Carbohydrate Polymers 121 (2015) 99–106

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Synthesis and characterization of pH-responsive N-naphthyl-N,O-succinyl chitosan micelles for oral meloxicam delivery

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ARTICLE INFO

Article history:
Received 9 October 2014
Received in revised form 6 December 2014
Accepted 9 December 2014
Available online 2 January 2015

Keywords:
pH-responsive chitosan
Polymeric micelles
Meloxicam
Physical entrapment methods

ABSTRACT

The aim of this study was to synthesize pH responsive chitosan and to evaluate the influence of drug-loaded micelle methods on loading efficiency, particle size and micelle stability. N-naphthyl-N,O-succinyl chitosan (NSCS) was successfully synthesized and meloxicam (MX) was loaded into the inner core of the NSCS micelles by physical entrapment methods (dialysis, O/W emulsion, dropping and evaporation) with a regular spherical shape (particle size 84–382 nm). MX-loaded micelles by evaporation method showed the highest entrapment efficiency. The stability of the drug-loaded micelles depended on not only the methods but also the initial of drug. NSCS micelles are less toxic on Caco-2 cells. In acidic medium at 0–2 h, percentage cumulative release of MX from MX-loaded micelles was similar to free drug. When the pH was adjusted to pH 6.8, the MX release was increased significantly. Therefore, this NSCS micelle would be desirable to develop MX carrier for oral drug delivery.

1. Introduction

Oral delivery is the most widely used and most readily accepted form of drug administration due to convenience, the absence of pain, good compliance and the ability to treat chronic disease. However, an important obstacle to the development of a successful and effective therapy that frequently results in low bioavailability of the orally administered drug is water insolubility (Lu & Park, 2013). Polymeric micelles have been investigated intensively as oral drug delivery applications because they are more convenient and preferred by the patient (Zhang et al., 2012). Polymeric micelles are comprised of amphiphilic block or graft copolymers that consist of hydrophobic and hydrophilic segments. In aqueous solutions, block or graft copolymers form structure containing hydrophobic drugs in the inner core surrounded by hydrophilic segments to protect the drug from the harsh environment of the gastrointestinal (GI) tract and stabilizes the micelles (Yokoyama, 2011). Using micelles, poorly soluble drugs can be successfully solubilized in aqueous media, which increases the water solubility of the drug by 10- to 5000-fold (Kedar, Phutane, Shidhaye, & Kadam, 2010). In the GI tract, variation in pH has been utilized to control drug release from carriers (Bromberg, 2008). The pH response of polymeric micelles as carriers has been designed to prevent GI degradation and to promote absorption into the intestine, which improves oral bioavailability by selectively releasing the drug into target sites or target organs. Several techniques have been employed for the preparation of drug-loaded micelles, and the efficiency of drug loading depends on the utilized tools such as chemical conjugates, physical entrapments, electrostatic techniques, and others. Physical entrapment is the simplest and most convenient technique and can be implemented *via* dialysis, O/W emulsion, dropping and evaporation. Thus, this method was selected to study PEGylated poly-4-(vinylpyridine) micelles loaded with dexamethasone by dialysis, O/W emulsion or evaporation (Miller et al., 2013). However, the preparation of these micellar systems with high drug loads remains challenging (Kim, Shi, Kim, Park, & Cheng, 2010) because different preparation methods may affect the efficiency of drug loading.

Chitosan is produced by Mucorales and other fungi, but in general it is manufactured by the deacetylation of chitin isolated from

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http://dx.doi.org/10.1016/j.carbpol.2014.12.039
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by-products of the marine fisheries, it is soluble in certain aqueous acidic solutions (pH <6.0) but also in ammonium carbonate (pH 10.2) (Muzzarelli, Tosi, Francescangeli, & Muzzarelli, 2003; Muzzarelli et al., 2012). However, chitosan cannot form micelles in water. Chitosan derivatives are widely used in pharmaceutical applications because they are nontoxic, biodegradable and less expensive (Bonferoni et al., 2014). Currently, chitosan derivatives have been converted to polymeric micelles for drug delivery. Several modified chitosans such as *N*-lauryl-carboxymethylchitosan, N-phthaloylchitosan-g-mPEG and N-octyl-O-sulfate chitosan have been utilized for the preparation of polymeric micelles (Miwa et al., 1998; Zhang, Qineng, & Zhang, 2004).

Meloxicam (MX), 4-hydroxy-2-methyl-N-(5-methyl-2-thiazolyl)-2H-1,2-benzothiazine-3-carboxamide-1,1-dioxide (Fig. 1a), is a non-steroidal anti-inflammatory drug, that acts as an inhibitor of prostaglandin synthetase. MX has been used in the treatment of pain and inflammation caused by arthritis. However, the adverse effects on the GI tract caused by MX after oral administration include indigestion and stomachache (Duangjit, Opanasopit, Rojanarata, & Ngawhirunpat, 2013), and these effects may reduce the life expectancy of patients with chronic diseases such as rheumatoid arthritis. Based on its structure, MX is an acidic drug that is poorly soluble in water (0.009 mg/mL at 25 °C) (Kim & Lee, 2007). Based on the Biopharmaceutics Classification System Guidance has defined four categories of drugs, and MX is in class II. During oral administration, the low solubility of drugs in classes II and IV limits the drug dissolution rate and affects absorption in GI tracts, resulting in low bioavailability. Therefore, the aim of this study was to synthesize a pH-responsive graft copolymer chitosan backbone and to evaluate the influence of preparation methods and organic solvents on MX-loading efficiency. Moreover, the effects of treatment with the drug on the morphology, particle sizes, and *in vitro* cytotoxicity of Caco-2 cells as well as their release behavior were investigated.

2. Materials and methods

2.1. Materials

MX was kindly provided by Siam Pharmaceutical Co., Ltd. (Bangkok, Thailand). Chitosan was purchased from OilZac Technologies Co., Ltd. (Bangkok, Thailand). The degree of deacetylation (DDA = 96%) was determined by NMR. The number-averaged molecular weight (M_n), weight average molecular weight (M_w), and polydispersity index (PDI; M_w/M_n) of chitosan were determined to be 7633 g/mol, 15,746 g/mol and 2.06, respectively, using gel permeation chromatography. 2-Naphthaldehyde, succinic anhydride, and sodium borohydride ($NaBH_4$) were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA). The dialysis bag (CelluSep®, 6000–8000 MWCO) was purchased from Membrane Filtration Products (Seguin, TX, USA). The Human colon adenocarcinoma cells (Caco-2 cells) line were obtained from American Type Culture Collection (Rockville, MD, USA). All other reagents and solvents were of analytical grade and used without further purification.

