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Development of Zein Nanoparticles Coated with Carboxymethyl Chitosan for Encapsulation and Controlled Release of Vitamin D3

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ABSTRACT: In this study, zein nanoparticles coated with carboxymethyl chitosan (CMCS) were prepared to encapsulate vitamin D3 (VD3). VD3 was first encapsulated into zein nanoparticles using a low-energy phase separation method and coated with CMCS simultaneously. Then, calcium was added to cross-link CMCS to achieve thicker and denser coatings. The nanoparticles with CMCS coatings had a spherical structure with particle size from 86 to 200 nm. The encapsulation efficiency was greatly improved to 87.9% after CMCS coating, compared with 52.2% for that using zein as a single encapsulant. The physicochemical properties were characterized by differential scanning calorimetry and Fourier transform infrared spectroscopy. Nanoparticles with coatings provided better controlled release of VD3 in both PBS medium and simulated gastrointestinal tract. Photostability against UV light was significantly improved after encapsulation. Encapsulation of hydrophobic nutrients in zein nanoparticles with CMCS coatings is a promising approach to enhance chemical stability and controlled release property.

KEYWORDS: *vitamin D3, zein, carboxymethyl chitosan, nanoparticles, encapsulation, controlled release*

INTRODUCTION

Vitamin D (VD) is an essential nutrient to human health. It is one of the fat-soluble vitamins and consists of two major physiologically forms, vitamin D2 (ergocalciferol) (VD2) and vitamin D3 (cholecalciferol) (VD3). VD is a prohormone and can be synthesized from a precursor in the skin under sunlight exposure. Dietary sources of VD3 are very limited, and only fish are found to be an abundant source.[1] Recently, VD has been intensively investigated to be not only important for calcium absorption and homeostasis regulation but also essential for the prevention of many other chronic diseases, such as type 2 diabetes, hypertension, and cardiovascular disease.[1,2] It was found that for elderly people with chronic disease and those with insufficient exposure to sunlight, VD deficiency is very frequent, especially in the United States and Europe, where 30−60% of the population have been diagnosed as having VD deficiency.[3] Furthermore, on the basis of data drawn from the National Health and Nutrition Examination Surveys (NHANES), it has been shown that the mean serum VD status over the years of 2000−2004 was significantly lower than that over the years of 1988−1994.[4] As a result, the U.S. FDA encouraged the addition of VD to milk and cereals to prevent rickets in children and osteoporosis in adults. Also, the Recommended Daily Allowance of VD in the Dietary Guideline for Americans newly released by the USDA in 2010 has increased to 600 IU per day for children and 800 IU per day for most adults who have minimal sun exposure.[5] A large amount of evidence has demonstrated that increasing attention has been given to improving VD status.

As a lipophilic vitamin, VD is not soluble in water and very sensitive to various environmental factors. For example, light, heat, and oxygen could rapidly induce isomerization or oxidation of VD3 and then adversely affect its chemical structure and physiological benefits.[6] In recent developments in the food and nutraceutical industries, encapsulation of lipophilic bioactive compounds has received tremendous interest as this technology is able to constitute an appropriate means to increase the stability and preserve the health-promoting properties during processing and storage.[7] Another benefit of encapsulation is that it might further provide target delivery and controlled release of nutraceuticals, thus enhancing bioactivity and diminishing side effects of potential hypervitaminosis due to high administration doses.[8] Although the encapsulation of VD has been reported since 1998,[9] many disadvantages still exist, inhibiting further application and industrialization of VD supplementation. For instance, encapsulation of VD3 into cyclodextrin involved high temperature (50 °C) during the preparation, which would cause loss of VD3.[9] Microencapsulation of VD2 was later developed using a chitosan/ethylcellulose complex microcapsule by Shi and Tan.[10] In their study, although high encapsulation efficiency (>95%) and controlled release were achieved, toxic solvents were used, such as tetrachloride and petroleum ether, which would pose potential side effects to final products due to residues of these solvents. A new study reported a novel method to encapsulate VD2 into a natural nanocapsular vehicle prepared with bovine casein micelle.[11] However, it was found that the loading capacity (vitamin/protein ratio) was as low as 0.05%, which means large amounts of protein are needed to encapsulate a low amount of vitamin, and only around 27.5% of VD2 was incorporated into micelles and the rest of the vitamin was bound only to the surface of casein molecules, resulting in little protection against UV exposure.

Zein is a maize prolamine protein with three-fourths lipophilic and one-fourth hydrophilic amino acid residues,

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leading to its unique aqueous-alcohol solubility and film-forming property. Zein has been extensively investigated for its capability to form self-assembled nanoparticles encapsulating bioactive compounds in food and pharmaceutical applications, such as fish oil,[12] essential oil,[13] gitoxin,[14] and fluorouracil.[15] Chitosan (CS), a natural polysaccharide, has been considered to be a versatile polymer for encapsulation and delivery of active ingredients. To improve its solubility in neutral pH, its derivative, carboxymethyl chitosan (CMCS), is widely used for biomedical applications. It is soluble in neutral aqueous solution, and it has been shown that negatively charged CMCS is able to form nanoparticles spontaneously with positively charged ions.[16] Several bioactive compounds have already been successfully encapsulated into CMCS nanoparticles to achieve controlled release and target delivery properties.[17,18] CMCS has also been modified by cholesterol to form self-assembled nanoparticles able to deliver anticancer drugs with higher targeting efficiency and longer retention time in the liver than those of pure drug solution.[19] In addition, it is newly elucidated that negatively charged CMCS nanoparticles with particle size around 150 nm had a better in vitro cellular uptake and more efficient in vivo targeting delivery of entrapped drug to tumor.[20] In our previous studies, zein/CS complex nanoparticles were developed as delivery systems for both hydrophilic and hydrophobic nutrients.[21,22] The physicochemical properties, including encapsulation efficiency and controlled release profile, of complex nanoparticles were greatly improved relative to those of nanoparticles prepared by the single polymer (i.e., zein or CS).

