Carbohydrate Polymers 90 (2012) 1469–1479

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

Electrosprayed polyelectrolyte complexes between mucoadhesive *N,N,N,*-trimethylchitosan-homocysteine thiolactone and alginate/carrageenan for camptothecin delivery

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ARTICLE INFO

Article history:
Received 23 March 2012
Received in revised form 5 July 2012
Accepted 7 July 2012
Available online 16 July 2012

Keywords:
N,N,N,-trimethyl chitosan
Thiolated chitosan
Alginate
Polyelectrolyte complexes
Drug delivery
Electrospray ionization technique

ABSTRACT

Novel hydrogel polyelectrolyte complexes (PECs) between the *N,N,N,*-trimethylchitosan-homocysteine thiolactone (TM-HT-chitosan) and two anionic polymers were investigated. The particles of pure thiolated chitosan and its PECs with alginate and carrageenan were fabricated using the electrospray ionization technique. The hydrogel PEC particles were characterized by scanning electron microscopy, dynamic light scattering, Fourier transform infrared microscopy, thermogravimetric analysis, encapsulation efficiency (EE), mucoadhesive property and in vitro drug release behavior. TM-HT-chitosan/alginate particles could be loaded with camptothecin (CPT), employed as a model anti-cancer drug, at an over 70% EE, and revealed both a reduced burst effect and a prolonged release of CPT over 3 days. The resultant TM-HT-chitosan/alginate PEC particles displayed a 5.60-, 1.86- and 1.55-fold stronger mucoadhesive property compared to that of the unmodified chitosan/alginate PEC at pH 1.2, 4.0 and 6.4, respectively, and this was not affected by the CPT loading level.

1. Introduction

Chitosan is a cationic linear polysaccharide consisting of copolymers of D-glucosamine and *N*-acetyl-D-glucosamine units linked by β-(1–4)-glycosidic linkages, which is produced commercially by deacetylation of the naturally occurring chitin (Muzzarelli et al., 2012). Chitosan is a non-toxic, biodegradable, and biocompatible polysaccharide used in biomedical applications (Muzzarelli, 2009, 2011; Sæther, Holme, Maurstad, Smidsrød, & Stokke, 2008). Mucoadhesive polymers, modified by the introduction of a thiolated function, have been shown to have much higher adhesive properties than the parental unmodified polymer that is generally considered to be mucoadhesive (Langoth, Kalbe, & Bernkop-Schnürch, 2003). Thiolated chitosan is one such modified polymer that has been investigated for several biomedical applications. The mucoadhesive properties of thiolated chitosans, such as chitosan–thioglycolic acid conjugates (Kast & Bernkop-Schnürch, 2001), chitosan–cysteine conjugates (Schmitz, Grabovac, Palmberger, Hoffer, & Bernkop-Schnürch, 2008), and chitosan–iminothiolane (Bernkop-Schnürch, Hornof, & Zoidl, 2003), are strongly improved in comparison to the unmodified chitosan due to the formation of inter- and intra-molecular disulphide bonds within thiomers or with mucus glycoproteins. As a result, these thiolated chitosan derivatives display improved mucoadhesive properties, a prolonged disintegration time and a comparatively more controlled release of the incorporated drugs (Anitha et al., 2011; Bernkop-Schnürch, Guggi, & Pinter, 2004; Iqbal et al., 2012).

Previously, thiolated trimethylchitosan, as *N,N,N*-trimethylchitosan-homocysteine thiolactone (TM-HT-chitosan), was found to exhibit a higher mucoadhesiveness and swelling behavior than that of the unmodified chitosan (Juntapram, Praphairaksit, Siraleartmukul, & Muangsin, 2012). However, no polyelectrolyte (PEC) form of a thiolated polymer has been reported before.

The use of chitosan and chitosan derivatives as carriers for oral drug administration is restricted by its fast dissolution in the stomach and its limited capacity as a controlled drug delivery system (Risbud, Hardikar, Bhat, & Bhonde, 2000; Sivakumar, Manjubala, & Panduranga Rao, 2002). To overcome these disadvantages the use of PECs of chitosan with other polymers, such as chondroitin (Ganza-González, Anguiano-Igea, Otero-Espinar, &

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http://dx.doi.org/10.1016/j.carbpol.2012.07.017

Blanco Méndez, 1999; Huang, Sui, Wang, & Jiao, 2010), carrageenan (Briones & Sato, 2010; Pinheiro et al., 2012), xanthan gum (Argin-Soysal, Kofinas, & Lo, 2009; Fukuda, Peppas, & McGinity, 2006), sodium alginate (Sæther et al., 2008; Sankalia, Mashru, Sankalia, & Sutariya, 2007; Tapia et al., 2004), polyvinyl alcohol (Costa-Júnior, Barbosa-Stancioli, Mansur, Vasconcelos, & Mansur, 2009; Don, King, Chiu, & Peng, 2006), pectin (Kim, Park, Kim, & Cho, 2003; Oliveira, Ferrari, Carvalho, & Evangelista, 2010), and polyuronans (Muzzarelli, Stanic, Gobbi, Tosi, & Muzzarelli, 2004) amongst others, have been evaluated.

Our interest in polymer thiolated chitosan, carrageenan, and alginate is based on the fact that these three polymers have mucoadhesive properties (Grabovac, Guggi, & Bernkop-Schnürch, 2005). Carrageenan is a naturally occurring linear polysaccharide that is extracted from red seaweed and consists of the sulfate esters of galactose and 3,6-anhydrogalactose copolymers (Dai, Dong, & Song, 2007). Alginate is an anionic and biodegradable copolymer of 1,4-linked β-D-mannuronic acid and α-L-gluturonic acid (Sankalia et al., 2007)

Various methods for the preparation of composite micro- or nano-particles have been reported, such as 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 (EI), where the 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 (Xu & Hanna, 2006).

The objectives of this study was to prepare PECs between thiolated chitosan and either carrageenan or alginate, by EI using the anti-cancer agent camptothecin (CPT) as a model drug.

2. Materials and methods

2.1. Materials

Chitosan, with an 85% degree of deacetylation and a M_W of 100 kDa, was provided by Bonafides Co. Ltd. (Thailand). Homocysteine thiolactone (HT) hydrochloride, imidazole, mucin (type II) from porcine, iodomethane, *N*-methyl pyrrolidone, basic fuchsin (pararosaniline), sodium metabisulphite, periodic acid, acetic acid and lactic acid were obtained from Aldrich Co. (USA), and used without further purification. Cellulose dialysis tubing (molecular weight cut off (M_WCO) of 3.5 kDa) was obtained from membrane filtration products Inc. (USA). Sodium alginate, calcium chloride, potassium chloride and CPT were from Sigma Aldrich (USA). κ-Carrageenan was obtained by Facobis Co. Ltd.

