PHARMACEUTICAL TECHNOLOGY

DEVELOPMENT OF NEW DRUG FORMULATIONS: CETIRIZINE-POLYMERS NANOPARTICLES

SAMER HASAN HUSSEIN-AL-ALI^{1,2*}, MOHD ZOBIR HUSSEIN³, RAMI AYOUB¹,
SHARIDA FAKURAZI^{4,5}, QAIS IBRAHIM ABDALLAH ABUALASSAL¹,
and YOUSEF AL-DALAHMEH¹

¹Faculty of Pharmacy, Isra University, P.O. Box 22, Amman 11622, Jordan

²Faculty of Science, Isra University, P.O. Box 22, Amman 11622, Jordan

³Materials Synthesis and Characterization Laboratory, Institute of Advanced Technology (ITMA),
Universiti Putra Malaysia, 43400UPM, Serdang, Selangor, Malaysia

⁴Laboratory of Vaccines and Immunotherapeutics, Institute of Bioscience, Universiti Putra Malaysia,
43400UPM, Serdang, Selangor, Malaysia

⁵Department of Human Anatomy, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia,
43400UPM, Serdang, Selangor, Malaysia

Abstract: Cetirizine (Cet)-encapsulated chitosan- (CSNP), alginate- (AlgNP) and chitosan-alginate- nanoparticles (CSNP-AlgNP) were prepared using the ion-ion interaction method to form Cet-CSNPs, Cet-AlgNPs, Cet-CSNPs-AlgNPs nanoparticles, respectively. The three nanoparticles were then subjected to drug delivery studies. The nanoparticles were prepared by the dropwise addition of Cet or CaCl₂ or TPP solution to Alg and CS solution, depending on the nanoparticles prepared. These Cet-CSNPs, Cet-AlgNPs, Cet-CSNPs-AlgNPs were around 96, 95, and 109 nm in size, respectively, with zeta potential values of +6.7, -5.6, and -6.4 mV, respectively. The percentage loading efficiency was found to be 20, 45, and 35% for Cet-CSNPs, Cet-AlgNPs, and Cet-CSNPs-AlgNPs, respectively. Fourier transform-infrared spectroscopy and thermogravimetric analysis data indicate the successful formation of Cet-CSNPs, Cet-AlgNPs, Cet-CSNPs-AlgNPs. In vitro release of Cet shows about 83%, 90%, and 75% release in 30 h for Cet-CSNPs, Cet-AlgNPs, and Cet-CSNPs-AlgNPs, respectively, and were best fitted into Hixson-Crowell, Pseudo-second, and Higuchi models for Cet-CSNPs, Cet-AlgNPs, Cet-CSNPs-AlgNPs, respectively. The in vitro cytotoxicity data for the three nanoparticles does not show any toxic effect toward 3T3 cell lines, at least up to 100 µg/mL. The nanoparticles have good potential to be used as the extended-release formulation of Cet.

Keywords: cetirizine, chitosan nanoparticles, alginate nanoparticles, chitosan-Alginate nanoparticles, sustained release, 3T3 cell lines

One of the major research areas in the drug delivery system is the preparation of polymer nanoparticles that are capable to deliver drugs with high efficacy and smart targeted sites which prevent the spread of antibiotic resistance and improve patient compliance (1). Polymer nanoparticles can improve drug stability (2), improve the time of the therapeutic effect (3), and prevent or minimize drug degradation (4). There is a great interest in the application of nanoparticles for the synthesis of biodegradable polymers, natural biopolymers, lipids, and polysaccharides as biomaterials for the delivery of drugs.

Recently, the use of alginate and chitosan nanoparticles to deliver drugs has become of great interest. Both of them have been often used in the

pharmaceutical industry for controlling the kinetics of the drug release (5).

Alginate (Alg) is a linear polysaccharide with alternating blocks of 1-4 linked α -L-guluronic and β -D-mannuronic acid residues. Alg has the potential to be used for drug delivery and cell encapsulation. AlgNPs can be prepared easily by gelation with crosslinkers such as calcium ions (6).

Chitosan (CS) is a linear polysaccharide polymer with glucosamine and N-acetyl glucosamine units. It is biocompatible, biodegradable, and non-toxic (7). CS contains three reactive functional groups; both primary and secondary hydroxyl groups at the C-2, C-3, and C-6 positions, and also the amino group and acetamide group. CS has

* Corresponding author: e-mail: samer.alali@iu.edu.jo, sameralali72@yahoo.com

very good potential for various biomedical applications, such as for treatment of recalcitrant lesions of cutaneous leishmaniasis (8), antibacterial wound dressing (9), delivery of drugs (10-12), and antioxidant and metal chelating. Different drugs were loaded on chitosan nanoparticles; antibiotic drugs like ampicillin, doxycycline, metronidazole, and ceftriaxone, anticancer drugs such as 5-fluorouracil, saponin, letrozole, doxorubicin, and paclitaxel; Parkinson's disease drugs such as selegiline; central nervous system drugs such as thiocolchicoside and anti-hypertension drugs such as perindopril erbumine, etc.

The opposite charge network of CSNPs is formed by the interaction between the protonated amine functional groups (NH_3^+) with an anionic phosphate group. In the case of AlgNPs, the anionic carboxyl group of alginate and cationic calcium ions reacted with each other. The polyelectrolyte network of CSNPs-AlgNPs is formed by the interaction between the dissociated anionic carboxyl groups of alginate with the protonated cationic amino group of chitosan. Intra- and inter-chain hydrogen bonding between different parts of the polysaccharides in CSNPs, AlgNPs, and CSNPs-AlgNPs structures will form (13). The formation of these nanoparticles depends on the order of mixing, the chemical composition of polymers, temperature, the flexibility of polymers, ionic strength, pH, and reagent concentration (14).

Cetirizine dihydrochloride (Cet), 2-[2-[4-[(4-chlorophenyl)-phenyl methyl] piperazin-1-yl] ethoxy] acetic acid dihydrochloride is one example of a second-generation antihistamine used to treat allergic rhinitis (hay fever), dermatitis, and urticaria (15). Different nanocarriers such as zinc-layered hydroxide nanoparticles (16) and carbon nanotubes (17) have been used, where Cet was used as the guest of these nanocarriers.