In the present study, VD3 was first encapsulated into zein nanoparticles prepared by phase separation. Then, CMCS was applied to coat zein nanoparticles and hardened by calcium ions. The preparation conditions, such as zein concentration and zein/CMCS ratio, as well as CMCS/calcium ratio, were optimized to obtain nanoparticles with small particle size, high zeta potential, and high encapsulation efficiency. The encapsulation of VD3 was evidenced by diffraction scanning calorimetry (DSC). The in vitro release profile and stability of VD3 in nanoparticles were also studied.

MATERIALS AND METHODS

Materials. VD3 (99%) was purchased from Alfa Aesar (Ward Hill, MA). Zein sample with a minimum protein content of 97% was provided by Showa Sangyo (Tokyo, Japan). CMCS was purchased from Nantongxingcheng Biological Product Inc. (Nantong, Jiangsu Province, China), with a deacetylation degree of 96% and a carboxylation degree of 65%. Phosphate buffer saline was bought from EMD Chemicals Inc. (Gibbstown, NJ). Simulated gastric fluid (SGF) and simulated intestinal fluid with pancreatin (SIF) were purchased from RICCA Chemical Co. (Arlington, TX). All other materials, including pepsin, hexane, and ethyl acetate, were of analytical grade and purchased from Sigma-Aldrich Chemical Co. Ltd. (St. Louis, MO).

Preparation of Nanoparticles. VD3 (1 mg/mL) was dissolved in pure ethanol as stock solution. Zein was dissolved in 70% aqueous-ethanol solution. The nanoparticles were prepared using a phase separation method as previously reported with some modifications.[15] Briefly, 0.3 mL of VD3 solution (1 mg/mL) was added dropwise into 2 mL of zein solution (2 mg/mL) with mild stirring for 30 min. Then, the above VD3 encapsulated zein solution was rapidly poured into 5 mL of CMCS solution (0.8, 1.6, or 0.4 mg/mL), dissolved in pure water. After 30 min of stirring, 1 mL of calcium solution in different concentrations was then dropwise added into the above solution. After another 30 min of stirring, the obtained opaque single phase solution was then freeze-dried for 48 h. The control nanoparticles without CMCS and/or calcium were also prepared in parallel. Please refer to Table 1 for detailed description of various formulations. All samples were prepared in triplicate, and all procedures were performed in darkness under room temperature.

Table 1. Formulations of Nanoparticle Complexes[a]

sample	VD3 loading (%)	Z/CMCS (m/m)	CMCS/Ca (m/m)
ZV	7.5	N/A	N/A
A0	7.5	1:1	N/A
A5			5:1
A10			10:1
A20			20:1
B0	7.5	1:2	N/A
B5			5:1
B10			10:1
B20			20:1
C0	7.5	2:1	N/A
C5			5:1
C10			10:1
C20			20:1

[a]Z, zein, used as 2 mg/mL; CMCS, carboxymethyl chitosan; VD3, vitamin D3. ZV represents VD3 encapsulated zein nanoparticles. VD3 loading percentage was calculated as mass ratio of VD3 to zein polymer. A0−A20 represent formulations with different mass ratios of CMCS/calcium, prepared with mass ratio zein/CMCS as 1:1. B0−B20 represent formulations with different mass ratios of CMCS/calcium, prepared with mass ratio zein/CMCS as 1:2. C0−C20 represent formulations with different mass ratios of CMCS/calcium, prepared with mass ratio zein/CMCS as 2:1.

Scanning Electron Microscopy (SEM). Morphological structures of nanoparticles were observed by a SEM (Hitachi SU-70, Pleasanton, CA). Samples were first cast-dried on an aluminum pan before cutting into an appropriate size and then adhered to conductive carbon tapes (Electron Microscopy Sciences, Fort Washington, PA). Subsequently, they were mounted on specimen stubs and coated with a thin (<20 nm) conductive gold and platinum layer using a sputter coater (Hummer XP, Anatech, Union City, CA). Representative SEM images were reported.

Fourier Transform Infrared Spectroscopy (FTIR) and Differential Scanning Calorimetry (DSC). The chemical structures of preparation ingredients (i.e., zein and CMCS) and nanoparticles (i.e., ZV, B0, and B20) were monitored by FTIR of Jasco 4100 series with an attenuated total reflection (ATR) cell (Jasco Inc., Easton, MO). Samples were first cast-dried on an aluminum tray for 24 h and then mounted onto ATR crystal directly. The spectra were acquired at 750−4000 cm^{-1} wavenumbers with a 4 cm^{-1} resolution.

