

## Research Article

# Development of Chitosan-Based pH-Sensitive Polymeric Micelles Containing Curcumin for Colon-Targeted Drug Delivery

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**Abstract.** pH-sensitive *N*-naphthyl-*N,O*-succinyl chitosan (NSCS) and *N*-octyl-*N,O*-succinyl chitosan (OSCS) polymeric micelles carriers have been developed to incorporate curcumin (CUR) for colon-targeted drug delivery. The physical entrapment methods (dialysis, cosolvent evaporation, dropping, and O/W emulsion) were applied. The CUR-loaded micelles prepared by the dialysis method presented the highest loading capacity. Increasing initial amount of CUR from 5 to 40 wt% to polymer resulted in the increase in loading capacity of the polymeric micelles. Among the hydrophobic cores, there were no significant differences in the loading capacity of CUR-loaded micelles. The particle sizes of all CUR-loaded micelles were in the range of 120–338 nm. The morphology of the micelles changed after being contacted with medium with different pH values, confirming the pH-responsive properties of the micelles. The release characteristics of curcumin from all CUR-loaded micelles were pH-dependent. The percent cumulative release of curcumin from all CUR-loaded micelles in simulated gastric fluid (SGF) was limited to about 20%. However, the release amount was significantly increased after contacted with simulated intestinal fluid (SIF) (50–55%) and simulated colonic fluid (SCF) (60–70%). The released amount in SIF and SCF was significantly greater than the release of CUR from CUR powder. CUR-loaded NSCS exhibited the highest anti-cancer activity against HT-29 colorectal cancer cells. The stability studies indicated that all CUR-loaded micelles were stable for at least 90 days. Therefore, the colon targeted, pH-sensitive NSCS micelles may have potential to be a prospective candidate for curcumin delivery to the colon.

**KEY WORDS:** pH-sensitive polymeric micelles; curcumin; NSCS; OSCS; oral colon-targeted drug delivery.

## INTRODUCTION

Polymeric micelles (PMs) are nano-sized carriers prepared by self-assembly of amphiphilic copolymers in an aqueous solution. Generally, they have a spherical inner core and an outer shell (1). The pH levels in the gastrointestinal (GI) tract vary from 1 to 3 in the stomach to 6–7.5 in the small intestine, and the variation of the pH has been utilized to control drug release from drug delivery carriers (2–4). The pH-sensitive PMs offer many advantages for oral drug delivery including protecting drug from being destroyed in

the upper part of GI tract, increasing drug solubilization and releasing the drug in spatially controlled manner (4–6).

Curcumin (CUR) is a hydrophobic polyphenolic compound from the obtained rhizome of turmeric (*Curcuma longa* Linn), one of the most widely used natural active constituents. It exhibited numerous biological activities, for example, antibacterial, antifungal, anti-inflammatory, antioxidant, etc. (7,8). In addition, anti-cancer activity of curcumin has been extensively investigated for its potential to be used in chemoprevention and the treatment of a wide variety of tumors (9). The recent studies have revealed the inhibition effect of CUR on tumor formation by affecting the initiation and progression stage of carcinogenesis of colorectal cancer *in vivo* (10–12). Curcumin can interfere multiple cell signaling pathway involved in carcinogenesis, for instance, inhibiting cell cycle progression, slowing proliferation, reducing angiogenesis, and inducing cell apoptosis in colorectal carcinoma cell (13,14). However, the potential of curcumin is hindered by its low aqueous solubility (0.011 µg/mL in aqueous buffer at pH 5 and 0.4 µg/mL at the physiological pH, pH 7.4), low

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bioavailability, and rapid metabolism (9,15). In our previous study, *N*-naphthyl-*N,O*-succinyl chitosan (NSCS) and *N*-octyl-*N,O*-succinyl chitosan (OSCS) were successfully synthesized and used to form pH-sensitive polymeric micelles. The micelles could increase the dissolution of poorly soluble drug like meloxicam (16,17). Moreover, previous study showed that CUR-loaded *N*-benzyl-*N,O*-succinyl chitosan (BSCS) micelles prepared by dialysis method displayed intense cytotoxicity to cervical cancer cells with the high amount of the drug release in physiological pH 5.5–7.4 (18). Herein, we purposed the entrapment of CUR into pH-sensitive NSCS and OSCS PMs in order to targeted release of the drug at the colon. The physical entrapment methods including dialysis, O/W emulsion, dropping, and co-solvent evaporation were applied to prepare PMs. The entrapment efficiency, loading capacity, particle size, morphology, and stability of CUR-loaded micelles were investigated. In addition, the anti-cancer activity of CUR-loaded micelles against human colorectal adenocarcinoma cells (HT-29 cells) and their release behaviors were also evaluated.

## MATERIALS AND METHODS

### Materials

Chitosan (MW 10–13 kDa; 96% deacetylated) was obtained from National Nanotechnology Center (NANOTEC) (Pathumthani, Thailand). Curcumin, 2-naphthaldehyde, *N,N*-Dimethylformamide (DMF), Octaldehyde, sodium borohydride ( $\text{NaBH}_4$ ), and succinic anhydride were purchased from Sigma Aldrich (St. Louis, USA). Dialysis tube (Cellu Sep T1, MW cut-off = 6000–8000 Da) was obtained from Membrane Filtration Products INC (Texas, USA). The Caco-2 cell lines and HT-29 cell lines were obtained from the American Type Culture Collection (Rockville, USA). HPLC grade acetonitrile and methanol were purchased from RCI Labscan Limited (Bangkok, Thailand). All other chemicals and reagents were of analytical grade.