2.2. Synthesis of N-naphthyl-N,O-succinyl chitosan (NSCS)

NSCS was synthesized by successive reductive N-amination and N,O-succinylation. Briefly, 2.0 g of chitosan was dissolved in 150 mL of 1% (v/v) aqueous acetic acid, and 100 mL of ethanol was added to the solution. 2-Naphthaldehyde (2.0 meq/GlcN) was added, and the reaction mixture was stirred at room temperature for 24 h. At this point, the pH of the solution was adjusted to 5 by adding 1 M NaOH, and 2.0 g of $NaBH_4$ (52.9 mmol) was added. The reaction mixture was then stirred at room temperature for 24 h. The precipitate was collected by filtration, washed several times with ethanol, and followed by drying under a vacuum at room temperature. This protocol created N-naphthyl chitosan (NCS). The N,O-succinylation of NCS was conducted using succinic anhydride. Briefly, 1.0 g of NCS was dispersed in 40 mL of N,N-dimethylformamide (DMF)/dimethyl sulfoxide (DMSO), (1:1, v/v), and 3.0 g of succinic anhydride (5.0 meq/GlcN) was added. The reaction was heated at 100 °C in a nitrogen atmosphere for 24 h. Next, the reaction mixture was cooled to room temperature and filtered to remove undissolved NCS. The clear solution was dialyzed with distilled water for 3 days to remove excess succinic anhydride and DMF. The powdered NSCS was then obtained by lyophilization. The M_n of NCS and NSCS was evaluated based on the theoretical molecular weight increase of the derivatives and the degree of substitution.

2.3. Characterization of NSCS

The ^1H NMR spectra of chitosan and its derivatives were measured in D_2O/CD_3COOD (99:1, v/v) solution and DMSO-d6, respectively, using a Bruker AVANCE 500 spectrometer (Bruker, Switzerland). Tetramethylsilane was used as an internal standard. Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectra were collected using a Nicolet 6700 spectrometer (Thermo Company, USA) using a single-bounce ATR-FTIR spectroscopy accessory with a diamond internal reflection element at ambient temperature (25 °C). Steady-state fluorescence spectra were obtained using a LS 55 fluorescent spectrometer (PerkinElmer, USA).

2.4. Preparation of polymeric micelles with and without MX

2.4.1. Dialysis

Overall, 5 mg of NSCS and MX (0–40%, w/w to polymer) was dissolved in 2 mL of DMSO or DMF in a glass bottom container. The mixture was stirred at room temperature until completely dissolved. The mixture was then placed in a dialysis bag and dialyzed against distilled water overnight (exchanged once after 4 h).

2.4.2. O/W emulsion

NSCS without MX was prepared as for dialysis. For drug-loaded micelles, MX (5–40%, w/w to polymer) was dissolved in dichloromethane (DCM) and was injected under constant stirring into 2 mL of aqueous empty micellar solution. Next, DCM was evaporated by overnight stirring at room temperature.

2.4.3. Dropping

In total, 5 mg of NSCS and MX (0–40%, w/w to polymer) was dissolved in 0.5 mL DMSO or DMF. The solution was slowly dropped into stirred water, and the mixed solution was stirred overnight. The final ratio of DMSO:water and DMF:water was 1:5 and 1:10, respectively. The mixture was then placed in a dialysis bag and dialyzed against distilled water overnight.

2.4.4. Evaporation

Overall, 5 mg of NSCS and MX (0–40%, w/w to polymer) was dissolved in DMF in a glass bottom container. The solution was mixed with acetone (1/3 of DMF) and stirred at room temperature under nitrogen gas flow until the solvent completely evaporated. Next, 3 mL of distilled water was added, and the solution was sonicated using a probe-type sonicator (CV 244, Sonics VibraCell™, Newtown, CT, USA) in a cycle with a sonication time of 5 min and a standby time of 5 min at 80 °C for 20 min.

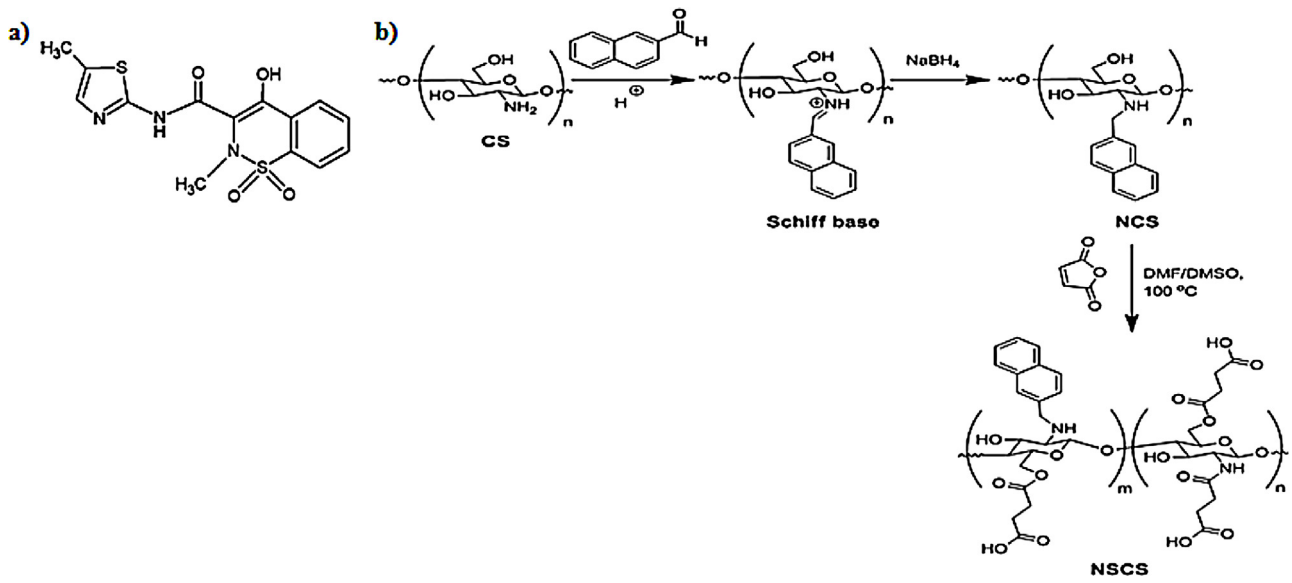

Fig. 1. The chemical structure of (a) MX and (b) the synthesis of NSCS.

Finally, the remaining aqueous solution of each method was centrifuged at 1000 rpm for 2 min. The supernatant was filtered through a 0.45-μm membrane filter and collected.

2.5. Characterization of micelles with and without MX

2.5.1. The morphology

The morphology of the micelles was investigated using atomic force microscopy (AFM). AFM samples were prepared by dropping an aqueous nanoparticle suspension onto a mica surface, followed by air drying. Subsequently, the nanoparticles were imaged by scanning a 1 μm × 1 μm area in tapping mode using an NSG01 cantilever with 115–190 kHz resonance frequencies and a constant force ranging from 2.5 to 10 N/m. All images were recorded in air at room temperature at a scan speed of 1 Hz, and the phase and topology images were used to determine the morphology and particle size of the nanoparticles.

