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Original Research Article

Preparation and characterization of N-benzyl-N,O-succinyl chitosan polymeric micelles for solubilization of poorly soluble non-steroidal anti-inflammatory drugs

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Abstract

Purpose: *To investigate the solubilization of poorly water-soluble non-steroidal anti-inflammatory drugs (NSAIDs) in N-benzyl-N,O-succinyl chitosan (BSCS) polymeric micelles*
Methods: *BSCS was synthesized by reductive amination and succinylation, respectively. NSAIDs; meloxicam (MX), piroxicam (PRX), ketoprofen (KP) and indomethacin (IND) were entrapped in the hydrophobic inner cores by evaporation method. The effects of drug structure on loading efficiency, particle size and surface charge of micelles were investigated.*
Results: *The critical micelle concentration of BSCS micelles was 0.0385 mg/mL and cytotoxicity on Caco-2 cells depends on the polymer concentration (IC_{50} = 3.23 ± 0.08 mg/mL). BSCS micelles were able to entrap MX, PRX, KP and IND and also improve the solubility of drugs. Drug loading efficiency was highly dependent on the drug molecules. The drug loading capacity of these BSCS micelles was in the following rank order: KP (282.9 µg/mg) > PRX (200.8 µg/mg) > MX (73.7 µg/mg) > IND (41.2 µg/mg). The highest loading efficiency was observed in KP-loaded BSCS micelles due to the attractive force between phenyl groups of KP and benzyl groups of the polymer. Particle size and surface charge were in the range of 312 - 433 nm and -38 to -41 mV, respectively.*
Conclusion: *BSCS copolymer presents desirable attributes for enhancing the solubility of hydrophobic drugs. Moreover, BSCS polymeric micelles might be beneficial carrier in a drug delivery system.*

Keywords: *BSCS, polymeric micelles, solubilization, non-steroidal anti-inflammatory drugs*

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INTRODUCTION

One-third of the drugs identified are poorly water soluble, which is one of the important hindrances for successful development and the therapy of the orally administered drug effective [1,2]. Based on the Biopharmaceutics Classification System (BCS), these drugs could be classified as class II and class IV. The rate and extent of absorption as well as the bioavailability of class II and IV drugs are ultimately depends on solubility of the drugs[3].The most commonly used techniques for increasing the drug solubility are solid dispersions, cosolvency, pH adjustment, micellization, etc [4].

The core-shell polymeric micelles is an alternative technique to dissolve hydrophobic

drugs. Drug-incorporated polymeric micelles can be formulated from amphiphilic copolymer with entrapped drug in the hydrophobic inner core and hydrophilic segment surrounding aqueous medium to stabilize the micelles. There has been great importance in the use of polymeric micelles as drug carriers [5]. The successful of hydrophobic drugs loaded into polymeric micelles can solubilize 10-5000 fold in aqueous solutions [6].

Chitosan (CS) and its derivatives are currently receiving attention in medical and pharmaceutical applications because of biodegradable, biocompatible and less expensive [7,8]. Generally, CS dissolve in aqueous acidic media. However, it cannot formulate polymeric micelles in water. CS derivatives have been modified in order to improve the solubility and employed in various formulations for the delivery of hydrophobic drugs such as microspheres, nanoparticles [9-11]. In our previous work, pH sensitive polymers from chitosan-based were successfully synthesized, and applied in oral curcumin [12] and oral meloxicam (MX) deliveries [13]. The evaporation method presented higher MX incorporated into NSCS polymeric micelles than other entrapment methods. In addition, the cytotoxicity of BSCS micelles had low on Caco-2 cells [14]. Therefore, in this experiment, BSCS was synthesized and used to entrap four NSAIDs with poorly water solubility, including MX, piroxicam (PRX), ketoprofen (KP) and indomethacin (IND) as model drugs in the hydrophobic core of the polymeric micelles by evaporation method to investigate the effect of drug structure on the loading efficiency and solubility improvement.

EXPERIMENTAL

Materials

Chitosan (CS) (DDA = 96±2 % as investigated using NMR) was obtained from OilZac Technologies (Bangkok, Thailand) [12,13]. Benzaldehyde, sodium borohydride ($NaBH_4$), succinic anhydride, MX, PRX, KP and IND were procured from Sigma Aldrich (St. Louis, USA). Dialysis tube (MWCO = 3500 Da) was obtained from Cellu Sep T1 (Segiun, USA).The Caco-2 cell line was obtained from the American Type Culture Collection (Rockville, USA). All other substances were of analytical grade.