### Methods

#### *Synthesis Amphiphilic Derivatives of Chitosan*

NSCS and OSCS were synthesized by reductive amination (17). Firstly, 2-Naphthaldehyde or octaldehyde (2.0 meq/GlcN) was attached onto primary amino groups of chitosan *via* Schiff base reaction. The imine groups were transformed into the more stable amine with formation of the corresponding aryl or alkyl chitosan derivatives (*i.e.* *N*-naphthyl chitosan (NCS) and *N*-octyl chitosan (OCS)) by adding sodium borohydride. Secondly, *N,O*-succinylation was performed by reacting with succinic anhydride. The NCS or OCS was dispersed in the solvent mixture of dimethyl sulfoxide (DMSO)/DMF, (1:1, *v/v*). The succinic anhydride (5.0 meq/GlcN) was then added to the solution mixture. The reaction occurred under nitrogen atmosphere at 100°C for 24 h. After cooled to the room temperature, the excess organic solvent and succinic anhydride were removed through dialyzed against distilled water, and then lyophilized to obtain powder of NSCS or OSCS. The successful synthesis was

confirmed by  $^1\text{H}$  NMR, ATR-FTIR, and elemental analysis as previously reported (17).

The critical micelle concentration (CMC) in aqueous media of NSCS and OSCS was established using a fluorescence spectroscopy with pyrene as fluorescent probe. Briefly, an aliquot of 1 mM pyrene solution in acetone was added to the polymer solutions (4 mL,  $0.5\text{--}3.9 \times 10^{-3}$  mg/mL). The final concentration of pyrene in each polymer solution was  $2.5 \times 10^{-6}$  M. The mixtures were then sonicated, heated at 50°C for 2 h, and subsequently kept in the dark overnight. Fluorescence spectra were recorded at an excitation wavelength of 335 nm, and the emission spectra were observed over a range of 350–500 nm. The CMC was estimated by fitting the semi-log plot of the intensity ratio of the first and third vibration bands at 373 nm ( $I_1$ ) /382 nm ( $I_3$ ) *versus* the concentration as previously reported (17).

#### *Preparation of Polymeric Micelles*

CUR-loaded and blank polymeric micelles were prepared by various physical entrapment methods as reported in our previous work (16). In the dialysis method, 5 mg of grafted-copolymer and curcumin (0 to 40 wt% to polymer) was dissolved in 2 mL of DMSO. Then the polymer solution was dialyzed against deionized (DI) water for 24 h. In O/W emulsion method, the blank polymeric micelles were prepared using dialysis method. Then, curcumin (5 to 40 wt% to polymer) dissolved in dichloromethane (DCM) was injected into the micellar solution. After that, DCM was evaporated by stirring at room temperature overnight. In the dropping method, the copolymer (5 mg) and curcumin (0 to 40 wt% to polymer) were dissolved in 0.5 mL of DMSO, and the solution was slowly added into DI water (2.5 mL). The solution was stirred overnight and dialyzed against DI water for 24 h. In the co-solvent evaporation method, the copolymer (5 mg) and curcumin (0 to 40 wt% to polymer) were dissolved in a solvent mixture of DMF and acetone (3:1). The mixed solution was stirred under nitrogen gas at room temperature until completely evaporated. Afterwards, DI water (3 mL) was added before the solution was sonicated using a probe sonicator (Sonics Vibra Cell TM, CT). The mixture was centrifuged at 1000 rpm for 10 min and filtered through 0.45- $\mu\text{m}$  membrane filters to obtain the micellar solution.

#### *In vitro Cytotoxicity Assay*

The cell viability of the Caco-2 cells and HT-29 cells after being treated with the blank polymeric micelles was determined using methyl tetrazolium (MTT) assay. The Caco-2 cells and HT-29 were cultured and seeded in 96-well plate at a density of  $10^4$  cells per well. Then, the cells were incubated with the micelles at varied concentrations. After 24 h of incubation, the solution was replaced by the fresh culture medium. The MTT solution was added (final concentration 1 mg/mL) and incubated for 4 h. The medium was removed, and 100  $\mu\text{L}$  of DMSO was added to solubilize the formazan crystal formed in the living cells. Relative cell viability was determined by measuring the absorbance at 550 nm using a microplate reader (Universal Microplate Analyzer, USA).

### CUR-Content Determination

To determine the quantity of curcumin in the CUR-loaded polymeric micelles, 100  $\mu\text{L}$  of the CUR-loaded micelles was added into 900  $\mu\text{L}$  of DMSO:H<sub>2</sub>O (9:1) to break the micelles. The content of curcumin was quantified using a high-performance liquid chromatograph (HPLC) (Agilent Technologies, USA) with a UV-visible detector operating at 428 nm and a Phenomenex® C18 column (150  $\times$  4.60 mm, 5  $\mu\text{m}$  particle size). The injection volume and the flow rate were 20  $\mu\text{L}$  and 1 mL/min, respectively. The mobile phase was acetonitrile:1% *v/v* acetic acid (43:57, *v/v*). The entrapment efficiency (EE) and loading capacity (LC) were determined according to Eqs. (1) and (2), respectively.

$$\text{EE (\%)} = \left( \frac{\text{amount of CUR in micelles/}}{\text{initial of amount CUR used}} \right) \times 100\% \quad (1)$$

$$\text{LC (\mu g/mg)} = \left( \frac{\text{amount of CUR in micelles/}}{\text{(amount of copolymer + amount of CUR)}} \right) \quad (2)$$

### Particle Size, Zeta Potential, and Morphology of CUR-Loaded Polymeric Micelles

The particle size and zeta potential of CUR-loaded polymeric micelles in different pH medium (pH 1.2, 5.0, and 6.8) were determined using a dynamic light scattering (DLS) (Malvern, Worcestershire, UK) at 25°C. On the other hand, the morphology of CUR-loaded OSCS polymeric micelles was observed under different pH conditions (pH 1.2, 5.0, and 6.8) using an atomic force microscopy (AFM).

### Structural Stability of CUR-Loaded Polymeric Micelles

The structure stability of CUR-loaded micelles was investigated using gel permeation chromatography (GPC) as previously reported (19). GPC was performed using an Agilent HPLC system equipped with a Shodex® GFC SB804 HQ column at 40°C. Deionized water was used as the mobile phase with a flow rate of 1 mL/min. Detection was performed using refractive index (RI) and UV detector at 428 nm. The micelles were freshly prepared and filtered through a 0.45- $\mu\text{m}$  membrane filter prior to the measurement.

