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Enhanced anti-topoisomerase II activity by mucoadhesive 4-CBS–chitosan/poly (lactic acid) nanoparticles

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ABSTRACT

In this study, the biodegradable mucoadhesive 4-carboxybenzensulfonamide chitosan (4-CBS–chitosan)/poly (lactic acid) (PLA) nanoparticles were fabricated by the electrospray ionization technique for enhancing anti-topoisomerase II (Topo II) activity. The obtained (4-CBS–chitosan/PLA)-DOX nanoparticles were characterized using SEM, particle size analyzer. We emphasis on encapsulation efficiency, *in vitro* drug release behavior and also performed *in vitro* studies of Topo II inhibitory activity using gel electrophoresis. In addition, the cytotoxicity of the 4-CBS–chitosan/PLA nanoparticles using MTT assay was also studied. The mean particle size of spherical shaped (4-CBS–chitosan/PLA)-DOX is less than 300 nm. The DOX loaded 4-CBS–chitosan/PLA composite nanoparticles produced high entrapment efficiency of 85.8% and provided the prolonged release of DOX extended to 26 days and also still had strong Topo II inhibitory activity up to 77.4%. Overall, it was shown that 4-CBS–chitosan/PLA nanoparticles could be promising carriers for controlled delivery of anticancer drugs.

1. Introduction

Topoisomerase II (Topo II) is a nuclear enzyme that passes an intact helix through a transient double-stranded break in DNA to modulate the DNA topology using ATP and plays a key role in transcription, replication, and chromosome segregation (Kılıçay et al., 2011). DNA is a cellular target for a number of widely used antitumor agents, such as doxorubicin (DOX) and daunorubicin (anthracycline class) as well as etoposide (Zunino & Capranico, 1990). Moreover, antitumor agents also intercalate into DNA and form reactive metabolites that interact with many intracellular molecules. Thus, Topo II inhibitors are important in cancer chemotherapy. A considerable interest has therefore arisen in combining Topo II inhibitors with a mucoadhesive polymer for enhancement of the Topo II activity.

Recently, mucoadhesive biodegradable and biocompatible polymeric nanoparticles have become a topic of great interestin biomedical applications to increase the specificity and localization at the target site, enhance the permeability property, improve the delivery efficiency and reduce the side-effects of drug toxicity (Andrews, Laverty, & Jones, 2009; Bernkop-Schnurch, 2005; Dodou, Breedveld, & Wieringa, 2005). One of the most promising biodegradable polymers is chitosan, which is a mucopolysaccharide derived from chitin by alkaline deacetylation that is soluble in dilute aqueous acidic solution (pH < 6.5) (Xu & Hanna, 2007). Chitosan has gained increasing attention in the pharmaceutical field due to its favorable biological properties, *i.e.*, non-toxic, biocompatible, and biodegradable, and is also applicable for oral or non-paraenteral delivery routes (Songsurang, Pakdeebumrung, Praphairaksit, & Muangsin, 2011; Wan, Wu, Yu, & Wen, 2006). Moreover, chitosan chains show good adhesive properties on mucosal surfaces, a property that can be exploited for the development of bioadhesive delivery devices. In our previous work, we developed mucoadhesive chitosan by modification with 4-carboxybenzenesulfonamide (4-CBS) for stomach-specific drug delivery, specifically for the treatment of *Helicobacter pylori* infections. In that study, the mucoadhesive 4-CBS–chitosan was shown to have enhanced mucoadhesive and swelling properties in the simulated gastric fluid (SGF; 0.1 M HCl, pH 1.2). Furthermore, the modified chitosan displayed antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* and appeared non-toxic to Vero, KB, MCF-7 and NCI-H187 cell lines in tissue culture (Suvannasara, Juntapram, Praphairaksit, Siralertmukul, & Muangsin, 2013).

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Despite these advantageous characteristics of chitosan, its insolubility in either water or common organic solvents and its poor mechanical properties for supporting tissue cells have limited basic research and various bio-fabrication applications (Feng & Dong, 2007; Huang, Yu, Guo, & Huang, 2010). Several approaches have been taken to overcome the limitations of chitosan, *i.e.*, graft polymerization (Liu, Wang, Shen, & Fang, 2005; Yao et al., 2003), blending (Suyatma, Copinet, Tighzert, & Coma, 2004; Wang, Wang, Lu, & Young, 2012; Zhang, Xu, & Jiang, 2012) and mixing with other natural or synthetic polymers (Chouwatat, Polsana, Noknoi, Siralertmukul, & Srikulkit, 2010) such as poly (lactic acid) (PLA) (Wu, Li, & Gao, 2009), which can impart desirable mechanical properties to the resultant copolymer composite while retaining the desirable properties of chitosan.

Delivering a potent drug with nanoparticles can reduce the toxicity and lessen side effects. Recently, various methods for the preparation of composite micro- or nano-particles have been reported, such as molecular probe fabrication (Wu et al., 2009), ionotropic gelation (Bigucci et al., 2008) and solvent emulsification/internal gelation (Jimenez-Kairuz, Allemandi, & Manzo, 2004). One of the novel techniques for the preparation of particles is electrospray ionization in which a liquid is atomized by means of electrical forces. The polymer flows out of the nozzle in the form of a droplet when a high voltage is applied. The advantages of this method are the ability to obtain a uniform particle size and lower polydispersity and the convenience of a one-step process (Xu & Hanna, 2006).

