl and imine nitrogen atom. On the other hand, due to the anticancer activity of coumarin compounds and the effects of low cytotoxicity of normal cells and in contrast to the high level of cytotoxicity on cancer cells, it is expected that the incorporation of 8-FHMC into chitosan polymer produces novel nanoparticles with the modified biological activity. The CS-COU-NPs were fabricated via the O/W nanoemulsion method. After ethanol evaporation, the CS-COU-NPs solutions were centrifuged to remove the
photo-crosslinking of coumarin moieties via [2π + 2π] cycloaddition reaction is an effective method. To achieve this objective, half of the CS-COU-NPs aqueous solution was irradiated under UV illumination at \( \lambda = 365 \text{ nm} \) at room temperature for the preparation of photo-crosslinked CS-COU-NPs (Scheme 2 A). On the other hand, the CS-COU-NPs and photo-crosslinked CS-COU-NPs can be conveniently cleaved at pH 5.8 (Scheme 2 B). As such, the use of harmful 265 nm UV-light is avoided and new pH-responsive de-crosslinking is integrated in this research.

3.2. Particle size and size distribution of CS-COU nanoparticles

CS-COU nanoparticles were prepared by employing Schiff-base bonding reaction based on oil in water nanoemulsion method. The size of crosslinked CS nanoparticles is impressed by a number of factors including chitosan concentration and molecular weight, pH and crosslinker concentration. Among them, concentration of chitosan and crosslinker are the most significant factors that control the size of the chitosan nanoparticles. The effect of these parameters on the CS-COU nanoparticles properties was assayed using varying one parameter while the other parameter was held constant.

3.2.1. Effect of 8-FHMC (cross linker) concentration

Whereas the concentration of chitosan was fixed at (1%, w/v) and the concentration of 8-FHMC was varied from (0.2–1.2%, w/v) as shown in Table 1. It is obvious that the increase in concentration of the 8-FHMC from 0.2 to 0.6% (w/v) decreases particle size, which can be attributed to the high degree of crosslinking of chitosan at higher 8-FHMC concentration. Therefore, compact networks are formed and the particles size is reduced. In addition, increase in the concentration of the 8-FHMC from 0.6 to 1.2% (w/v) led to a considerable increase in the particle size. Since increasing the number of aldehyde groups increases the chemical interaction with chitosan molecule, a decrease in free residual amine groups as well as increase in particle size occur concomitantly. Utilization of 8-FHMC in higher concentration than 1.2%, w/v leads to the formation of the aggregate solution. This in turn gives a gel-like or solid-like structure. Recall that the concentration of 8-FHMC has slight effect on the polydispersity index of particles and it is below 0.5 that indicates particle size uniformity.

3.2.2. Effect of chitosan concentration

The concentration of 8-FHMC was fixed at 0.6% (w/v) and the concentration of chitosan varied from 1 to 5% (w/v) as shown in Table 1. It is observed that the increase in chitosan concentration from 1 to 5% (w/v) increases the size of nanoparticles due to the linear enhancement in chitosan solution viscosity and the increase in the number of chitosan amine groups. 8-FHMC interacts with one amino groups of chitosan. The high viscosity inhibits efficient interaction between 8-FHMC and chitosan molecule. Moreover, polydispersity index of the chitosan nanoparticles are less than 0.5 that indicates particle size uniformity.

Table 1

<table>
<thead>
<tr>
<th>Factors</th>
<th>Concentration (% w/v)</th>
<th>Particle size (nm)</th>
<th>Polydispersity Index (PDI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-FHMC a</td>
<td>0.2</td>
<td>161.6 ± 3.42</td>
<td>0.321 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>110.3 ± 1.05</td>
<td>0.327 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>335.8 ± 8.34</td>
<td>0.252 ± 0.01</td>
</tr>
<tr>
<td>Chitosan b</td>
<td>1</td>
<td>110.3 ± 1.05</td>
<td>0.327 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>374.9 ± 2.33</td>
<td>0.273 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>452.9 ± 13.11</td>
<td>0.323 ± 0.05</td>
</tr>
</tbody>
</table>

a Mean ± S.D., n = 5, Concentration of chitosan: (1%, w/v).

b Mean ± S.D., n = 5, Concentration of 8-FHMC: (0.6%, w/v).