### In vitro Release Study

The release characteristics of curcumin from CUR-loaded micelles were evaluated using a dialysis method. One milliliter of CUR-loaded micelles was transferred to a dialysis bag. The dialysis bag was immersed into 0.1 N HCl containing 30% (*v/v*) methanol and 1% (*v/v*) Tween 20 under constant stirring with sink conditions at 37  $\pm$  0.5°C for 2 h. Afterwards, the pH was adjusted to 6.8 for 3 h and followed by pH 7.4 for 8 h. At the predetermined time intervals of 1, 2, 3, 4, 5, 6, and 8 h, 1 mL aliquot of the medium was withdrawn, and fresh

medium in the same volume was added. The sample solution was analyzed by HPLC (18).

### Chemical Stability Test

The stability of 20% initial CUR-loaded polymeric micelles after lyophilization process was evaluated according to ICH guideline for storage under accelerated condition (25°C  $\pm$  2°C and 60  $\pm$  5% relative humidity; RH) comparing with long-term condition in refrigerator (5°C  $\pm$  3°C) for 90 days. The amount of curcumin from CUR-loaded polymeric micelles was determined after being kept for 0, 30, and 90 days.

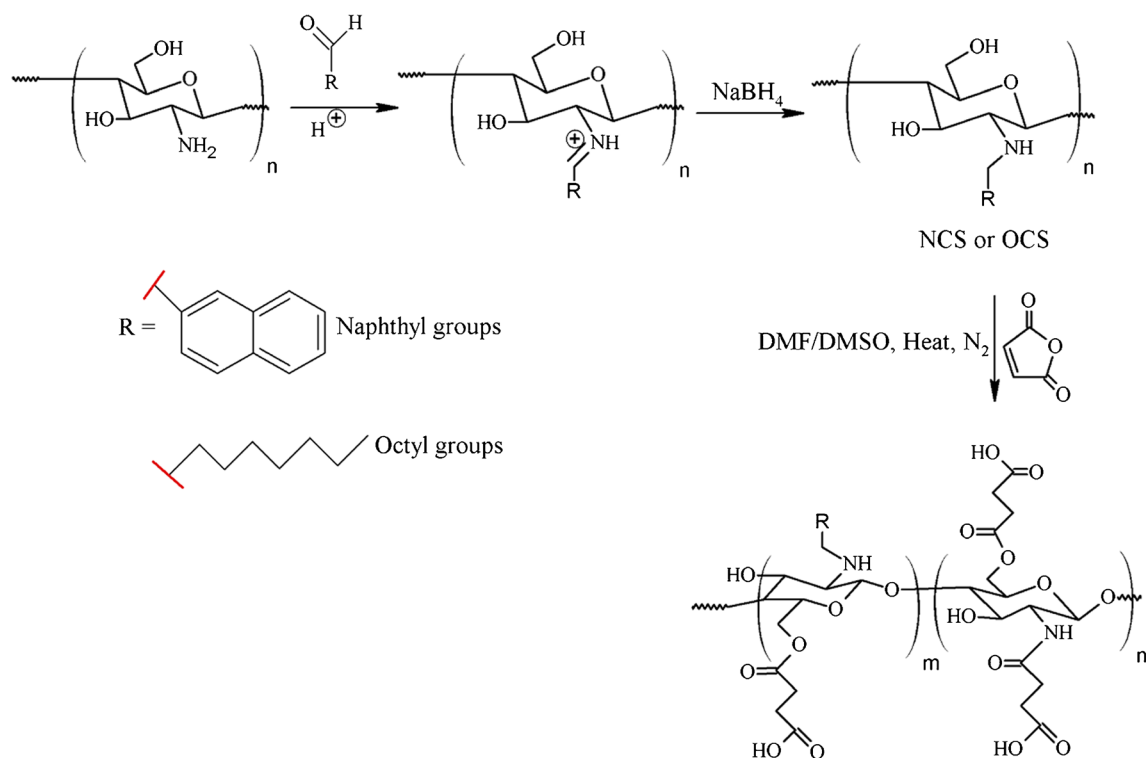
### Anti-cancer Activity

The anticancer activities of the CUR-loaded polymeric micelles and pure CUR were performed using MTT assay. HT-29 cells were cultured and seeded into 96-well plates. The cells were incubated for 24 h at a density of 10<sup>4</sup> cells per well. After 24 h, the cells were treated with fresh medium containing different concentrations of samples and incubated at 37°C and 5% CO<sub>2</sub> for 24 h. The cells were investigated using MTT test as mentioned above.

## RESULTS AND DISCUSSION

### Preparation of Blank and CUR-Loaded Polymeric Micelles

The NSCS and OSCS were successfully produced by reductive N-amination and N,O-succinylation reactions, as illustrated in Fig. 1. The NSCS and OSCS are composed of hydrophobic and hydrophilic segments on the chitosan backbones, being able to form micelles through self-assembly. The CUR-loaded polymeric micelles were prepared by four different methods including dialysis, co-solvent evaporation, dropping, and O/W emulsion. The CMC for NSCS and OSCS was observed to be 0.068 and 0.086 mg/mL, respectively, which was lower than the CMC value of low molecular weight surfactants as reported in our previous publications (16,17,19). The copolymer used to prepare the polymeric micelles should be non-toxic. Generally, chitosan is considered to be biocompatible and safe. However, some studies reported about the cytotoxic effects of chitosan derivatives (20,21). The Caco-2 cells, appeared to be functionally similar to intestinal epithelium, are commonly used as *in vitro* model for cytotoxicity investigation or prediction of intestinal drug absorption (22,23). Moreover, HT-29 cells were selected as model for the study relating to colon cancer. The viability of Caco-2 cells and HT-29 treated with the various concentrations of blank NSCS and OSCS micelles is displayed in Fig. 2. The half maximal inhibitory concentration (IC<sub>50</sub>) value of the NSCS and OSCS micelles in Caco-2 cells was 3.08  $\pm$  0.15 and 2.95  $\pm$  0.06 mg/mL, respectively. No significant difference in cytotoxic effect of NSCS and OSCS micelles in the Caco-2 cells has been observed. Chae *et al.* (2005) reported that low molecular weight chitosan (3.8–13 kDa; DDA 87–92%) with concentrations lower than 5 mg/mL had low toxicity to Caco-2 cell after 2 h of incubation (24). This revealed that chitosan micelles had low cytotoxic effect on Caco-2 cells, indicating the excellent biocompatible. Moreover, the blank NSCS and

