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ORIGINAL ARTICLE

Folate-targeted drug-delivery systems prepared by nano-comminution

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Abstract

Background: The size reduction ability of conventional wet comminution has been improved by proper polymeric stabilizer systems, and the resulting nano-comminution methods have led to the commercialization of many poorly water-soluble drugs after improving their bioavailability. During nano-comminution, polymer steric stabilizers physically adsorb onto the surface of drug particles. *Method:* In this study, the cross-linking and subsequent functionalization methods of the physically adsorbed polymers were used to widen the applicability of the nano-comminution. Chitosan was used as a steric stabilizer for two hydrophobic drugs, naproxen and paclitaxel. *Results:* Chitosan was successfully cross-linked (immobilized) by tripolyphosphate. The cross-linked stable polymer layer on drug nanoparticles was conjugated with folic acid, a model targeting moiety. The chemical reactions were performed without destroying the stabilities of drug nanosuspensions. The cross-linking and conjugation reactions significantly modified the release profiles of drug nanoparticles. *Conclusion:* This simple preparation method can be utilized to prepare novel drug encapsulations and folate-targeted delivery systems.

Key words: Drug release, milling, nanoparticles, particle size, suspensions

Introduction

Nano-comminution is a successful method for preparing drug nanoparticles, which improves the dissolution rates and subsequently the bioavailabilities of drugs[1–10]. Owing to the efficiency and drug stability advantages of the method, it has produced many successful commercial products such as Rapamune®, Emend®, Tricor®, and Megace® ES. More novel drugs will benefit from the further development of nano-comminution.

The particle size reduction method of comminution uses shear force to fracture crystalline drug particles. Subsequently, and more importantly, the surface of the drug is stabilized by a proper steric stabilizer with or without an ionic stabilizer. The minimum size attainable by comminution is determined not by shear force but by steric stabilization[3].

Polymer chains physisorbed onto the surface of drug nanocrystals can be treated as an encapsulation layer. In general, encapsulation provides drug particles with protection (stabilization), release control, and other functions such as drug-targeting ability. However, polymer steric stabilizers do not successfully provide all of these functions, mainly because they are not stationary. They are not chemically anchored, but are reversibly adsorbed onto and desorbed from the surfaces of drug nanoparticles. For example, targeting moieties such as folate can be attached to the polymer chains on a drug surface, but polymer chains having targeting moieties can then be detached from the surface. Eventually, equilibrium between the polymer concentration on the surface and that in solution will be reached, and this equilibrium condition is not favorable for drug-delivery systems in blood. Furthermore, at target sites, the polymers having targeting moieties preferentially bind to receptors on the surfaces of the target sites over binding to the drug surface, resulting in the binding of polymer alone to the receptors.

Therefore, to realize the full advantages of encapsulation using the physisorbed polymers in drug nano-comminution, a method for forming stable polymer layers with subsequent functionalization is needed. The concentrations of polymer chains in solution and on the

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drug surface are in kinetic equilibrium. Therefore, the chemical reactions of the polymer chains with small molecules are not straightforward. Herein, we developed a methodology to achieve chemically modified chitosan-stabilized nanoparticles by nano-comminution. Chitosan was successfully used as a polymeric steric stabilizer for model drug particles, and the conjugation reaction of chitosan with folic acid was used on the surfaces of the drug particles. This approach is expected to be the basis for novel applications of the well-established drug nano-comminution technology, which has never before been reported for the preparation of targeting and controlled release systems.

Chitosan (deacetylated chitin) is an abundant polysaccharide that supports numerous living organisms[11–13]. Its biocompatible, biodegradable, and antimicrobial nature has resulted in significant research activities in the drug-delivery field[14]. The cross-linking of chitosan by tripolyphosphate (TPP) or genipin is an important tool for encapsulating an active component and has widened the applications of chitosan polymers[15]. In a previous report on the cytotoxicity of folic acid–chitosan–DNA nanoparticles, the conjugate of folic acid and chitosan was reported to have good delivery performance and biocompatibility[16]. Folate-targeted drug delivery has emerged as an alternative therapy for the treatment and imaging of many cancers and inflammatory diseases[17]. This moiety has advantages such as small molecular size and high-binding affinity for cell-surface folate receptors.