2.2. Synthesis of TM-HT-chitosan

The TM-HT-chitosan derivatives (Fig. 1) were prepared using HT by covalent attachment as previously reported (Juntapram et al., 2012). Briefly, 100 mL of a 1% (w/v) of TM-chitosan in 1% (v/v) lactic acid solution was added to an aqueous solution of imidazole (0.68 g in 2.5 mL water), followed by the dropwise addition of HT (0.15 (w/v) aqueous) to the desired final concentration and stirred at room temperature under a nitrogen atmosphere for 12 h. The reaction mixture was adjusted to pH 7 and then precipitated, dialyzed, lyophilized and stored at 4 °C before use.

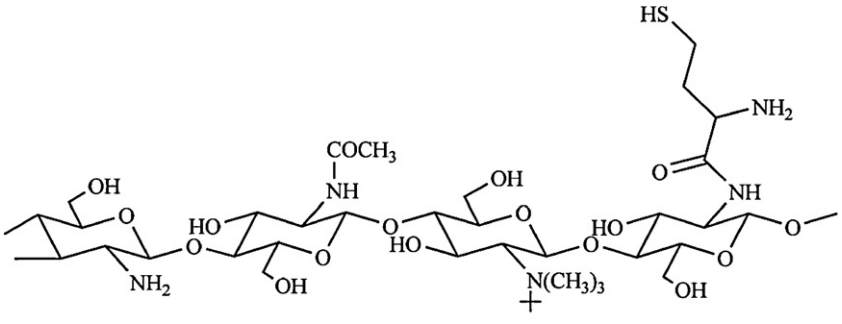
2.3. Preparation of TM-HT-chitosan PEC particles

TM-HT-chitosan/carrageenan or TM-HT-chitosan/alginate PEC particles were formed by the ionic crosslinking of the cationic TM-HT-chitosan to the anionic partner of either carrageenan or alginate, which is schematically shown in Fig. 1. For the EI process, using the set up schematically shown in Fig. 2, an electrospray voltage of 12 kV, an 8 cm working distance, a 400 rpm stirring rate 400 rpm, 26 G needle and a flow rate 1.2 mL/h flow rate were utilized. Carrageenan and alginate were selected as the anionic components to interact with the cationic TM-HT-chitosan to form the PECs. Firstly, carrageenan and alginate (10 g) were separately dissolved in 100 mL deionized water (DW) at room temperature with stirring for 24 h. After that the carrageenan or alginate solution was loaded into a 100 mL syringe and the needle was connected to a high-voltage generator. The droplets from the needles fell into 50 mL of a 4 mg/mL coagulant bath (3% mM KCl for carrageenan or (3% $CaCl_2$ for alginate) mixed with 2.5 mL of a 1 g/mL TM-HT-chitosan solution in 1% (v/v) lactic acid. The particles were then separated by centrifugation at 12,000 × *g* for 5 min, washed with DI three times, and then freeze dried at 0.01 mbar and −30 °C for 24 h.

For CPT-loading of the respective chitosan/alginate or TM-HT-chitosan/alginate PEC particles, CPT was first dissolved in 10% (v/v) dimethyl sulfoxide (DMSO) and then transferred to the 1% (w/w) alginate solution and stirred overnight. The particles were then prepared using the same conditions as for the drug-free particles (Section 2.3) except using the CPT/alginate solution in place of the alginate solution.

2.4. Characterization of the particles

The morphology and surface appearance of the different particles (before and after CPT loading) were examined by scanning electron microscopy (SEM). Each sample was mounted onto an aluminum stub using double-sided carbon adhesive tape and coated



Thiolated Chitosan = *N,N,N,*-Trimethylchitosan-homocysteine thiolactone (TM-HT-Chitosan)

Fig. 1. Chemical structure of *N,N,N*-trimethylchitosan-homocysteine thiolactone (TM-HT-Chitosan).

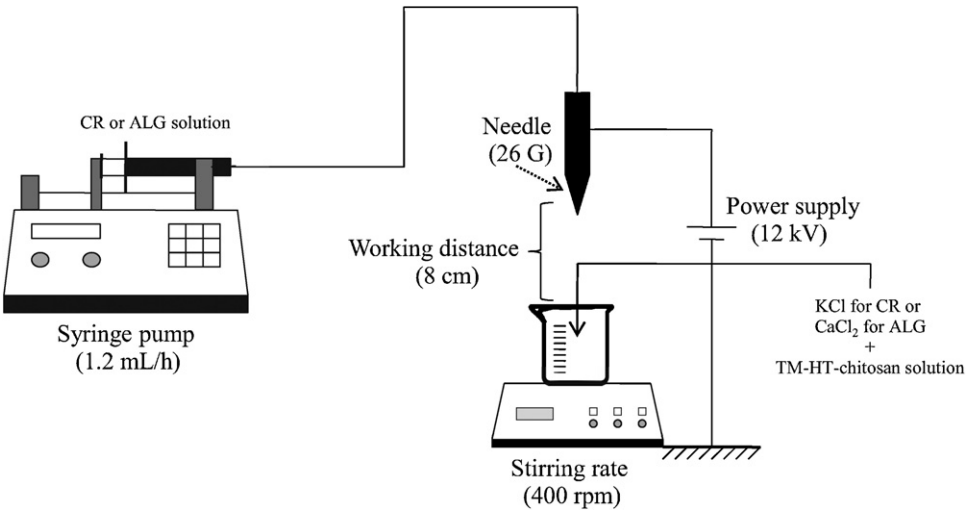


Fig. 2. Schematic diagram of the instrumentation for the electrospray ionization (EI) technique. CR and ALG represent carrageenan and alginate, respectively.

with gold–palladium. Coating was achieved at 18 mA for at least 4 min. Scanning was performed under high vacuum at an ambient temperature with a beam voltage of 10–20 kV.

The zeta potential, size and size distribution (polydispersity or PDI) of the different types of particles were evaluated by dynamic light scattering (DLS) on a particle size analyzer after suspension of the microspheres in DW. The scattered light was collected at an angle of 90° and all samples were sonicated and run in triplicate with the number of runs set to five and run duration set to 10 s.

The fluorescence transform infrared spectroscopy (FTIR) derived spectra of the particles were examined using the potassium bromide disk (KBr) method with a FTIR in the range of 4000–400 cm^{-1}.

The thermal stability of each of the particles was evaluated by thermogravimetric analysis (TGA) using a PerKinElmer Pyris Diamond TG/DTA machine under a nitrogen flow rate of 30 mL/min. Approximately 5 mg of samples were placed in the aluminum pan, sealed and heated at 10 °C/min from 25 to 500 °C.