The quick disintegration of traditional, cetirizine tablets may result in primary "burst-released" kinetics, which may cause rapid drug absorption leading to high serum concentration with possible adverse effects such as local irritation to gastrointestinal (GI) mucous membranes (18-20). Disadvantages could be avoided by control the release rate of cetirizine dihydrochloride and cover the bitter taste, using design and develop a rational delivery system as modified chitosan nanocarriers (20, 21).

The purpose of the current study was to assess the possibility of CSNPs, AlgNPs, and CSNPs-AlgNPs nanoparticles to be used as carriers for cetirizine. The challenge of this work was to encapsulate cetirizine drugs onto CSNPs, AlgNPs, and

CSNPs-AlgNPs, which were prepared by ionotropic gelation. Cet was selected as a model drug for the preparation of the formulations for better-controlled release properties.

MATERIALS AND METHODS

Materials

Cetirizine hydrochloric acid ($\text{C}_{21}\text{H}_{25}\text{ClN}_2\text{O}_3 \cdot 2\text{HCl}$, molecular weight: 461.81) was purchased from Sigma-Aldrich, at 99.79% purity. CS with low molecular weight (10-120 kDa, 90% deacetylation, Sigma-Aldrich), low viscosity sodium alginate (10-100 kDa), and sodium tripolyphosphate (TPP), was purchased from Sigma Aldrich, at 98.0% purity.

Cells and cytotoxicity reagent

Normal mouse fibroblast cells (3T3) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The MTT assay kit was obtained from Sigma-Aldrich (Steinheim, Germany).

Preparation of the nanoparticles

Preparation of solutions

CS solution was prepared by dissolving about 300 mg of CS into 1.0% (v/v) of acetic acid and the solution was then stirred at 500 rpm for 3 h, and heated to 30°C until a viscous solution of CS was obtained.

Sodium TPP solution was prepared by dissolving about 300 mg of TPP into 10 mL distilled water.

Cet and sodium TPP solution was prepared by mixing 300 mg of TPP with 60 mg of Cet. Both of them were dissolved into 10 mL distilled water.

For Alg and CaCl_2 solutions, about 300 mg of Alg powder was dissolved into 100 mL distilled water with overnight stirring, where the pH of this solution was around 7.1. The CaCl_2 solution was prepared by dissolving 335 mg of CaCl_2 into 100 mL of distilled water.

For CS and CaCl_2 solutions, 150 mg of CS were dissolved into 1.0% (v/v) acetic acid for the CS solution. At the same time, the CaCl_2 solution was prepared by dissolving 167 mg CaCl_2 into distilled water, giving a clear solution.

Finally, for the Alg and TPP solutions, 150 mg of Alg with 150 mg of TPP were dissolved into 100 mL distilled water, giving a clear solution.

Preparation of CSNPs and Cet-CSNPs

The CSNPs nanoparticles were prepared by adding the TPP solution into the CS solution and adjusting the pH of the CS solution to 5.4 using NaOH.

The prepared CSNPs were stirred for 18 h and the resulting nanoparticles were centrifuged at 15000 rpm for 10 min and dried at 50°C for 24 h. The Cet-CSNPs were prepared by adding a mixture of TPP and Cet to the CS solution followed by the procedure for the preparation of the CSNPs.

Preparation of the AlgNPs and Cet-AlgNPs

The AlgNPs were prepared by adding the solution of Alg to CaCl_2 . The reaction content was adjusted to pH 5.4 using NaOH solution. Prepared AlgNPs were stirred for 18 h and the resulting nanoparticles were centrifuged at 15 000 rpm for 10 min and dried at 50°C for 24 h.

The Cet-AlgNPs were prepared using 60 mg of Cet and 335 mg of CaCl_2 . The mixture solution of Cet and CaCl_2 was added to the Alg solution. The pH of the three components was increased from 4.5 to 5.4 using NaOH solution. The resulting Cet-AlgNPs were collected by centrifugation at 15000 rpm for 10 minutes and dried at 50°C for 24 h.

Preparation of the CSNPs-AlgNPs and Cet-CSNPs-AlgNPs

The CSNPs-AlgNPs were prepared by a method briefly describe as follows: the homogeneous mixture solution of Alg-TPP was dropped slowly into the CS- CaCl_2 solution for around 3 h while stirring at a high speed. The pH of the resulting solution was increased to pH 5.4 using NaOH solution and the stirring was continued overnight. The CSNPs-AlgNPs formed were centrifuged at 15 000 rpm for 10 min and dried at 50°C for 24 h. The Cet-CSNPs-AlgNPs were prepared as follows: the solution of Alg-TPP and Cet was dropped slowly into the CS- CaCl_2 solution for around 3 h while stirring at a high speed. The pH of the resulting solution was increased to pH 5.4 using NaOH solution and the stirring was continued overnight. The Cet-CSNPs-AlgNPs formed were centrifuged at 15 000 rpm for 10 minutes and dried at 50°C for 24 h.

The release of Cet from its nanoparticles

Cet release profiles from the Cet-CSNPs, Cet-AlgNPs, and Cet-CSNPs-AlgNPs were determined using a phosphate buffer solution (PBS) of 0.01 mole/L at pH 7.4. About 100 mg of the Cet-CSNPs, Cet-AlgNPs, and Cet-CSNPs-AlgNPs were added separately into 500 mL of the PBS media. The cumulative amount of Cet released into the PBS solution was measured at preset time intervals using a UV-Vis spectrophotometer at 231 nm. The release medium (2 mL) was removed

for the UV-vis analysis at given time intervals and it was then replaced with 2 mL of the buffered solution.

To compare the release behavior of Cet from the nanoparticles with that from a physical mixture (prepared for this purpose), 10.0 mg of the physical mixture of Cet (5.0 mg) and nanoparticles (50 mg) were used and placed into a cuvette and subjected to the UV-Vis spectrophotometer measurement.