Materials and methods

Materials

Naproxen [(±)-2-(6-methoxy-2-naphthyl)propionic acid] from Tokyo Kasei Kogyo (Tokyo, Japan), and paclitaxel from Samyang Genex (Sequl, South Korea) were used without purification. Chitosan with a 95% deacetylation and number-average molecular weight of 10 kg/mol (KITTO LIFE, Korea) was dried at 60°C for 2 days under vacuum before use. TPP, folate, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), sodium tetraborate decahydrate, and hydroxypropyl cellulose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cremophore® RH 40 from BASF (Ludwigshafen, Germany), methanol, hydrochloric acid, sodium chloride, monobasic sodium phosphate, dibasic sodium phosphate from SamCheon Chemicals (Seoul, South Korea), water high-performance liquid chromatography (HPLC), methanol (HPLC), and methylene chloride (MC, HPLC) from J.T. Baker (Phillipsburg, NJ, USA) were used without purification.

Preparation of the targeting drug-delivery systems

The targeting moiety, folic acid, was introduced to the surfaces of drug particles prepared by nano-comminution as follows (Figure 1). First, water-insoluble drugs were mixed with a prepared polymer solution and yittria-stabilized zirconia beads (1 mm diameter) in 30 mL vials. The amount of drug in water was 4 wt%, and the polymer to water weight ratio was 1:6, unless otherwise specified. At room temperature, media milling at 108 rpm proceeded for 5 days to produce polymer-stabilized drug nanosuspensions. Then, the chitosans on the surfaces of the drugs were cross-linked using TPP. Specifically, 2.5 mL of paclitaxel nanosuspension was slowly dropped into 50 g of an aqueous solution of TPP (0.02 wt%) and the cross-linking reaction progressed for 1 hour. In the dark, 0.025 g (0.06 mmol) folate and 0.011 g (0.06 mmol) ECD were dissolved in 10 g of buffer (pH 9). The cross-linked chitosan/drug particles were slowly dropped into the folate solution, followed by stirring for 20 hours[18]. After the chemical reactions, the unreacted compounds were removed using an osmosis membrane (MWCO 1000 g/mol) for 3 days and centrifuging at 4383 × *g* and 20°C for 30 minutes. The remaining solution was added drop-wise into liquid nitrogen and was freeze-dried for 24 hours (FD-1000, EYELA, Tokyo, Japan).

Characterization

Transmission electron microscopy (TEM) was used to investigate the cross-linked chitosan layers. The cross-linked nanosuspension was centrifuged at 4383 × *g* and 20°C for 30 minutes using a Micro 17 R PLUS centrifuge (Hanil, South Korea). The supernatant (ca. 2/3, v/v of total solution) was removed and was stirred to again achieve a homogeneous solution. This concentrated nanosuspension was added drop-wise to liquid nitrogen and was then freeze-dried for 24 hours (FD-1000, EYELA, Tokyo, Japan). To compare the chitosan layers with and without a drug core, the freeze-dried particles were mixed with a drop of methanol on a TEM Cu grid and vacuum-dried for 24 hours. TEM was performed using a JEM-4010 (JEOL, Tokyo, Japan) at 200 kV.

The chemical structure of the folate–chitosan conjugate was analyzed by ^{1}H-NMR (500 MHz, UI500, Varian, Palo Alto, CA, USA). The sample was prepared without cross-linking. After the reaction of folate and chitosan, unreacted compounds were removed using an osmosis membrane (MWCO 1000 g/mol) for 3 days, followed by centrifugation at 4383 × *g* and 20°C for 30 minutes. The remaining solution was freeze-dried following the same method described above, and the freeze-dried powders were re-constituted in D_2O for NMR characterization. For comparison, pure folate and chitosan were analyzed in DMSO-d6 and D_2O, respectively.

UV–Vis spectroscopy was employed to measure the amount of folate conjugated with chitosan. The concentration of chitosan was varied from 0.07 to 0.28 mmol, and EDC was used at a 1:1 molar ratio. After carrying out the procedure described above, the freeze-dried powders of 10 mg was dispersed in 20 mL water, and the amount of folate was measured using a UV–Vis spectrometer (V-500, ASCO, Tokyo, Japan) (λ = 370 nm).