2.5.2. Determination of the critical micelle concentration

The critical micelle concentration (CMC) of NSCS in aqueous media was determined using fluorescence spectroscopy with pyrene employed as a fluorescent probe (Liu, Desai, Chen, & Park, 2005; Opanasopit et al., 2006). An aliquot (10 μL) of 1 mM pyrene solution in acetone was added to each vial of a series of aqueous polymer solutions (4 mL, 0.5–3.9 × 10^{-3} mg/mL). The final concentration of pyrene in each sample solution was 2.5 × 10^{-6} M. The mixtures were sonicated for 15 min, heated at 50 °C for 2 h, and then kept in the dark at room temperature overnight to equilibrate. Fluorescence spectra were recorded at an excitation wavelength of 335 nm, and the emission spectra were monitored over a range of 350–500 nm. The change in the intensity ratio of the first and third vibration bands (I_1/I_3) at 373 nm (I_1) and 382 nm (I_3) in the emission spectra were used to investigate the shift in NSCS hydrophobic microdomains. The CMC was calculated after fitting the semi-log plot of the intensity ratio I_1/I_3 *vs.* the concentration.

2.5.3. Particle size

The mean particle size and size distribution of the polymeric micelles with and without MX were determined in triplicate at 25 °C using the dynamic light scattering (DLS) (Malvern, Worcestershire, UK). The micelle samples were diluted with distilled water and were passed through a 0.45-μm membrane filter prior to use.

2.5.4. Gel permeation chromatography (GPC)

The molecular weight of the polymer and the stability of drug-loaded micelles were determined by GPC. High performance liquid chromatography (HPLC) was conducted using an Agilent HPLC system (Santa Clara, CA, USA) equipped with a Shodex® GFC SB804 HQ column at 40 °C. The samples (50 μL) were passed through a 0.45-μm membrane filter, injected into the column and eluted with distilled water at a flow rate of 1 mL/min. The detection was performed using an evaporative light scattering detector.

2.6. Determination of entrapment efficiency

The MX-loaded NSCS micelles of each method were dissolved in a mixture solution of DMSO:H_2O (9:1). The amount of MX loaded into the polymeric micelles was determined using the UV-vis Spectrophotometer (8453E, Santa Clara, CA, USA) at 365 nm. The incorporation efficiency and loading capacity were calculated with the following Eqs. (1) and (2), respectively.

$$\text{Incorporation efficiency}(\%) = \left(\frac{C_1}{C_2}\right) \times 100 \quad (1)$$

where C_1 is the amount of MX in NSCS micelles and C_2 is the initial of amount MX used for preparation

$$\text{Loading capacity}\,(\text{mg/mg}) = \frac{L_1}{L_2} \quad (2)$$

where L_1 is the amount of MX in micelles and L_2 is the amount of graft copolymer used for preparation.

2.7. In vitro cytotoxicity

In vitro cytotoxicity of tested NSCS was performed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Mosmann, 1983). Briefly, Caco-2 cells were seeded 10,000 cells/well in 96-well plates and preincubated for 24 h in atmospheric humidity (5% CO_2, 95% air, 37 °C). Then, the Caco-2 cells were treated with bare NSCS micelles at various concentrations (0.01–5 mg/mL) or pure MX (concentrations 1–250 μg/mL). After treatment, the solution was removed, fresh medium was added and the mixture was incubated with MTT solution (final concentration 1 mg/mL) for 4 h. The medium was removed, and the formazan crystals formed in the living cells were solubilized in 100 μL DMSO. Relative cell viability was calculated based on the absorbance at

550 nm using a microplate reader (Universal Microplate Analyzer, AOPUS01 and AI53601, Packard BioScience, CT, USA). The viability of non-treated control cells was arbitrarily defined as 100%.

2.8. In vitro release

The release of MX from the 40% (w/w) initial MX-loaded NSCS micelles prepared by evaporation method (particle size 293.80 ± 6.84 nm) was determined using a dialysis bag. In total, 20 mL of medium (supplemented with 0.1 N HCl (pH 1.2) for 2 h, then the pH of the medium was changed to 6.8 with trisodium phosphate for 6 h) was maintained under constant stirring with sink conditions at 37 °C, and 1 mL of MX-loaded NSCS micelles in water was placed in a dialysis bag and immersed in the medium. At certain time intervals, 1 mL aliquots of the medium were withdrawn, and the same volume of fresh medium was added. The sample solution was analyzed by HPLC (Santa Clara, CA, USA) using an Eclipse XDB-C18 column (particle size 5 μm; column dimension 4.6 mm × 150 mm) according to Duangjit et al. (2013). A mobile phase was comprised of potassium dihydrogen phosphate pH 4.4:methanol:acetonitrile (45:45:10, v/v/v), and the flow rate was 1 mL/min. The injection volume was 20 μL, and the wavelength was set at 365 nm. The sample was filtered through a 0.45-μm membrane filter prior to injection.

2.9. Statistical analysis

The results are depicted as the mean ± standard deviation of triplicate experiments. One-way ANOVA and the LSD *post hoc* test were used to analyze the different of amounts of MX-loaded polymeric micelles. The significance level was set at $p < 0.05$.

3. Results and discussion

3.1. Synthesis and characterization of NSCS

The amphiphilic chitosan derivative, NSCS was synthesized by reductive N-naphthylation and N,O-succinylation (Fig. 1b). Overall, the N-naphthyltion of chitosan occurred *via* the corresponding Schiff base intermediate, which is easily reduced by sodium borohydride or sodium cyanoborohydride to obtain NCS (Sajomsang, Tantayanon, Tangpasuthadol, Thatte, & Daly, 2008). The homogenous N,O-succinylation of NCS was conducted using succinic anhydride in mixture solvents and DMF and DMSO at 100 °C. The degree of substitution (DS), defined as the number of N-naphthyl groups or N,O-succinyl groups per hundred glucosamine units of chitosan, was determined by elemental analysis. The DS of the N-naphthyl groups (DSN) and N,O-succinyl groups (DSS) was calculated by comparing the C/N molar ratio obtained from elemental analysis using the following Eqs. (3) and (4), respectively (Huo et al., 2012).

$$\text{DSN of naphthyl group}\,(\%) = \frac{(\text{C/N})_{\text{NCS}} - (\text{C/N})_{\text{CS}}}{11} \times 100 \quad (3)$$

$$\text{DSS of succinyl group}\,(\%) = \frac{(\text{C/N})_{\text{NSCS}} - (\text{C/N})_{\text{NCS}}}{4} \times 100 \quad (4)$$

In this study, the naphthyl groups were effectively substituted onto the primary amino groups; however, the succinyl groups were introduced to both the primary amino and hydroxyl groups in the chitosan backbone. The M_n of NCS and NSCS was calculated based on the theoretical molecular weight increase of the derivatives due to the DSN and DSS. The M_n of chitosan was determined to be 7633 g/mol (~47 repeating units) by GPC. The DSN was calculated to be 0.54 (~25 repeating units), while the DSS was 0.52 (~24 repeating units) (Table 1). The hydrophobic/hydrophilic ratio on the chitosan backbone was calculated to be 1.04. After N-naphthylation and N,O-succinylation, the M_n was calculated to increase from 7633 g/mol to 10,907 and 13,351 g/mol, respectively.