DSC analyses of pure ingredients (i.e., zein, CMCS, and VD3), mixture of zein and VD3, and nanoparticles (i.e., ZV and B20) were performed using a TA Q100-DSC thermal analyzer (TA Instruments, New Castle, DE), calibrated with indium. The mass ratio of zein and VD3 in the physical mixture was the same as that in nanoparticles. Each sample (5 mg) was placed onto a standard aluminum pan, crimped, and heated from room temperature to 230 °C, with a constant heating rate of 10 °C/min under continuous purging of nitrogen (20 mL/min). An empty sealed aluminum pan was applied as the baseline.

Particle Size and Zeta Potential. The freshly prepared nanoparticle samples were used for particle size and zeta potential measurement. Hydrodynamic diameters of different treatments were measured by a dynamic light scattering (DLS) instrument (DLS, BI-200SM, Brookhaven Instruments Corp., Holtsville, NY), which was equipped with a 35 mW HeNe laser beam at a wavelength of 637 nm.

Table 2. Particle Size, Polydispersity Index (PDI), Zeta Potential, Loading Capacity (LC), and Encapsulation Efficiency (EE) of Nanoparticles in Different Formulations[a]

sample	particle size (nm)	PDI	zeta potential (mV)	LC (%)	EE (%)
ZV	120.2 ± 2.2bc	0.21 ± 0.02d	−26.5 ± 2.8f	3.9 ± 0.1f	52.2 ± 1.7a
A0	200.9 ± 5.9e	0.19 ± 0.01d	−26.8 ± 2.3f	2.4 ± 0.3bcd	64.1 ± 7.3ab
A5	156.6 ± 5.4d	0.07 ± 0.01ab	−13.1 ± 0.5ab	2.2 ± 0.2abc	65.1 ± 3.1ab
A10	116.7 ± 2.9b	0.16 ± 0.02d	−17.0 ± 0.8abc	2.0 ± 0.2abc	56.9 ± 6.6ab
A20	86.3 ± 2.0a	0.18 ± 0.01d	−20.5 ± 1.2cde	2.5 ± 0.1 cd	69.5 ± 3.6b
B0	139.5 ± 5.8cd	0.17 ± 0.01d	−25.8 ± 0.6ef	1.8 ± 0.1ab	71.5 ± 2.5b
B5	large aggregates				
B10	145.3 ± 6.4d	0.15 ± 0.01 cd	−16.5 ± 1.9bcd	1.7 ± 0.1a	74.7 ± 5.7c
B20	109.5 ± 11.3b	0.20 ± 0.01d	−20.2 ± 1.7cde	2.1 ± 0.1abc	87.9 ± 1.8c
C0	119.1 ± 5.5bc	0.19 ± 0.02d	−27.2 ± 1.8def	3.2 ± 0.3e	63.5 ± 6.9ab
C5	200.8 ± 16.7e	0.04 ± 0.01a	−11.8 ± 0.3a	2.5 ± 0.2 cd	53.1 ± 3.7a
C10	113.1 ± 1.9b	0.10 ± 0.00bc	−16.3 ± 1.4abcd	3.0 ± 0.4ed	62.9 ± 2.8ab
C20	109.8 ± 2.9b	0.15 ± 0.01 cd	−21.0 ± 1.2cef	3.5 ± 0.2ef	72.1 ± 4.3bc

[a]Z, zein; CMCS, carboxymethyl chitosan; VD3, vitamin D3. ZV represents VD3 encapsulated zein nanoparticles. A0−A20 represent formulations with different mass ratios of CMCS/calcium, prepared with mass ratio zein/CMCS as 1:1. B0−B20 represent formulations with different mass ratios of CMCS/calcium, prepared with mass ratio zein/CMCS as 1:2. C0−C20 represent formulations with different mass ratios of CMCS/calcium, prepared with mass ratio zein/CMCS as 2:1. Please refer to Table 1 for detailed description of formulations. Values with different letters represent significant difference within the column ($P < 0.05$).

All DLS measurements were performed at 25 °C. The polydispersity index (PDI) reflecting the particle size distribution of nanoparticles was also reported. Electrophoretic mobility of different samples was measured by a laser Doppler velocimetry (Zetasizer Nano ZS90, Malvern, U.K.), using a fold capillary cuvette (Folded Capillary Cell DTS1060, Malvern, U.K.). Zeta potential was obtained by converting the measured electrophoretic mobility using the Smoluchowski theory. All measurements were performed in three replicates.

Encapsulation Efficiency (EE). EE was measured according to the method of Wang et al.[23] with minor modifications. Briefly, 10 mg of lyophilized nanoparticle samples was flushed with 1 mL of ethyl acetate three times, using no. 1 Whatman filter paper. Then, the washed nanoparticle samples were vacuum-dried in a vacuum oven (VWR International, Radnor, PA). The ethyl acetate elute containing free VD3 was dried using a vacuum rotary evaporator (Büchi, DE), and then VD3 was extracted by hexane and determined by a UV−vis spectrophotometer (Beckman Coulter, DU-730, Fullerton, CA) at 264 nm. The VD3 encapsulated in lyophilized nanoparticles was also extracted according to the method described by Wang et al.[24] to calculate recovery rate. Five milliliters of ethanol was added and vigorously shaken on a vortex mixer for 30 s, and then 5 mL of hexane was added. The obtained mixture was vigorously shaken for another 30 s. Five milliliters of pure water was then added into the above mixture, and the tube was then sealed tightly and shaken on a multipurpose rotator/rocker (Scientific Industries Inc., Bohemia, NY) for 30 min. After the mixture had been centrifuged at 800*g* for 5 min at 4 °C to quickly separate the hexane phase and water/ethanol phase, the VD3 in the hexane layer was measured as described above. The free VD3 in the elute and encapsulated VD3 extracted from lyophilized nanoparticles were added to calculate the recovery rate of VD3 measurement, which was found to be >95%. The EE was calculated by using the following equation:

$$\text{EE}\ (\%) = \frac{\text{total VD amount} - \text{free VD amount}}{\text{total VD amount}} \times 100$$

$$\text{LC}\ (\%) = \frac{\text{encapsulated VD weight}}{\text{nanoparticles weight}} \times 100$$