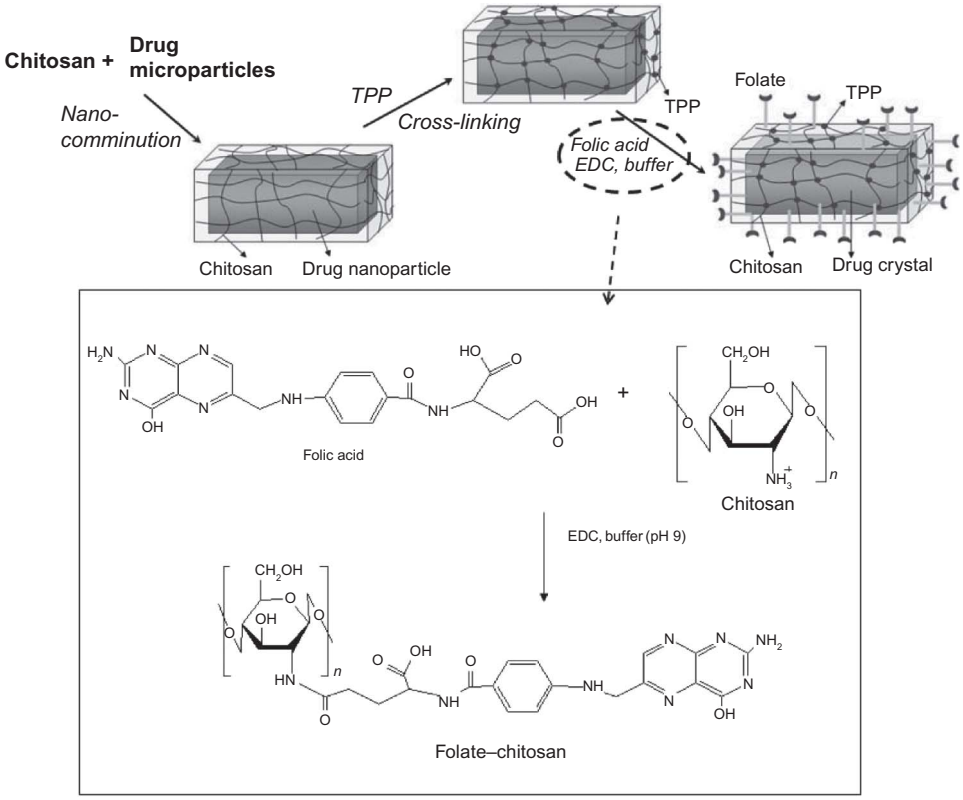


Figure 1. Schematic representation of the preparation of folate–chitosan-stabilized drug nanoparticles and the folate–chitosan conjugation reaction.

At each preparation step, volume-averaged particle sizes of chitosan were measured in 150 mL water using a light scattering particle size analyzer (LA-910, Horiba Co., Kyoto, Japan) (relative refractive index = 1.06, Mie & Fraunhofer). Sonication was applied for 1 minute (40 W, 39 Hz) prior to measurement, and the stirring speed was 340 mL/min. For the examination of particle morphology, a scanning electron microscope S-4800 (Hitachi, Tokyo, Japan) was used at 4 kV, and the specimens for SEM were coated with carbon (ca. 3 nm).

For in vitro release tests using the paddle method[19–22], 20 mg nanosuspensions in an osmosis membrane tube (MWCO 12,000–14,000) were immersed in 500 mL phosphate buffer solution (pH 7.4) having 0.1% (w/v) cremophor RH40 at 37 ± 0.5°C and 100 rpm. At each time point, a 3 mL aliquot was collected for HPLC analysis, and a fresh phosphate buffer solution release medium of the same amount was added. The aliquot was mixed with 2 mL of MC for 3 minutes to dissolve the paclitaxel. After 15 minutes, centrifugation (using the same conditions described above) and vacuum-drying (24 hours, 30°C) produced paclitaxel powders. The powders were dissolved in 0.5 mL of acetonitrile:water (1:1) for HPLC characterization. The HPLC instrument was an NS4000 (Futecs, Daejeon, South Korea), and the column used was a RP-C18 (5 μm, 150 × 4.6 mm, Shodex, Kawasaki, Japan). As the mobile phase, a 1:1 mixture of acetonitrile and water was used, and its flow rate was 1 mL/min. The injection volume was 20 μL, and the 227 nm UV peak was used. For each data point, three repeated measurements were performed.