Synthesis of amphiphilic derivatives of chitosan

The modified chitosan derivative, *N*-benzyl-*N,O*-succinyl chitosan (BSCS), was produced using reductive benzylation and succinylation, respectively, for grafting hydrophobic and hydrophilic functional groups onto the CS structure as described in the literature [12,14]. Firstly, Benzaldehyde (2.0 meq/GlcN) was added in dissolving CS solution. The mixture solution was then stirred for 24 h at room temperature, and adjusted pH to 5 by 1 M sodium hydroxide. The reaction mixture was converted by sodium borohydride (52.9 mmol) to obtain the N-benzyl chitosan (BCS). Next step, BCS was dispersed in N,N-dimethylformamide (DMF). Succinic anhydride (5.0 meq/GlcN) was added in BCS solution via *N,O*-succinylation reaction under nitrogen atmosphere for 24 h at 100°C. After cooling process, the reaction mixture was filtered and dialyzed against deionized water to obtain the clear solution. Finally, the obtained amphiphilic CS derivative was dried to collect by freeze dehydration.

Characterization amphiphilic derivatives of chitosan

The CS or BSCS sample was measured ^{1}H NMR spectrometer (Bruker, Switzerland). Tetra-methylsilane (TMS) was exploited as an internal standard [13]. Fourier transform infrared (FTIR) was carried out using a Nicolet 6700 spectrometer (Thermo Company, USA) at 25 °C. The molecular weight of amphiphilic CS derivative was evaluated via gel permeation chromatography (GPC) analysis utilizing a waters 600 E series instrument equipped with an ultrahydrogel column, and a water 2410 refractive index (RI) detector [12].

Fluorescence spectroscopy was used to investigate the critical micelle concentration (CMC) of BSCS using hydrophobic pyrene as a fluorescent probe [15]. An aliquot (10 µL) of 1 mM pyrene solution in acetone was placed in the small glass bottles containing a concentration sequence of aqueous copolymer media from 0.5 × 10^{-3} to 3.9 × 10^{-3}mg/mL (4 mL). The amount was adjusted to give a pyrene concentration in the final solution of each sample at 2.5×10^{-6} M. The mixture solutions were sonicated for 15 min, heated at 50°C for 2 h to equilibrate the pyrene and the micelles, and then stored to cool overnight in dark at ambient temperature. Fluorescence excitation was set at 335 nm, and the emission band was observed from 350 to 500 nm. After measuring, the intensity ratio of I_{373}/I_{382} was plotted as a function of logarithm the concentration of polymer and the CMC was calculated.

In vitro cytotoxicity assay

The *in vitro* cytotoxicity of blank BSCS against Caco-2 cells was investigated by an MTT assay [16]. Overall, The Caco-2 cells were incubated in DMEM solution at pH 7.4 and were kept at 37 °C in 5 % CO_2 atmosphere. The cells were seeded into each well of 96-well plates with a density of 10,000 cells/well and pre-incubated for 24 h to allow cell attachment.

Thereafter, blank micelles in 100 µL of DMEM were added to each well of the Caco-2 cells. After 24 h, the medium was removed and the wells were washed. Then, the wells were filled with 200 µL of new medium, and incubated the cells were with MTT solution (1 mg/mL) for additional 4 h. After removal of the medium, 100 µL of DMSO was added to dissolve the formed formazan crystals. The cell viability was calculated based on the absorbance measurements at 550 nm using a microplate reader (Packard BioScience, USA). The viability of non-treated control cells was arbitrarily defined as 100 %.

Preparation of drug-loaded polymeric micelles

A mixed-solvent evaporation method was selected to prepare polymeric micelles as previously described [13]. Briefly, 5 mg of BSCS and drugs (MX, PRX, KP, IND) (at 40 % concentration of the drug to polymer) were dissolved in DMF in a small glass container. After dissolving, small amount of acetone was added and stirred at ambient temperature. The solvent of the mixed solution was then completely evaporated under nitrogen gas flow. Then, deionized water (3 mL) was added, and the mixture was sonicated for 20 min at 80 °C using a probe sonicator (model CV 244, USA). The micellar solution was then centrifuged at 1000 rpm for 2 min, filtered through a 0.45-µm membrane filter to remove insoluble drug.