Release Profile. The lyophilized samples after removal of free VD3 were used for in vitro kinetic release test in simulated gastrointestinal tract (SGI). For a kinetic release test in PBS, a certain amount of sample (10 mg) was resuspended in PBS (pH 7.4) containing Tween 20 (Tw, 0.5%, w/v) that was to provide sink condition and increase VD3 solubility, at 37 °C. At designated time intervals, samples were centrifuged at 10000*g* for 5 min to withdraw the supernatant medium, and equivalent fresh medium was added in. The released VD3 in the withdrawn medium was extracted and measured as described under Encapsulation Efficiency. The VD3 percentage released was calculated and plotted as a function of time (up to 6.5 h). The accumulated release profiles of the nanoparticles in the SGI with digestive enzymes were obtained using the method as previously reported.[21] The weighted samples (10 mg) were first incubated in 30 mL of SGF with 0.1% pepsin (w/v) for 0.5 h. Digestion was stopped by raising the pH to 7.5 using NaOH, and the sample was then centrifuged to separate aggregates from supernatant, which was collected for VD3 measurement. Subsequently, the 30 mL of SIF with 1.0% pancreatin (w/v) at 37 °C was added and digested for 6 h under mild stirring. After digestion, the supernatant was collected by centrifugation and used for VD3 measurement. All measurements were performed in three replicates.

Photochemical Stability Measurement. The freshly prepared samples (i.e., ZV, B0, and B20) as described under Preparation of Nanoparticles together with VD3 dispersion in water as control were used for VD3 stability measurement. VD3 dispersion was prepared by dissolving a small amount of VD3 in ethanol followed by dispersion into water, with the final concentration of VD3 equivalent to nanoparticle samples. Samples in transparent glass vials were placed in a light-proof cabinet and exposed to two 352 nm UV light bulbs (15 W) for up to 9.5 h. At exposure time intervals of 0.5, 1.0, 1.5, 3.5, 6.5, and 9.5 h, 200 μL of sample was withdrawn from each treatment and then VD3 was extracted and measured according to the method described above. All measurements were performed in triplicate.

Statistical Analysis. All of the experiments were conducted in triplicate with data reported as the mean ± standard deviation. Experimental statistics were performed using the SAS software (version 9.2, SAS Institute Inc., Cary, NC). The analysis of variance (ANOVA) with Tukey's multiple-comparison test was used in the analysis of differences between physicochemical properties of nanoparticles in different formulations. The significance level (P) was set at 0.05.

RESULTS AND DISCUSSION

Optimization of the Formulation. *Particle Size, Zeta Potential, LC, and EE.* Particle size and zeta potential are both paramount parameters to prepare stable nanoparticles targeting nutritional and medical applications. The effects of preparation parameters on particle size and zeta potential in different formulations are summarized in Table 2. The particle size of