Results and discussion

Nano-comminution

Nano-comminution technology has been widely applied to water-insoluble drugs. It usually produces drug particles of 100–300 nm in size and cannot reduce the sizes of drug crystals far below 100 nm, since this size is limited by the fracture characteristics of the materials[18,23,24]. Nano-comminution uses a polymer to stabilize the hydrophobic surfaces of water-insoluble drugs in water, accomplished mainly through steric stabilization. The polymer is physically adsorbed onto the surfaces of the drugs and produces a steric repulsion force, thereby preventing particle aggregation. Therefore, strong surface adsorption at full coverage, long-time scale for desorption, and relatively large steric repulsion are necessary for the successful preparation of nanoparticles by nano-comminution[25]. In a previous report, the amounts of polymers on the surfaces of drugs were found to be sufficiently large, that is, larger than the minimum layer thickness often required for steric stabilization, ca. (0.05–0.2) × particle size (solid). The calculated thickness of the polymer layer was about 1.5–16 nm, whereas the surface coverage was 0.15–1.6 μg/cm^2.[26] These values

indicate that the surface chemical modification can be performed after the nano-comminution process.

In this study, chitosan was successfully employed to produce significant particle size reductions of paclitaxel and naproxen, although chitosan has not been previously reported as a steric stabilizer for nano-comminution. The volume-averaged particle sizes were 330 (±90) and 250 (±60) nm, respectively. The relatively poorly soluble characteristics and the existence of side functional groups of chitosan may help the stabilizing ability[27].

Conjugation between folic acid and chitosan

Figure 1 shows the schematic diagram of the simple conjugation reaction between folic acid and chitosan. The reaction can be carried out on the surfaces of drugs, since the reaction conditions are not detrimental to the drug nanoparticles. Figure 2 shows the ^{1}H-NMR results before and after the conjugation reaction between chitosan and folic acid without drugs (at the same conditions). After the conjugation, the major peaks of folic acid (6.5, 7.5, and 8.6 ppm) were visible in the folate–chitosan conjugate polymers. The quantitative analysis of the NMR results showed that more than 40% of the chitosan monomeric units were reacted with folic acid. However, the reaction yield was not directly applicable to the actual conjugation reaction between the chitosan and the folic acid on the drug surfaces, as will be discussed later.