The aim of this work was to prepare a mucoadhesive polymer composite for non-paraenterally administered routes, particularly in mucoadhesive sites, *i.e.*, buccal, nasal, rectal or vaginal. Therefore, the present work focuses on the preparation, characterization and *in vitro* dug release behavior; Topo II inhibitory activity; and evaluation of the cytotoxic activity of doxorubicin (DOX)-loaded 4-CBS–chitosan/PLA nanoparticles formed by electrospray ionization.

2. Materials and methods

2.1. Materials

Chitosan with a deacetylation level of 95% (M_w = 400 kDa) was purchased from Biolife (Thailand). The PLA, with a $\overline{M_w}$ of 121.4 kg/mol and consisting of a 1:24 mole ratio of D-lactide:L-lactide, was purchased from NatureWorks 2000D (Thailand). The 4-CBS and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) were obtained from Sigma (St. Louis, USA). Cellulose dialysis tubing (Membrane Filtration Products, Inc., USA) with a 12–14 kDa molecular weight cut-off was used to purify all of the modified chitosan. The doxorubicin (DOX) hydrochloride, etoposide, PBR322 plasmid, sodium tripolyphosphate (TPP) and SDS were purchased from Sigma Aldrich. Human Topo II was purchased from Amersham (UK), andlactic acid was purchased from Union chemicals (Thailand). All other chemicals used in this work were of reagent grade.

2.2. Synthesis of 4-CBS–chitosan

The 4-CBS–chitosan (Fig. 1) was prepared *via* the coupling reaction of 4-CBS to chitosan in the presence of EDAC as a coupling reagent as previously reported (Suvannasara et al., 2013). Briefly, 1 g of chitosan was dissolved in 100 mL of 1% (v/v) acetic acid solution at room temperature overnight, and 4-CBS and EDAC at a mole ratio of 1.2:1 EDAC:4-CBS were added at a 0.05:1 (w/w) ratio of 4-CBS:chitosan. The reaction was refluxed for 6 h to form the 4-CBS–chitosan using EDAC as the coupling agent. Excess EDAC

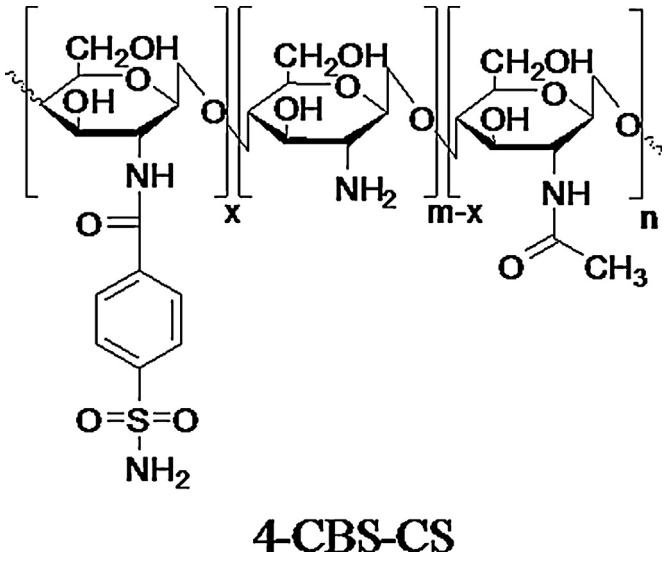

Fig. 1. Chemical structure of 4-CBS–chitosan.

was removed by precipitation following the addition of 1 M HCl to the reaction mixture followed by centrifugation. The supernatant was subsequently neutralized with 1 N NaOH, and the residual free acetic acid, NaCl, *o*-acylurea derivative and unreacted 4-CBS were eliminated by dialysis against three changes of 1000 mL of ethanol. The reaction mixture was filtered, washed with water and lyophilized at 198 mbar and −45 °C.

2.3. Preparation of 1:1 (w/w) DOX-loaded 4-CBS–CS/PLA nanoparticles

The 4-CBS–chitosan/PLA composite was prepared according to the previous work. The preparation of DOX-loaded 4-CBS–chitosan/PLA nanoparticles was formed by ionic cross-linking. Briefly, DOX was added to a solution of 1% (w/v) 4-CBS–chitosan/PLA at three different (w/w) ratios of 4-CBS–chitosan to PLA (1:1, 1:2, and 2:1), selected for two different amounts of DOX (1% and 2%, w/v) in 4:1 (v/v) dichloromethane: toluene, and subsequently electrosprayed into 80 mL of a 5% (w/v) aqueous solution of TPP. An applied voltage of 10 kV, an 8-cm working distance, a 800-rpm stirring rate, 20 G needle, and a flow rate of 10 mL/h were used for the electrospray ionization process.

The DOX-loaded hydrophobic 4-CBS–chitosan/PLA colloidal nanoparticles formed spontaneously under mild agitation at room temperature, and after 30 min of stirring, the colloidal nanoparticles were harvested by centrifugation at 15,000 × *g* for 30 min. The supernatant was discarded, and the deposit was re-dispersed in distilled water for further use.

2.4. Characterization of the 4-CBS–chitosan/PLA nanoparticles (with or without DOX loading)

2.4.1. Anhydrous particle size and morphology: scanning electron microscopy (SEM)

Samples for scanning electron microcopy (SEM) analysis were prepared by sprinkling the dried nanoparticles on one side of a double adhesive stub. The stub was subsequently coated with gold under vacuum conditions. The microcapsules were observed *via* SEM (Phillips XL30CP) operating in the range from 10 kV to 20 kV.