3.3. FT-IR characterization

Fig. 2 shows the FT-IR spectrum of 8-FHMC, pure chitosan, blank CS-COU-NPs and photo-crosslinked CS-COU-NPs. The IR spectrum of 8-FHMC reveals absorption at 1596 cm\(^{-1}\) which may be attributed to alkene C=C stretching vibration. Peaks appearing at 1639 cm\(^{-1}\) and 1742 cm\(^{-1}\) may be due to C=O stretching vibrations. Compared to the pure chitosan that displays a weak shoulder at 1654 cm\(^{-1}\) due to the amide bands, blank and photo-crosslinked CS-COU nanoparticles FTIR spectrum exhibit the characteristic imine band detected as a sharp peak at 1627 and 1631 cm\(^{-1}\) respectively, implying the formation of the chemical crosslinking by nucleophilic addition reaction between the 8-FHMC aldehyde group and chitosan chain amino groups. On the other hand, significant information concerning the physical crosslinking could be seen in the 2800–3500 cm\(^{-1}\) region of the nanoparticles FTIR spectrum in comparison to that of pure chitosan. This FTIR domain has been represented to be specific to the presence of hydrogen bonded OH stretching vibrations either intra- or inter-molecular (Marin et al., 2014).

Whereas the intra- and inter-molecular H-bonds of pure chitosan could be observed at 3352 and 3289 cm\(^{-1}\) respectively, in the FTIR spectrum, the corresponding vibrations for CS-COU-NPs moves to the higher wavenumbers at the range of 3403 cm\(^{-1}\), perhaps due to the presence of a new intramolecular hydrogen bond between 8-FHMC OH and the electron rich imine nitrogen of the nanoparticles. As such, this new intramolecular hydrogen bond stabilizes the imine bond. The CS-
COU-NPs also revealed a peak for C=O at 1729 cm⁻¹.

3.4. Photo-crosslinking of chitosan nanoparticles via UV-irradiation

In this work, the process of photo-crosslinking of coumarin moieties was traced by measuring the changes of UV intensity of nanoparticles solution at λ = 321 nm at different exposure times (Fig. 3). Fig. 3A displays the UV–vis spectrum change of the crosslinked chitosan nanoparticles solution under irradiation with 365 nm UV light. As it is observable, upon increasing the irradiation time, the maximum absorption intensity of coumarin groups observed at λ = 321 nm clearly decreases, indicating the formation of cyclobutane ring via photocycloaddition reaction. After about 12 min, the crosslinking process reached equilibrium while the final degree of photo-dimerization for this system was about 46% (Fig. 3B). No further photo-dimerization occurred when the polymer chains became more rigid due to the exclusion of the coumarin units (Jia et al., 2015). Water-soluble chitosan (MW = 3000 Da) was used to compare the structures of CS-COU-NPs and photo-crosslinked CS-COU-NPs by ¹H NMR. It was illustrated in Fig. 3C that the further dimerization can be identify by comparing the ¹H NMR spectrum of CS-COU-NPs and photo-crosslinked CS-COU-NPs. From the ¹H NMR spectrum of photo-crosslinked CS-COU-NPs, we can see that after UV-irradiation, the peak intensity of the double bond of coumarin groups (C=O) at δ 6.2 ppm significantly decreased and the protons of g and f appeared at δ 1.3 and 3.4 ppm, respectively, suggesting the photo-dimerization of coumarin groups via the [2π + 2π] cycloaddition reaction.