2.5. In vitro bioadhesion of mucin to the composites particles

In this study, the periodic acid Schiff's (PAS) colorimetric assay was used as previously reported (Juntapram et al., 2012) for the determination of the free mucin concentration, so as to (indirectly) evaluate the amount of mucin adsorbed onto the test particles (chitosan and its three derivatives). Schiff's reagent contained 100 mL of 1% (w/v) basic fuchsin (pararosaniline) in an aqueous solution and 20 mL of 1 M HCl. To this was added sodium metabisuphite (1.67% (w/v) final) just before use, and the resultant solution was incubated at 37 °C until it became colorless or pale yellow. Periodic acid reagent was freshly prepared by adding 10 μL of 50% (v/v) periodic acid solution to 7 mL of 7% (v/v) acetic acid solution.

Standard calibration curves were prepared from four mucin standard solutions (0.125, 0.25, 0.375 and 0.5 mg/mL). After adding 0.1 mL of periodic acid reagent, the solutions were incubated at 37 °C for 2 h before 0.1 mL of Schiff reagent was added and incubated at room temperature for 30 min. Next 0.1 mL aliquots of the solution were transferred in triplicate into separate wells of a 96-well microtiter plate and the absorbance at 555 nm (λ_{555}) was recorded. The mucin contents were then calculated by reference to the standard calibration curve.

A 0.5% (w/v) mucin solution in each of three broadly isoosmotic solutions that differ in pH, namely simulated gastric fluid (SGF; 0.1 M HCl, pH 1.2) simulated duodenum fluid (SDF; 0.1 M sodium acetate buffer, pH 4.0) and simulated intestine fluid (SIF; 0.1 M sodium acetate buffer pH 6.4) media, were prepared. Note these buffers are intended to simulate the gastric, duodenum and small intestine in terms of the pH only, and not the other components. The composite particles were dispersed at 20 mg/1.5 mL (final concentration) in the above mucin solutions, vortexed, and shaken at 37 °C for 2 h. The dispersions were then centrifuged at 12,000 rpm (give the rotar radius or else as × *g* rcf) for 2 min to pellet the polymer–mucin complexes, the supernatant was harvested and the free mucin content evaluated by measuring the λ_{555} (Section 2.5.1) with reference to the calibration curve. The amount of mucin adsorbed to the microspheres was then calculated as the difference between the total amount of mucin added and the free mucin content in the supernatant.

2.5.1. Determination of drug encapsulation efficiency (EE)

UV spectroscopy was used for the determination of the CPT encapsulation efficiency (EE). Briefly, an exact weight of dried particles (~2 mg) was dissolved in 10 mL of 10% (v/v) DMSO with stirring at room temperature for 30 min. The amount of CPT released into the DMSO (W_E) was then examined by measuring the absorbance at 370 nm (A_{370}). The CPT contents were then calculated by reference to the standard curve, formed from 10 different concentrations (0.1–10 μM) in the same solvent. The standard curve was found to be highly accurate and reproducible, with a coefficient of determination of 0.999. All experiments were performed in triplicate. The percentage EE of CPT was obtained as in Eq. (1):

$$\text{EE } (\%) = \frac{100 W_E}{W_T} \quad (1)$$

where W_E is the weight of the drug (CPT) in the particles and W_T is the total weight of the CPT added to the particle loading solution in their synthesis (Section 2.3)

The profile of CPT release from the TM-HT-chitosan/alginate and chitosan/alginate PEC particles was evaluated in three different buffers (SGF at pH 1.2, SDF at pH 4.0 and SIF at pH 6.4) at 37 ± 2 °C using the membrane dialysis technique. An accurately weighed quantity (~10 mg) of CPT-loaded TM-HT-chitosan/alginate or CPT-loaded chitosan/alginate PEC particles were enclosed in a dialysis bag with a M_WCO of 3.5 kDa and immersed into 50 mL of the appropriate buffer in a flask. The flask was placed in a shaking water bath at 100 rpm and incubated 37 ± 2 °C for 3 days. At 2 h intervals a 1 mL aliquot of the incubated solution was withdrawn for analysis of the CPT concentration by A_{370} and replaced with an equal volume of fresh buffer. The amount of released CPT over the 24-h period from

the two types of particles was then plotted against time for direct comparison.

2.6. Data analysis

Results are shown as the mean ± one standard deviation (SD) from the indicated number of independent repeats. Tests of statistical significance for differences between means were performed by Analysis of Variance (ANOVA) and Duncan's multiple means test for parametric data, and Kruskall–Wallis and Mann–Whitney *U*-tests with Holm correction for non-parametric data. *P* values of less than 0.05 were accepted as significant.

3. Results and discussion

3.1. Morphology

The SEM based analysis of the morphology of the particles obtained from the EI technique (Section 2.3) is shown in Fig. 3. The surface appearance of the TM-HT-chitosan particles were typically in a generic spherical shape and appeared smooth on the surface without visible pores when seen at a high magnification (Fig. 3a and b). Thus, TM-HT-chitosan can be fabricated as microspheres by EI, and so the EI technique under these conditions (Section 2.3) was used to further investigate the possible preparation of PECs between TM-HT-chitosan and the anionic polymers, carrageenan and alginate.

The SEM micrographs of the TM-HT-chitosan/carrageenan PEC particles revealed that the particles had a smooth surface without visible pores, but they were not spherical and uniform in shape (Fig. 3c and d). Rather the PECs with κ-carrageenan were found to be dependent on the specific types of cations and anions used (data not shown). κ-Carrageenan has one sulfate group per two galactose residues (Tapia et al., 2004), which leads to reduced electrostatic repulsion forces between the potassium ion and the participating chains.

The CPT-free TM-HT-chitosan/alginate PEC particles were almost spherical in shape with a smooth surface and without visible pores (Fig. 4e and f). Therefore, TM-HT-chitosan/alginate PEC was chosen to load with CPT and then to study the release behavior of the CPT anticancer drug. The morphology of the CPT-loaded TM-HT-chitosan/alginate PEC particles were of an acceptable spherical shape, although slightly flattened, and were smooth surfaced without visible pores (Fig. 4g–f). The absence of the more ideal spherical morphology can probably be attributed to the drying process that causes certain invaginations in the particles (Mladenovska et al., 2007). From these results a preparation of TM-HT-chitosan PECs with alginate was seemingly prepared by the EI process.