The ATR-FTIR spectrum for NCS is similar to chitosan except for the appearance of additional absorption bands at 1632, 1490, 817 and 746 cm^{-1} (Lim et al., 2013). These bands were assigned to C=C stretching and C—H deformation (out of plane) from the naphthyl groups. The NSCS spectrum exhibited characteristic bands at 1704, 1642, and 817 cm^{-1} due to C=O stretching of the succinic acid moiety, C=C stretching, and C—H deformation (out of plane) of the naphthyl groups, respectively (Fig. 2a) (Lim et al., 2013; Sajomsang et al., 2014). In comparison with the ^{1}H NMR spectra of chitosan that contains no aromatic protons, the ^{1}H NMR NCS spectra exhibited broad multiple protons at δ ranging from 7.42 to 7.89 ppm due to the presence of the naphthyl groups, while the NSCS spectra was similar to NCS except for the additional proton signal at δ (2.45 ppm) due to the methylene protons of the succinyl moiety (Fig. 2b) (Lim et al., 2013; Sajomsang et al., 2014). Based on ^{1}H NMR spectra, the DSN value was calculated using Eq. (5) shown below (Table 1). The DSN value was calculated as 0.52 using the ^{1}H NMR method and was in agreement with the values obtained from the elemental analysis method.

$$\text{DSN} = \frac{(I_{\text{Nap}}/7)}{(I_{\text{H2-H6}}/6)} \quad (5)$$

I_{Nap} represents the total area (integration) of N-naphthyl protons, and $I_{\text{H2–H6}}$ represents the peak area of protons C2–C6 in the chitosan backbone.

In contrast, the methylene protons of the succinyl groups overlapped with the DMSO-d6 used as a solvent; therefore, the DSS was determined using the elemental analysis method instead of ^{1}H NMR. These results in conjunction with the IR and ^{1}H NMR spectra discussed above indicate the successful introduction of both these functionalities onto the chitosan backbone.

3.2. Preparation and characterization of NSCS micelles with and without MX

Novel pH-responsive chitosan micelles were developed based on an introduction of hydrophobic and hydrophilic moieties into a chitosan backbone. NSCS is both hydrophobic (naphthyl moieties) and hydrophilic (succinyl moieties) and can be induced to undergo self-aggregation or micelle formation in aqueous media prepared by physical entrapment methods (dialysis, O/W emulsion, dropping and evaporation). The micellar behavior of NSCS in aqueous media was investigated by fluorescence spectroscopy using pyrene as a fluorescence probe. The fluorescence spectra of pyrene in the presence of various concentrations of the NSCS polymer were recorded (Fig. S1a). The CMC was identified by plotting the intensity ratio I_1/I_3 of the pyrene excitation spectra at various concentrations of NSCS and determining the intercept between two straight lines (Fig. S1b). When the concentration reached the CMC, the I_1/I_3 ratio dramatically decreased. The CMC for NSCS was determined to be 0.0678 mg/mL, which is lower than the CMC value of low molecular weight surfactants (Jiang, Quan, Liao, & Wang, 2006).

The particle sizes and PDI of NSCS micelles without MX were prepared using multiple methods (dialysis, dropping and evaporation) and were measured by the DLS method. The particle sizes of empty micelles ranged from 84 to 233 nm. The different methods influenced the particle size of the micelles, and the micelles prepared *via* the dropping method were the smallest particle sizes in both the DMSO and DMF solvents. The rank of particle sizes of the micelles created using the different methods are as follows: dropping (84.31 ± 0.88 nm) < dialysis (196.20 ± 1.51 nm) < evaporation (233.50 ± 7.07 nm). The type and ratio of mixed solvents was also an important factor that affected the micelle particle size prepared by the dropping method. When using DMSO as a single solvent, the

Table 1
Elemental analysis data for chitosan, NCS and NSCS.

Sample	%C	%H	%N	C/N	DSN[a]	DSN[b]	DSS[c]	M_n (Da)
Chitosan	44.30	7.87	8.38	5.28	–	–	–	7633[d]
NCS	66.79	11.29	5.97	11.18	0.52	0.54	–	10,907[e]
NSCS	54.97	10.41	4.14	13.27	–	0.54	0.52	13,351[e]

[a] The DSN determined by ^{1}H NMR.
[b] The DSN determined by elemental analysis.
[c] The DSS determined by elemental analysis.
[d] Measured by GPC.
[e] Determined by elemental analysis.

particle size of the micelles (84.31 ± 0.88 nm) was smaller than the micelles prepared with DMF (98.35 ± 0.46 nm), with a similar narrow size distribution. In the case of mixed solvents, the reduction in the ratio of organic solvent to water from 1:5 to 1:10 resulted in an increase in the particle size of the micelles from 84.31 to 97.97 nm for DMSO:water and from 98.35 to 107.40 nm for DMF:water. Based on these results, DMSO and DMSO mixed with water at a ratio of DMSO:water (1:5) were selected to prepare the MX-loaded NSCS micelles.

Fig. 3 showed the effect of entrapment methods on the incorporation efficiency (Fig. 3a) and loading capacity (Fig. 3b) of the MX-loaded NSCS micelles. The results revealed that the evaporation method had the highest incorporation efficiency (22–52%) and loading capacity (25–75 μg/mg) compared to the other methods. The loading capacity increased from 25 to 75 μg/mg with an increase in the initial MX loading from 5% to 40%. This high content indicates the successful incorporation of water-insoluble drugs to polymeric self-assemblies with good water solubility. Micelle formation and drug incorporation into the micelles were expected to occur simultaneously. The hydrophobic interactions among the hydrophobic N-phthaloylchitosan chain, MX, and solvent may be an important key to controlling this incorporation process. This result was similar to previous studies that reported that the incorporation efficiency of stearoyl gemcitabine-loaded poly(ethylene glycol)–poly(D,L-lactide) (PEG–PLA) polymeric micelles and dexamethasone-loaded PEGylated poly-4-(vinylpyridine) polymeric micelles were also dependent on the incorporation methods (Daman, Ostad, Amini, & Gilani, 2014; Miller et al., 2013). The co-solvent evaporation method had a higher drug loading percentage than direct dialysis, O/W emulsion and the dropping method; therefore, the incorporation method affected the drug-loading efficiency. The particle sizes of the MX-loaded NSCS micelles are shown in Table 2. Micelles with MX were larger in size compared to the empty micelles. The particle sizes of the MX-loaded micelles had a tendency to increase with an increase in the weight ratio of MX to polymer (Kumar, Kulkarni, Nagesha, & Sridhar, 2012). The larger particle size of the MX loaded into the polymeric micelles may be due to the increase of MX in the micelles and the aggregation of the micelles (Ngawhirunpat et al., 2009). The stability of MX-loaded NSCS micelles at various concentrations was characterized by GPC. The result showed the ratio of the micelle peak area/MX concentration [MX] (Fig. 3c), and if the value of the ratio was larger, MX more stably incorporated into the micelles (Opanasopit, Ngawhirunpat, Rojanarata,