Determination of entrapment efficiency

To measure drug loading efficiency and loading capacity, 0.1 mL of the drug-incorporated polymeric micelles were mixed with 0.9 mL of a mixture solvent of dimethyl sulfoxide (DMSO) : H_2O (9:1). The drug content incorporated into BSCS micelles was measured using HPLC (Agilent technologies, USA). The entrapment efficiency (EE) and loading capacity (LC) of the drugs were calculated according to Eqs.1 and 2, respectively. All samples were analyzed in triplicate.

$$EE\ (\%) = (C_1/C_2)100 \quad \text{..... (1)}$$

where C_1 is the amount of drug loaded into the polymeric micelles, and C_2 is the initial amount of drug used for preparation.

$$LC\ (\mu g/mg) = (L_1/L_2) \quad \text{..... (2)}$$

where L_1 is the amount of drug incorporated into the polymeric micelles, and L_2 is the amount of BSCS used for the preparation.

High performance liquid chromatography (HPLC) conditions

The amount of the drugs encapsulated into the polymeric micelles was investigated using by HPLC with a Luna® 5 µm C18 (2) column according to a calibration curve generated from a series of standard solutions ranging from 5 to 50 µg/mL. The injection volume was 20 µL. The mobile phase used for each drug was described in Table 1 [17-19].

Table 1: HPLC experimental conditions used to quantify NSAIDs concentrations [17-19]

Drug	Mobile phase	Flow rate (mL/min)	Wavelength (nm)
MX	Potassium dihydrogen phosphate (pH 4.4):MeOH:ACN (45:45:10, v/v/v)	1.0	365
PRX	Water: methanol (45:55) pH3.2	1.0	360
KP	0.2% phosphoric acid:acetronitrile (50:50)	1.2	255
IND	0.2% phosphoric acid:acetronitrile (50:50)	1.8	230

Polymeric micelle size and surface charge measurement

The mean particle diameter and surface charge of the BSCS polymeric micelles with and without drugs were assessed using Zetasizer Nano ZS (Malvern, UK) in triplicate at 25°C. The diluted micellar solution was passed through a 0.45-µm membrane filter before measurement.

Statistical analysis

All data are presented as mean ± standard deviation (SD, n = 3). A one-way analysis of variance (ANOVA) was used to test the statistical significance between means using SPSS 17.0 statistical software program. A *p*-value of 0.05 or less was considered as statistically significant.

RESULTS

Characteristics of BSCS

Grafted copolymer, BSCS, was successfully synthesized onto a CS backbone via reductive benzylation and succinylation, respectively (Figure 1). Overall, the N-benzylation of CS occurred through the corresponding Schiff base intermediate, then the N,O-succinylation of BCS was performed by reacting with succinic anhydride in DMF at 100 °C.

BSCS was characterized using ^{1}H-NMR spectroscopy, FTIR and GPC. The ^{1}H-NMR spectrum of BSCS exhibited proton signal of the benzyl groups at δ 7.32 ppm and the methylene protons of the succinyl moiety at δ 2.45 ppm, as shown in Figure 2 (a). FTIR spectrum of BCS, the result revealed that the features of additional absorption bands at 1600, 1494, 743 and 695 cm^{-1}, compared with spectrum of CS. These bands were assigned to C=C stretching and C—H deformation (out of plane), respectively, arising from the benzyl groups [20]. The BSCS spectrum exhibited a characteristic band at 1715 cm^{-1} that corresponds to C=O stretching of the succinic acid moiety (Figure 2 (b)). The number average molecular weight (M_n) of CS by GPC analysis was 7633 g/mol. After modification, the M_n of BCS and BSCS was increased from 7633 to 10,552 and 15,628 g/mol, respectively. Moreover, the degree of substitution (DS) of copolymer was investigated using elemental analysis. The DS of the N-benzyl groups (DSB) and N,O-succinyl groups (DSS) was computed based on the C/N molar ratio achieved from elemental analysis according to Eqs 3 and 4, respectively [14].

$$\text{DSB of benzyl group } (\%) = \frac{(C/N)_{BCS} - (C/N)_{CS}}{7} \times 100 \quad \ldots. (3)$$

$$\text{DSS of succinyl group } (\%) = \frac{(C/N)_{BSCS} - (C/N)_{BCS}}{4} \times 100 \quad \ldots (4)$$

DSB was calculated to be 0.69 (approximately 32.4 repeating units) while the DSS was 1.08 (approximately 50.7 repeating units) [12,14].