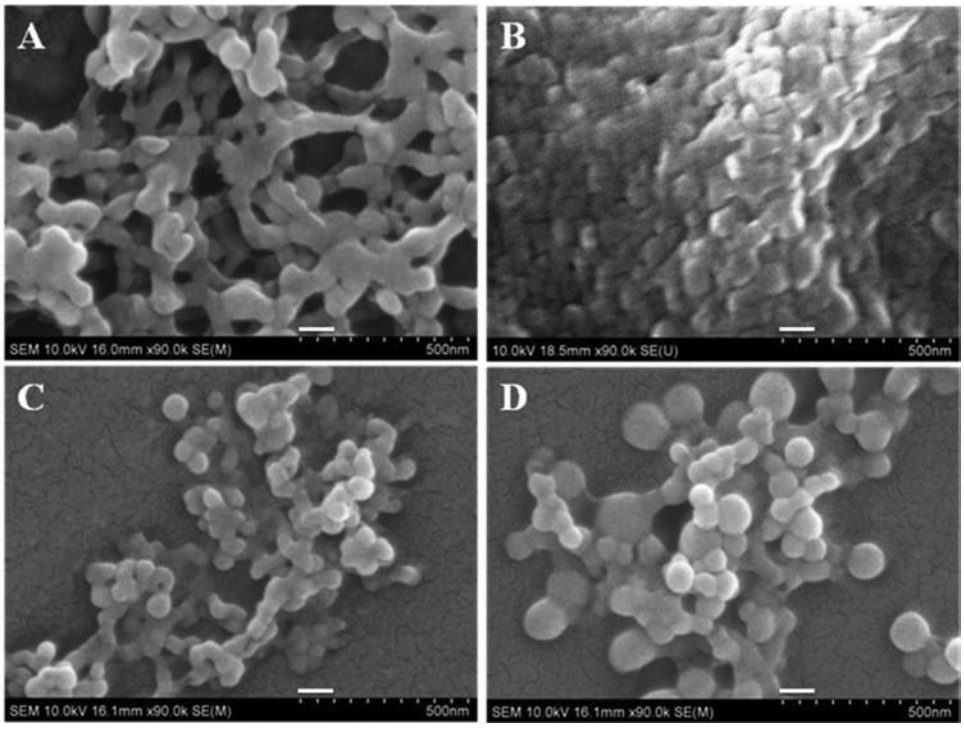

Figure 1. Scanning electron microscopy (SEM) images of prepared nanoparticles: (A) ZV sample, VD3 encapsulated zein nanoparticles; (B) B0 sample, VD3 encapsulated zein−CMCS nanoparticle complex without calcium; (C) B20 sample, VD3 encapsulated zein−CMCS nanoparticle complex with calcium; (D) B20 sample after 1 week of storage under room temperature. Please refer to Table 1 for detailed information of formulations. The white bar in each image represents 100 nm.

VD3-encapsulated zein nanoparticles without CMCS coating was as small as 120 nm with relatively small polydispersity (PDI), indicating the size distribution was uniform. After CMCS coating was applied on zein nanoparticles, the particle size varied with calcium concentration added. Generally, the largest particle size was obtained without either CMCS or calcium (A0). The lower the calcium concentration added, the smaller the particle size was. Sample A20 (with a CMCS/calcium ratio of 20:1) had the smallest particle size of 86.3 nm in all formulations ($P < 0.05$). At higher concentration of CMCS, particles precipitated at a CMCS/calcium ratio of 5:1 (B5), suggesting the aggregation would occur at high calcium concentration, a phenomenon that was also observed previously.[16] However, with the same CMCS concentration, more calcium ions resulted in significantly smaller PDI (A5 and C5, $P < 0.05$), indicating that particles in these formulations may have more uniform particle size. The phase separation method has been considered to be a reliable and simple method to produce zein nanoparticles. The preparation of zein nanoparticles was previously reported as shearing zein stock solution into water using a high-speed homogenizer,[13,25] with particle size around 100 nm. Although homogenization with high shearing force is an effective approach to produce zein nanoparticles, particularly when the zein concentration is high, this high-energy method generates heat during homogenization, which might cause loss of labile encapsulated ingredients as we have observed when preparing VD3-loaded zein nanoparticles. To avoid this potential disadvantage, an alternative method to produce zein nanoparticles in low concentration was adopted in this study by pouring zein stock solution into water with stirring. It was found that this low-energy method was applicable to the production of zein nanoparticles with a diameter of 100 nm, and a similar observation was also reported previously.[15]

The zeta potential of VD3-encapsulated zein nanoparticles without CMCS coating was −26.5 mV and remained the same as CMCS coating was added. As more calcium was added and cross-linked with CMCS through electrostatic interaction, the zeta potential became less negative, ranging from −21.0 to −11.8 mV. It was suggested from these results that higher calcium content caused a decrease of the absolute value of the zeta potential, thus resulting in polymer aggregation. It was reported that different CMCS/calcium ratios were required to form nanoparticles when the molecular weight of CMCS varied,[16] showing that CMCS with a molecular weight of 38.9 kDa needed only a small amount of calcium to form nanoparticles and excessive calcium would cause aggregation. It is suggested in that study that a longer molecular chain was much easier to roll up when coordinated with calcium ions, compared to shorter chains. On the basis of the above results, due to the much higher molecular weight of CMCS (67−79 kDa) used in our study, it was observed that a CMCS/calcium ratio of 20:1 could be an appropriate formulation, which generated nanoparticles with particle size of <110 nm and absolute value of zeta potential >20 mV.

After removal of unencapsulated or loosely attached VD3 from nanoparticles, the effects of different parameters on EE are shown in Table 2. The EE of zein nanoparticles without CMCS was around 52.2% and increased to 71.5% after CMCS was added to the system depending on the CMCS concentration ($P < 0.05$). With the addition of calcium to the system, the EE increased further to 87.9%, significantly at 20:1 CMCS/calcium and 2:1 CMCS/zein ratios ($P < 0.05$). This could be ascribed to the cross-link between calcium and CMCS through electrostatic interactions, resulting in thicker

and denser coatings on the surface of zein nanoparticles and therefore an increase of EE. Due to the decreased surface charges of nanoparticles, too much calcium could cause aggregation of CMCS/calcium particles, resulting in lower EE of VD3 (i.e., B5), compared to the formulations with lower calcium concentration (i.e., B20). The LC of all samples was within the range of 1.7−3.9%, almost 40 times higher than the previously reported LC of VD2 using bovine casein micelle, which was 0.05%.[11] The LC values of ZV and samples in group C were higher than those of samples in groups A and B, which were made with higher CMCS concentration at constant VD3 loading percentage. Therefore, consistent with the observation of particle size and zeta potential, the results of EE also suggested that the optimal formulation was the sample B20.