2.4.2. Hydrated particle size, size distribution and zeta potential: dynamic light scattering (DLS)

The particle size, size distribution and zeta potential of the nanoparticles were evaluated by dynamic light scattering (DLS) with a particle size analyzer (Zetasizer nano series, Malvern instruments). The nanoparticles were suspended in an aqueous solution and sonicated for 30 min at room temperature before loading into the instrument for analysis. The scattered light was collected at an angle of 90° through fiber optics and converted to an electrical signal by an avalanche photodiode array. All samples were run in triplicate with the number of runs set to five and the run duration set to 10 s using a refractive index of 1.59 and an absorbance of 633 nm.

2.5. DOX encapsulation efficiency (EE)

The obtained nanoparticles were frozen and lyophilized by freeze-drying to obtain dried nanoparticles. The DOX content in the DOX-loaded 4-CBS–chitosan/PLA nanoparticles was quantitatively analyzed by suspending 2 mg of the nanoparticles in 20 mL distilled water, and the particles were clarified by centrifugation at 15,000 × *g* for 30 min. The supernatant was collected, and the amount of DOX in the supernatant was measured by UV–vis spectrophotometry at 480 nm. The weight of DOX was calculated using a calibration curve derived from known concentrations (range10–200 μM) of DOX. The percentage encapsulation efficiency (EE) was calculated as shown in the following equation:

$$\text{DOX encapsulation efficiency} = \frac{\text{weight of DOX in nanoparticles}}{\text{weight of the DOX initially}} \times 100\% \quad (1)$$

2.6. Evaluation of in vitro DOX release

The DOX release from the 4-CBS–chitosan/PLA nanoparticles was studied as a suspension in phosphate buffer (PBS) at pH 7.4 using the dialysis bag diffusion technique. Accurately weighed quantities (~10 mg) of nanoparticles were enclosed in a dialysis bag (molecular weight cutoff of 3.5 kDa) and dialyzed against 50 mL of PBS at pH 7.4 in a flask, which was shaken at 100 rounds per minute (rpm) and incubated at 37 ± 1 °C. At designated time points, 200 μL of the dialyzed PBS was removed for analysis of the DOX content (spectrophotometrically at 480 nm, as detailed above) and replaced by an equal volume of fresh PBS. The amount of released DOX was determined over 30 days, correcting for the PBS replacements each time.

2.7. Inhibition of topoisomerase II (Topo II) activity

The 4-CBS–chitosan/PLA nanoparticles, with or without DOX-loading, were screened for *in vitro* inhibitory activity against Topo II activity in terms of the ability of the nanoparticles to inhibit the conversion of supercoiled PBR322 plasmid double-stranded (ds) DNA to its relaxed form by human Topo II.

The Topo II reaction was performed in 20 μL of reaction mixture containing 10 mM Tris–HCl (pH 7.9), 175 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 2.5% (v/v) glycerol, 1 mM ATP, 0.5 mM dithiothreitol, 30 μg/mL bovine serum albumin, 0.2 μg pBR322 plasmid DNA, 0.3 units (U) of human DNA Topo IIα and the test compound (100 μM) in a final volume of 50 μL. An addition of etoposide instead of the test sample was used as a positive control. The reaction mixtures were incubated at 37 °C for 30 min and terminated by the addition of 3 μL of an aqueous solution of 0.77% (w/v) SDS/77 mM EDTA. The samples were mixed with 2 μL of an aqueous solution of 30% (w/v) sucrose, 0.5% (w/v) bromophenol blue and 0.5% (w/v) xylene cyanol and subsequently resolved by electrophoresis on a 1% (w/v) agarose-TBE gel at 1.5 V/cm for 10 h. The gels were stained for 30 min in an aqueous solution of ethidium bromide (0.5 μg/mL) and rinsed. The DNA bands were visualized by UV transillumination and quantified using an image analyzer and Syngene software (Lin, Fang, Wang, Cheng, & Wang, 2006).

2.8. Evaluation of cytotoxic activity by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to evaluate the inhibition of cell growth (IC_{50}) due to treatment with DOX-free and DOX-loaded nanoparticles compared with that offree DOX on a normal (non-transformed) human foreskin fibroblast (HS27) cell line and human cancer (transformed) cells lines originally derived from lung (CHAGO), hepato- (HepG2), gastric (KATO3) and colon (SW620) carcinomas cells. In addition, the effect on the cell viability rate of the DOX as released from the DOX-loaded nanoparticles compared with that of free DOX was evaluated on the four different types of cells assessed at 24-, 48- and 72-h exposure.

2.9. Statistical analysis

Data are presented as the mean ± one standard deviation (S.D.) derived from the indicated number of independent repeats and were subject to statistical analysis by ANOVA and Duncan's multiple means tests after transformation to parametric values where required. The differences were considered to be significant at the level of $p < 0.05$.

3. Results and discussion

3.1. Preparation of the 4-CBS–chitosan/PLA complex

The first modification in this study was achieved *via* the covalent attachment of 4-CBS to chitosan using EDAC as the activating agent, as described previously (Suvannasara et al., 2013). The 4-CBS–chitosan was white in color and odorless. The polymer displayed a fibrous structure and was easily dissolved in 1% (v/v) acetic acid solution to form a high viscosity pale yellow gel. The obtained 4-CBS–chitosan was synthesized and verified by ^{1}H NMR and FTIR measurements (data not shown) accordingly with the methodologies described in the previous work, Suvannasara et al. (2013). The 4-CBS–chitosan samples with % DS values of 4.1–13.3 were obtained by changing the weight ratio of 4-CBS to the primary amine groups (—NH_2) of the CS from 0.05 to 1.