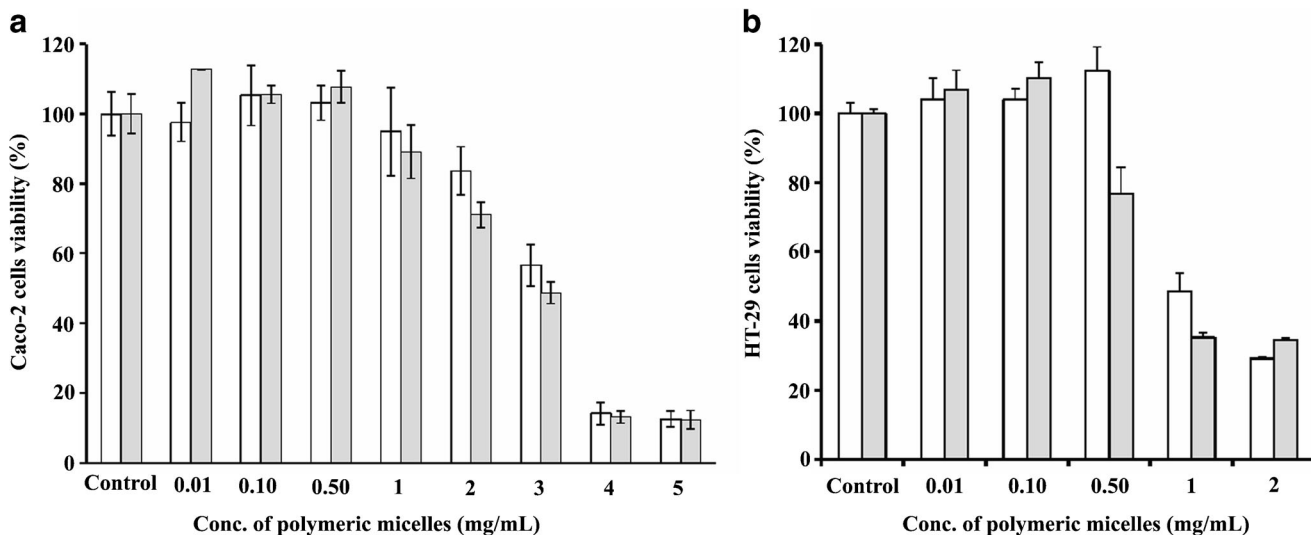


**Fig. 1.** Schematic illustration of the synthesis of *N*-naphthyl-*N,O*-succinyl chitosan (NCS) and *N*-octyl-*N,O*-succinyl chitosan (OSCS)

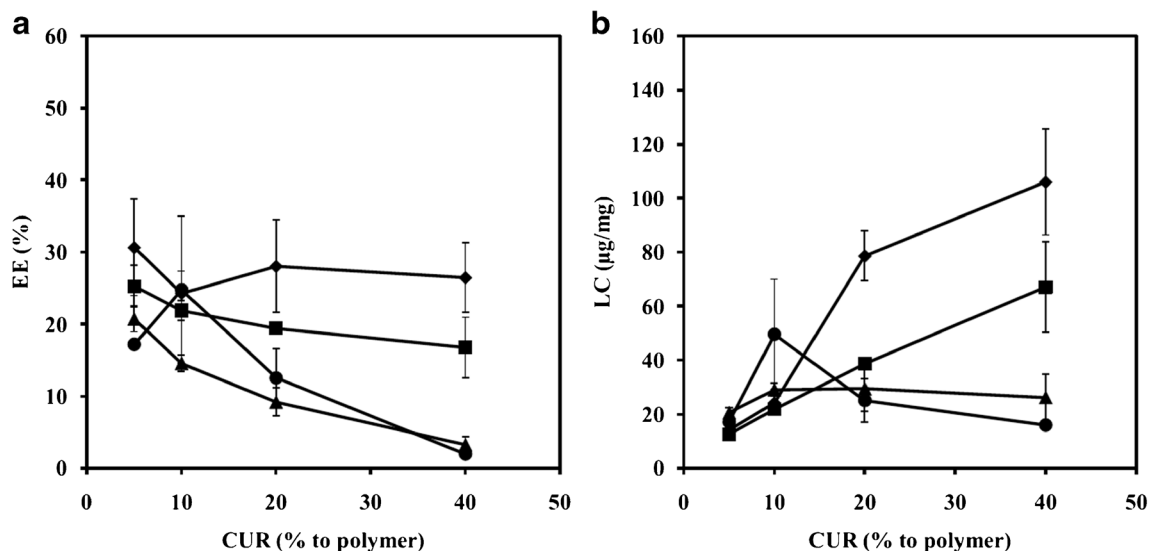
OSCS micelles showed minimal cytotoxicity in HT-29 cells at the concentration up to 0.5 mg/mL. These findings indicated that the blank micelles may be regarded as a safe drug carrier.

Figure 3 showed %EE and LC of CUR-loaded NCS micelles prepared by various physical entrapment methods. The preparation methods and initial drug concentrations (5 to 40 wt% to polymer) had an influence on the %EE and LC. The CUR-loaded NCS prepared by dialysis method showed the highest EE (24–30%) and LC (14–105  $\mu\text{g}/\text{mg}$ ), followed by evaporation method (%EE 16–25%; LC 12–67  $\mu\text{g}/\text{mg}$ ), dropping method (%EE 2–24%; LC 16–49  $\mu\text{g}/\text{mg}$ , and O/W

emulsion method (%EE 3–20%; LC 20–29  $\mu\text{g}/\text{mg}$ ), respectively. It was found that various factors in drug incorporation process are important factors in controlling incorporation efficiency (25,26). The hydrophobic interactions force between hydrophobic moieties of copolymers and CUR, and the miscibility between copolymers and drugs are involved in drug encapsulation (27,28). If CUR interacts more favorably with the hydrophobic polymer chain than with solvent, high incorporation efficiency can be obtained. However, if CUR molecules interact with each other with greater affinity than with the hydrophobic polymer chain, CUR will precipitate