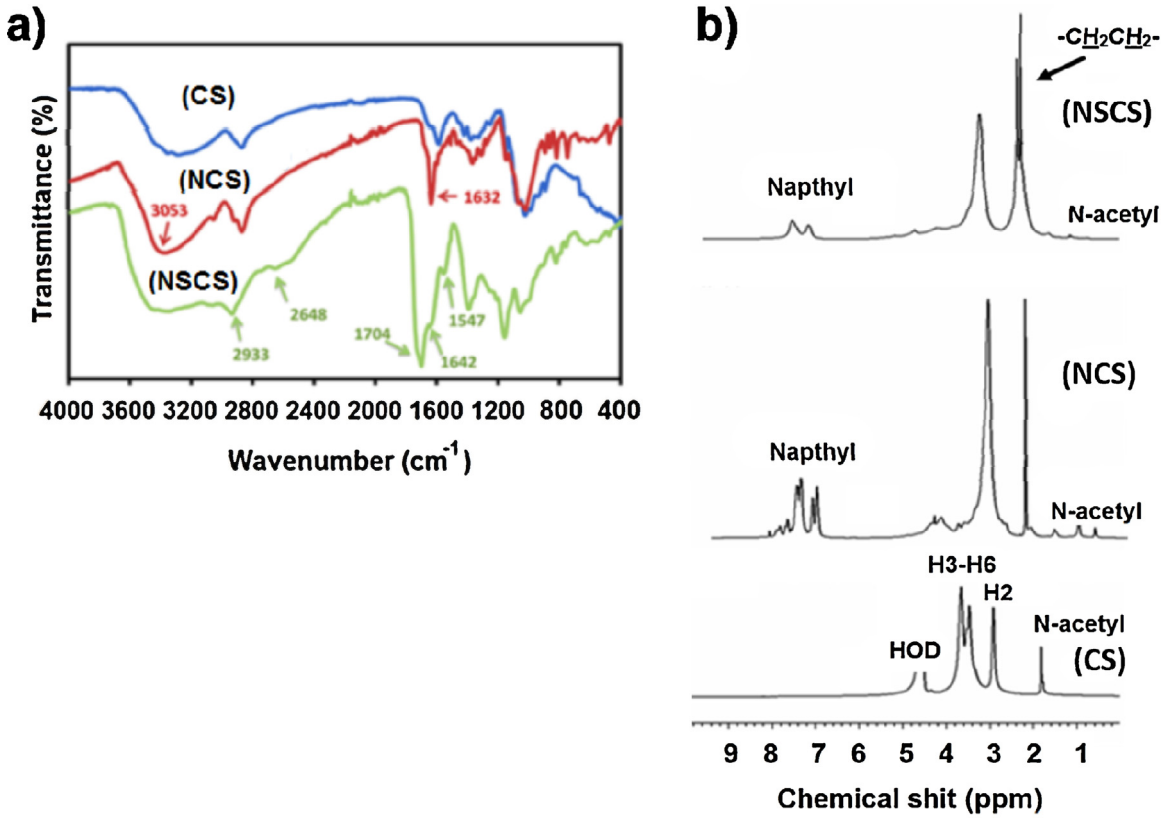

Fig. 2. Characterization of chitosan, NCS and NSCS, (a) ATR-FTIR spectra; (b) ^{1}H NMR spectra.

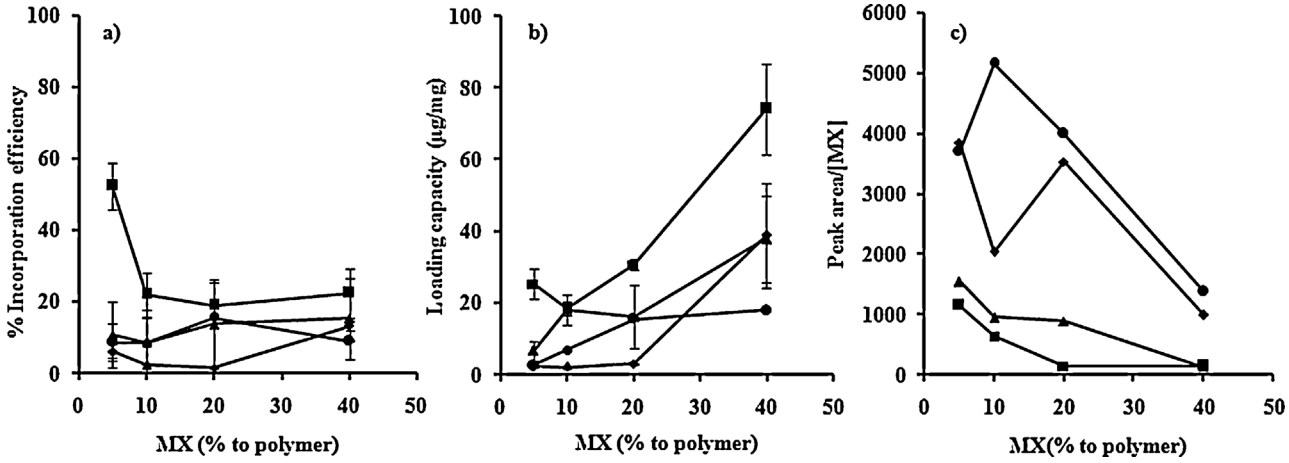

Fig. 3. Effect of the incorporation technique and initial drug concentration (5–40% to polymer) on (a) the incorporation efficiency, (b) loading capacity of MX-loaded NSCS micelles, and (c) the micelles stability by ratio of peak area/[MX]; (♦) dialysis method; (●) dropping method; (■) evaporation method; (▲) emulsion method. Data are plotted as the mean ± S.D. of three measurements.

Table 2
The particle sizes and PDI of NSCS micelles with and without MX. Each value represents the mean ± SD from three independent measurements.

MX to polymer (%)	Dialysis		Dropping		Evaporation		O/W emulsion	
	Particle size (nm)	PDI	Particle size (nm)	PDI	Particle size (nm)	PDI	Particle size (nm)	PDI
0	196.20 ± 1.51	0.061	84.31 ± 0.88	0.198	233.50 ± 7.07	0.384	–	–
5	275.03 ± 6.12	0.169	108.77 ± 4.03	0.217	291.77 ± 6.35	0.383	192.93 ± 0.85	0.070
10	266.17 ± 6.91	0.223	113.87 ± 1.32	0.156	382.17 ± 12.02	0.523	194.83 ± 3.02	0.100
20	311.80 ± 11.39	0.215	127.07 ± 0.29	0.153	312.17 ± 12.00	0.381	192.13 ± 2.54	0.076
40	310.30 ± 0.78	0.166	142.73 ± 0.57	0.174	293.80 ± 6.84	0.310	195.37 ± 2.17	0.120

Choochottiros, & Chirachanchai, 2007). The micelles were more stable at the high drug concentration compared to the initial low drug concentration. The hydrophobic N-phthaloylchitosan chain (N-phthaloyl group) showed high micelle stability upon the MX incorporation. This suggests a large contribution of π–π interaction between aromatic groups of MX molecules and the hydrophobic inner core (N-phthaloyl group) of the NSCS polymers (Opanasopit et al., 2006). In addition, the different techniques utilized influenced micelle stability. The result revealed that micelle stability was higher when the dropping method was used compared to the other method.