3.2. Fourier transform infrared spectroscopy (FTIR)

The interaction of the TM-HT-chitosan and alginate in the PEC particles, and CPT loading, was confirmed by FTIR analysis, comparing the spectra of the TM-HT-chitosan/alginate with or without CPT loading with those of TM-HT-chitosan, pure alginate and CPT. The FTIR spectrum of alginate (Fig. 4a) showed distinct peaks at 3422 cm^{-1}, 1618 cm^{-1}, 1412 cm^{-1} and 1024 cm^{-1} which are due to the O—H groups, the symmetric and symmetric COO^- stretching vibration and C—O stretching, respectively (Mladenovska et al., 2007). The FTIR spectrum of TM-HT-chitosan (Fig. 4b) showed peaks at 3428 cm^{-1}, 1675 cm^{-1} and 1599 cm^{-1} that were ascribed to the O—H stretching, the vibration of the amide bond and the partial *N*-deacetylation of chitin, respectively (Juntapram et al., 2012). The TM-HT-chitosan/alginate PEC showed a new absorption peak of the composite at 1719 cm^{-1} (Fig. 4c), which is the C=O stretching of TM-HT-chitosan was shifted from 1675 cm^{-1}. The stretching vibration at 1434 cm^{-1} was due to the symmetric stretching of $-COO^-$ groups. The band appearing at 1249 cm^{-1} in the spectrum of polyion complexes can be assigned to a symmetric $-NH_3C$ and $-N(CH_3)_3C$ deformation, and the broad bands that appeared at 2500 cm^{-1} confirm the presence of the $-NH_3C$ group in the polyion complex particles. These results indicate that the carboxylic groups of alginate are associated with the ammonium groups of the TM-HT-chitosan through electrostatic interactions to form the PECs. Moreover, as the polyion complex formation proceeds, O—H bonding would also be expected because of an increase in intermolecular interactions, such as hydrogen bonding, between sodium alginate and modified chitosan (Smitha, Sridhar, & Khan, 2005).

The FTIR spectrum of the CPT-loaded TM-HT-chitosan/alginate PEC particles is shown in Fig. 4e. As can be seen, the characteristic peaks at 2986 cm^{-1} and 1728 cm^{-1} that correspond to the amino-quinoline and ketone peak of CPT, and the peaks at 1314 cm^{-1} and 962 cm^{-1} due to the C—H in-plane bending vibrations of CPT, appeared in the FTIR of the composite. These results indicate that CPT was loaded onto the TM-HT-chitosan/alginate PEC particles.

3.3. Thermogravimetric analysis (TGA)

Fig. 5 shows the TG (Fig. 5a) and corresponding DTG (Fig. 5b) thermograms of the particles with and without CPT loading of the TM-HT-chitosan/alginate PEC particles compared with TM-HT-chitosan and pure alginate. Here, the pure alginate showed a weight loss of water of about 16.3% (30–104 °C), and degraded in the temperature range of 203–307 °C with 35.4% weight loss. The TGA thermograms for the CPT-free TM-HT-chitosan/alginate PEC particles showed the expected three stages of weight loss. The first decomposition peak, due to water evaporation, was followed by a two-step decomposition process occurring at 193.1–278.4 °C for HT decomposition and final weight loss curve for alginate and TM-HT-chitosan revealed two additional broad peaks at 274.8 °C and 317.0 °C, respectively. We suggest that the shift of the last decomposition temperature of the TM-HT-chitosan/alginate to a higher value, compared to that seen with TM-HT-chitosan, is the result of inter- and intra-molecular disulfide interactions, which result in a higher polymer thermal stability. It is well established that intramolecular interactions increase the thermal stability of polymers, and so leads to a higher decomposition temperature (Davidovich-Pinhas & Bianco-Peled, 2011b).

However, the CPT inclusion complexes with TM-HT-chitosan/alginate PEC showed a weight loss (decomposition) at lower temperatures (317 °C) compared to that without CPT (380 °C), indicating that CPT is dissolved in the polymer solution which then results in a lower polymer thermal stability (Barreiro-Iglesias et al., 2004). Moreover, the particles presented a degradation peak at around 70 °C, a likely indication of the interaction between the alginate and calcium ion.

The different composite particles had a hydrated size range of between ~2.3 μm and ~3.4 μm, as determined by DLS analysis (Table 1). When CPT was loaded into the PEC polymer it resulted in an increase in the mean size of the hydrated particles of 1.33- and 1.15-fold for the chitosan/alginate and TM-HT-chitosan/alginate PEC particles, respectively, due to the attachment of CPT on the composites. The PDI, which measures the width of the particle size distribution (from 0 = monodisperse to 1 = polydisperse), revealed that the particles were of a fairly broad size range with a PDI of 0.69–0.88. The zeta potential, a measurement of the overall surface charge of the particles, is affected by changes in the environment, such as the pH, presence of counter-ions and adsorption and can greatly influence particle stability in suspension through the electrostatic repulsion between particles. The CPT-free composite particles had a weakly negative zeta potential,