The mean dissolution time (MDT) as a dissolution parameter was calculated for different formulation types according to Equation (1),

$$\text{MDT}_{\text{in-vitro}} = \frac{\sum_{i=1}^n t_{\text{mid}} \Delta M}{\sum_{i=1}^n \Delta M} \quad (\text{Eq. 1})$$

where I is the dissolution sample number, n is the number of dissolution times, t is the time at the mid-point between times t_i and t_{i-1} , M is the amount of drug dissolved between time t_i and t_{i-1} .

The loading efficiency of Cet

The loading efficiency of Cet in various nanoparticles was determined using Equation (2) as given below. The amount of free Cet in the supernatant was collected by a centrifuge at 15000 rpm and measured by a UV-vis spectrophotometer. The Cet loading efficiency in the nanoparticles was calculated as follows:

$$\begin{aligned} \text{Loading Efficiency \%} &= \\ &= \frac{C_i - C_f}{\text{mass of nanoparticles}} \times 100 \end{aligned} \quad (\text{Eq. 2})$$

where C_i is the initial mass of the drug used in the experiment and C_f is the mass of Cet in the supernatant, therefore $(C_i - C_f)$ is the mass of Cet which binds with the formulation.

The procedure which was used to determine the C_f is as follows,

- (i) centrifuge the nanoparticles at 15 000 rpm for 10 min and collect the remaining unloaded Cet in the supernatant
- (ii) measure the absorbance for the supernatant and calculate the mass of Cet in the supernatant using a calibration curve.

After centrifuging, washing, and drying, the collected mass of the final sample was kept in a sample bottle and the loading efficiency of the drug was calculated according to Equation 1.

The effect of pH on the Zeta potential of the nanoparticles

pH is the most important factor that affects the Zeta potential of the nanoparticles. To confirm these changes, the pH of titration was done using the dynamic light scattering (DLS) (Malvern Zetasizer Nano ZS) and the change in pH was plotted as a function of zeta potential.

Cell culture and MTT cytotoxicity assays

3T3 cells

The 3T3 cells were obtained from Swiss albino mice (primary mouse embryonic fibroblast cells). The '3T3' designation refers to the abbreviation of "3-day transfer, inoculum 3×10^5 cells".

Culture media

The 3T3 cells were maintained at 37°C and 5% CO₂ in a humidified incubator with a cultured medium which consisted of five different components: Dulbecco's Modified Eagles Medium (DMEM) appended with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 unit/mL Penicillin and 100 microgram/mL streptomycin. After two days, the cells had grown to approximately 90% confluence. Then, they were detached with trypsin and seeded into a 96-well plate at 1×10^5 cells/mL and kept overnight for cell attachment.

After two days, the medium of each well was removed and 100 µg/mL of the prepared samples suspensions (pure Cet, CSNPs, AlNPs, CSNPs-AlNPs, Cet-CSNPs, Cet-AlNPs, and Cet-CSNPs-AlNPs) in varying concentrations in the cell culture medium were added to the wells (0.0781, 1.562, 3.124, 6.25, 12.5, 25, 50 and 100 µg/mL). The 3T3 cells were cultivated for three more days after which the viability of the cells was determined by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide) assay (22).

Cytotoxicity test using the MTT assay

The viability of the 3T3 cells was analyzed by the MTT assay. It is a colorimetric assay based on the reaction of the yellow tetrazole by mitochondrial dehydrogenase enzymes and leads to an insoluble purple formazan product (23). Firstly, the medium was removed from each well. Secondly, 100 µL of fresh medium and 10 µL of MTT solution (5 mg/mL PBS) were added to each well and incubated for 4 h at 37°C/5% CO₂. Two hours later, the dimethyl sulfoxide was added to the cells as a detergent reagent to stop the conversion and solubilize the formazan. The amount of purple product is directly correlated with the number of viable cells. The absorbance of the purple color that is formed is taken at a wavelength

of 570 nm using a multiwell microplate reader. The experiment was performed in triplicates, and the result was expressed as the mean±SD. The cell viability calculated according to equation 3.

$$\text{Cell Viability \%} = \frac{\text{Average of treated}}{\text{Average Control}} \times 100 \quad (\text{Eq. 3})$$

Characterization of the nanoparticles

Powder X-ray diffraction

The entity and crystallinity of the nanoparticles were evaluated by the XRD diffraction technique. XRD patterns were taken with an XRD-6000 model diffractometer (Shimadzu, Tokyo, Japan) using the CuK_α radiation ($\lambda = 1.5406 \text{ \AA}$), at a high accelerating voltage of 40 kV and 30 mA with $2\theta = 2-70$ degrees.

Fourier transform infrared analysis

To confirm the presence of chemicals or functional groups that constitute the nanoparticles, a Fourier-transform infrared (FTIR) analysis was done. The FTIR spectra were recorded over the range of 400–4000 cm⁻¹ on a Perkin Elmer spectrometer (model Smart UAIR-two) with 4 cm⁻¹ resolutions.

Measurement of the Zeta potential

The measurements of the zeta potential and particle size distribution of the nanoparticles were performed using a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK) at 25°C.

Transmission electron microscopy

The particle shape, internal structure, and size of the nanoparticles were examined using transmission electron microscopy (TEM), (Hitachi H-7100, Tokyo, Japan) with an accelerating voltage of 80 and 200 kV.

Thermogravimetric analysis

The thermogravimetric analysis (TGA) was carried out using a Mettler-Toledo 851e instrument (Switzerland) with a heating rate of 10°C min⁻¹, in 150 µL alumina crucibles at 30-900°C.

RESULTS AND DISCUSSION

X-ray diffraction

Powder XRD patterns of Cet, CSNPs, Cet-CSNPs, AlNPs, Cet-AlNPs, CSNPs-AlNPs and Cet-CSNPs-AlNPs are presented in Figure 1. Free Cet (Figure 1A) shows crystalline properties due to the presence of sharp reflection peaks at $2\theta = 7.8^\circ$, 14.4° , 18.2° , 21.0° , 24.2° , 28.2° , 30.8° and 33.0° (24).