The second modification was performed between 4-CBS–CS and PLA *via* a complexation method by mixing of aqueous 4-CBS–chitosan with SDS followed by solution mixing of the hydrophobic 4-CBS–chitosan complex with PLA (with or without the DOX to be encapsulated), and electrospray ionization was carried out to obtain the 4-CBS–chitosan/PLA composite nanoparticles. It was noticeable that the hydrophobic 4-CBS–chitosan complex appeared as a gel and could be dissolved in such low- or non-polar solvents as toluene, hexane and dichloromethane (data not shown), thus revealing the likely hydrophobic nature of the 4-CBS–chitosan complex. Therefore, the obtained hydrophobic 4-CBS–chitosan was likely compatible with PLA.

Accordingly, the 4-CBS–chitosan/PLA composite was synthesized by the solution mixing method using the hydrophobic 4-CBS–chitosan prepared from complexation of 4-CBS–chitosan and SDS. For DOX-loaded 4-CBS–chitosan/PLA composites, 1% (w/v)

DOX was added to a solution of 1% (w/v) 4-CBS–chitosan/PLA in 4:1 (v/v) dichloromethane: toluene and electrosprayed into 80 mL of a 5% (w/v) aqueous solution of TPP.

3.2. Characterization of the 4-CBS–chitosan/PLA nanoparticles with or without DOX loading

3.2.1. Anhydrous particle morphology: SEM analysis

Fig. 2 shows representative SEM images of the surface morphology of anhydrous 4-CBS–chitosan/PLA and DOX-loaded 4-CBS–chitosan/PLA nanoparticles at three different (w/w) ratios of 4-CBS to PLA (1:1, 1:2, and 2:1, w/w). The anhydrous 4-CBS–chitosan/PLA nanoparticles (Fig. 2a and b) were uniformly spherical in shape, with visible pores and an average particle size of 228 ± 39 nm (Table 1). In the case of the DOX-loaded 4-CBS–chitosan/PLA nanoparticles (Fig. 2c–f), the majority of the particles were also spherical in form and devoid of aggregates, with a slightly larger size than that of the unloaded 4-CBS–chitosan/PLA nanoparticles except for 1% DOX-loaded (1:2, w/w) 4-CBS–chitosan/PLA. Therefore, the size of the particles decreased with increasing PLA ratio. Moreover, the size of the particles increased with increasing DOX ratio (Table 1).

3.3. Hydrated particle size, size distribution and zeta potential: DLS analysis

The hydrated particle sizes and size distributions of the 4-CBS–chitosan/PLA and DOX-loaded 4-CBS–chitosan/PLA nanoparticles (Table 1) were in the range of 357 nmto 410 nm with a PDI range of 0.455–0.705. The average size of the hydrated DOX-loaded 4-CBS–chitosan/PLA nanoparticles was larger than that of the corresponding unloaded nanoparticles. Furthermore, the size of the particles decreased with increasing PLA and increased with increasing DOX ratio, producing essentially the same magnitude of size difference as observed in the anhydrous particles (Table 1). Thus, DOX-loading of the 4-CBS–chitosan/PLA particles caused a size increase, and the addition of PLA caused a size decrease.

The zeta potential or surface charge can greatly influence the particle stability in suspension *via* the degree of electrostatic repulsion between particles (Arya, Chakraborty, Dube, & Katti, 2009). The surfaces of the 4-CBS–chitosan/PLA and DOX-loaded 4-CBS–chitosan/PLA nanoparticles were found to possess a negative charge, as shown in Table 1, which suggests that they could be fairly resistant to agglomeration in aqueous TPP (pH 7.4) suspensions. The negative surface charge is due to the effect of PLA and TPP (Koo & Jang, 2008).

3.4. Encapsulation efficiency of DOX in 4-CBS–chitosan/PLA nanoparticles

The EE of DOX in the 4-CBS–chitosan/PLA nanoparticles was analyzed using UV/vis microplate reader spectroscopy at λ_{max} = 480 nm.

Generally, the EE of drugs into nanoparticles is one of the limiting factors in their effectiveness and often display slow levels