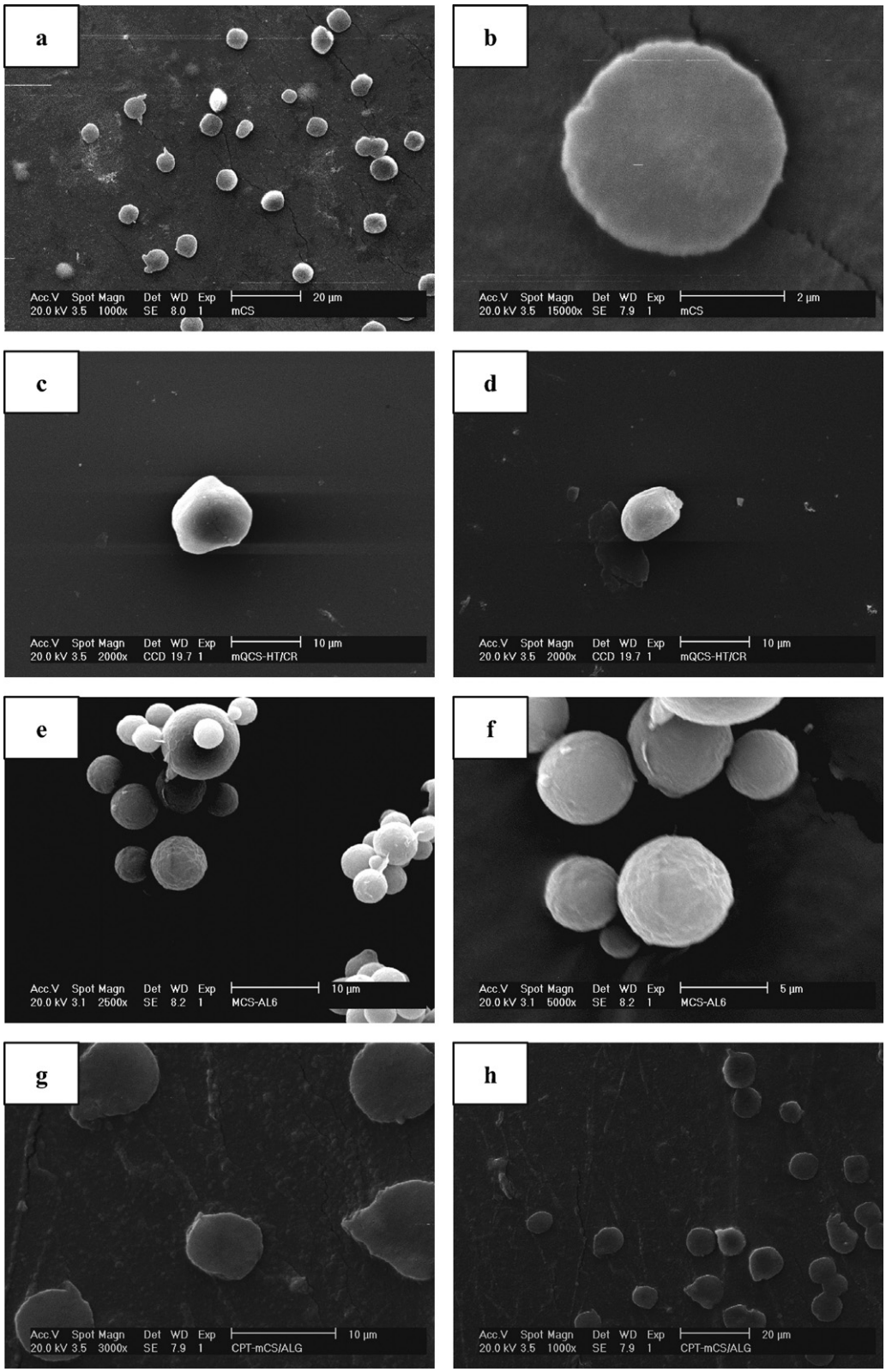


Fig. 3. Representative SEM images of (a and b) TM-HT-chitosan, (c and d) TM-HT-chitosan/carrageenan PEC, (e and f) TM-HT-chitosan/alginate PEC, and (g and h) CPT-loaded TM-HT-chitosan/alginate PEC.

with the TM-HT-chitosan/alginate PEC composite (−4.9 mV) being less negatively charged than the chitosan/alginate PEC (−6.1 mV). This is because the positive charge on the amino group of chitosan is pH dependent and so not all are positively charged at this pH, in contrast to the pH-independent positive charge of the quaternary ammonium group of the TM-HT-chitosan (Sadeghi et al., 2008). However, since both types of CPT-loaded PEC particles displayed a higher negative zeta potential value, at about −17.2 and −10.9 mV for TM-HT-chitosan/alginate and chitosan/alginate PEC portals, respectively, then the CPT is likely to be on the surface of

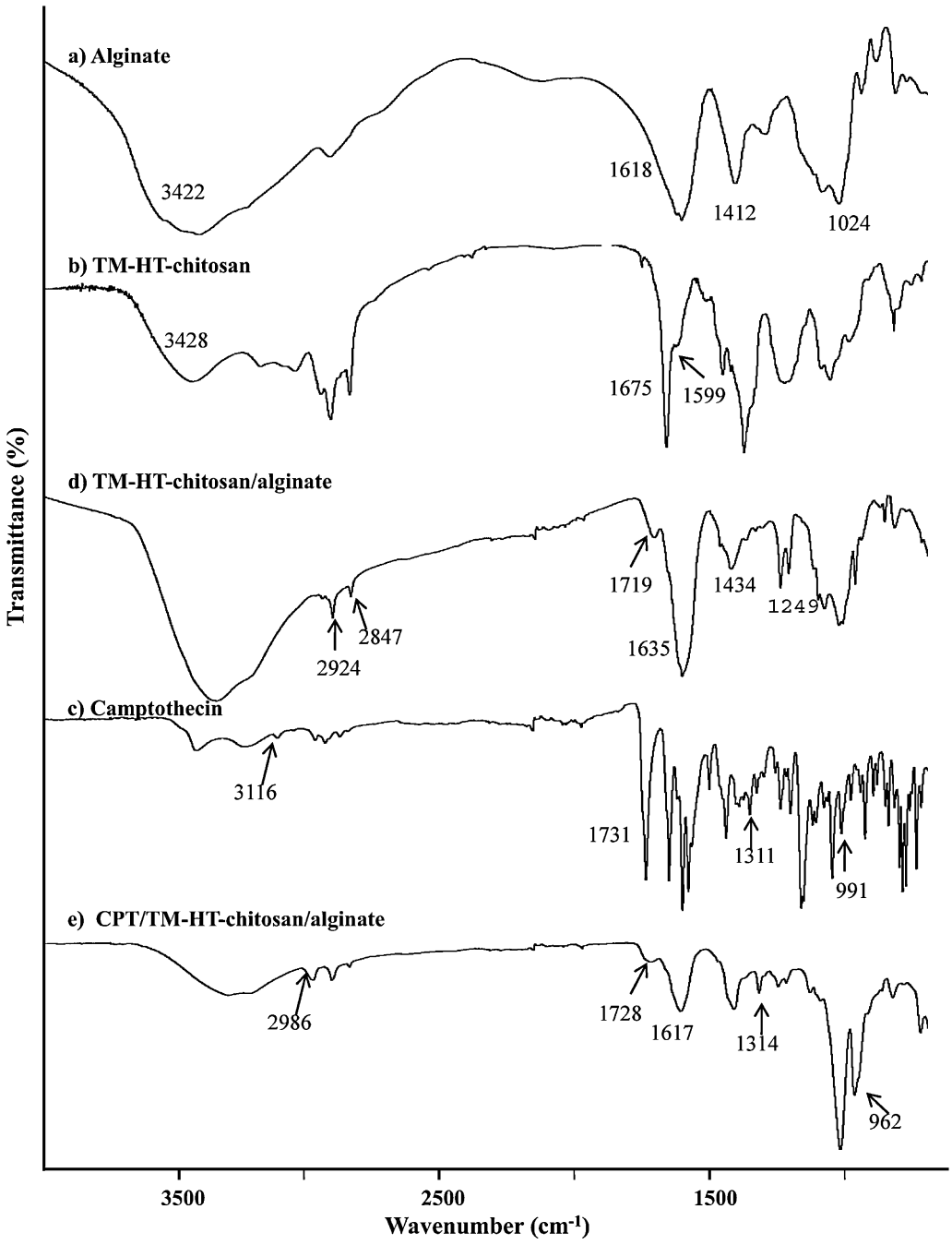


Fig. 4. FTIR spectra of (a) alginate, (b) TM-HT-chitosan, (c) TM-HT-chitosan/alginate PEC, (d) camptothecin (CPT), and (e) CPT-loaded TM-HT-chitosan/alginate PEC.