Self-assembly micelle formation of the BSCS copolymer occurs when the concentration is above the CMC, which determined by plotting the intensity ratio I_1/I_3 of pyrene versus concentration. The CMC value of BSCS was 0.0385 mg/mL.

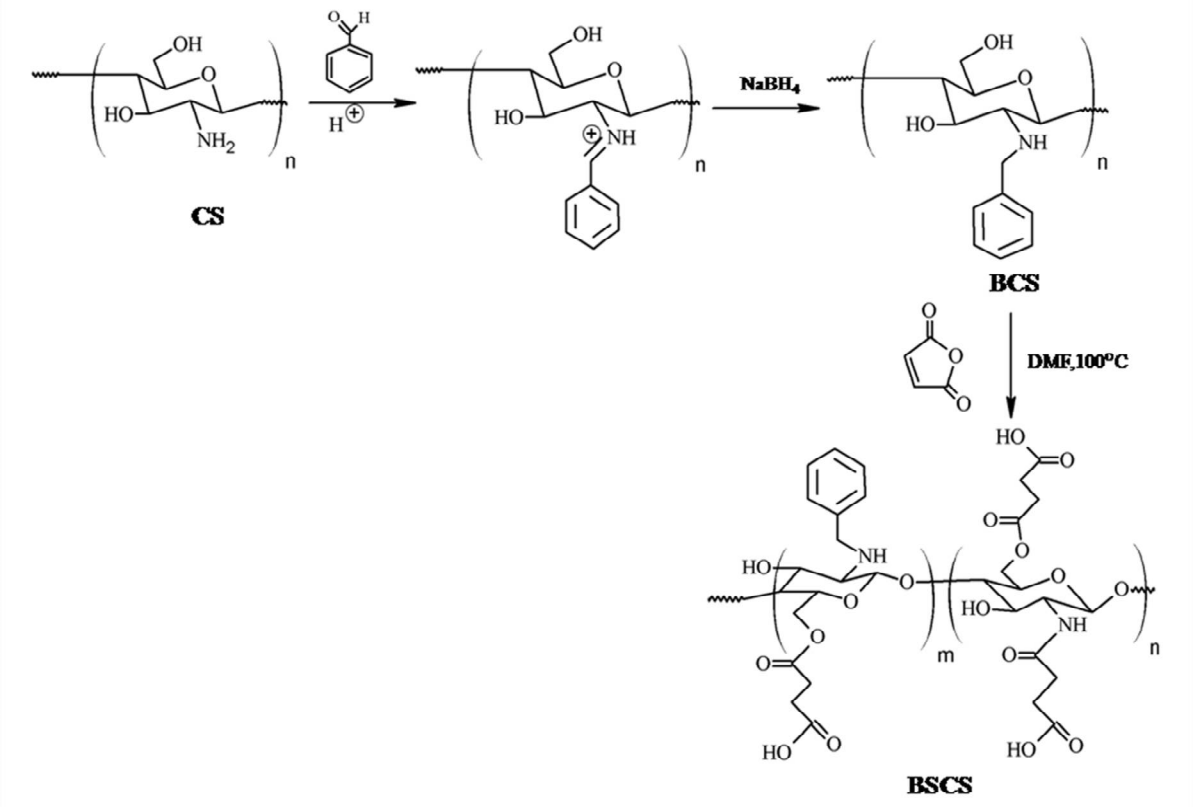


Figure 1: Schematic illustration of the synthesis of *N*-benzyl-*N,O*-succinyl chitosan (BSCS) graft copolymer