Physicochemical Characterization. *Morphological Observation.* As shown in Figure 1A, the VD3-encapsulated zein nanoparticles without CMCS coatings shared features of a spherical shape and smooth surfaces, with uniform particle size around 100 nm. However, most of the particles were clumped and connected to each other, and it was hard to see individual ones, unlike other reported studies in which the individual zein nanoparticles were clearly observed in SEM pictures.[13,25] This difference might be caused by the low-energy method used in our study instead of the high-energy method using a high-speed homogenizer in other studies. After zein nanoparticles were coated by CMCS, it was still not possible to see individual particles clearly (Figure 1B), which might be attributed to the highly hydrophilic CMCS coating on the surface of zein nanoparticles. Interestingly, the addition of calcium made clearer partition of nanoparticles (Figure 1C), showing the effect of cross-linking between CMCS and calcium ions. Figure 1D shows the SEM image of B20 nanoparticles after 1 week of storage under room temperature. Whereas the particle size increased a little compared to that of the original nanoparticles (Figure 1C), it maintained the spherical shape and smooth surface, suggesting VD3-encapsulated B20 nanoparticles were stable over time. The freeze-dried nanoparticles maintained intact structure and shape after reconstitution into 25% ethanol-aqueous solution (data not shown).

FTIR Study. Figure 2 shows the representative FTIR spectra of zein, CMCS, and their corresponding VD3 encapsulated complex nanoparticles in different formulations (i.e., B0 and B20). In the infrared spectra, an interesting characterization peak was in the range of 3200−3400 cm^{-1}, indicating the hydrogen bonding. The hydrogen bonding in zein and CMCS polymer was at 3312 and 3279 cm^{-1}, respectively, and shifted to 3323 and 3291 cm^{-1} after VD3 was encapsulated, suggesting the hydrogen bonding was formed between VD3 and zein/CMCS. Therefore, the hydrogen bonding among zein, CMCS, and VD3 was considered to be one of the major forces facilitating nanoparticle formation. The vibration peaks of 1500−1700 cm^{-1}, corresponding to amide I and II bonds, had no obvious shift in all formulations. The vibration peak at 1419 cm^{-1} in CMCS could be assigned to the symmetric stretching vibrations of carboxyl groups,[26] and it shifted significantly to 1468 cm^{-1} in sample B20, indicating the strong electrostatic interactions between carboxyl groups in CMCS and calcium ions. Because both zein and VD3 are highly hydrophobic molecules, the hydrophobic interactions could also contribute to the formation of nanoparticles, besides the hydrogen bonds and electrostatic interactions.

Thermal Property Study. The DSC thermograms corresponding to zein, CMCS, VD3, the physical mixture of zein and

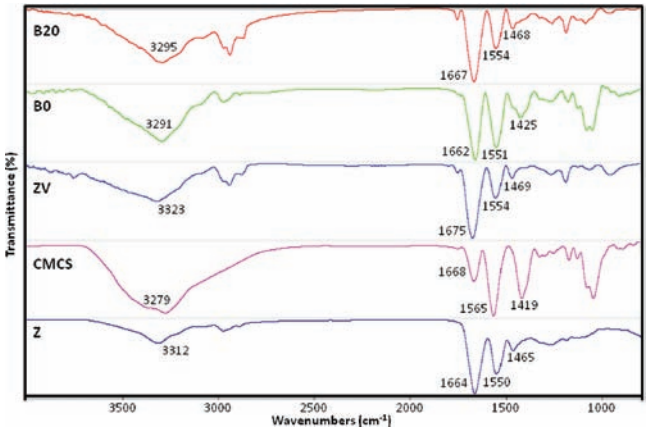

Figure 2. Fourier transform infrared spectroscopy (FTIR) spectra of different samples. Z, zein powder; CMCS, carboxymethyl chitosan powder; ZV, VD3 encapsulated zein nanoparticles; B0, VD3 encapsulated zein−CMCS nanoparticle complex without calcium; B20, VD3 encapsulated zein−CMCS nanoparticle complex with calcium. Please refer to Table 1 for detailed information of formulations.

VD3 (i.e., sample Z+VD3), ZV, and B20 nanoparticles are shown in Figure 3. The DSC curves of zein and CMCS exhibited broad endothermic peaks at 74.8 and 91.1 °C (Figure 3A), respectively. These characteristic endotherms could correspond to the evaporation of bound water from polymer molecules, and the peak for hydrophobic polymer zein was at lower temperature than hydrophilic polymer CMCS, due to the stronger affinity between CMCS and water. A similar phenomenon was also reported in our previous study.[21] The DSC curve of VD3 displayed a single melting peak at 88.1 °C (Figure 3A). For the physical mixture of zein and VD3, both the melting peak of VD3 and the endothermic peak of zein had been detected (Figure 3B). However, only endothermic peaks of zein and CMCS were detected in samples of ZV and B20 nanoparticles, respectively (Figure 3B), giving evidence that VD3 was molecularly dispersed in the polymeric matrix and hence encapsulated in the nanoparticles. A similar observation was also reported by Lai and Guo, showing that the absence of the endotherm peak of drugs provided evidence of encapsulation.[15] Interestingly, the small characteristic peak of zein at around 180 °C was also observed in Z + VD3 and ZV nanoparticles. However, this endotherm peak is not shown in B20 nanoparticles, suggesting that zein might be molecularly dispersed and entrapped in the CMCS−calcium matrix.