Table 1
Mean particle diameter, particle size distribution (PDI), zeta potential, and CPT encapsulation efficiency (EE) of chitosan/alginate and TM-HT-chitosan/alginate PEC particles with or without CPT-loading.

Abbreviations	Bead size ± SD ((m) by nanosizer	Polydispersity (PDI)	Zeta potential (mV)	Encapsulation efficiency (%EE)
Chitosan/alginate	2.33 ± 0.05	0.82 ± 0.10	−6.06 ± 0.67	–
TM-HT-chitosan/alginate	2.98 ± 0.36	0.87 ± 0.08	−4.87 ± 1.31	–
CPT/chitosan/alginate	3.15** ± 0.37	0.69 ± 0.18*	−17.23 ± 1.36	74.3 ± 2.11
CPT/TM-HT-chitosan/alginate	3.42** ± 0.09	0.88 ± 0.07	−10.90 ± 0.90	69.6 ± 0.71

*The mean difference is insignificant ($P > 0.01$) compare to chitosan/alginate particles.
** The mean difference is significant ($P < 0.01$) compare to chitosan/alginate particles.

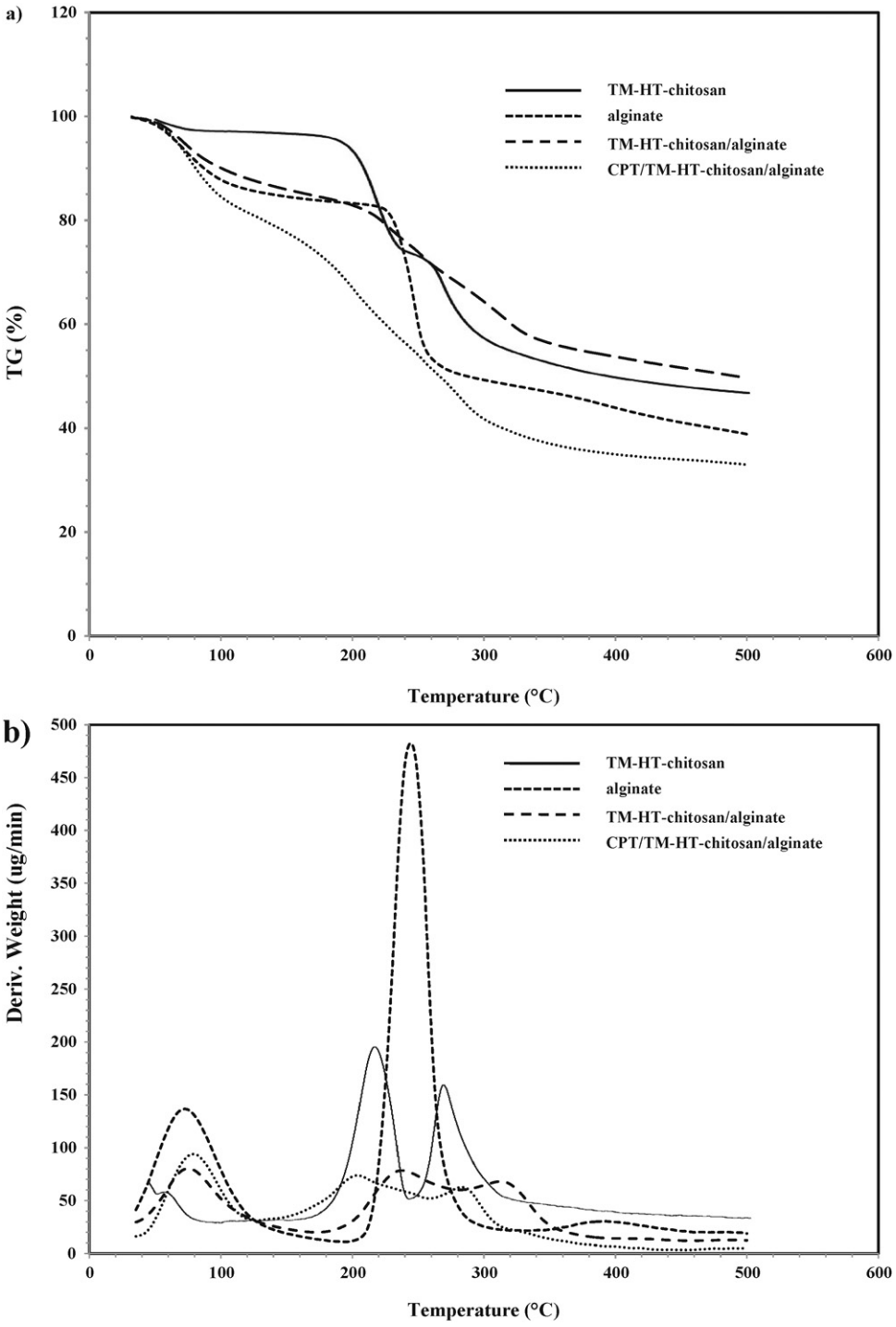


Fig. 5. TGA thermograms, shown as the (a) TG and (b) TGA curves of TM-HT-chitosan (solid line), alginate (large dot line), TM-HT-chitosan/alginate PEC (dashed line), and CPT-loaded TM-HT-chitosan/alginate PEC (fine dot line).

the PEC particles (Cirpanli, Bilensoy, Lale Dogan, & Calis, 2009). This can be understood by associating the polymer solidification process with the CPT encapsulation process. Chitosan derivatives and alginate gradually form a homogeneous network of many cages. CPT could easily enter inside this because of the loose structure outside the particles. As the solidification process then proceeds, CPT would be hindered because the structure of the particles becomes more compact due to the matrix formation between chitosan and alginate. Moreover, this result would explain how the EI technique can produce such a high EE (about 70%).

3.4. Mucoadhesiveness of the different PEC particles with or without CPT-loading

The chitosan/alginate PEC particles without CPT-loading absorbed mucin at about 2.67-, 1.42- and 1.21-fold more than

Table 2
Comparison of the mucoadhesive property in different pH solutions of the TM-HT-chitosan/alginate and chitosan/alginate PECs with or without CPT loading.