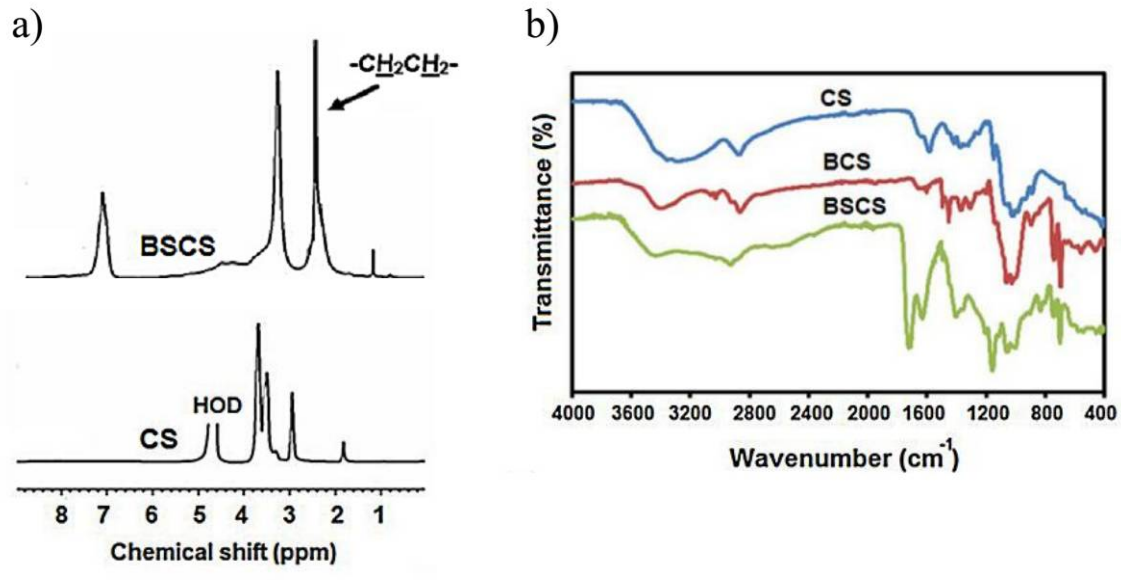


Figure 2: 1H NMR spectra of CS and BSCS (a) and ATR-FTIR of CS, BCS and BSCS (b)

In vitro cytotoxicity

The cytotoxicity of BSCS micelles was determined against Caco-2 cells. Figure 3 shows the viability of Caco-2 cells after treatment with various the concentration (0.01–5 mg/mL) of the modified polymer. The IC_{50} value was calculated to be 3.23 ± 0.08 mg/mL.

Drug-loaded polymeric micelles

In this study, four model NSAIDs drugs (MX, PRX, IND and KP) with poor aqueous solubility, presented the physicochemical properties in Table 2 [21,22], were selected to load into BSCS polymeric micelles. The EE (a) and LC (b) of all NSAIDs loaded into BSCS polymeric micelles are shown in Figure 4.

Polymeric micelle size and surface charge

The particle sizes, size distribution and surface charge of blank and four NSAIDs drugs loaded BSCS polymeric micelles are displayed in Table 3.

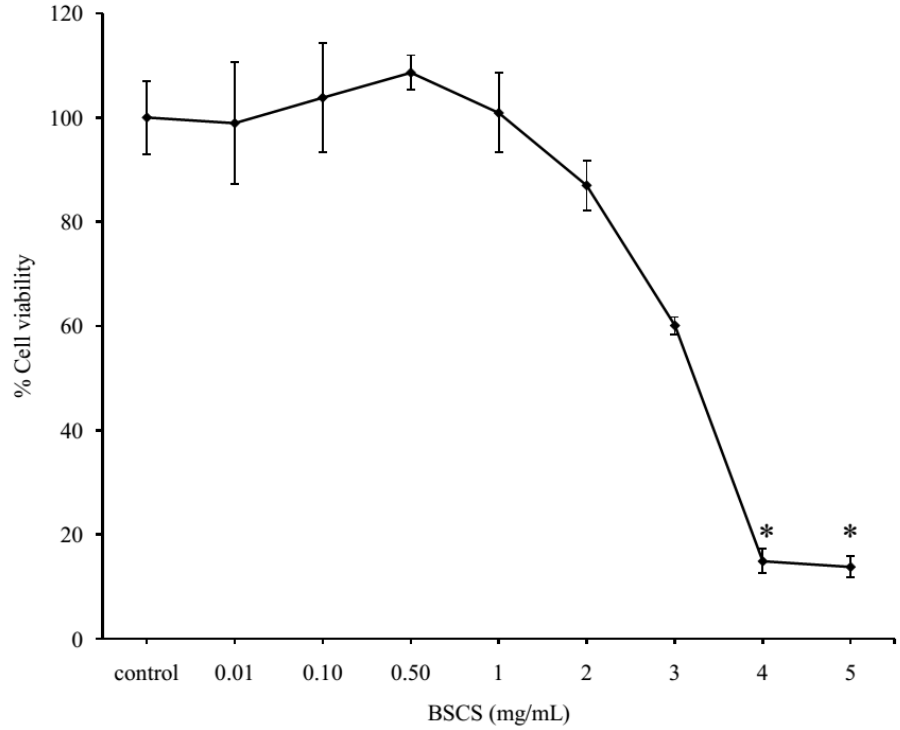
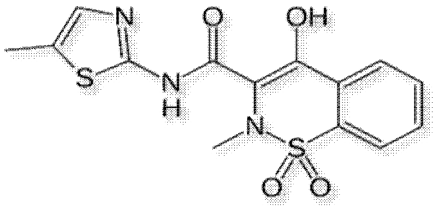
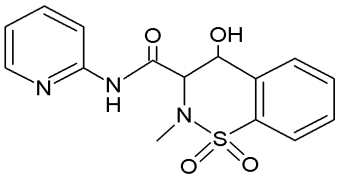
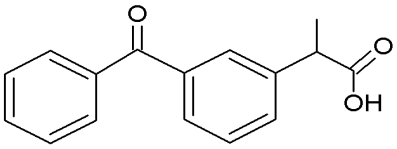
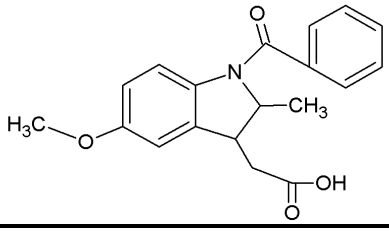