The XRD patterns of CSNPs (Figure 1B) appeared at $2\theta = 25.2^\circ$ and 49.7° with amorphous patterns, which is due to the cross-linkages of CSNPs with TPP (25). The Cet-CSNPs (Figure 1C) show a weak peak at $2\theta = 25.2^\circ$ and 50.4° , with amorphous characteristics. This amorphous phase of the weak peak can be related to the loading of the Cet into CSNPs nanoparticles (26).

Figure 1D shows the XRD patterns of AlgNPs. The nanoparticles exhibited two characteristic reflection peaks at $2\theta = 8.6^\circ$ and 42.4° with amorphous characteristics. Similar to AlgNPs, Cet-AlgNPs also show two characteristic reflection peaks at $2\theta = 10.2^\circ$ and 40.0° with amorphous properties due to the presence of Cet in the Alg polymer (27). In addition, Figure 1E (Cet-AlgNPs) shows the presence of trace-free Cet, which is indicated by the (♦) symbol at $2\theta = 16.2^\circ$.

XRD patterns of CSNPs-AlgNPs and Cet-CSNPs-AlgNPs are illustrated in Figures 1F and 1G, respectively. The XRD patterns of CSNPs-AlgNPs shows very low-intensity peaks at $2\theta = 24.4^\circ$, 39.2° , and 45.2° , revealing the amorphous nature of the polymers. The Cet-CSNPs-AlgNPs (Figure 1G) shows a weak peak at $2\theta = 24.6^\circ$, 38.6° , and 44.2° , with amorphous characteristics. The characteristic peaks of the Cet were absent in Cet-CSNPs-AlgNPs. This indicates that Cet was dispersed into both CS and Alg polymeric nanoparticles and there could be less or no free Cet in its crystalline form on the surface of the CSNPs-AlgNPs.

Infrared spectroscopy

Figure 2 shows the FTIR spectra and their assignment, respectively for Cet, Cet-CSNPs, Cet-AlgNPs, and Cet-CSNPs-AlgNPs. The FTIR spectra of Cet (Figure 2A) show broadband at 3432 cm^{-1} , corresponding to the stretching of the hydroxyl groups of the $-\text{COOH}$. A band at 3046 cm^{-1} is due to C-H symmetry and asymmetry stretching in phenyl and chlorophenyl groups. A band at 1436 cm^{-1} was assigned to the CO bending, C-C stretching, and C-H wagging at piperazinyl ethoxy acetic acid. A band at 1187 cm^{-1} is suggested due to the presence of C-O-H bending and C-O stretching vibration at piperazinyl ethoxy acetic acid groups. A band at 1093 cm^{-1} shows the C-H rocking in the phenyl group and chlorophenyl group, as well as the C-Cl stretching group. A band at 847 cm^{-1} was assigned to the C-C stretching and C-O stretching at piperazinyl ethoxy acetic acid and due to the C-N stretching (28).

The assignments of the FTIR spectra of CSNPs and Cet-CSNPs. A band at 3447 cm^{-1} is due to the stretching vibrations of the O-H group. The presence of the absorption band at 1640 cm^{-1} for CSNPs

Figure 1. Powder X-ray diffraction patterns of Cet (A), CSNPs (B), Cet-CSNPs (C) AlgNPs (D) Cet-AlgNPs (E), CSNPs-AlgNPs (F) and Cet-CSNPs-AlgNPs (G).

and 1635 cm^{-1} for Cet-CSNPs is due to the protonated NH_2 groups (NH_3^+). An absorption band at 1548 cm^{-1} for CSNPs and 1547 cm^{-1} for Cet-CSNPs is due to the C-H bending vibration of the alkyl group. The $\text{P}=\text{O}$ group for TPP was observed at 1093 cm^{-1} for CSNPs and 1100 cm^{-1} for Cet-CSNPs (29).

The presence of Cet in Cet-CSNPs can be derived from two factors; first, the shift in some bands of Cet-CSNPs compared to CSNPs; the shift from 2948 to 2913 , 1640 to 1635 , and from 895 to 883 cm^{-1} . This indicates that there exists an interaction between CSNPs and Cet. Secondly, some of the bands appeared in Cet-CSNPs and Cet FTIR spectra but were not observed in the FTIR spectrum of CSNPs. For example, a band at 1436 cm^{-1} which is due to the C-O bending, C-C stretching, and C-H wagging at piperazinyl ethoxy acetic acid, and another band at 847 cm^{-1} which is assigned to C-C stretching, and C-O stretching at piperazinyl ethoxy acetic acid, and C-N stretching.

Figure 2. FTIR spectrum of the Cet (A), Cet-CSNPs (B), Cet-AlgNPs (C) and Cet-CSNPs-AlgNPs (D).

The absorption bands of AlgNPs show important bands for carboxylic, ether, and hydroxyl functional groups. The stretching vibration of the O-H bond is observed at 3446 cm^{-1} , while the stretching vibration of the aliphatic C-H bond is observed at 2930 cm^{-1} . Bands at 1631 and 1425 cm^{-1} were attributed to asymmetric and symmetric stretching vibrations of COO^- of the salt ion, respectively. A band at 1108 cm^{-1} is attributed to the C-O stretching vibration of pyranosyl ring (30).

The hydroxyl group (OH) present in Cet-AlgNPs (Figure 2C) exhibits broadband at around 3446 cm^{-1} . A band at 2921 cm^{-1} is due to the $-\text{CH}_2$ group. A band at around 1034 cm^{-1} is attributed to the C-O-C group of the saccharide structure. In addition, bands at 1631 cm^{-1} and 1424 cm^{-1} are due to asymmetric and symmetric stretching of carboxylate salt groups (COO^-) for both Cet anion and Alg.