Composite particles	Adsorbed mucin level[a] (mg) at		
	pH 1.2	pH 4.0	pH 6.4
Chitosan	0.06 ± 0.01	0.31 ± 0.05	0.42 ± 0.03
Chitosan/alginate	0.16 ± 0.01	0.44 ± 0.03	0.51 ± 0.01
TM-HT-chitosan/alginate	0.30 ± 0.01	0.61 ± 0.01	0.65 ± 0.02
TM-HT-chitosan	0.38 ± 0.01	0.79 ± 0.03	0.75 ± 0.01
CPT-chitosan/alginate	0.17 ± 0.03	0.53 ± 0.01	0.59 ± 0.03
CPT-TM-HT-chitosan/alginate	0.31 ± 0.01	0.60 ± 0.01	0.66 ± 0.02

[a] Data are shown as the mean + SD and are derived from three independent repeats.

native chitosan at pH 1.2, 4.0 and 6.4, respectively, whilst for the TM-HT-chitosan/alginate PEC particles without CPT-loading the mucin absorption levels were 5.00-, 1.97- and 1.55-fold higher, respectively, than chitosan (Table 2).

The PECs formed between chitosan or TM-HT-chitosan and alginate showed an increase in their mucoadhesion, and this is likely to be due to the increased flexibility of the chitosan/alginate PEC structure and polar functional groups. As a result of this, they are able to interact with the mucus chains and so show a good mucoadhesiveness. This notion is supported by the studies of Wittaya-areekul, Kruenate, and Prahsarn (2006), who demonstrated that the adhesive capabilities of chitosan/alginate hydrogels can be improved by tethering of long flexible chains to the particle surface. The resulting modified hydrogels then exhibited increased mucoadhesive properties due to enhanced anchoring of the flexible chains with the mucosa (Wittaya-areekul et al., 2006).

Furthermore, one of the most important factors which affect the mucoadhesion ability of the composite particles is the electrostatic interactions, and in accord a statistically significant decrease in the mucoadhesion level was observed as the pH of the medium increased.

Much of the apparent mucoadhesion dependence upon the pH can be explained by the relative charge density. The TM-HT-chitosan/alginate PEC particles tend to shrink when exposed to the acidic environment of the SGF or SDF buffers (pH values < 4), since the carboxylate groups of the alginate backbone are protonated and hence the interaction by hydrogen bonds with mucin-type glycoproteins through carboxyl-hydroxyl interactions are reduced (Davidovich-Pinhas & Bianco-Peled, 2011a). Moreover, in the chitosan part of the composite, the interactions between the composite and mucus are also stronger at an acidic pH due to the amine units being protonated to NH_3^+, and so chitosan can interact with either the COO^- or SO_3^- group on the mucin glycoprotein side chain. In addition, other hydrogen bonds and ionic interactions, might also be involved. In the case of the TM-HT-chitosan/alginate PEC, although the reactivity of the thiol groups on the polymer were oxidized before contact with the mucus gel layer in the acidic media (Palmberger, Hombach, & Bernkop-Schnürch, 2008), the mucoadhesive ability of this PEC was increased by the addition of the HT moieties and the positive quaternary amines because of their electrostatic and hydrophobic effects. The electrostatic effect is mainly due to the remaining protonated amine ($^+NH_3$) of the chitosan backbone and the positive quaternary amine ($^+N(CH_3)_3$) of the HT side chain being able to interact with either the COO^- or SO_3^- groups on the mucin carbohydrate side chain in an acidic media. For the hydrophobic effect, the $-CH_2$ moieties of HT interact in part with the $-CH_3$ groups on the mucus layer.

Increasing the pH value up to 6.4, which is then above the pK_a of alginate (3.38–3.65), causes the carboxylate groups (—COOH) of alginate to become deprotonated into the anionic form ($-COO^-$) (Xu, Zhan, Fan, Wang, & Zheng, 2007), allowing hydrogen bonds to form between the $-COO^-$ and the hydrogen part of the mucus layer (John, 2005), resulting in an increase in the mucoadhesive property. However, at pH 6.4, the alginate/chitosan PEC particles exhibited a statistically significant ($P < 0.01$) decrease in their mucoadhesion level compared to that in media of pH 1.2 or 4. This is due to the reduced average positive charge density at pH 6.4, since this is principally derived from the chitosan backbone amine groups in an acidic environment. For the thiolated derivative polymer (TM-HT-chitosan/alginate), at a pH above 6 there is an increased concentration of the reactive form of the thiolate anion ($-S^-$) (Leitner, Walker, & Bernkop-Schnürch, 2003), and so not only electrostatic and hydrophobic effects are at play, as discussed above, but also the level of covalently linked disulfide bonds between the thiolated polymer and the cysteine rich chain of the mucus glycoproteins comes into play and is an important determinant of the polymer's mucoadhesiveness.

Previous work has suggested the use of TM-HT-chitosan as a new approach for adhering chitosan polymers to mucosal surfaces, with a reported absorbed mucin level of about 6.33-, 2.55- and 1.79-fold higher than that for chitosan at pH 1.2, 4.0 and 6.4, respectively (Juntapram et al., 2012). However, the TM-HT-chitosan/alginate PEC particles of this study revealed a reduced mucoadhesion level compared to pure TM-HT-chitosan at all three-tested pH conditions. Presumably after the TM-HT-chitosan formed the PEC with alginate, the carboxylate groups of alginate interacted with protonated amino groups of chitosan through electrostatic interactions, and so the influence of the positive and negative charges of the TM-HT-chitosan/alginate PEC particles were then insufficient to increase the mucoadhesion compared to that of the TM-HT-chitosan.

Encapsulation of CPT into either the chitosan/alginate or the TM-HT-chitosan/alginate PEC particles caused no statistically significant ($P > 0.05$) changes in their mucoadhesive property (Table 2).

3.5. In vitro CPT release profiles

To investigate the effect of the TM-HT modification of chitosan in chitosan/alginate PEC particles on the release of CPT, the in vitro release of CPT from CPT-loaded TM-HT-chitosan/alginate and chitosan/alginate PECs were performed in three different pH buffers, namely pH 1.2 (SGF), 4.0 (SDF) and 6.4 (SIF) at 37 °C. The CPT was slowly released from the TM-HT-chitosan/alginate PEC particles in all pH mediums in the initial release stage (Fig. 6a). However, the release rate of CPT from the TM-HT-chitosan/alginate PEC particles at pH 1.2 was much higher than at either pH 4.0 or 6.4. In the pH 1.2 medium the cumulative release ratio of CPT from the TM-HT-chitosan/alginate PEC particles was approximately 78% after 6 h and then increased very little over the remaining time, whereas at the higher pH values (4.0 and 6.4), the cumulative CPT release level was about 28% and 15%, respectively, at the same time and then continued to increase to either plateau at ~60% at 10 h in pH 4, or to attain ~70% by 12 h at pH 6.4 and continued to sustained drug release up to 3 days. The differences in the CPT release kinetics are likely to be due to the swelling ratio of the particles with the changing pH of the medium. Even though alginate with its carboxylate groups shrinks