3.3. *In vitro cytotoxicity test*

The polymer is used to prepare the micelles that should be non-toxic following the majority requirement. Although chitosan is generally considered to be a biodegradable and safe polymer, several case studies were identified the toxic effects of chitosan derivatives on Caco-2 cells using the MTT assay (Ngawhirunpat et al., 2009). Thus, the cytotoxicity of NSCS micelles and pure MX on Caco-2 cell viability was evaluated. The result showed that after treating the Caco-2 cells with various concentrations of NSCS micelles (Fig. 4a) and pure MX (Fig. 4b), the IC_{50} value after 72 h was

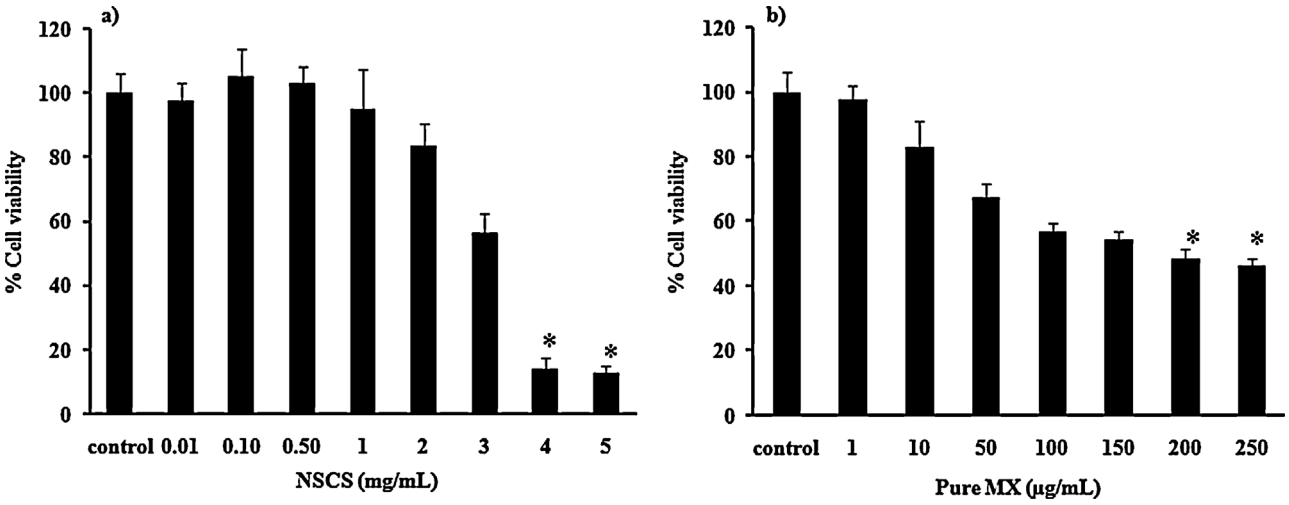

Fig. 4. Effect of (a) NSCS micelles and (b) pure MX on cytotoxicity using the MTT assay. Data are plotted as the mean ± S.D. of five measurements.

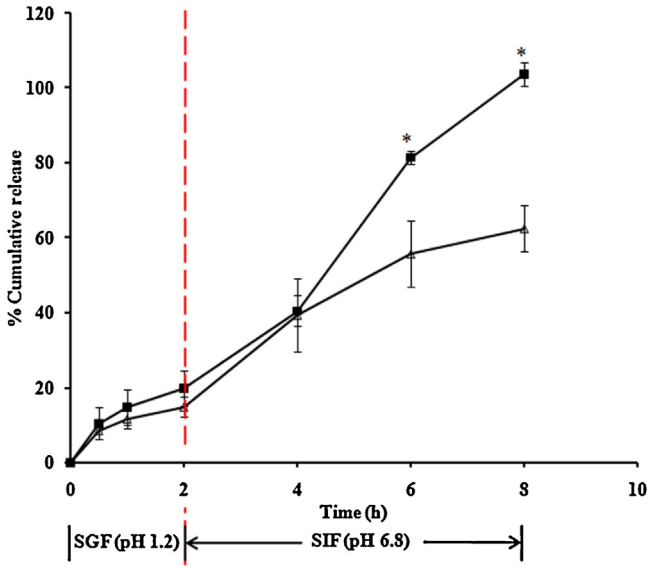

Fig. 5. Release profiles of (■) MX-loaded NSCS micelles and (△) MX free drug in 0.1 N HCl pH 1.2 (0–2 h) then in PBS pH 6.8 (2–8 h). Data are plotted as the mean ± S.D. of three measurements. * Statistically significant ($p < 0.05$).

3.15 ± 0.15 mg/mL and 190.22 ± 16.24 μg/mL, respectively. NSCS micelles are less toxic, indicating excellent biocompatibility *in vitro* with the Caco-2 cells (Bastakoti et al., 2013).

3.4. *In vitro* MX release from NSCS micelles

In vitro release of MX from MX free drug and MX-loaded NSCS micelles was determined using the dialysis bag diffusion method shown in Fig. 5. Due to its high entrapment level compared to the other methods, 40% initial MX-loaded NSCS micelles prepared by evaporation method were selected to test release. The release profile of MX from the NSCS micelles was obtained in 0.1 N HCl to simulate gastric fluid (SGF) medium (pH 1.2) (2 h) and phosphate buffer (pH 6.8) to simulate the intestinal fluid (SIF) medium (6 h) as follow gastrointestinal transit time. In SGF medium between 0 and 2 h, the percent of cumulative release of MX loaded into NSCS micelles was almost drug free (approximately 20%). When the pH was changed to 6.8 (as SIF medium), the percentage cumulative release of MX from the MX-loaded NSCS micelles significantly increased due to the ionization of the succinic acid moiety. Succinic acid has a pK_{a1} value of approximately 4.21 at 25 °C (Haynes, 2012); therefore, succinic acid is ionized when the pH is greater than the pK_{a1} value, leading to micelle dissociation. At pH 6.8, MX is release is greater from the NSCS micelles than at pH 1.2. Thus, the NSCS micelles were designed for site-specific drug release in the small intestine to improve bioavailability (Hunter, Elsom, Wibroe, & Moghimi, 2012). To obtain a better understanding of the release behavior of MX from the NSCS micelles as a function of pH, the morphologies and particle sizes of the micelles before and after MX was loaded was investigated using AFM and DLS (Fig. 6). AFM and DLS studies confirmed that the morphologies and particle sizes of micelles before and after MX was loaded changed when the pH varied due to the ionizable succinic acid groups on the micelle surface. At pH 1.2, which is lower than the pK_{a1} of succinic acid, the micelles before and after MX was loaded were present as aggregates with particle sizes ranging from 3622.00 ± 315.23 to 7000.00 ± 680.24 nm (Fig. 6a, b, c, j, k, and l, Table S1 and Fig. S2a). At pH 5.0, the particle size of the micelles before and after MX was loaded decreased in the range of 352.13 ± 30.37 to 433.43 ± 38.42 nm as a result of partial ionization of succinic acid on the micelle surface compared to pH 1.2 (Fig. 6d, e, f, m, n and o, Table S1 and Fig. S2b). Then, the particle sizes of the micelles before and after MX was loaded increased from 387.77 ± 1.52 to 551.63 ± 7.09 nm when the pH increased from 5.0 to 6.8, while AFM images indicated that the micelles had a spherical morphology at the higher pH (>5) and no aggregation (Fig. 6g, h, i, p, q and r, Table S1 and Fig. S2c), implying substantial swelling to promote the release of MX from the micelles.