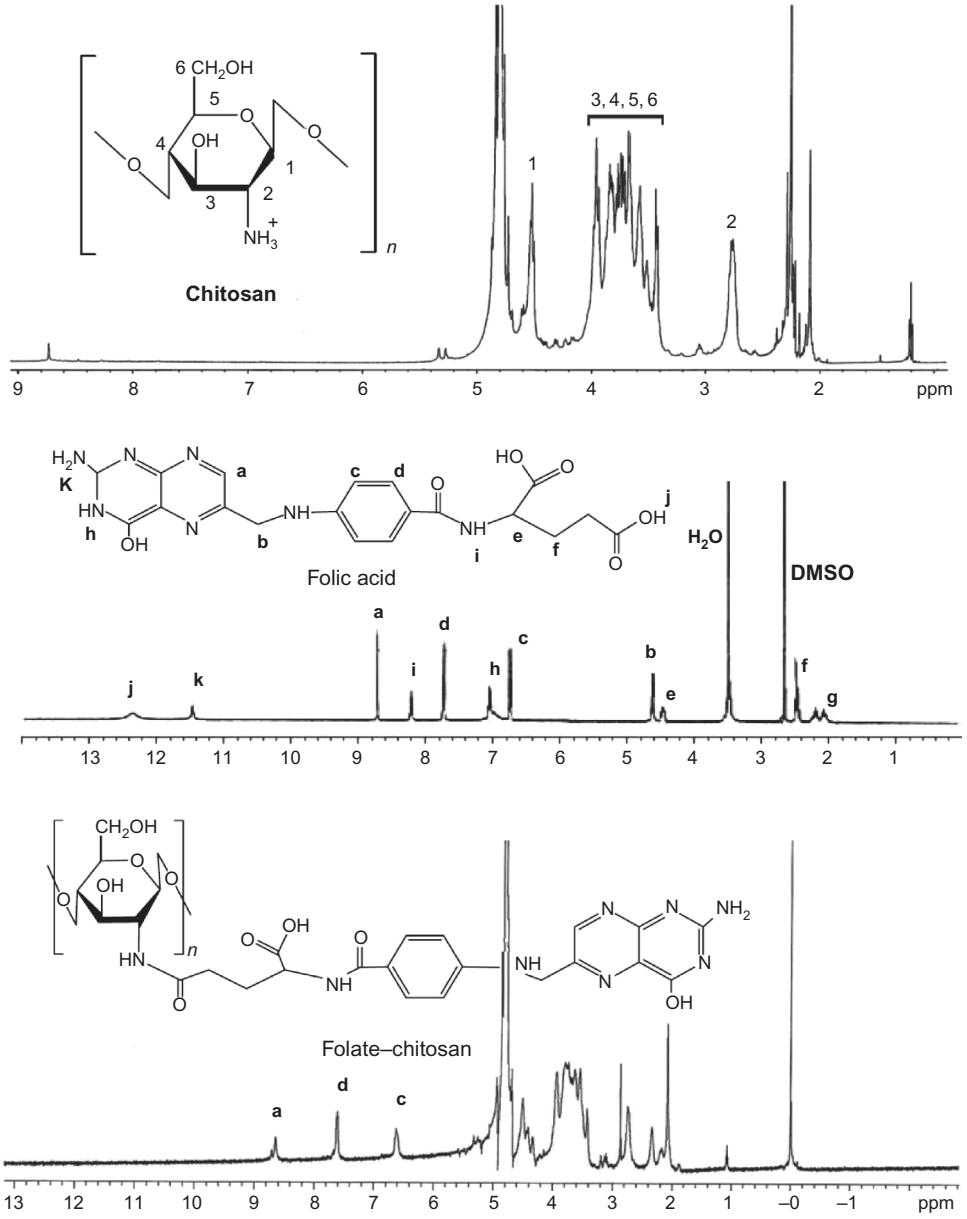


Figure 2. ^{1}H-NMR spectra of chitosan, folic acid, and folate–chitosan conjugate. After the conjugation of chitosan and folic acid, the characteristic peaks of folate at 6.7, 7.6, and 8.6 ppm are visible (lower spectrum).

An important problem is the fact that the polymers on the drug surfaces are not covalently anchored; they are merely physically adsorbed and can be readily detached. Therefore, the chemical modification of chitosan by folic acid can happen both in solution and also on the drug surfaces. According to our TGA measurement on supernatant, 58.0 wt% of polymers were on the drug surfaces, and the remaining 42.0 wt% existed in solution (data not shown, same method in Ref.[26]). This result is consistent with our previous results[26], namely, that polymers can undergo chemical reactions on the surfaces of drugs and also detach back into solution.

To better control the surface modification, the polymers physically adsorbed onto the drug surfaces were first chemically cross-linked with TPP. TPP was used for deprotonation and the slight ionic cross-linking between the positively charged amino group of chitosan and the negatively charged counterion of TPP ($HP_3O_{10}^{4-}$ and $P_3O_{10}^{5-}$), following the methods of previous studies[28–30]. The cross-linking was confirmed by determining the insolubilities of the chitosan layers in water and methanol. The cross-linked layer was stable throughout the conjugation reaction and the sample preparation steps.

The cross-linking reaction before the conjugation reaction was difficult to characterize because of its insolubility and the relatively small amount of chitosan adsorbed onto the drug surfaces. Therefore, direct evidence was obtained using TEM (Figure 3). The cross-linked polymer layers successfully survived out the extraction process using methanol. Chitosan-stabilized particles in Figure 3a show dark drug cores, but extraction with a solvent produced more porous structures. The drying step experienced by the TEM samples induced the aggregation of particles and also the aggregation of the remaining empty shells of the cross-linked chitosan after extraction of the drug core, as can be seen in Figure 3.