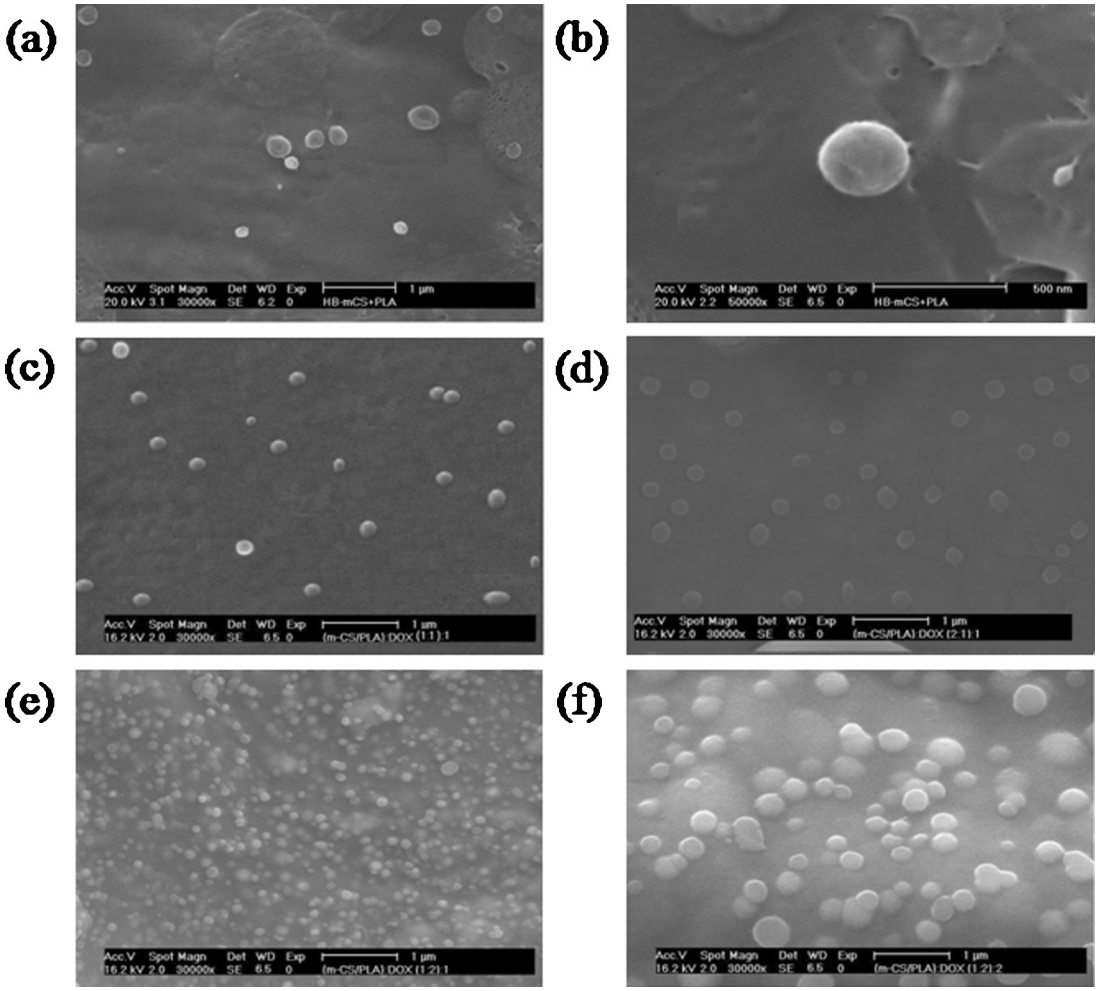

Fig. 2. SEM images of particles: (a) 4-CBS–chitosan/PLA at wide screen, (b) 4-CBS–chitosan/PLA (capture of one particle), (c) (4-CBS–chitosan/PLA)-DOX (1:1, w/w)-1%, (d) (4-CBS–chitosan/PLA)-DOX (2:1, w/w)-1%, (e) (4-CBS–chitosan/PLA)-DOX (1:2, w/w)-1% and (f) (4-CBS–chitosan/PLA)-DOX (1:2, w/w)-2%.

Table 1
Encapsulation efficiency (%EE) of DOX and the particle morphology in terms of the hydrated and anhydrous spherical size, polydispersity index (PDI and SPAN) and zeta potential of polymer composite nanoparticles produced under the optimized electrospray ionization conditions.

Formulation	EE (%)	Particle size[a] (nm ± SD)	Particle size[b] (nm ± SD)	PDI[c]	Zeta potential (mV ± SD)
4-CBS–chitosan/PLA	–	228 ± 39	361 ± 17	0.455 ± 0.034	−22.6 ± 0.8
(4-CBS–chitosan/PLA)-DOX (1:1, w/w)-1%	64.4 ± 0.8	233 ± 8	410 ± 9	0.570 ± 0.082	−31.5 ± 1.0
(4-CBS–chitosan/PLA)-DOX (2:1, w/w)-1%	68.3 ± 1.4	250 ± 10	437 ± 14	0.645 ± 0.035	−15.6 ± 1.1
(4-CBS–chitosan/PLA)-DOX (1:2, w/w)-1%	60.2 ± 2.6	119 ± 10	357 ± 38	0.602 ± 0.010	−17.1 ± 0.7
(4-CBS–chitosan/PLA)-DOX (1:2, w/w)-2%	85.8 ± 2.9	278 ± 15	407 ± 11	0.705 ± 0.016	−15.8 ± 1.1

[a] Particle size measured by SEM.
[b] Particle size measured by particle size analyzer.
[c] Polydispersity index measured by particle sizer.

(*e.g.*, 21.9%) (Janes, Fresneau, Marazuela, Fabra, & Alonso, 2001), and therefore, the %EE of DOX into 4-CBS–chitosan/PLA nanoparticles was investigated. In this study, DOX was successfully loaded at high levels into 4-CBS–chitosan/PLA nanoparticles at over 60% EE (Table 1). Moreover, the %EE was found to increase as the amount of DOX loaded was increased (nonlinearly), yielding encapsulation efficiencies of 60.2% to 85.8% at the same 4-CBS–chitosan:PLA ratio, showing that the electrospray technique can produce a high %EE.

3.5. In vitro DOX release profiles

The release of the DOX from encapsulated nanoparticles is another important factor that determines their suitability. The release behavior of DOX from hydrophobic chitosan/PLA (our previous study, data not shown) and 4-CBS–chitosan/PLA nanoparticles was evaluated in PBS (pH 7.4) by dialysis until 100% of the DOX had been released (Fig. 3).

3.5.1. Effect of CS/PLA and 4-CBS–CS/PLA with DOX

The release behavior of DOX from the nanoparticles can be described in a graph, and the release rate of DOX-loaded chitosan/PLA and 4-CBS–chitosan/PLA is shown in Fig. 3.