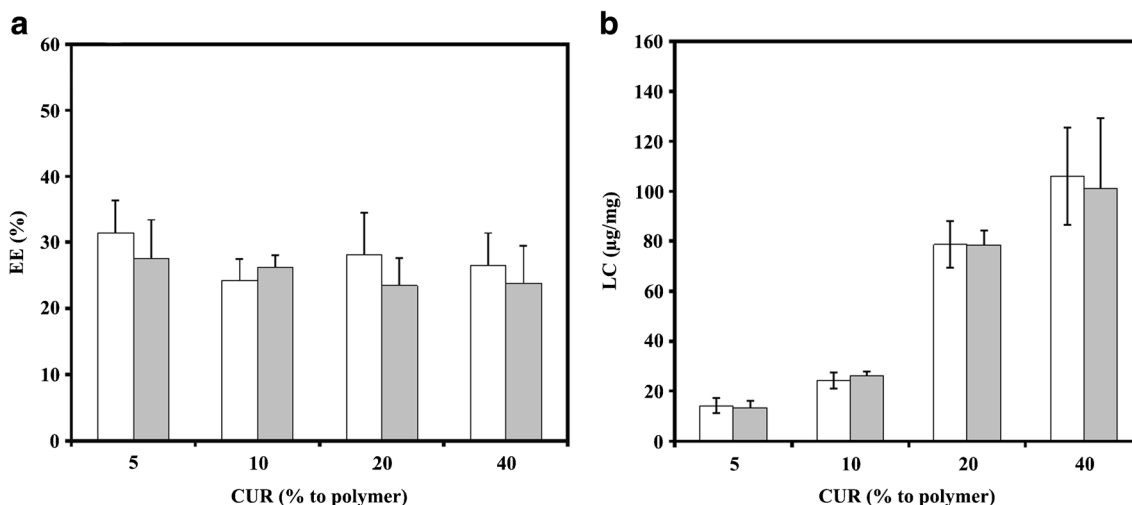
**Fig. 2.** The percent cell viability in **a** Caco-2 cells and **b** HT-29 cells at varying concentrations of polymeric micelles; NCS (white bar graph), OSCS (shaded bar graph). Each value represents the mean  $\pm$  SD of five wells



**Fig. 3.** Effect of entrapment method and initial drug concentration (5–40% to polymer) on **a** the entrapment efficiency (EE), **b** loading capacity (LC) of CUR-loaded NSCS micelles (circle) dialysis method; (square) dropping method; (diamond) co-solvent evaporation method; (triangle) emulsion method. Data are plotted as the mean  $\pm$  S.D. of three measurements.

rather than being incorporated into micelles. In this study, NSCS and OSCS were employed as micelle forming polymers because chitosan is hydrophilic and cannot interact with CUR molecules *via* hydrophobic interactions. On the other hand, NSCS and OSCS showed high-micelle structure stability upon the CUR incorporation. This suggests a large contribution of hydrophobic interaction between CUR molecules and the hydrophobic inner core (naphthyl and octyl group) of the NSCS and OSCS polymers. In addition, the type of organic solvent and preparation technique had a strong influence on drug loading as well as particle sizes (25). As can be noticed from Fig. 3, two different patterns of the graphs can be observed. The %EE of CUR-loaded NSCS micelles prepared by both dropping method and emulsion method decreased after increasing the initial concentration of CUR. This may be due to the saturation of the drug in the micelles at high initial

drug concentration. However, CUR-loaded NSCS micelles prepared by dialysis method and evaporation method demonstrated different pattern. Increasing the initial drug concentration (from 5 to 40 wt% to polymer) led to the further increase in LC with the similar %EE (Fig. 4). This is because the NSCS and OSCS micelles prepared by dialysis method and evaporation method can encapsulate CUR and form drug nanocrystals (> 100 wt% to polymer) (unpublished data). The loading capacity of CUR-loaded NSCS micelles prepared by both dropping method and emulsion method reaches a plateau at the initial CUR concentrations of 10 wt% to polymer. On the other hand, the CUR-loaded NSCS prepared by dialysis method and evaporation method showed the incessant increase in loading capacity after increasing the initial amount of CUR (5 to 40 wt% to polymer). These results indicated that these later two methods might be



**Fig. 4.** Effect of hydrophobic moieties and initial drug loading (5–40% to polymer) on **a** the entrapment efficiency (EE), **b** loading capacity (LC) of CUR-loaded polymeric micelles, CUR-loaded NSCS (white bar graph), and CUR-loaded OSCS (shaded bar graph). Data are plotted as the mean  $\pm$  SD of three measurements

suitable for CUR loaded into NSCS micelles. However, adding greater initial amount of CUR (> 40 wt% to polymer) would lead to the creation of other nanocarriers such as nanocrystals as mentioned above. At the same initial curcumin concentration to polymer, the dialysis method exhibited higher drug loading percentage than any other methods. It was, therefore, selected for the further studies.

### Characterization of Polymeric Micelles

The particle sizes, PDI, and zeta potential of CUR-loaded NSCS PMs and blank NSCS PMs were determined, and the results are presented in Table I. The different physical methods influenced the size of the polymeric micelles. The mean particle size of CUR-loaded NSCS micelles prepared by dropping method was smallest ranged from 120 to 181 nm, followed by O/W emulsion (148–166 nm), dialysis (201–321 nm), and co-solvent evaporation (289–338 nm). The results showed that the mean particle sizes of drug-loaded micelles were larger than those of blank micelles, because the CUR was entrapped in the copolymer micelles. The particle sizes of CUR-loaded OSCS micelles prepared by the dialysis method were in the range of 163–255 nm. Increasing the initial amount of CUR resulted in the increase in the sizes of the CUR-loaded micelles. The mean particle sizes of the CUR-loaded NSCS and OSCS were different, that might be due to the difference in the copolymer composition (29). The blank and CUR-loaded micelles possess negative charges (−27 to −32 mV) due to carboxyl groups (COO<sup>−</sup>) of the succinic acid (Table I).