A conjugation reaction between chitosan and folic acid followed the cross-linking step. The amount of conjugated folic acid was analyzed by UV spectroscopy, and the contents were between 2.1 and 3.1 wt% (Table 1). Compared to the yield of the conjugation reaction in a

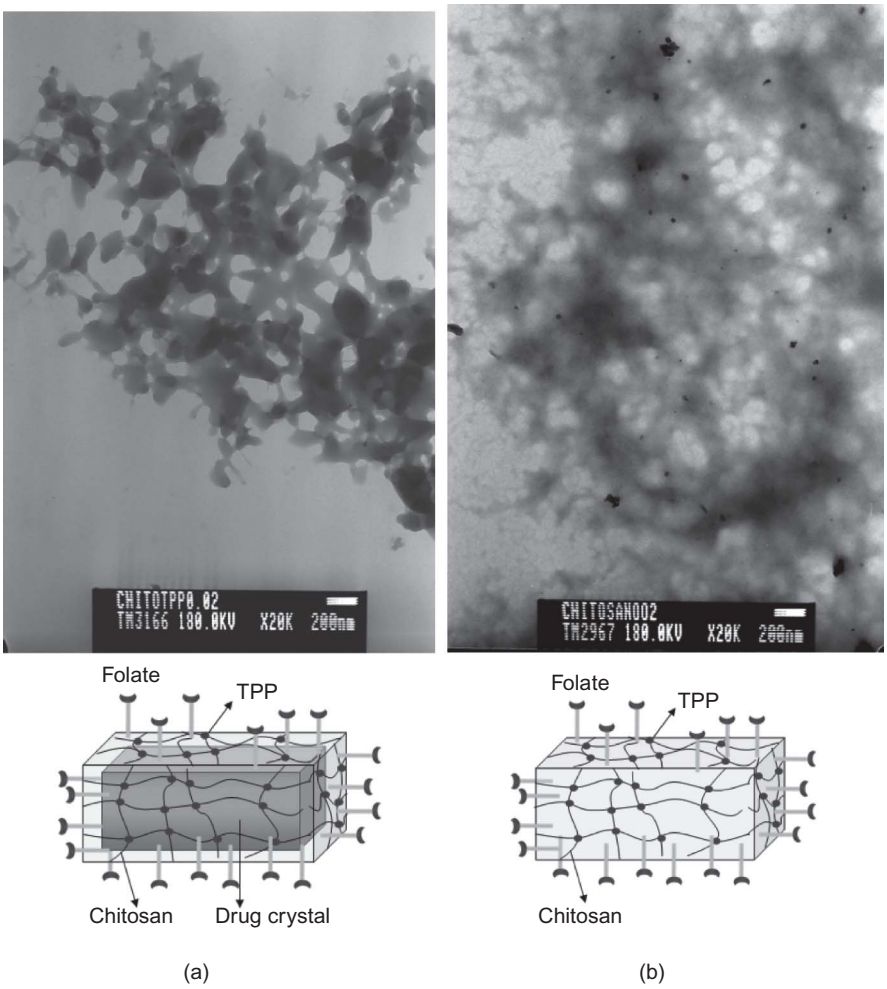


Figure 3. TEM observations of cross-linked chitosan after the extraction of the drug core using methanol confirms the cross-linking of physisorbed chitosan: (a) before and (b) after cross-linking in 0.002 wt% TPP solution and extraction.

Table 1. Amount of folic acid conjugated with chitosan.

Input amount of folic acid (g)	0.125	0.0625	0.031
Folate content (wt%) in folate–chitosan conjugate	2.1	3.8	3.1

solution without drugs, the values were quite low. However, considering the TPP cross-linking reaction and the reversible adsorption and desorption process of the polymer chains, the value was not surprising. The cross-linking reaction with TPP reduced the possible reactive sites of chitosan. As expected, the conjugation yield was not significantly dependent on the input amount of folic acid (Table 1). This indicates that the reaction was not limited by the concentrations of folic acid but by other factors. The available chitosan amine sites on the drug surfaces seem to be a limiting factor.

One thing that needs to be mentioned is the possibility of conjugation between naproxen and chitosan. Although naproxen is in a solid crystalline state, the conjugation between naproxen and chitosan is possible during the EDC conjugation step. This possibility could decrease the desorption of chitosan and the number of available amines for folic acid conjugation.