The cumulative release of DOX from (chitosan/PLA)-DOX (1:1, w/w)-1% exhibited the two release stages. In first stage, the drug was burst-released by approximately 35% within 8 h, followed by the slower release rate of the second stage over8 days and a slow sustained release rate to 100% by 12–14 days (data not shown). However, in the case of the 1:1 (w/w) DOX-loaded 4-CBS–chitosan/PLA nanoparticles, also at 1% (w/v) in PBS pH 7.4, a much slower biphasic and sustained release of DOX was observed. In the first stage, the drug was released by approximately 20% within 8 h. In the second stage, the release rate was slower and was sustained up to 18 days.

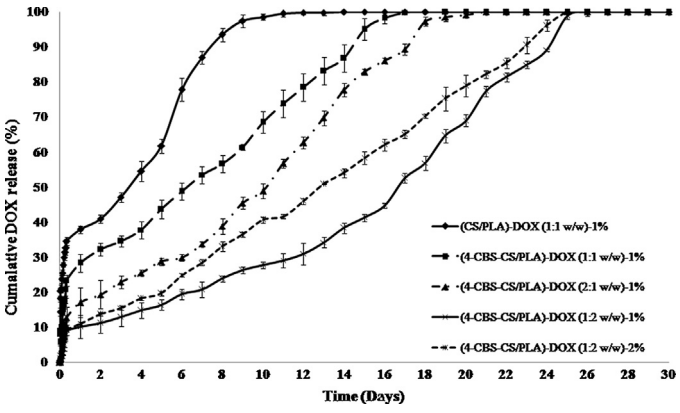

Fig. 3. Cumulative DOX release profile from (chitosan/PLA)-DOX (1:1, w/w)-1%, (4-CBS–chitosan/PLA)-DOX (1:1, w/w)-1%, (4-CBS–chitosan/PLA)-DOX (2:1, w/w)-1%, (4-CBS–chitosan/PLA)-DOX (1:2, w/w)-1% and (4-CBS–chitosan/PLA)-DOX (1:2, w/w)-2% nanoparticles at pH 7.4 and 37 °C.

It was clearly observed that (4-CBS–chitosan/PLA)-DOX (1:1, w/w)-1% showed a significantly prolonged release profile of DOX up to18 days compared with that of (chitosan/PLA)-DOX (1:1, w/w)-1%. Thus, 4-CBS–chitosan/PLA was selected for further studies to improve the release profile.

3.5.2. Effect of4-CBS–CS and PLA with DOX

The release rates for DOX-loaded (4-CBS–chitosan/PLA)-DOX (2:1, w/w)-1%, (4-CBS–chitosan/PLA)-DOX (1:2, w/w)-1% and (4-CBS–chitosan/PLA)-DOX (1:2, w/w)-2% nanoparticles are given in Fig. 3.

The *in vitro* release profiles of (4-CBS–chitosan/PLA)-DOX (2:1, w/w)-1%, (4-CBS–chitosan/PLA)-DOX (1:2, w/w)-1% and (4-CBS–chitosan/PLA)-DOX (1:2, w/w)-2% nanoparticles were investigated in phosphate buffer solution (PBS, pH 7.4) until 100% of the drug was released, and these formulations showed sustained release profiles. The (4-CBS–chitosan/PLA)-DOX (2:1, w/w)-1% nanoparticles exhibited nearly 50% and 80% release within 10 and 15 days, respectively, followed by continuing release. The release profiles of the (4-CBS–chitosan/PLA)-DOX (1:2, w/w)-1% nanoparticles exhibited nearly 50% and 80% release within 17 and 22 days, respectively, and DOX displayed subsequent sustained released. It was observed that the release rate of (4-CBS–chitosan/PLA)-DOX (1:2, w/w)-1%was slower than that of the (4-CBS–chitosan/PLA)-DOX (2:1, w/w)-1%nanoparticles. If the amount of PLA was increased, the release rate was slower and the release was prolonged from 18 days to 26 days. Therefore, (4-CBS–chitosan/PLA)-DOX (1:2, w/w)-1% nanoparticles were selected for further studies.

With increases in the loaded DOX, the periods of release remained the same (26 days), but the release rate was faster due to the higher loading of DOX.

It was obvious that the cumulative release of DOX increased with the increasing amount of DOX in the formulation. The higher level of DOX corresponding to a lower level of the polymer in the formulation resulted in an increase in the cumulative percentage release. As additional drug was released from the nanoparticles, more channels were produced, thus contributing to faster drug release. In addition, higher drug levels in the nanoparticle formulation produced a higher drug concentration gradient between the nanoparticles and dissolution medium, and thus the cumulative release of drug was also increased (Zhao, Holl, Pith, & Lambla, 1987).