The FTIR spectrum of the Cet-CSNPs-AlgNPs (Figure 2D) revealed the presence of characteristic bands of CSNPs and AlgNPs. This is because, during the preparation, we used TPP as a reagent for CS as well as the CaCl_2 as a precipitating reagent for Alg. This also can be seen from the XRD results. In the FTIR spectra of mixed CSNPs and AlgNPs, as shown in Figure 2D, the Cet-CSNPs-AlgNPs has a narrower, more intense band at about 3446 cm^{-1} , which is due to the forming of new hydrogen bonds between

the functional groups in CSNPs and the functional groups of AlgNPs (31). In addition, the characteristic absorption band of Cet that appeared in the Cet-CSNPs-AlgNPs spectra indicates that Cet was encapsulated in the CS and Alg polymeric system.

The interaction between Cet and the nanocarriers

As indicated in the literature, CSNPs have been prepared by several methods such as ionotropic gelation methods, spray drying, emulsion cross-linking, template polymerization, and precipitation techniques (31). The ionic gelation method was adopted in this work, where gelation depends on the ionic

Figure 3. A schematic model showing plausible interaction between Cet and CSNPs (A), and Cet and AlgNPs (B).

interaction between the amino groups (NH_3^+) of CS which is positively charged, and TPP which is negatively charged (32). As shown in Figure 3A, one of the NH_3^+ groups react with TPP and the other one reacted with the negatively charged group of Cet.

During the formation of Cet-AlgNPs, the Ca^{2+} ions have two binding sites. The first binding site will interact with the Alg molecule and the second binding site will interact with Cet (Figure 3B).

Transmission Electron Microscopy

A typical TEM micrograph of the CSNPs, Cet-CSNPs, AlgNPs, Cet-AlgNPs, CSNPs-AlgNPs, and Cet-CSNPs-AlgNPs is shown in Figure 4A, B, C, D, and E, respectively. In this study, TEM images show physical aggregation of the CSNPs, AlgNPs, CSNPs-AlgNPs (Figure 4A, C, and E), and Cet-CSNPs, Cet-AlgNPs, and Cet-CSNPs-AlgNPs (Figure 4B, D, and F). As shown in Figures 3A and B, the nanoparticles were seen to be spherical, distinct, and regular. The mean particle size diameter of the CSNPs was found to be 63 nm, compared to 96 for Cet-CSNPs, similar to the one reported in the literature (33). The mean particle size of AlgNPs was found to be 112 nm (Figure 4C) which is slightly larger than that of Cet-AlgNPs, 94 nm (Figure 4D). The TEM image was found to be similar to the one in the literature (34).

The mean particle diameter measured from the TEM images for CSNPs-AlgNPs and Cet-CSNPs-AlgNPs is shown in Figures 4 E and F, respectively. The Cet-CSNPs-AlgNPs were found to be smaller than CSNPs-AlgNPs. The mean size for the CSNPs-AlgNPs is around 121 nm with a round shape (Figure 4E) while the mean size for Cet-CSNPs-AlgNPs was found to be 109 nm, which is similar to the one reported in the literature (35).

Figure 4. TEM images of CSNPs (A), Cet-CSNPs (B), AlgNPs (C), Cet-AlgNPs (D), CSNPs-AlgNPs (E) and Cet-CSNPs-AlgNPs (F).

Table 1. Zeta potential of CSNPs, Cet-CSNPs, AlgNPs, Cet-AlgNPs, CSNPs-AlgNPs and Cet-CSNPs-AlgNPs.

Sample	Zeta potential/mV
CSNPs	+15.0 ± 2.5
Cet-CSNPs	+6.7 ± 4.2
AlgNPs	-4.2 ± 1.1
Cet-AlgNPs	-5.6 ± 2.0
CSNPs-AlgNPs	+8.2 ± 4.3
Cet-CSNPs-AlgNPs	-6.4 ± 3.3

The effect of pH on the Zeta potential of the nanoparticles

Particle charge is one of the stability-determining factors and their Zeta potential value is usually between $> +30$ mV or < -30 mV. This is the ideal value for the physical stability of any nanoparticles (36). The zeta potential of CSNPs and Cet-CSNPs was determined in this work and it was decreased from +15.0 mV in CSNPs to +6.7 mV in Cet-CSNPs as shown in Table 1. During the synthesis of CSNPs, the CS and TPP were mixed into acid media solutions, such as acetic acid. They formed solid nanoparticles with a positive surface charge, and the density of the surface charge is measured by the zeta potential values (36).

The zeta potential for AlgNPs and Cet-AlgNPs was found to be -4.2 mV and -5.6 mV, respectively as shown in Table 1. The surface charges of AlgNPs and Cet-AlgNPs were negative, due to the electric potential of the nanoparticles. It was influenced by the composition of the starting materials and the medium they dispersed into (37). This result was in agreement with the previous study (38). The AlgNPs and Cet-AlgNPs with a small value of zeta potential (-4.2 mV and -5.6 mV) are due to higher Alg content and the amounts of CaCl_2 , and Cet was

still insufficient to interact with all the carboxylic groups of Alg (39).

Zeta potential of CSNPs-AlgNPs and Cet-CSNPs-AlgNPs was determined and it was changed from +8.2 mV for CSNPs-AlgNPs to -6.4 mV for Cet-CSNPs-AlgNPs as shown in Table 1. The positive charge for CSNPs-AlgNPs was dependent on the total protonated NH_3^+ groups of CS and their neutralization with carboxylic groups of Alg, i.e. the Alg/CS ratio. When the amount of CS exceeded the amount of Alg, the nanoparticles showed positively charged zeta potential, which indicated that the NH_3^+ groups of CS were sufficiently neutralized by the carboxylic groups of Alg. The Cet-CSNPs-AlgNPs have a negative charge which is due to the presence of Cet with carboxylate anion.

Figure 5 shows the change in the zeta potential of three nanoparticles from acidic (pH 4) to alkaline (pH 8.5). For Cet-CSNPs, when the pH was increased by the addition of NaOH solution, the zeta potential was decreased from +23.4 mV to -4.4 mV. In the case of Cet-AlgNPs, the potential was changed from -15.1 mV to -3.9 mV, whereas for Cet-CSNPs-AlgNPs, the potential also was changed from +15.3 mV to -9.4 mV. The isoelectric point (pI) can be defined as the pH in which the net charge of the particle is zero. It is the point where the positive charges are completely neutralized by the negative charges. The pI value for Cet-CSNPs, Cet-AlgNPs, and Cet-CSNPs-AlgNPs are 7.9, 8.3, and 5.4, respectively.