Particle size and morphology

In the early development of preparation methods, the volume-averaged particle size was used as a guide. Any significant change in the particle size could be related to possible interparticular cross-linking and aggregation, desorption of chitosan chains, deactivation of steric stabilization, or damage to the drug crystals. Following the method described in the experimental section, the mean particle sizes of the drug nanosuspensions were successfully maintained throughout the preparation steps. Table 2 shows the mean particle sizes measured at each step of preparation. The changes in particle size were negligible, and the particle size distribution was also maintained as unimodal. Figure 4 shows the particle size distributions of nanoparticles at each preparation step, where only slight changes were observed. No significant changes in particle or size distribution were found when the chitosan-to-drug ratio was changed from 1:6 to 1:12 or 1:24.

No significant changes in particle morphology such as particle aggregation were observed in SEM observations before and after the cross-linking and conjugation reactions (Figure 5). This is possible, since the surface

Table 2. Volume-averaged particle sizes of paclitaxel for each step of the folate–chitosan conjugate preparation.

	Particle size (μm)	Standard deviation (μm)
After nano-comminution	0.33	0.09
After cross-linking	0.35	0.10
After conjugation	0.35	0.10

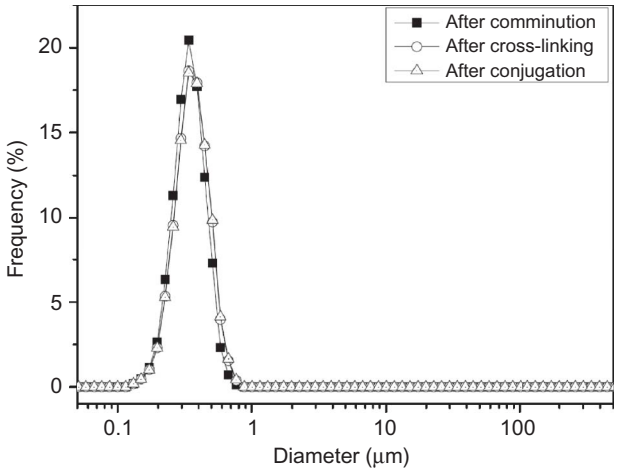
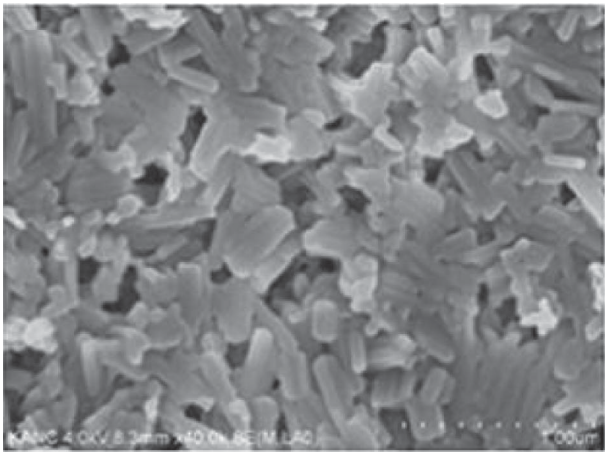
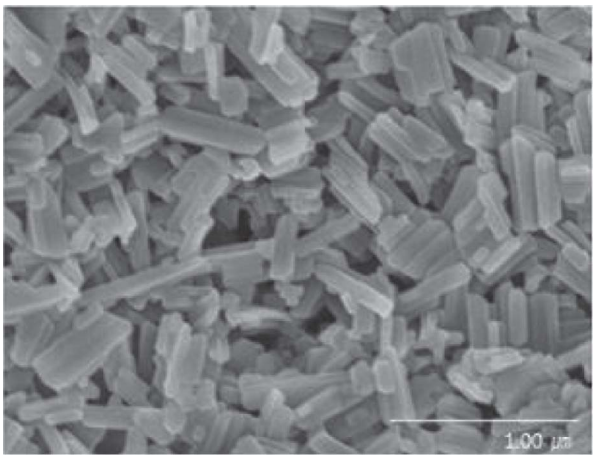


Figure 4. Particle size distributions of paclitaxel stabilized by chitosan. The curves were obtained at each step of the folate–chitosan conjugate preparation.



(a)



(b)

Figure 5. SEM images of drug particles stabilized with chitosan: (a) after comminution and (b) after conjugation.

layer of polymer is fairly thin[18,26,27]. The particle sizes and distributions before and after the reactions also seemed to be similar, as shown in Figure 5, which is consistent with Table 2 and Figure 4.