The morphology of the pH-sensitive PMs was observed under different pH conditions (pH 1.2, 5.0, and 6.8) by AFM and DLS techniques. In this case, the CUR-loaded OSCS was selected for the investigation, and the results are shown in Fig. 5. At pH 1.2, the aggregation of the micelles with particle sizes of about 1072 nm has been observed. This may be due to the unionized carboxyl groups and intermolecular hydrogen bond formation of succinic acid. As it can be seen from AFM images, self-assembled micelles at pH 5.0 appeared to be spherical shape, and the size was around 237 nm. As the pH increased to 6.8, the mean particle size of CUR-loaded OSCS increased to 285 nm due to the effect of deprotonation. This result was in accordance with the previously reported (17) and revealed the pH-sensitivity property of CUR-loaded micelles.

The structure stability of CUR-loaded PMs was evaluated by GPC equipped with reflective index (RI) and UV detector. This *in vitro* GPC method was found to well correlate with *in vivo* study, and it is a good technique for screening the structure stability of drug-loaded PMs (19,30). With this GPC method, PM peak and CUR peak can be detected by the RI detector and the UV detector, respectively. If CUR is loaded into PMs, both CUR (from UV detector) and PMs (from RI detector) will be detected at the same retention time. The result showed that the peak of micelles detected by RI displayed the similar retention time (4.2 min) to the peak detected by UV absorption at 428 nm. This revealed that all the samples (CUR-loaded NSCS or OSCS PMs) had polymeric micelle structures and contained CUR inside the micelles. In addition, the peak area obtained from UV detector at 428 nm represents the amount of CUR loaded into the micelles. The higher ratio of the peak area to CUR concentration, [CUR], (determined by HPLC) indicates

**Table I.** The Particle Sizes, PDI, and Zeta Potential of NSCS Micelles With and Without curcumin. Each Value Represents the Mean ± SD From Three Independent Measurements

CUR to polymer	Dialysis			Dropping			O/W emulsion			Co-solvent evaporation		
	Particle size (nm)	PDI	Zeta potential	Particle size (nm)	PDI	Zeta potential	Particle size (nm)	PDI	Zeta potential	Particle size (nm)	PDI	Zeta potential
0	196.20 ± 1.51	0.061	-29.23 ± 1.25	84.31 ± 0.88	0.198	-31.53 ± 0.81	—	—	—	233.50 ± 7.07	0.384	-30.37 ± 0.38
5	201.17 ± 1.90	0.239	-28.73 ± 1.36	129.97 ± 3.79	0.297	-31.57 ± 0.42	148.37 ± 2.93	0.239	-31.37 ± 0.38	289.13 ± 27.71	0.453	-30.80 ± 0.36
10	224.53 ± 2.54	0.237	-27.43 ± 0.65	181.47 ± 18.69	0.451	-31.23 ± 0.61	150.73 ± 2.24	0.214	-31.00 ± 1.39	326.47 ± 21.66	0.402	-31.67 ± 0.21
20	274.43 ± 3.20	0.353	-30.43 ± 0.59	120.20 ± 6.46	0.452	-32.40 ± 1.56	156.50 ± 1.99	0.230	-29.37 ± 0.33	317.40 ± 23.61	0.374	-31.50 ± 1.73
40	321.13 ± 2.41	0.239	-31.03 ± 0.47	124.30 ± 5.68	0.455	-32.50 ± 0.70	166.93 ± 0.59	0.215	-30.63 ± 0.61	338.47 ± 6.36	0.358	-31.73 ± 1.46

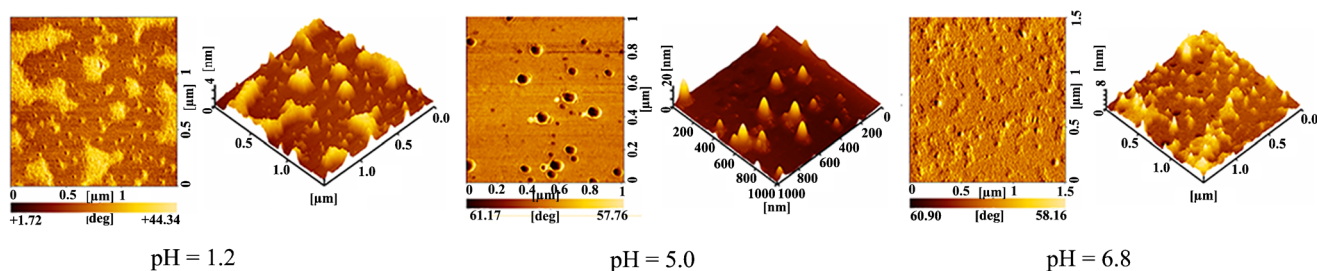


Fig. 5. AFM images of CUR-loaded OSCS micelles at different pH

that CUR was more stably incorporated into the micelles. In contrast, the low ratio of the peak area to [CUR] implies that most of the CUR was adsorbed to the GPC column by hydrophobic interactions due to unstable packing of CUR in the micelles (31). Figure 6 showed the structural stability of CUR-loaded PMs as evaluated by GPC. The ratios of the peak area to [CUR] of all CUR-loaded PMs decreased with the increase in the initial amount of CUR. PMs prepared from NSCS showed greater CUR stability than those prepared from OSCS at the similar initial amount of CUR (Fig. 6). At 5% CUR-loaded, the NSCS micelles exhibited the highest values of peak area/[CUR] which indicated that the initial drug used in the preparation and hydrophobic moieties were necessary to form stable CUR-loaded PMs. To be more specific, the naphthyl group of NSCS could generate more stable micelles than the octyl group of OSCS. This implied that not only the hydrophobic force but also a large contribution of  $\pi$ - $\pi$  interaction between the phenyl groups of CUR molecules and the naphthyl groups of NSCS is involved in the structure stability of the drug-loaded micelles.