3.5. XRD analysis

Crystalllographic structures of CS, blank CS-COU NPs as well as drug-loaded crosslinked CS-NPs were studied by X-ray diffraction and illustrated in Fig. 4A. The two characteristic peaks in the diffractogram of CS at 2θ = 12.05 and 20.39 indicates the crystalline form II of hydrogen-bonded and highly deacetylated chitosan chains (Leceta, Guerrero, Ibarburu, Dueñas, & de la Caba, 2013). Upon the crosslinking of chitosan with 8-FHMC, disappearance of the diffraction peak at 2θ = 12.05 as well as the decrease in the peak intensity at 2θ = 20.39 indicate that the CS crystalline structure is destructed as soon as the crosslinking between the CS amino group and 8-FHMC aldehyde groups occurs. This in turn restricts the movement of the molecular chain of CS-COU-NPs. Observation of a broad diffraction peak upon loading of quercetin within CS-COU-NPs indicates that quercetin is diffused in an amorphous state in the chitosan–coumarin packing structure.

3.6. Morphology and size of drug-loaded chitosan particles

The morphological characteristics of the CS-COU and drug-loaded CS-COU nanoparticles before and after UV-irradiation were imaged by
the SEM technique. As shown in Fig. 5, the blank CS-COU-NPs (A) and drug-loaded CS-COU-NPs (B) before UV-irradiation have semi-spherical shape and nanosize structure, also reveal nearly uniform distribution with no intense particle agglomeration.

After irradiation at 365 nm, the photo-crosslinked microspheres with the diameter of 8 μm could be obtained by photodimerization of coumarin moieties (Fig. 5C). Actually, inter- and/or intra-particle crosslinking would be occurred when the samples were UV-irradiated and consequently results in the formation of micro and nanogel, respectively. By increasing the irradiation time, crosslinkages between the particles in the shell could be cleaved leading to release the embedded nanoparticles. The beginning of the photo-crosslinked microspheres cleavage as well as the cleavage of the microspheres and release of nanoparticles are observed in Fig. 5D and E, respectively. Finally, the formation of hollow polymeric microspheres can be seen in Fig. 5F.

Investigation of AFM images also confirmed the spherical shape and nanostructured surface of quercetin-loaded CS-COU nanoparticles (Fig. 6). On the other hand, this analysis obviously confirms the existence of cleaved microspheres and the release of the nanoparticles. The roughness parameter generated from this test represents a considerable difference between the non-photo and photo-crosslinked CS-COU-NPs. Whereas the non-photo-crosslinked nanoparticles had a comparatively low level of surface roughness (about 100 nm) the photo-crosslinked nanoparticles had rougher surfaces (approximately 350 nm). This may have been due to the existence of the thick shells and hollow particles formed in the polymer network upon the UV irradiation of photo-crosslinking. As demonstrated in Fig. 6, In compound (1) the dipole moment of the molecule is the vector sum of the dipole moments of C=C, C=O and C==O bonds. The magnitude of resultant dipole moment not only depends upon the values of the individual dipole moment of the bonds but also on their arrangement. In compound (1), C=C and C=O bonds are in the s-cis form and the resultant dipole moment of them (red arrow) is opposite to dipole moment of C==O bond and hence nearly cancel out. In case of compound (2), the dipole moment of the molecule is the vector sum of the dipole moments of C=C, C=O and C==O bonds. Therefore, due to the absence of the C=C bond, the compound (2) shows higher dipole moment in comparison to that of the compound (1), and hence a more negative charge on the compound (2) is concentrated. Consequently, the overall charge of photocrosslinked nanoparticles become more negative, so as, its zeta potential was converted to negative value as aforementioned.