Figure 3: Cell viability in Caco-2 cells at varying concentrations of polymeric micelles (n=5; *p < 0.05)

Table 2: Physicochemical properties of the drugs [21,22]

Drug	Structure	MW (g/mol)	pK_a	Solubility (mM)
Meloxicam (MX)		351.41	1.09,4.18	0.20[a]
Piroxicam (PRX)		331.35	1.86,5.46	0.48[a]
Ketoprofen (KP)		254.38	4.4	24.59[a]
Indomethacin (IND)		357.79	4.5	0.04[b]

[a] Solubility at pH 6; [b] Solubility at pH 5.7

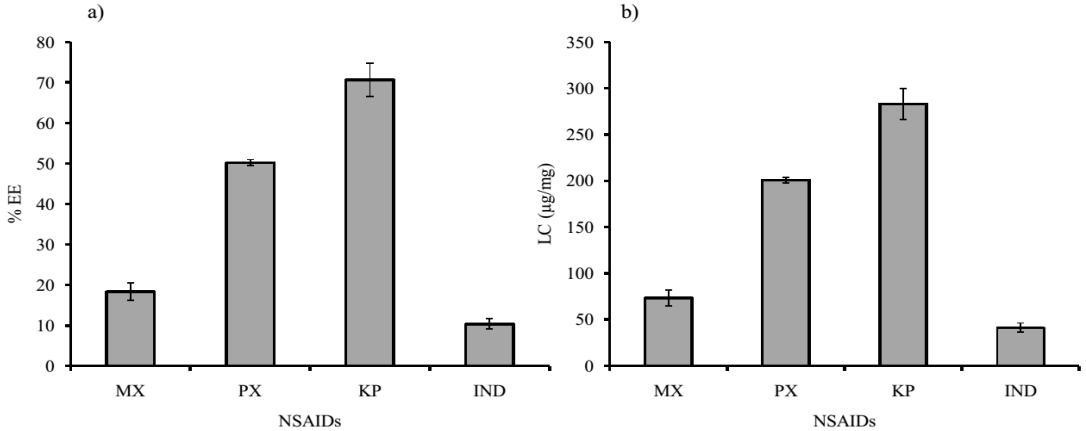


Figure 4: Entrapment efficiency (a) and loading capacity (b) of NSAIDs drugs (MX, PRX, KP and IND). Each value represents mean ± SD (n = 3)

Table 3: Particle size with polydispersity index (PDI) and surface charge of polymeric micelles with and without drugs. Each value represents mean ± SD (n = 3)

Polymeric micelles (PMs)	Particle size (nm)	PDI	Zeta potential (mV)
Blank-PMs	226.63±9.57	0.304	-39.60±1.91
MX -loaded PMs	312.63±11.75	0.386	-40.67±0.47
PRX-loaded PMs	402.70±24.81	0.420	-41.53±2.94
KP-loaded PMs	349.23±7.89	0.430	-38.13±1.92
IND-loaded PMs	433.53±2.43	0.280	-39.13±0.75