Thermogravimetric analysis

Thermogravimetric analyses (TGA) were obtained for Cet, CSNPs, Cet-CSNPs, AlgNPs, Cet-AlgNPs, CSNPs-AlgNPs, and Cet-CSNPs-AlgNPs and are shown in Figure 6. For Cet, the TGA thermogram shows a single sharp weight loss starting at 160°C with a maximum temperature of 301°C and a total weight loss of 91.2%. Therefore Cet was found to be stable up to 160°C (40). The TGA curve of the CSNPs is shown in Figure 6A. The initial weight loss at 60°C is due to the evaporation of H_2O , while the second weight loss between 200-400°C is due to decomposition and the dehydration of the saccharide structure, depolymerization, and finally, decomposition of the acetylated and deacetylated polymer, with a peak observed at 228°C. The TGA curve of Cet-CSNPs in Figure 6A shows the same weight loss steps for CSNPs. The difference between them lies in the total weight loss, as the total weight of the Cet-CSNPs is higher than the CSNPs.

TGA analysis in Figure 6B gives some information about the thermal behavior of the AlgNPs.

Figure 5. The effect of pH on the zeta potential of the nanoparticles.

Figure 6. TGA thermograms of Cet, CSNPs, Cet-CSNPs, AlgNPs, Cet-AlgNPs, CSNPs-AlgNPs and Cet-CSNPs-AlgNPs nanoparticles.

The first weight loss between 100–300°C is due to the loss of bound (residual) and unbound (moisture) water molecules from the polymer matrix, as well as the decomposition of the saccharide structure. The second stage of weight loss observed between 300–800°C could be related to the removal of saccharide molecules from the polymer structure and the complete degradation and decomposition of the polymer main chain. The TGA thermogram of Cet-AlgNPs shown in Figure 6B shows the same thermal behavior as that of AlgNPs. The second stage of weight loss in the temperature range of 300–800°C is due to the degradation of Cet as well as AlgNPs with 53% weight loss.

The decomposition of Cet-CSNPs-AlgNPs shows two major and one minor weight loss (Figure 6C). The thermograms of Cet, CSNPs, and AlgNPs are shown in Figure 4E. The first weight loss at 62°C is due to moisture that is present from the polymer structure with 14.3% weight loss. However, when the

temperature was reached at 227°C, 42.2% weight loss was observed, which is due to the decomposition of Cet and the complexes of CSNPs-AlgNPs. The second major step of weight loss occurred at 750–800°C, which is due to CSNPs.

***In vitro* study of Cet release from its nanoparticles**

The release behaviors for Cet from Cet-CSNPs, Cet-AlgNPs, and Cet-CSNPs-AlgNPs, and the physical mixture of Cet with CSNPs, AlgNPs, and CSNPs-AlgNPs in a phosphate-buffered solution at pH 7.4 are shown in Figure 7. It can be seen that the physical mixture, when exposed to the phosphate-buffered solution at 7.4, released Cet quickly and reached 90%, 95%, and 86% at 25 min for CSNPs, AlgNPs, and CSNPs-AlgNPs, respectively. The release behaviors of Cet from nanoparticles were very much lower than that from the physical mixture, indicating that the nanoparticles are

Figure 7. The release profiles of Cet from its Cet-CSNPs (A), Cet-AlgNPs (B), and Cet-CSNPs-AlgNPs (C) into PBS solution at pH 7.4 and from its physical mixture.

a potential controlled-release system. This may be due to the electrostatic attraction between the Cet and the nanoparticles.

Drugs can be released from their polymer hosts by three mechanisms; swelling-diffusion (41), diffusion and erosion, or degradation of polymers (42). The swelling mechanism occurs for hydrophilicity polymer and it depends on the pH (43). The polymer nanoparticles are initially dry, and when they are added into the release media, they swell to increase their porosity, leading to the diffusion of the drug molecules (44). In the diffusion mechanism, the drug moves from the inner polymeric matrix to the release medium. Polymer chains form the diffusion barrier, and this barrier will make the release of the drug difficult and therefore drug is released in a controlled manner.

Erosion and degradation of polymers take place when the polymer chains convert to lower molecular weight, effectively releasing drug molecules that were trapped by the polymer chains. The erosion mechanism occurs in two different ways: homogenous and heterogeneous. In homogenous erosion, the polymer is eroded at the same rate throughout the matrix. On the other hand, in heterogeneous erosion, polymer erosion takes place from the surface towards the inner core. Polymer degradation may be caused due to the pH surrounding the media or the presence of enzymes (45).

Release studies of Cet from Cet-CSNPs, Cet-AlgNPs, and Cet-CSNPs-AlgNPs were carried out using PBS medium at pH 7.4. Figure 7B shows that about 70% of the drug is fast released in the first 2 h. This result indicates that part of the drug is localized

as a free drug on the surface of the nanoparticles. In addition, AlgNPs are unstable at pH 7.4 (46), and the release of the drug follows the swelling mechanism (47). After this initial release, the drug release is almost constant, and around 85% of the Cet was released from the Cet-AlgNPs in 27 h.

Figures 7A and C show that both nanoparticles, Cet-CSNPs and Cet-CSNPs-AlgNPs, are able to release about 90% and 75% of the Cet, respectively, in 30 h. Also in both nanoparticles, the release of the Cet drug was found to follow the diffusion through the polymers matrix. This is because CSNPs is pH-dependent, it is faster at a lower pH than around neutral pH ($\text{pH } 5.2 > \text{pH } 6.8 > \text{pH } 7.4$) (48).

The mean dissolution time value is used to describe the drug release rate from the polymer, and the efficiency of the polymer. A higher value of MDT indicates a slower release rate (49). The MDT value for Cet-CSNPs, Cet-AlgNPs, and Cet-CSNPs-AlgNPs was found to be 10.5, 37.4, and 22.5 h, respectively. These results reflect modification of the release performance from rapid for Cet-AlgNPs to sustained release for Cet-CSNPs, and Cet-CSNPs-AlgNPs.