3.7. Drug loading capacity and in vitro release profile

The hydrophobic anticancer drug, quercetin, was used to evaluate the drug loading and release properties of photo- and non-photo-crosslinked chitosan nanoparticles. The drug loading capacity and the encapsulation efficiency of photo-crosslinked nanoparticles determined...
as 10.2% and 78.4%, respectively, which were higher than those of 6.3% and 53.2% for the non-photo-crosslinked nanoparticles. The in vitro quercetin release profile from photo-crosslinked as well as non-photo-crosslinked CS-COU-NPs prepared via O/W nanoemulsion method was studied in a dialysis setup at 37 °C with pHs 7.4 and 5.8. As shown in Fig. 7A, less than 40% of quercetin was released from the non-photo-crosslinked nanoparticles at pH 7.4 within 100 h. On the other hand, approximately 20% quercetin release from photo-crosslinked nanoparticles under similar conditions. These results demonstrate the release decreased by aggregation of nanoparticles during and after photo-crosslinking. The surface-positioned nanoparticles were photo-dimerized with each other and make a thick shell around small-sized nanoparticles via inter-particles crosslinking. It is obvious that the rate of drug release decreases by photo-crosslinking because of entrapment of nanoparticles within microparticles. The accumulative release of photo- and non-photo-crosslinked chitosan nanoparticles was about 50.3% and 74.1%, respectively, within 100 h upon acidic conditions (pH 5.8). Since both of the photo- and non-photo-crosslinked nanoparticles contain pH-sensitive imine bond, these imine linkages can be quickly cleaved and disappeared in the acidic intracellular
compartments (pH 5.8), which leading to the entrapped quercetin release acceleration at the tumor site. The results indicate that the pH-sensitive crosslinked chitosan nanoparticles have the potential application and the impressive drug release in cancer cells.

3.8. Cell viability assays

MTT assay was applied to investigate the cytotoxicity of quercetin-loaded crosslinked chitosan nanoparticles with and without UV irradiation, the free quercetin and free 8-FHMC at various concentrations of 10–100 μg mL⁻¹, by using HeLa cells as model. All the formulations showed remarkable anticancer activity against HeLa cells after 48 h incubation (Fig. 7B). As the concentration of released quercetin increased, the cell viability was further reduced. Whereas The half maximal inhibitory concentration (IC₅₀) value for crosslinked chitosan nanoparticles without light irradiation was determined as 10 μg mL⁻¹, that with irradiation was found to be about 31 μg mL⁻¹. These results demonstrated that non-photo-crosslinked chitosan nanoparticles showed higher cytotoxicity for cancer cell proliferation in comparison to that of the photo-crosslinked nanoparticles. The lower cytotoxicity of photo-crosslinked CS nanoparticles can be attributed to the accumulation of nanoparticles within micrometer-thick shells, resulting in the slower release of quercetin as it is observable in the in vitro release too (Fig. 7A). In addition, it should be noted that there no meaningful difference in cell viability was observed between the free quercetin and photo-crosslinked chitosan nanoparticles in our experiments. All these results indicated that quercetin-loaded crosslinked chitosan nanoparticles have potential application in cancer chemotherapy.

4. Conclusions

In summary, novel ultraviolet- and pH-responsive iminocoumarin-chitosan nanoparticles were successfully designed and synthesized. The coumarin-containing chitosan nanoparticles could be readily fabricated via an oil-in-water emulsion system, using 8-formyl-7-hydroxy-4-methyl coumarin, 8-FHMC as a new friendly crosslinking agent. Spherical nanoparticles, cleaved microspheres and hollow polymeric microspheres could be observed in SEM and AFM images. The dimerization process of coumarin groups was monitored using UV spectrophotometer. The LC and EE results revealed the improved stability of nanoparticles after photo-crosslinking. The coumarin-containing chitosan nanoparticles have been employed as vehicles for efficient encapsulation of quercetin, exposing a pH-responsive controllable release. Due to the cleavage of Schiff base linkage between CS and 8-FHMC at lower pH condition, in vitro results indicated an accelerated drug release at endosomal pH than that under physiological conditions. The quercetin-loaded CS-COU nanoparticles exhibited effective comparable cytotoxicity with free quercetin by MTT results. This new type of chitosan nanoparticles with pH-triggered de-cross-linking property is promising and hopes to have a great potential in cancer therapy applications.
Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.carbpol.2018.08.074.

References