### *In vitro* Release Study

Although several studies have shown that curcumin may possess as a potential preventive or therapeutic agent for colorectal cancer, its poor bioavailability is the important drawback which limits its efficacy (13,14). To

overcome this issue, pH-sensitive polymeric micelles were developed for curcumin delivery to the colon cancer. Based on the fact that the pH in the stomach is 1–2, in the small intestine is 5.1–7.5, and in the colon is 7–7.5 (26,32), the release behavior of curcumin at the fixed amount of drug from the pH-sensitive PMs with different grafted copolymers and CUR free drug was evaluated at in these pH environments (simulated gastric fluid (SGF) pH 1.2, simulated intestinal fluid (SIF) pH 6.8, and simulated colonic fluid (SCF) pH 7.4) in order to mimic the GI tract. The release profiles in different media are presented in Fig. 7. The time interval for three different stages was at 1–2 h in SGF, then 3–5 h in SIF, and 6–8 h in SCF. The results revealed that the release rate of curcumin from all pH-sensitive CUR-loaded PMs was relatively low at acidic pH 1.2 (SGF), with about 20% of amount of curcumin released after 2 h. This may be because of the poor solubility of the drug. After that, the increased amount of curcumin released in SIF (approximately 50–55%; 5 h) and SCF (approximately 60–70%; 8 h) due to the swelling and dissociation of PMs caused by the ionization of the carboxyl groups from succinic acid moiety at the higher pH conditions. Moreover, CUR has three ionizable protons caused by the enolic proton (approximate pKa of 8.54) and two phenolic OH<sup>-</sup> groups (pKa of 9.30 and 10.69) (33,34). As considered from the pKa value, CUR is not ionized at pH 1.2. However, at the

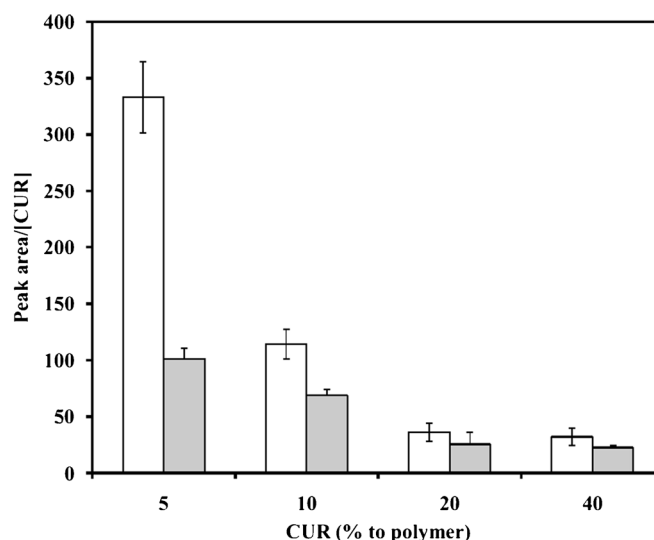
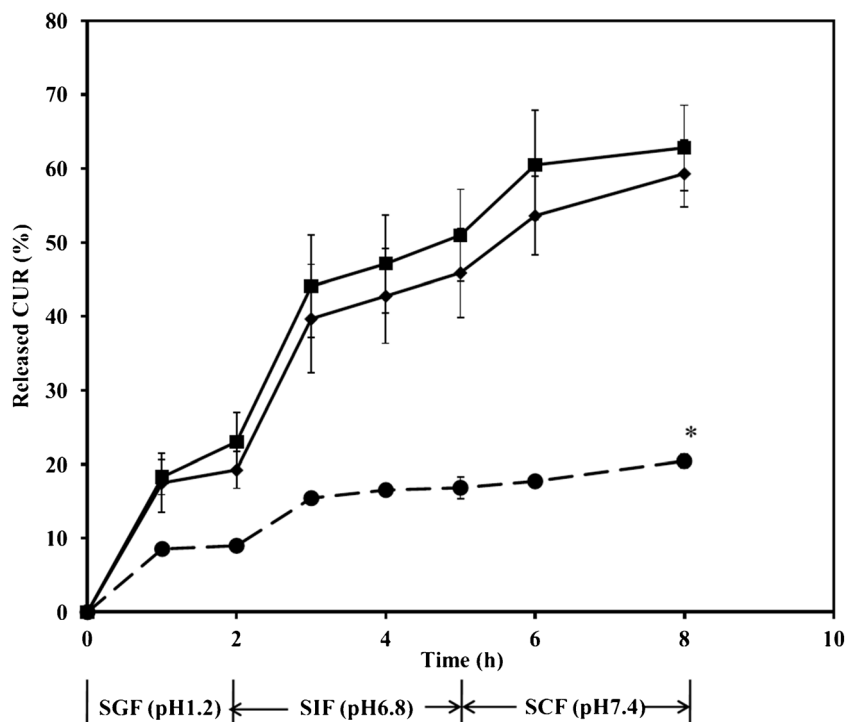


Fig. 6. Structure stability of CUR-loaded NSCS (white bar graph) and CUR-loaded OSCS (shaded bar graph) evaluated by gel permeation chromatography (GPC). Data are plotted as the mean  $\pm$  SD of three measurements



**Fig. 7.** CUR release profiles from the (diamond) CUR-loaded NSCS, (square) CUR-loaded OSCS, and (circle) CUR suspension in 0.1 N HCl, pH 1.2 (0–2 h), then in PBS pH 6.8 (2–5 h) and then, in PBS pH 7.4 (5–8 h). Data are plotted as the mean  $\pm$  SD of three measurements. \*Statistically significant ( $p < 0.05$ )

higher pH value of 6.8 and 7.4, CUR may partially ionized leading to the increase in the release of CUR from the micelles. Previous study reported that the apparent solubility values of CUR were 132, 152, and 348  $\mu\text{g/mL}$  in 0.1 N HCl, water, and solution pH 7.4, respectively (35). Therefore, it is possible that higher release at high pH might be also due to the change of CUR structure or dissociation of CUR. The accumulative release of curcumin from the all CUR-loaded PMs in SCF was significantly higher than that of CUR from free drug (approximately 20%). These results indicated that all pH-