Release kinetics of Cet from its nanoparticles

The data of the cumulative release of Cet from its nanoparticles was fitted to four kinetic models which are generally described in Table 2 (50).

Table 3 and Figure 8 show that the release of Cet from Cet-CSNPs was best fitted to the Hixson-Crowell model with $R^2 = 0.9631$. On the other hand, for Cet-AlgNPs, Cet release was best fitted to the pseudo-second-order, with $R^2 = 0.9998$. In addition, the release of Cet from Cet-CSNPs-AlgNPs

Table 2. Kinetic models that were used in the fitting of the cumulative release of Cet from its nanoparticles (Dong et al., 2010, Ho and Ofomaja, 2006, Sakore and Chakraborty, 2013).

Kinetic model	Equation
First-order	$\ln (q_e - q_t) = \ln q_e - k_1t$
Second-order	$t/q_t = 1/k_2q_e^2 + t/q_e$
Higuchi	$q_t = K_H \sqrt{t}$
Hixson-Crowell	$\sqrt[3]{M_o} - \sqrt[3]{q_t} = Kt$
q_e is the quantity released at equilibrium, q_t is the quantity released at any time t , k_n is the rate constant of the n -order release kinetics, and M_o is the initial quantity of drug in the nanoparticles	

Table 3. The correlation coefficients (R^2) and constant rate model obtained by fitting the Cet release data from the Cet-CSNPs, Cet-AlgNPs, Cet-CSNPs-AlgNPs in PBS solutions at pH 7.4.

Samples	Release (%)	Pseudo-first order		Pseudo-second order		Higuchi model		Hixson-Crowell model	
		R^2	K_o	R^2	K_2	R^2	K_H	R^2	K_{HC}
Cet-CSNPs	90	0.921	7.0×10^{-2}	0.593	2.1×10^{-4}	0.943	5.0×10^{-2}	0.963	7.9×10^{-2}
Cet-AlgNPs	85	0.736	3.5×10^{-2}	0.999	1.5×10^{-2}	0.661	1.0×10^{-1}	0.670	3.3×10^{-2}
Cet-CSNPs-AlgNPs	75	0.972	5.1×10^{-2}	0.946	8.0×10^{-4}	0.980	6.2×10^{-2}	0.960	6.0×10^{-2}

Figure 8. Data for the cumulative release of Cet from its Cet-CSNPs, Cet-AlgNPs, Cet-CSNPs-AlgNPs into PBS solution at pH 7.4 fitted to various kinetic models.

was found to follow the Higuchi model with $R^2 = 0.9802$.

Cytotoxicity studies

In this study, the potential cytotoxicity of nanoparticles on 3T3 cells was evaluated by the MTT assay. The results are shown in Figures 9A-C. In vitro, MTT results showed that blank CSNPs, AlgNPs, and CSNPs-AlgNPs, as well as the nanoparticles of Cet-CSNPs, Cet-AlgNPs, and Cet-CSNPs-AlgNPs, show no significant cytotoxicity after 24 h. These results were consistent with the previous reports that CS and Alg are natural carriers with low toxicity and biodegradability (51).

CONCLUSION

The findings of this study show that various nanoparticles, Cet-CSNPs, Cet-AlgNPs, and Cet-CSNPs-AlgNPs, can be prepared by the ionic gelation method. The resulting nanoparticles are generally of amorphous phase with a mean size of 96, 95, and 109 nm, respectively, where there exists an interaction between Cet with CSNPs, AlgNPs, and CSNPs-AlgNPs. Cet was found to be less loaded on CSNPs than AlgNPs; 20 and 45%, respectively. The combination of CSNPs and AlgNPs i.e. CSNPs-AlgNPs gave almost the average value of the two polymers, i.e. 35%. However, there is no clear trend between the size of the nanoparticles with the polymer that can be observed. Similarly, there is no specific relationship between the release of Cet from its nanoparticles to the kinetic release mechanism; they follow the Hixson-Crowell, Pseudo-second, and

Figure 9. The cytotoxicity profiles for the Cet, Cet-CSNPs, Cet-AlgNPs, and Cet-CSNPs-AlgNPs toward 3T3 cells.

Higuchi models for Cet-CSNPs, Cet-AlgNPs, and Cet-CSNPs-AlgNPs, respectively. However, these preliminary results are useful to be used as the starting points for further in vivo studies.

Funding acknowledgment

The author would like to thank the project supported by the Faculty of Pharmacy, Isra University, and the Ministry of Higher Education of Malaysia (MOHE) under grant No. UPM/800-3/3/1/GPB/2019/9678800.

Conflict of interest

The authors declare no conflicts of interest.