DISCUSSION

The results presented indicate the successful synthesis of amphiphilic copolymer via reductive amination and succinylation, respectively, onto the CS backbone. The copolymer was confirmed by ^{1}H NMR spectroscopy, FTIR, GPC and elemental analysis. These results revealed the successful functionalization of *N*-benzyl groups or *N,O*-succinyl groups onto the CS backbone. The formation polymeric micelles in aqueous solution occur when the concentration of the copolymer increases above CMC. The CMC of BSCS polymeric micelles was very low. This value indicates the polymeric micelles are stable of structure. The major requirements for drug delivery to the body are the polymers used for micelle preparation should be non-toxic. Here, the cytotoxicity of BSCS micelles was determined by quantitative evaluation of cell viability using Caco-2 cells. As reported the BSCS copolymer micelles had low cytotoxicity and would be safe *in vivo*.

One important problem in drug delivery is the deficient solubility of drugs. Polymeric micelles are one of the most promising carriers for drug delivery and have improved the solubility of poorly water soluble drugs by entrap the drug into hydrophobic core. Generally, the solubility of drugs depends on particle size, the nature and composition of the solvent medium, temperature, physical form of solid, nature of solute, etc [23]. In Table 2, MX and PRX are classified of enolic acid derivatives. They have two acid-base dissociation constants pK_a (1.09, 4.18 for MX and 1.86, 5.46 for PRX). The isoelectric points (pI), which were computed from (pK_{a1} + pK_{a2})/2 of MX and PRX, are 2.63 and 3.66, respectively. The pI is defined as the pH at which the drugs have no net charge. When the pH < pI, drugs display a positive charge or become a cationic, but when pH > pI, drugs turn into a negative charge or become an anionic. KP and IND belong to propionic acid derivatives with $pK_a \approx 4.4$ and acetic acid derivatives with $pK_a \approx 4.5$, respectively. Both of them are negatively charged or anionic at pH > pK_a [22]. Thus, the solubility of NSAIDs (MX, PRX, KP and IND) increased with the increase in pH because of ionization of the drugs. However, these drugs present low solubility in aqueous solution (Table 2). The improvement in EE of the four model drugs through BSCS polymeric micelles can be ranked as KP (70.6 %) > PRX (50.2 %) > MX (18.3 %) > IND (10.3 %) (Figure 4a). In addition, the drug LC of these BSCS micelles for the four model drugs was in the similar rank order; KP (282.9 µg/mg) > PRX (200.8 µg/mg) > MX (73.7 µg/mg) > IND (41.2 µg/mg).

These high contents indicate successful encapsulation of poorly water soluble drug to polymeric micelles. The LC of polymeric micelles is influenced by several factors such as composition, molecular weight and structure of polymer and drug [24]. This result revealed that the chemical structure of drugs is the key factor to control EE and LC. It is thought that BSCS micellar encapsulation was occurred not only by hydrophobic interaction but also by the forces assembled via the attraction between benzyl groups in the copolymer and aromatic groups in drugs. These results were in agreement with previously studied encapsulation behaviors of anticancer drugs, octaethylporphine (OEP), meso-tetraphenyl porphine (mTPP) and camptothecin (CPT), in Pluronic and poly(ethyleneglycol)-distearoylphosphatidyl ethanolamine (PEG–DSPE) polymeric micelles. The results indicated that the high efficacy of drug loading depends on the type of polymer and drug used and their ratios. The phenyl groups in mTPP might lead to attraction between alkyl groups in the polymer and increase drug incorporation [25]. The particle sizes of the drugs encapsulated into polymeric micelles ranged from 312 to 433 nm and were larger than the blank polymeric micelles (226.63 nm) as a result of drug entrapment. The zeta potential of all micelles formation in deionized water (pH 5) exhibited negatively charge (-38 to -41 mV) of carboxyl group in hydrophilic moieties resulting in the particles tended to poorly aggregate [26].

CONCLUSION

BSCS grafted copolymer has been successfully synthesized and prepared as polymeric micelles in aqueous solutions. NSAIDs (MX, PRX, KP and IND) have also been successfully incorporated in the hydrophobic inner core by evaporation method. Among the drug-loaded micelles, KP-loaded BSCS polymeric micelles showed the highest loading capacity. Drug loading depends on the molecular structure of the drugs. Therefore, generation of BSCS polymeric micelle in this study represents an efficient and attractive approach for solubilization of NSAIDs.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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