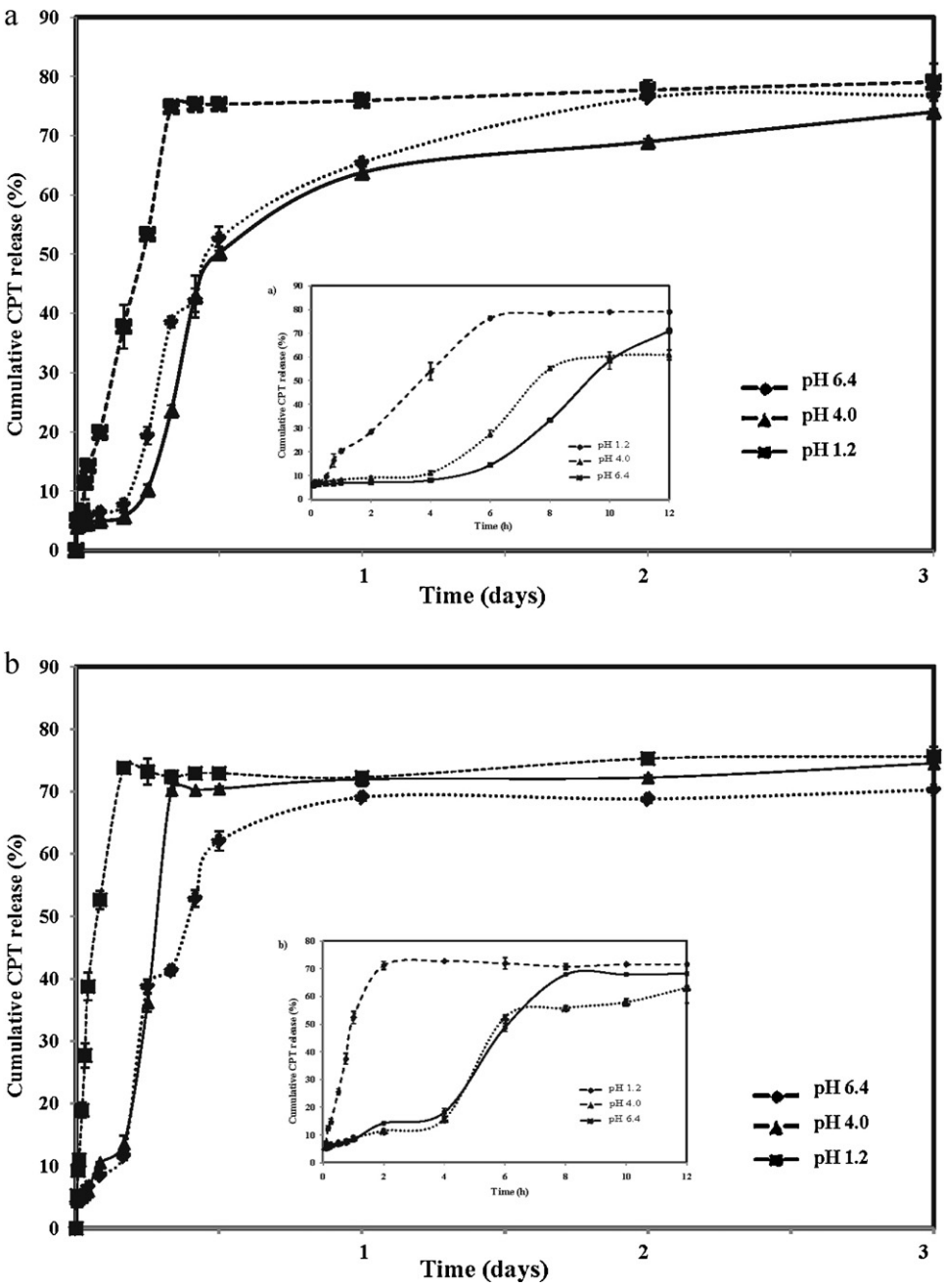


Fig. 6. In vitro drug release profiles of camptothecin (CPT) from (a) the TM-HT-chitosan/alginate and (b) chitosan/alginate PEC particles in different pH solutions. Data are shown as the mean + SD and are derived from three independent repeats.

at a low pH and dissolves at a high pH (Yu et al., 2008), in this study the alginate was composited with the TM-HT-chitosan derivative, which results in a decreased degree of swelling as the pH increases (Juntapram et al., 2012). The higher swelling ratios of the polymer creates a larger surface area and looser matrix for the CPT to diffuse out of (Huang & Brazel, 2001) and so led to its quick release from the particles at pH 1.2 and correspondingly the slower release kinetics at pH 4.0 and 6.4. Thus, the CPT release behavior was dominated by the swelling ratio of the TM-HT-chitosan. In accord, the CPT did not show any significant burst release from the TM-HT-chitosan/alginate PEC particles in all three media, including at pH 1.2 (Fig. 6a), compared to that seen from the chitosan/alginate PEC particles (Fig. 6b). The reason for the reduced and sustained release of CPT at a low pH was the formation of disulfide bonds within the matrix particles (Palmberger et al., 2008). This cross-linking process then provides a tightened three-dimensional polymeric network leading to a more controlled drug release. Hence, the advantage of the controlled drug release system based on mucoadhesive polymers can be improved by using their thiolated derivatives (Senyigit, Vetter, Guneri, & Bernkop-Schnurch, 2011).

4. Conclusion

New PEC matrixes for the oral delivery of PCT (as a model payload drug) based on TM-HT-chitosan/alginate PEC particles were prepared by EI. The resulting controlled release of CPT-loaded TM-HT-chitosan/alginate PEC particles demonstrated that the EI process is a facile technique for the production of chitosan/alginate and TM-HT-chitosan/alginate PEC carriers, and for their

CPT-loading. The size distribution of the CPT-loaded chitosan/alginate and the TM-HT-chitosan/alginate PEC microparticles was relatively narrow (PDI of 0.69–0.88) and yielded a high EE (~70–74%). Thus, the EI technique could be a promising technique for the fabrication of particle-based drug delivery carriers, at least as microparticles. The CPT-loaded TM-HT-chitosan/alginate PEC particles prolonged the release of CPT over 12 h and were greatly improved over that obtained using the unmodified chitosan/alginate PEC within 3 days in SIF.

Acknowledgments

This research was financially supported by the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) to K.J.; The Center for Petroleum, Petrochemicals and Advanced Materials, Program of Petrochemistry and Polymer Science; the Center of Innovative Nanotechnology (CIN); the National Research University Project of CHE; and the Ratchadaphiseksomphot Endowment Fund (AS613A) to N.M. The comments of three anonymous reviewers are gratefully appreciated. Thanks are also given to Dr. Robert Butcher of the PCU, Faculty of Science, Chulalongkorn University, for constructive comments and English corrections.

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