Kinetic Release in PBS and Cumulative Release in SGI. Both the kinetic release profile of nanoparticles in PBS and the cumulative release profile in SGI with digestive enzymes were evaluated and are shown in Figure 4, panels A and B, respectively. In PBS medium, all formulations showed a first-order release profile, with biphasic kinetic releasing trend, that is, a burst effect within 1.5 h followed by a sustained release for up to 7 h. ZV and B0 showed a similar burst effect at 1.5 h with nearly 60% VD3 released, whereas <40% VD3 was released for sample B20. In the following sustained release phase, VD3 from B20 was released much more slowly with only about 50% of total VD3 released after 6.5 h of incubation, compared with ZV and B0, both of which had >80% VD3 released. Under the SGI condition with digestive enzymes, ZV and B0 nanoparticles also performed similarly in the release profile, with almost 60% being released in gastric fluid and 40% in intestinal fluid.

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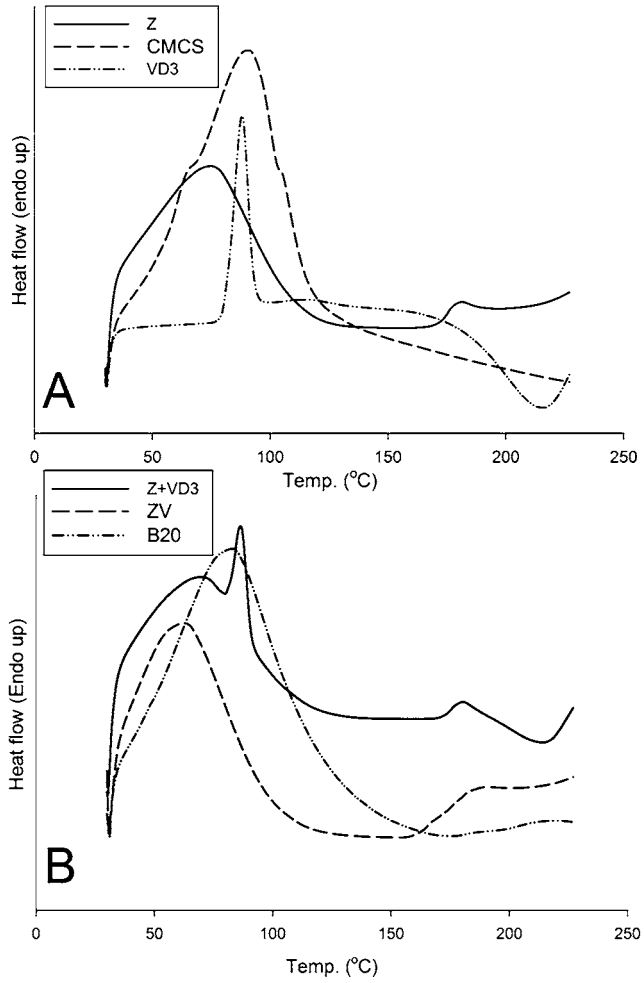

Figure 3. Differential scanning calorimetry (DSC) thermograms of different samples: (A) thermograms of each ingredient, that is, zein, CMCS, and VD3; (B) thermograms of nanoparticle complex, that is, ZV, VD3 encapsulated nanoparticles. Z+D, physical mixture of zein and VD3; B20, VD3 encapsulated zein−CMCS nanoparticle complex with calcium. Please refer to Table 1 for detailed information of formulations.

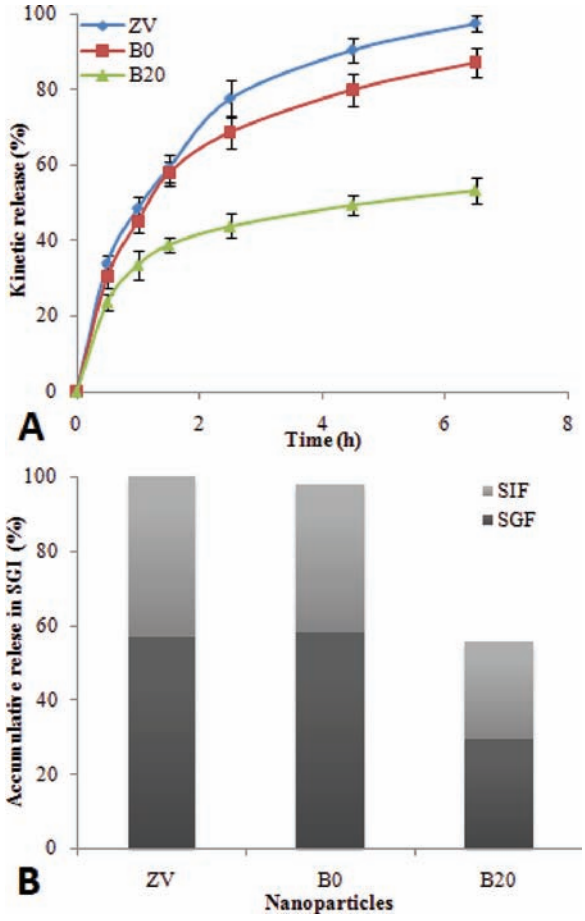

Figure 4. (A) Kinetic release of VD3 from nanoparticles in PBS. (B) Cumulative release of nanoparticles in simulated gastrointestinal (SGI) tract (the standard error of each measurement was within 10% of the mean, n = 3). SGF, simulated gastric fluid; SIF, simulated intestinal fluid; ZV sample, VD3 encapsulated zein nanoparticles; B0 sample, VD3 encapsulated zein−CMCS nanoparticle complex without calcium; B20 sample, VD3 encapsulated zein−CMCS nanoparticle complex with calcium. Please refer to Table 1 for detailed information of formulations.