It is indicated that non-pH-sensitive micelles may enhance drug solubilization but probably not necessarily the drug absorption (Rieux des, Fievez, Garinot, Schneider, & Préat, 2006). Acrylic-based polymers are widely used in oral pH-sensitive drug delivery such as poly(methacrylic acid) (PMAA) and acrylic acid. Our

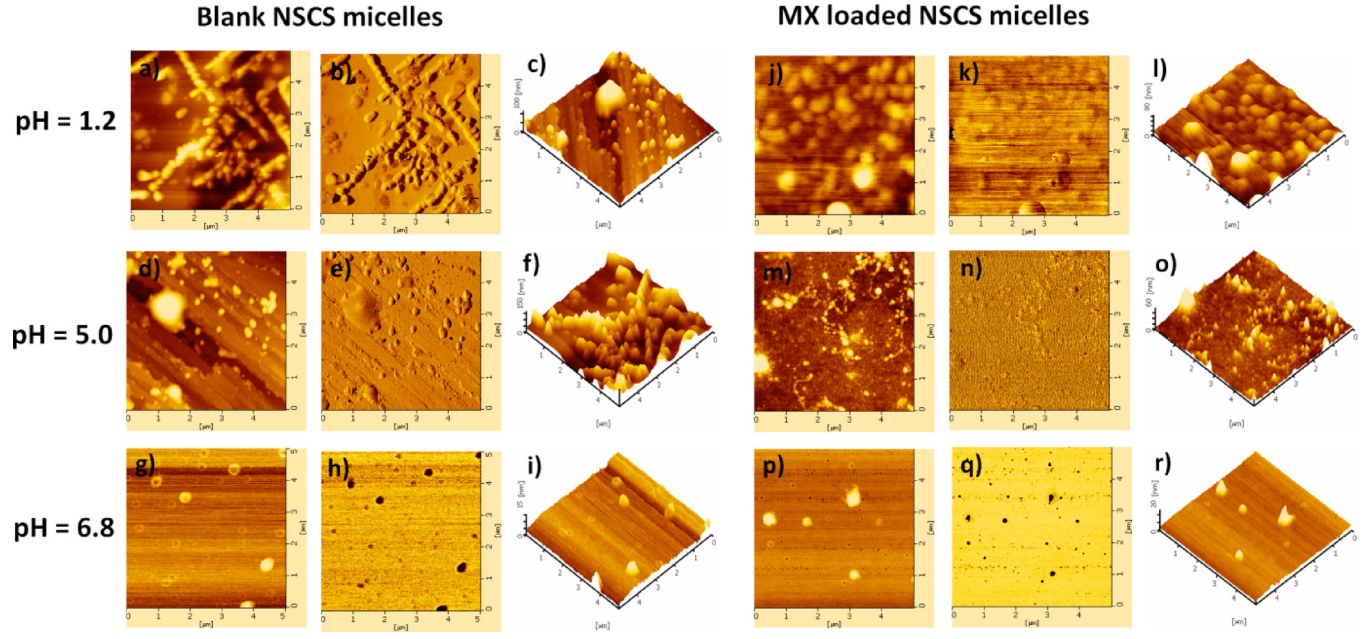

Fig. 6. AFM images of NSCS micelles; topology image (a), phase image (b), and 3D image (c) at pH 1.2, topology image (d), phase image (e), and 3D image (f) at pH 5.0, and topology image (g), phase image (h), and 3D image (i) at pH 6.8, and AFM images of MX-loaded NSCS micelles; topology image (j), phase image (k), and 3D image (l) at pH 1.2, topology image (m), phase image (n), and 3D image (o) at pH 5.0, and topology image (p), phase image (q), and 3D image (r) at pH 6.8.

novel pH-responsive graft copolymer chitosan backbone, NSCS, revealed the high MX-loaded and release profiles were adjusted by pH similar to previous studied using acrylic acid. Kim and his coworkers developed a pH-responsive polymer, PEG-b-(4-(2-vinylbenzyloxy)-N,N-(diethylnicotinamide-co-acrylic acid)) (PEG-*b*-P(VBODENA-*co*-AA)s), and testing paclitaxel loading/release profiles by changing the pH condition. The PTX release from micelles with more than 20% acrylic acid contents was completed within 12 h in a simulated intestinal fluid (pH = 6.5). The micelles without any acrylic acid moiety showed very slow release profiles (Kim, Kim, Huh, Acharya, & Park, 2008). Our results obtained from the release study lead us to assume that these MX-loaded NSCS micelles can be used to increase the bioavailability of MX upon oral delivery. Thus, the bioavailability of MX formulated in the NSCS micelles should be further examined by *in vivo* experiments.

4. Conclusion

In the present study, novel pH-responsive NSCS was successfully synthesized. NSCS was formed *via* self-assembly in aqueous media and successfully incorporated a poorly water-soluble drug in the hydrophobic inner core by various physical entrapment methods. Among the methods, the evaporation method and initial 40% MX to polymer percentage showed the highest MX incorporation efficiency. The micelle particle size and stability depended on the initial concentration of the drug and the entrapment method. The MX release behaviors were adjusted by pH; therefore, these NSCS micelles have promising potential as MX carriers for oral drug delivery.

Acknowledgements

The authors would like to acknowledge the Commission of Higher Education (Thailand) and the Thailand Research Funds through the Golden Jubilee Ph.D. Program (Grant No. PHD/0027/2556), the Thailand Research Fund (TRG5480019), Thailand Center of Excellence for Life Sciences (TCELS; P-14-50431), the Thailand Toray Science Foundation (TTSF) in 2010, the Silpakorn University Research and Development Institute, and the National Nanotechnology Center (NANOTEC), Thailand.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.12.039.

References

Bastakoti, B. P., Wu, K. C.-W., Inoue, M., Yusa, S., Nakashima, K., & Yamauchi, Y. (2013). Multifunctional core–shell–corona-type polymeric micelles for anticancer drug-delivery and imaging. *Chemistry: A European Journal*, *19*, 4812–4817.

Bonferoni, M. C., Sandri, G., Dellera, E., Rossi, S., Ferrari, F., Mori, M., et al. (2014). Ionic polymeric micelles based on chitosan and fatty acids and intended for wound healing. Comparison of linoleic and oleic acid. *European Journal of Pharmaceutics and Biopharmaceutics*, *87*, 101–106.

Bromberg, L. (2008). Polymeric micelles in oral chemotherapy. *Journal of Controlled Release*, *128*(2), 99–112.

Daman, Z., Ostad, S., Amini, M., & Gilani, K. (2014). Preparation, optimization and in vitro characterization of stearoyl-gemcitabine polymeric micelles: A comparison with its self-assembled nanoparticles. *International Journal of Pharmaceutics*, *468*, 142–151.