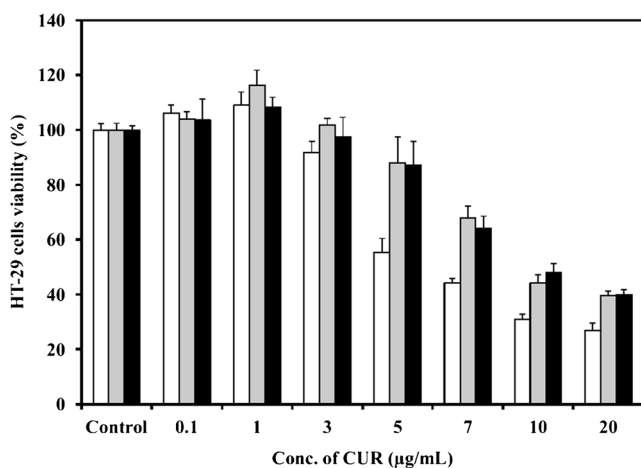
sensitive PMs may be a prospective candidate as colon delivery carrier for the efficient delivery of CUR.

#### Anti-cancer Activity

The CUR-loaded NSCS, OSCS, and CUR free drug were evaluated for their anti-cancer activity against the HT-29 cells using the MTT assay. As shown in Fig. 8, the different concentrations of the CUR-loaded PMs and CUR free drug (0.1–20  $\mu\text{g/mL}$ ) demonstrated cytotoxicity on HT-29 cells in dose-dependent manner. After treatment, the  $\text{IC}_{50}$  values of all the self-assembled PMs and free drug were calculated. The CUR-loaded NSCS exhibited higher extent of inhibition of cell viability ( $\text{IC}_{50}$  6.18  $\pm$  0.18  $\mu\text{g/mL}$ ) than CUR free drug ( $\text{IC}_{50}$  11.38  $\pm$  3.07  $\mu\text{g/mL}$ ). On the other hand, the CUR-loaded OSCS ( $\text{IC}_{50}$  9.29  $\pm$  0.44  $\mu\text{g/mL}$ ) showed the similar level of inhibition to the free CUR. This result indicated that the cytotoxicity of the CUR-loaded NSCS was more potent than that of CUR free drug in the growth suppression of the HT-29 cell lines studied.

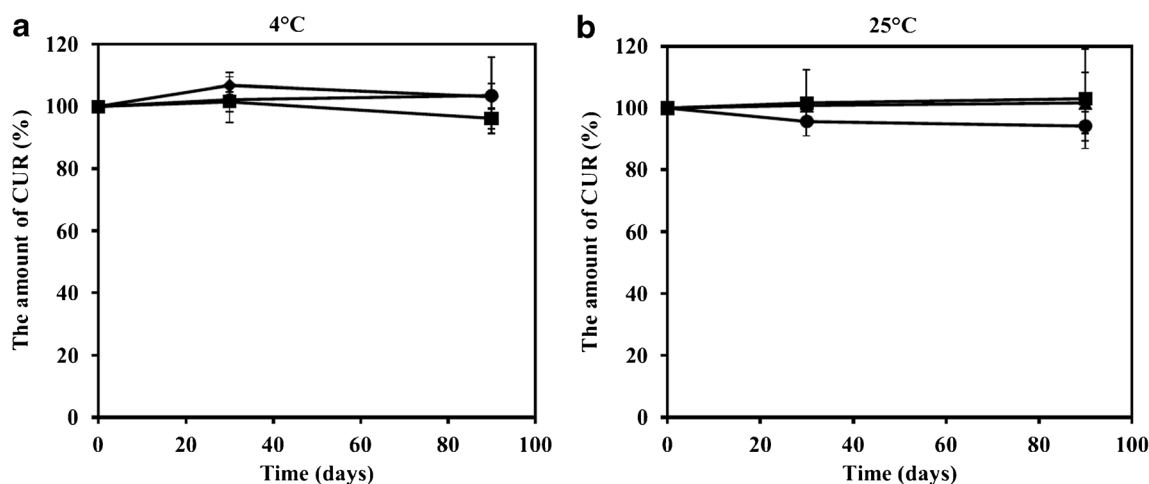
#### Chemical Stability Test

The chemical stability of CUR-loaded NSCS, OSCS, and CUR free drug was evaluated under the accelerated conditions (at 25°C) compared with the long-term conditions (at 4°C) for 90 days. Figure 9 showed that the percentage of an amount of CUR from all CUR-loaded polymeric micelles under both conditions was higher than 80%. There was no significant difference in the amount of in micelles and CUR free drug under this storage drug condition. This indicated that CUR-loaded polymeric micelles exhibited high stability.



**Fig. 8.** Anti-cancer activity of the CUR-loaded NSCS (white bar graph), the CUR-loaded OSCS (shaded bar graph), and CUR free drug (black bar graph) against HT-29 cells by MTT assay. Each value represents the mean  $\pm$  SD of five wells





**Fig. 9.** The chemical stability of (diamond) the CUR-loaded NSCS, (square) the CUR-loaded OSCS, and (circle) free CUR drug under the accelerated conditions (at 25°C) compared with the long-term conditions (at 4°C) for 90 days. Data are plotted as the mean  $\pm$  SD of three measurements

## CONCLUSION

The NSCS and OSCS were successfully synthesized and used for the incorporation of CUR. The CUR-loaded PMs prepared by the dialysis method presented the highest loading capacity. The release of CUR from the all PMs could be controlled by the pH values of the GI tract. The NSCS and OSCS PMs had low cytotoxicity on Caco-2 cells and HT-29 cells. However, the CUR-loaded NSCS exhibited desirable anti-cancer activity against HT-29 colorectal cancer cells. All CUR-loaded PMs were stable for at least 90 days. From these aspects, the pH-sensitive NSCS micelles may have the potential to be a desirable candidate for targeted drug delivery to the colon. Nevertheless, for clinical application of these CUR-loaded PMs, *in vivo* experiments are needed to be evaluated.

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## COMPLIANCE WITH ETHICAL STANDARDS

**Conflict of Interest** The authors declare that they have no conflict of interest.

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