However, the release profile in SGI was significantly improved after calcium was added into the system. For sample B20, only 30% of VD3 was released in gastric fluid and 25% of VD3 was released in intestinal fluid, indicating it had controlled release property in the SGI tract. However, almost 100% of VD3 was released for the ZV and B0 nanoparticles after 6.5 h of incubation.

The delivery system of VD3 in our study was considered to be the nanoparticles with hydrophobic core and hydrophilic shell, which has been extensively studied for encapsulating hydrophobic drugs and nutrients through hydrophobic interactions.[21,27] On the basis of the above results, the zein nanoparticles with CMCS and calcium could provide better controlled release of VD3 in PBS medium, which mimics the blood condition. In addition, a more significant effect in the SGI tract was observed in this study, which could be explained from several aspects. First, only the nanoparticles with addition of CMCS and calcium had the morphology of clear and sphere particles, which could provide a better barrier against release of VD3 than those particles with aggregated bulk morphology. Second, calcium ions served as a cross-linker, resulting in the formation of dense CMCS coatings on the surface of zein nanoparticles. It was previously reported that the addition of tripolyphosphate anions to self-assembled oleoyl-carboxymethyl chitosan nanoparticles was able to slow the release of entrapped drugs.[28] Third, the slow release of B20 in gastric fluid might be due to the fact that although CMCS was fully soluble in water at neutral pH, it formed gels when contacting acidic medium. Therefore, it could form a CMCS gel layer, being a barrier against diffusion of VD3 and also slowing the digestion of zein by enzymes. Compared with our previous study,[21] it was suggested that the zein nanoparticles coated with CMCS could provide better controlled release in gastric fluid than those coated with native chitosan, because native chitosan is highly soluble under the pH of gastric fluid and might decompose quickly. Additionally, the products of partially hydrolyzed zein in gastric fluid may act as emulsifying agents and adsorb onto oil droplets and hence increase its resistance to degradation in intestinal fluid, resulting in slow release of VD3 in intestinal fluid.[21,29]

Photochemical Stability against UV Light. The freshly prepared samples were put in a light-proof chamber and exposed to UV light for the UV stability measurement. As

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shown in Figure 5, the control sample VD3 underwent a photochemical degradation very quickly when exposed to 352

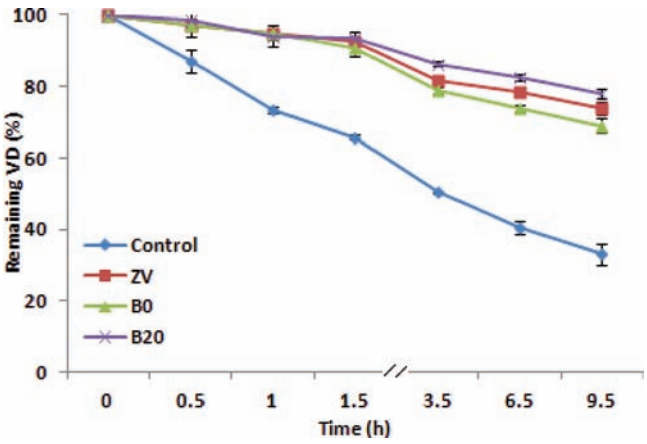

Figure 5. Photochemical stability of different samples against UV light. Control, VD3 dispersion in water; ZV sample, VD3 encapsulated zein nanoparticles; B0 sample, VD3 encapsulated zein−CMCS nanoparticle complex without calcium; B20 sample, VD3 encapsulated zein−CMCS nanoparticle complex with calcium. Please refer to Table 1 for detailed information of formulations.

nm UV light. In 9.5 h, only around 30% of VD3 remained in the control. All of the nanoparticle samples were able to provide great protection again UV light-induced degradation, with >70% of VD3 remaining in samples after 9.5 h of UV light exposure. Especially for sample B20, around 80% of VD3 had not been degraded, showing the greatest protection among the tested samples. The photochemical stability of VD3 is one of the major hurdles for commercialization of supplement or fortified food products, such as infant formula and milk.[6] Encapsulation of labile VD3 into polymeric matrix is a proper way to provide protection against a harsh environment. A previous study also demonstrated that encapsulation of vitamin D2 into casein micelles provided partial protection against UV light-induced degradation.[11] One possible protection mechanism of the protein matrix against photochemical degradation of VD3 was that proteins with aromatic side groups and double bonds can absorb UV light and hence reduce the absorption of UV light by VD3.

Conclusions. In conclusion, zein/CMCS complex nanoparticles were successfully developed as a novel delivery system for VD3, using a low-energy liquid−liquid dispersion method. Zeta potential, particle size, and encapsulation efficiency can be modulated with different preparation parameters. Hydrogen bonding and electrostatic interaction as well as hydrophobic interaction were considered to be the major forces facilitating the formation of complex nanoparticles. In terms of their release properties in PBS solution and at SGI conditions, addition of calcium to cross-link with CMCS was found to be critical for optimal performance. Photochemical stability against UV light was thought to be mainly contributed from zein protein, given the fact that no significant difference was observed between samples with and without CMCS/calcium. Thus, encapsulation of hydrophobic nutrients in zein/CMCS complex nanoparticles would achieve the controlled release property and improve the stability of labile nutrients. The bioavailability of such complex nanoparticles is currently being studied in our laboratory using both in vitro and in vivo approaches, and results will be published soon.

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