3.6. Topo II activity assay

Topo II is a nuclear enzyme that catalyzes changes in the topological state of DNA by single-strand breaking and rejoining of (ds)DNA. This enzyme plays important roles in DNA metabolism, including replication, recombination, transcription and chromosome condensation. The DNA is a double helix with a mostly

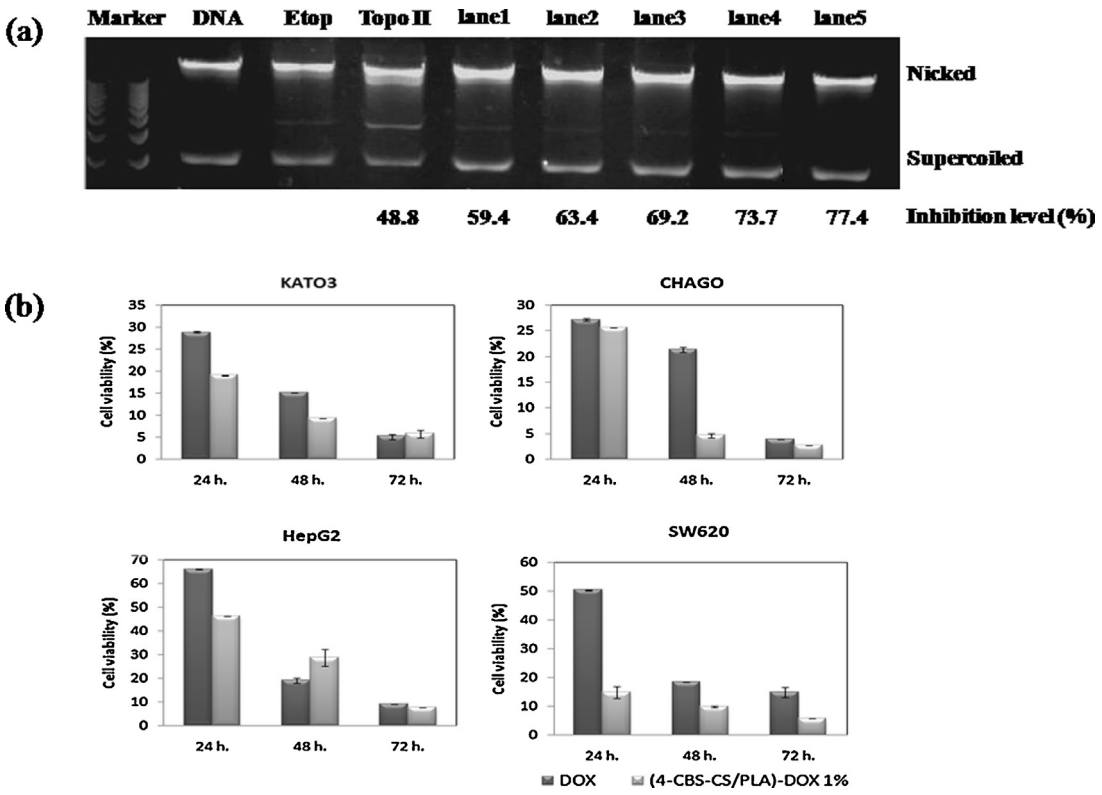

Fig. 4. (a) Inhibitory effects of tested compounds on human DNA topoisomerase II (lane 1): (chitosan/PLA)-DOX (1:1, w/w)-1%, (lane 2): (4-CBS–chitosan/PLA)-DOX (1:1, w/w)-1%, (lane 3): (4-CBS–chitosan/PLA)-DOX (2:1, w/w)-1%, (lane 4): (4-CBS–chitosan/PLA)-DOX (1:2, w/w)-1%, (lane 5): (4-CBS–chitosan/PLA)-DOX (1:2, w/w)-2%. (b) Effect on cell viability as determined by the MTT assay of various human transformed (cancer-derived) cell lines after 24- and 48-h exposure to 10 μg/mL DOX or DOX-loaded 4-CBS–chitosan/PLA at a net DOX level of 10 μg/mL.

supercoiled structure. However, supercoiled DNA must be relaxed by the Topo II enzyme before processing during transcription, repair or replication. DOX is known to inhibit Topo II activity, and thus the ability of the DOX-loaded polymer nanoparticles to inhibit the relaxation of supercoiled pBR322 plasmid dsDNA by human Topo II was evaluated as a measure of the release of functional DOX from the DOX-loaded hydrophobic chitosan/PLA (1:1, w/w) and 4-CBS–chitosan/PLA nanoparticles at three different (w/w) ratios of 4-CBS to PLA (1:1, 1:2, and 2:1, w/w).

As shown in Fig. 4a, the% inhibition was classified into strongly or weakly active, depending on whether the% inhibition was lower or higher than the activity of the reference control of100 μM etoposide (63.4% inhibition). All formulations of DOX-loaded 4-CBS–chitosan/PLA nanoparticles displayed a strong Topo II inhibitory activity with greater than 63.4% inhibition at 100 μM. The (4-CBS–chitosan/PLA)-DOX (1:1, w/w)-1% (63.4%) displayed stronger Topo II inhibition than (hydrophobic chitosan/PLA)-DOX (1:1, w/w)-1% (59.4%) nanoparticles at 100 μM. The results suggest that 4-CBS–chitosan enhanced the anti-Topo II activity. Furthermore, (4-CBS–chitosan/PLA)-DOX (1:2, w/w)-2% showed the strongest Topo II inhibition (over 77.4%) compared with etoposide as the reference (63.4%). Accordingly, the DOX-loaded 4-CBS–chitosan/PLA nanoparticles would appear to release functional DOX, and therefore, if the nanoparticles are internalized into the cells (or if the DOX is released nearby and taken up by the target cells), this delivery could be a potentially potent inhibitor of Topo II activity.