Duangjit, S., Opanasopit, P., Rojanarata, T., & Ngawhirunpat, T. (2013). Evaluation of meloxicam-loaded cationic transfersomes as transdermal drug delivery carriers. *American Association of Pharmaceutical Sciences Technology*, *14*(1), 133–140.

Haynes, W. M. (2012). *Handbook of chemistry and physics* (93rd ed.). New York: CRC Press.

Hunter, A. C., Elsom, J., Wibroe, P. P., & Moghimi, S. M. (2012). Polymeric particulate technologies for oral drug delivery and targeting: A pathophysiological perspective. *Maturitas*, *73*, 5–18.

Huo, M., Zou, A., Yao, C., Zhang, Y., Zhou, J., Wang, J., et al. (2012). Somatostatin receptor-mediated tumor-targeting drug delivery using octreotide-PEG-deoxycholic acid conjugate-modified N-deoxycholic acid-O,N-hydroxyethylation chitosan micelles. *Biomaterials*, *33*, 6393–6407.

Jiang, G. B., Quan, D., Liao, K., & Wang, H. (2006). Novel polymer micelles prepared from chitosan grafted hydrophobic palmitoyl groups for drug delivery. *Molecular Pharmaceutics*, *3*, 152–160.

Kedar, U., Phutane, P., Shidhaye, S., & Kadam, V. (2010). Advances in polymeric micelles for drug delivery and tumor targeting-review article. *Nanomedicine: Nanotechnology, Biology and Medicine*, *6*, 714–729.

Kim, S., Kim, J. Y., Huh, K. M., Acharya, G., & Park, K. (2008). Hydrotropic polymer micelles containing acrylic acid moieties for oral delivery of paclitaxel. *Journal of Controlled Release*, *132*(3), 222–229.

Kim, H., & Lee, I. (2007). A novel drug delivery system design for meloxicam. *Journal of Pharmacy and Pharmaceutical Sciences*, *10*(3), 288–298.

Kim, S., Shi, Y., Kim, J. Y., Park, K., & Cheng, J. X. (2010). Overcoming the barriers in micellar drug delivery: Loading efficiency, in vivo stability, and micelle–cell interaction. *Expert Opinion on Drug Delivery*, *7*, 49–62.

Kumar, R., Kulkarni, A., Nagesha, D. K., & Sridhar, S. (2012). In Vitro evaluation of theranostic polymeric micelles for imaging and drug delivery in cancer. *Theranostics*, *2*(7), 714–722.

Lim, E. K., Sajomsang, W., Choi, Y., Jang, E., Lee, H., Kang, B., et al. (2013). Chitosan-based intelligent theragnosis nanocomposites enable pH-sensitive drug release with MR-guided imaging for cancer therapy. *Nanoscale Research Letters*, *8*(467), 1–12.

Liu, C. G., Desai, K. G., Chen, X. G., & Park, H. J. (2005). Linolenic acid-modified chitosan for formation of self-assembled nanoparticles. *Journal of Agricultural and Food Chemistry*, *53*, 437–441.

Lu, Y., & Park, K. (2013). Polymeric micelles and alternative nanonized delivery vehicles for poorly soluble drugs. *International Journal of Pharmaceutics*, *453*(1), 198–214.

Miller, T., Colen, G. V., Sander, B., Golas, M. M., Uezguen, S., Weigandt, M., et al. (2013). Drug loading of polymeric micelles. *Pharmaceutical Research*, *30*, 584–595.

Miwa, A., Ishibe, A., Nakano, M., Yamahira, T., Itai, S., Jinno, S., et al. (1998). Development of novel chitosan derivatives as micellar carriers of taxol. *Pharmaceutical Research*, *15*, 1844–1850.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cyto-toxicity assays. *Journal of Immunological Methods*, *65*, 55–63.

Muzzarelli, C., Tosi, G., Francescangeli, O., & Muzzarelli, R. A. A. (2003). Alkaline chitosan solutions. *Carbohydrate Research*, *338*, 2247–2255.

Muzzarelli, R. A. A., Boudrant, J., Meyer, D., Manno, N., DeMarchis, M., & Paoletti, M. G. (2012). Current views on fungal chitin/chitosan, human chitinases, food preservation, glucans, pectins and inulin: A tribute to Henri Braconnot, precursor of the carbohydrate polymers science, on the chitin bicentennial. *Carbohydrate Polymers*, *87*, 995–1012.

Ngawhirunpat, T., Wonglertnirant, N., Opanasopit, P., Ruktanonchai, U., Yoksan, R., Wasanasuk, K., et al. (2009). Incorporation methods for cholic acid chitosan-g-mPEG self-assembly micellar system containing camptothecin. *Colloids and Surfaces B: Biointerfaces*, *74*, 253–259.

Opanasopit, O., Ngawhirunpat, T., Rojanarata, T., Choochottiros, C., & Chirachanchai, S. (2007). Camptothecin-incorporating N-phthaloylchitosan-g-mPEG self-assembly micellar system: Effect of degree of deacetylation. *Colloids and Surfaces B: Biointerfaces*, *60*, 117–124.

Opanasopit, P., Ngawhirunpat, T., Chaidedgumjorn, A., Rojanarata, T., Apirakaramwong, A., Phongying, S., et al. (2006). Incorporation of camptothecin into N-phthaloyl chitosan-g-mPEG self-assembly micellar system. *European Journal of Pharmaceutics and Biopharmaceutics*, *64*, 269–276.

Rieux des, A., Fievez, V., Garinot, M., Schneider, Y.-J., & Préat, V. (2006). Nanoparticles as potential oral delivery systems of proteins and vaccines: A mechanistic approach. *Journal of Controlled Release*, *116*(1), 1–27.

Sajomsang, W., Gonil, P., Rungsardthong Ruktanonchai, U., Saesoo, S., Srinuanchai, W., & Puttipipatkhachorn, S. (2014). Synthesis and anticervical cancer activity of novel pH responsive micelles for oral curcumin delivery. *International Journal Pharmaceutics*, *477*, 261–272.

Sajomsang, W., Tantayanon, S., Tangpasuthadol, V., Thatte, M., & Daly, W. H. (2008). Synthesis and characterization of *N*-aryl chitosan derivatives. *International Journal of Biological Macromolecules*, *43*, 79–87.

Yokoyama, M. (2011). Clinical application of polymeric micelle carrier systems in chemotherapy and image diagnosis of solid tumors. *Journal of Experimental and Clinical Medicine*, *3*(4), 151–158.

Zhang, C., Qineng, P., & Zhang, H. (2004). Self-assembly and characterization of paclitaxel-loaded N-octyl-O-sulfate chitosan micellar system. *Colloids and Surfaces B: Biointerfaces*, *39*, 69–75.

Zhang, Y., Chen, J., Zhang, G., Lu, J., Yan, H., & Liu, K. (2012). Sustained release of ibuprofen from polymeric micelles with a high loading capacity of ibuprofen in media simulating gastrointestinal tract fluids. *Reactive and Functional Polymers*, *72*, 359–364.