REFERENCES

- Khan G., Yadav S.K., Patel R.R., Nath G., Bansal M., Mishra B.: *AAPS PharmSciTech* 17, 1312 (2016).
- Lu Y., Zhang E., Yang J., Cao Z.: *Nano Res.* 11, 4985, (2018).
- Endo K., Ueno T., Kondo S., Wakisaka N., Murono S., et al.: *Cancer Sci.* 104, 369 (2013).
- Han J., Zhao D., Li D., Wang X., Jin Z., Zhao K.: *Polymers (Basel)* 10, 31 (2018).
- Sabbagh H.A.K., Hussein-Al-Ali S.H., Hussein M.Z., Abudayeh Z., Ayoub R., Abudoleh S.M.: *Polymers (Basel)* 12, 772 (2020).
- Bedade D.K., Sutar Y.B., Singhal R.S.: *Food Chem.* 275, 95 (2019).
- Gomes J., Belinha J., Jorge R.N., Eds.: *Application of chitosan in dentistry—a review. Biodental Engineering V: Proceedings of the 5th International Conference on Biodental Engineering, Porto, Portugal 2019.*
- Abdollahimajd F., Moravvej H., Dadkhahfar S., Mahdavi H., Mohebbi M., Mirzadeh H.: *Indian J. Dermatol. Venereol. Leprol.* 85, 609 (2019).
- Kenawy E., Omer A.M., Tamer T.M., Elmeligy M.A., Eldin M.S.M.: *Int. J. Biol. Macromol.* 139, 440 (2019).
- Newton A., Prabakaran L.: *Comparative efficacy of chitosan, pectin based Mesalamine colon targeted drug delivery systems on TNBS-induced IBD model rats. Anti-inflammatory & anti-allergy agents in medicinal chemistry* (2019).
- Shariatinia Z., Mazloom-Jalali A.: *J. Mol. Liq.* 273, 346 (2019).
- Sydow S., Aniol A., Hadler C., Menzel H.: *Biomolecules* 9, 573 (2019).
- Smitha B., Sridhar S., Khan A.A.: *Eur. Polym. J.* 41, 1859 (2005).
- Thünemann A.F., Müller M., Dautzenberg H., Joanny J.F., Löwen H.: *Adv. Polym. Sci.* 166, 113 (2004).
- Slater J.W., Zechin A.D., Haxby D.G.: *Drugs* 57, 31 (1999).
- Hasan S., Al Ali H., Al-Qubaisi M., Zobir Hussein M., Ismail M., et al.: *Int. J. Nanomedicine* 7, 3351 (2012).
- Gholivand M.B., Shamsipur M., Ehzari H.: *Microchem. J.* 146, 692 (2019).
- Goindi S., Kumar G., Kaur A.: *J. Liposome Res.* 24, 249 (2014).
- Kirimlioglu G.Y., Öztürk A.A.: *Turk. J. Pharm. Sci.* 17, 27 (2020).
- Li F.Q., Ji R.R., Chen X., You B.M., Pan Y.H., Su J.C.: *Arch. Pharm. Res.* 33, 1967 (2010).
- Yu X., Mu Y., Xu M., Xia G., Wang J., et al.: *Carbohydr. Polym.* 173, 600 (2017).
- Sambale F., Wagner S., Stahl F., Khaydarov R.R., Scheper T., et al.: *J. Nanomater.* 2015, 9 pages (2015).
- Mosmann T.: *J. Immunol. Methods* 65, 55 (1983).
- Hasan S., Al Ali H., Al-Qubaisi M., Zobir Hussein M., Ismail M., et al.: *Int. J. Nanomedicine* 7, 3351 (2012).
- Nagarwal RC, Singh PN, Kant S, Maiti P, Pandit JK.: *Chem. Pharm. Bull. (Tokyo)* 59, 272 (2011).
- Zhou H., Yu W., Guo X., Liu X., Li N., et al.: *Biomacromolecules* 11, 3480 (2010).
- Fang D., Liu Y., Jiang S., Nie J., Ma G.: *Carbohydr. Polym.* 85, 276 (2011).
- Paczkowska M., Mizera M., Lewandowska K., Kozak M., Miklaszewski A., et al.: *J. Incl. Phenom. Macrocycl. Chem.* 91, 149 (2018).
- de Souza Costa-Júnior E., Pereira M.M., Mansur H.S.: *J. Mater. Sci. Mater. Med.* 20, 553 (2009).
- Daemi H., Barikani M.: *Sci. Iran.* 19, 2023 (2012).
- Kulig D., Zimoch-Korzycka A., Jarmoluk A., Marycz K.: *Polymers (Basel)* 8, 167 (2016).
- Wu J., Wang Y., Yang H., Liu X., Lu Z.: *Carbohydr. Polym.* 175, 170 (2017).
- Hussein-Al-Ali S.H., Kura A., Hussein M.Z., Fakurazi S.: *Polym. Compos.* 39, 544 (2018).
- Biswal T., Barik B., Sahoo P.K.: *J. Mater. Sci. Nanotechnol.* 4, 203 (2016).
- Li P., Dai Y.N., Zhang J.P., Wang A.Q., Wei Q.: *Int. J. Biomed. Sci.* 4, 221 (2008).
- Nallamuthu I., Devi A., Khanum F.: *Asian J. Pharm. Sci.* 10, 203 (2015).

37. Sumathi R., Tamizharasi S., Sivakumar T.: *Int. J. App. Pharm.* 9, 60 (2017).
38. Morsi N., Ghorab D., Refai H., Teba H.: *Int. J. Pharm. Pharm. Sci.* 7, 234 (2015).
39. Ahdyani R., Novitasari L., Martien R., Danarti R.: *Int. J. App. Pharm.* 11, 48 (2019).
40. Hussein Al Ali S.H., Al-Qubaisi M., Hussein M.Z., Zainal Z., Hakim M.N.: *Int. J. Nanomedicine* 6, 3099 (2011).
41. Liu S., Yang S., Ho P.C.: *Asian J. Pharm. Sci.* 13, 72 (2018).
42. Singh R., Lillard J.W. Jr.: *Exp. Mol. Pathol.* 86, 215 (2009).
43. Fonseca-Santos B., Chorilli M.: *Mater. Sci. Eng. C Mater. Biol. Appl.* 77, 1349 (2017).
44. Bae K.H., Chung H.J., Park T.G.: *Mol. Cells* 31, 295 (2011).
45. Göpferich A.: *Biomaterials* 17, 103 (1996).
46. Sambu S., Xu X., Ye H., Cui Z.F.: *Proc. Inst. Mech. Eng. H* 225, 1092 (2011).
47. Bajpai S.K., Sharma S.: *React. Funct. Polym.* 59, 129 (2004).
48. Bhavin K.P., Rajesh H.P., Pooja S.A.: *J. Drug Delivery* 2013, 10 pages (2013).
49. Rinaki E., Dokoumetzidis A., Macheras P.: *Pharm. Res.* 20, 406 (2003).
50. Gouda R., Baishya H., Qing Z.J.J.D.D.: *J. Dev. Drugs* 6, 171 (2017).
51. Kumar S.P., Birundha K., Kaveri K., Devi K.T.: *Int. J. Biol. Macromol.* 78, 87 (2015).