3.7. Cytotoxic activity by MTT assays

The *in vitro* cytotoxicity of the 4-CBS–chitosan/PLA nanoparticles with and without DOX at 1% (w/v) was evaluated against the normal cell line (HS27) and the four cancer cell lines (CHAGO, KATO3, HepG2 and SW620) using the MTT assay. Free DOX (the final concentration range of 0.01–10 μg/mL) was used as a reference drug for comparison against the (4-CBS–chitosan/PLA)-DOX (1:2, w/w)-2% nanoparticles (1–1000 μg/mL) at the same equivalent net DOX concentration (0.01–10 μg/mL at 100% release). The inhibition of cell growth (IC_{50}) of the 4-CBS–chitosan/PLA nanoparticles with and without DOX and free DOX are given in Table 2. The IC_{50}values of both 4-CBS–chitosan/PLA with and without DOX against the normal cell line HS27are over 100 μg/mL, indicating that both nanoparticles are nontoxic to the normal HS27 cell line, in contrast to the free DOX, which showed an IC_{50} > 10 μg/mL under the same testing conditions.

The 4-CBS–chitosan/PLA with 2% DOX showed inhibition against all tested cell lines with IC_{50} values in the range of 0.01–3.86 μg/mL, similar to that of the free DOX with an IC_{50} in the range of 0.05–2.98 μg/mL. Interestingly, both the 4-CBS–chitosan/PLA nanoparticles with/without DOX-loading revealed the lowest IC_{50} values against the gastric carcinoma cell line (KATO3) and five-fold greater cytotoxicity than the free DOX, indicating that the nanoparticle delivery produced a stronger cytotoxicity than free DOX at the same tested dose. This result is most likelydue to the mucoadhesive property of the (4-CBS–chitosan/PLA)-DOX (1:2, w/w)-2% that promotes the charge interactions between the surface of the nanoparticles and the gastric cell membrane.

The inhibition rate of (4-CBS–chitosan/PLA)-DOX (1:2, w/w)-2% compared with free DOX on the cell viability of the four transformed cell lines (CHAGO, KATO3, HepG2 and SW620) was evaluated using the *in vitro* cytotoxicity MTT assay at 24, 48 and 72 h. The concentration of DOX for the free DOX and DOX-loaded 4-CBS–chitosan/PLA was fixed at a 10 μg/mL equivalent. The DOX released from the nanoparticles showed a greater cytotoxicity than the free DOX at

Table 2
Inhibition of cell growth (anti-proliferative plus cytotoxic activities) as determined by the MTT assay and expressed as IC_{50} valuesof free DOX and the 4-CBS–chitosan/PLA and DOX-loaded 4-CBS–chitosan/PLA nanoparticles against four cancer cell lines and one normal (HS27) human cell line.

Cnompound[a]	IC_{50} (μg/mL)				
	HS27	KATO3	HepG2	SW620	CHAGO
4-CBS–chitosan/PLA	>100[b]	5.46 ± 1.54	>100[b]	>100[b]	>100[b]
(4-CBS–chitosan/PLA)-DOX	>100 [b]	0.01 ± 0.003	3.63 ± 0.28	3.41 ± 0.96	3.86 ± 0.11
DOX	>10	0.05 ± 0.006	0.77 ± 0.03	0.72 ± 0.17	2.98 ± 0.16

[a] DOX-loaded4-CBS–chitosan/PLA polymer nanoparticles were formed at a 1:1 (w/w) DOX: polymer ratio and usedas a 1% (w/v) suspension.
[b] IC_{50} > 100 μg/mL = inactive.

the same net concentration against all four transformed cell lines (Fig. 4b). Increasing the exposure time caused a further reduction in the cell viability of all four cell lines but varied in magnitude among them.

The DOX-loaded nanoparticles exhibited a stronger effect on the cell viability of SW620 (14.84%), KATO3 (19.08%), and CHAGO (25.68%) than on HepG2 (46.16%), at the first 24 h exposure time. Moreover, the remarkable differences in the apparent cell viability between the free DOX and the (4-CBS–chitosan/PLA)-DOX (1:2, w/w)-2% displayed at 24 h for SW620 (35.40%) are greater than those for HepG2 (19.97%), KATO3 (9.67%) and CHAGO (1.54%) (one-way ANOVA $p < 0.05$). These results indicated that the DOX-loaded 4-CBS–chitosan/PLA nanoparticles have the greatest effect on the cell viability rate of SW620 in the first 24 h. However, the cytotoxic activity of the DOX-loaded 4-CBS–chitosan/PLA nanoparticles on cell viability is reduced by 4.84% based on the cell viability at 24 h. In contrast to SW620, the cell viabilities of HepG2 and CHAGO are reduced by 17.32% and 21.00%, respectively. This result is consistent with the release profiles of the DOX-loaded nanoparticles in the PBS (pH 7.4) (Fig. 4b), which showed the faster rate at the first day and the sustained release rate later on. From 48 h to 72 h, the cytotoxicity of free DOX and the (4-CBS–chitosan/PLA)-DOX (1:2, w/w)-2% showed a greater effect on the HepG2 cell viability, which changed by 21.12%, whereas the cell viability of the other cell lines changed less than 5% after 48 h, indicating that the DOX-loaded nanoparticles showed the lowest effect on the cell viability rate of HepG2.

4. Conclusion

The 4-CBS–chitosan/PLA nanoparticles were fabricated *via* electrospray ionization for the delivery of DOX as a model payload drug. The size distribution of the DOX-loaded 4-CBS–chitosan/PLA nanoparticles was relatively narrow (PDI of 0.455–0.705) and yielded a high EE (over 60%). The DOX-loaded 4-CBS–chitosan/PLA nanoparticles exhibited prolonged release of DOX and more sustained release, up to 26 days. The DOX-loaded 4-CBS–chitosan/PLA nanoparticles may be an advantageous alternative vehicle for sustained release formulation of DOX in the treatment of certain cancers.

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