In vitro release characteristics

Because of the dominant particle size reduction effect of comminution, the existence of thin polymer layers on the surfaces of drug crystals was hard to identify using the release characteristics of drugs. However, the cross-linking reaction and conjugation with folic acid significantly influenced the in vitro release behavior. Figure 6 shows the in vitro release profiles of paclitaxel after comminution, cross-linking, and conjugation. The cross-linking reaction itself decreased the initial release rate (slopes between 0 and 4 hours) by more than half, and the further chemical modification by folic acid decreased it even further, resulting in a release rate that was about 1/3 of the release rate of paclitaxel nanoparticles obtained by nano-comminution. The area under the curve reflected the release rate changes. The slowed release rate of the cross-linked and conjugated nanoparticles indicates that their complete release takes significantly more time than that of the particles obtained by nano-comminution.

As the sizes of drug particles decreases, both the release rate and the area under the curve increase[31]. In previous studies of drug nanocrystals, the influence of surface-adsorbed polymers on in vitro release has seldom been considered[31]. It is difficult for the thin physically adsorbed polymer layer to influence the release behavior unless it has a quite different hydrophobicity[31]. However, the polymer layer was successfully cross-linked here, and then acted as a diffusion barrier. The release behavior in Figure 6 clearly shows the effects of the polymer layer itself, its cross-linking reaction, and its conjugation with folic acid. The conjugation with folic acid further reinforced the diffusion-barrier properties since folic acid caused the polymer chain to become more hydrophobic. In all cases, no burst release was observed, contrary to that observed in previous nanocrystal studies[31].

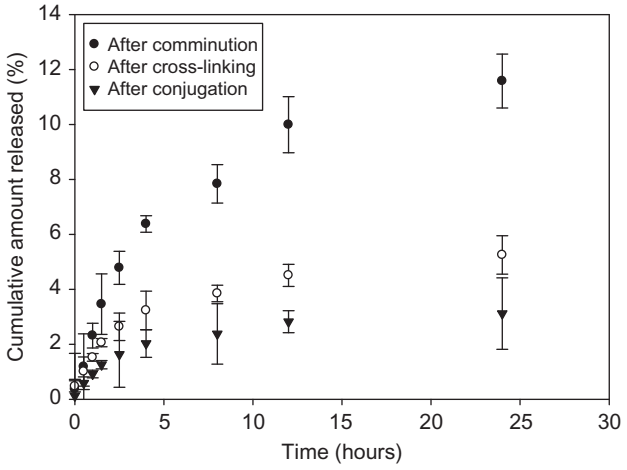


Figure 6. In vitro release profiles (37°C and pH 7.4) of paclitaxel particles stabilized by chitosan. The slopes between 0 and 4 hours for the cases after comminution, cross-linking, and conjugation were 1.52, 0.69, and 0.46, respectively.

The released amount of drug prepared by cross-linking and conjugation reactions was still significantly higher than that of the as-received paclitaxel particles. The release of the as-received particles could not be compared in Figure 6 because the amount released was too low to measure. The actual human oral absorption of poorly water-soluble drugs depends on both the dissolution and permeation parameters[31]. The release profile and targeting ability of the paclitaxel nanoparticles prepared by this reactive nano-comminution could offer a better solution for improved bioavailability and reduced side effects compared to conventional strategies. Currently, both of the commercially available formulations, Taxol and Abraxane, prepared using a conventional strategy, have serious adverse side effects such as severe hypersensitivity, neurotoxicity, nephrotoxicity, and hypotensive vasodilation[32].

Conclusions

The thickness of the polymer layers physically adsorbed onto the surfaces of hydrophobic drugs can be too thin to be used as an encapsulation shell. However, as the sizes of the drug particles decrease, the physically adsorbed layer becomes important. The polymer chains physically adsorbed onto the surfaces of drug nanoparticles were successfully cross-linked and functionalized in a subsequent step after nano-comminution. These layers were used to control the release rates of drugs and can contain targeting moieties such as folate. The cross-linking and surface functionalization reactions did not cause significant interparticular aggregation. This study showed that the nano-comminution of drugs, which has proved to be an efficient method for the preparation of solid dosage forms of drug nanoparticles, can be used as an encapsulation method to prepare intelligent drug-delivery systems.

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Declaration of interest

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