Preparation and Characterization of Nanoparticles Based on Hydrophobic Alginate Derivative as Carriers for Sustained Release of Vitamin D₃

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ABSTRACT: Hydrophobic alginate derivative was prepared by modification of alginate by acid chloride reaction using oleoyl chloride without organic solvents. The conjugate of oleoyl alginate ester (OAE) was confirmed by FT-IR and ^{1}H NMR. The degree of substitution (DS) of OAE was determined by ^{1}H NMR, and it ranged from 0.84 to 3.85. In distilled water, OAE formed self-assembled nanoparticles at low concentrations in aqueous medium, and nanoparticles retained their structural integrity both in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). The loading and release characteristics of nanoparticles based on OAE were investigated using vitamin D_3 as a model nutraceutical. As the concentration of vitamin D_3 increased, the loading capacity (LC) increased, whereas the loading efficiency (LE) decreased. Nanoparticles could release vitamin D_3 at a sustained rate in gastrointestinal fluid. These results revealed the potential of OAE nanoparticles as oral carriers for sustained release of vitamin D_3.

KEYWORDS: Alginate, oleoyl chloride, nanoparticles, gastrointestinal stability, vitamin D_3

INTRODUCTION

Although colloidal systems such as micelles, liposomes, nanoemulsions, and biopolymeric nanoparticles have found numerous applications as delivery vehicles for pharmaceutical compounds, their use as vehicles for functional food ingredients is relatively new in the food industry.[1] The use of biopolymer nanoparticles offers a promising means to improve the bioavailability of poorly soluble substances such as functional lipids (e.g., carternoids, phytosterals, and ω-3 fatty acids) and natural antioxidants, because of their subcellular size and the versatility in terms of compounds that can be encapsulated, etc.[2] Recently, nanoparticles based on food proteins such as casein and lactoglobulin have been studied for nutraceutical delivery.[3,4] However, there are few reports of the application of polysaccharide-based nanoparticles to the food industry.

Alginate, a natural linear polysaccharide, consists of two kinds of hexurunic acid residues including 1,4-β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues, which are arranged in repeating GG (MM) blocks or alternating MG blocks.[5] Alginate is widely used in the food industry as a thickener, emulsifier, and stabilizer. It also has been extensively studied for particle formation in the size range of 100 nm–2 mm for drug delivery because of several properties such as biocompatibility, bioadhesiveness, and pH sensitivity.[6−10] For example, hydrogel alginate particles, produced in aqueous media by cross-linking sodium alginate with divalent cations, were proposed for the encapsulation of cells, proteins, oligonucleitides, or DNA.[11,12] However, the cross-linking structures are easily destroyed in biological buffers containing chelators of calcium ions or monovaleat electrolytes. To enhance the stability, viscosity, and peculiar rheological properties, various hydrophobic groups such as n-octylamine groups, alkyl chains (C_{12} and C_{18}), and cholesteryl were introduced to the alginate backbone by different procedures.[13−15] Covalent modification of alginate by hydrophobic materials is an effective way to increase

drug loading and controlled release because hydrophobic segments may undergo intermolecular association to form multimolecular clusters. For example, microspheres prepared by alkyl chain-grafted sodium alginate show high protein encapsulation yields and controlled release properties.[5] Millimetric beads based on amphiphilic derivatives of alginate bearing poly(ε-caprolactone) (PCL) chains allowed the retention of a model poorly water-soluble drug (theophylline) and slowed its release considerably.[16,17] Recently, it has been found that cholesteryl-grafted sodium alginate was able to self-aggregate as nanoparticles through hydrophobic interactions, which are able to encapsulate the hydrophobic compound pyrene.[15] However, these amphiphilic derivatives of alginate bearing hydrophobic groups were synthesized in the presence of organic solvent, such as chloroform, dichloromethane, and dimethyl sulfoxide. If the residual solvents occur at levels higher than can be supported by safety data, there may be harm to the human body or to the environment.[18]

Vitamin D_3, which was chosen here as a model, is the naturally occurring form of vitamin D. Vitamin D is of great importance in health and disease prevention. It takes part in calcium and phosphate metabolism, in the formation of osteoblasts, in fetal development, etc. However, vitamin D insufficiency is a widespread public health problem, for it is a fat-soluble vitamin. It is minimally found in fat-free and low-fat dairy products, which are in growing demand.[19] Because the lining of the digestive tract is aqueous in nature, to be assimilated, vitamin D is absorbed in a bile–fat complex, which is a water-soluble globule with a fatty core.[20,21] The absorption is blocked when there are not enough lipids in the intestine. To improve the bioavailability of vitamin D, it is important to enhance the water solubility.

In the present study, a hydrophobic alginate derivative was prepared by modification of alginate by acid chloride reaction using oleoyl chloride. The key feature of this reaction is that the oleoyl alginate ester (OAE) was synthesized without the use of an organic solvent. We suppose that OAE can self-aggregate to form nanoparticles when dissolved in water and the hydrophobic nutraceutical compound may be entrapped within nanoparticles. The objective of this work was to elaborate nanoparticles from hydrophobically modified alginate to develop a carrier for oral administration of vitamin D_3.

■ MATERIALS AND METHODS

Materials. Sodium alginate, oleoyl chloride, formic acid, pyrene, pepsin, pancreatin, and vitamin D_3 were purchased from Sigma Chemicals and used without further purification. Methanol (HPLC grade) was purchased from Merck Chemicals. All other chemicals used in this study were of analytical grade. Low molecular weight alginate was obtained according to the method of acid hydrolysis.[22] Its relative molecular mass was 1.72×10^4 Da measured with high-permeation chromatography with a column of TSKgel G3000 SWXL on an Agilent 1100 system equipped with a refraction index detector.[23]

Preparation of OAE. OAE was prepared by reacting low molecular weight alginate with oleoyl chloride through esterification in the presence of formic acid derived from a previous work.[24] The reaction was carried out in a three-neck flask equipped with a magnetic bar, a tube for introducing reactant via a peristaltic pump, a tube for bubbling nitrogen, and a cooling system, and the flask was placed in a homothermal magnetic stirrer (GuoSheng Co., China). The formylation reaction was carried out by impregnating 1 g of alginate with 10 mL of formic acid for 10 min at 25 °C under stirring. The OAE with different degrees of substitution (DS) were prepared by controlling the reaction between oleoyl chloride and formated alginate. A volume of 8 mL of oleoyl chloride was then added dropwise via the peristaltic pump. The reaction mixture was then heated to 50 °C for 20 min. Another two samples of OAE were obtained by adding 16 mL of oleoyl chloride and heating to 30 and 50 °C, respectively. At the end of the reaction, 100 mL of ethanol (95% v/v) was added to terminate the reaction, and the mixture was filtered to obtain the solid phase. The solid phase was washed with ethanol (95% v/v) several times and dried under vacuum at room temperature.

Fourier Transform Infrared (FT-IR) and ¹H Nuclear Magnetic Resonance (NMR) Spectroscopy. The FT-IR and NMR methods were used to demonstrate the presence of an ester bond and oleoyl groups to the alginate. The FT-IR spectra of alginate and OAE were recorded on a Fourier transform infrared spectrometer (Nicolet Co.). About 2 mg of the sample was mixed with 100 mg of KBr and made into pellets for spectroscopic analysis at 20 °C.

¹H NMR spectra of alginate and OAE were recorded on a nuclear magnetic resonance spectrometer (JEOL Co., Japan) using a 5 mm NMR tube at 25 °C. The samples were dissolved in pyridine-d_5 to the concentration of approximately 10 mg/mL. ¹H NMR spectra were measured at 298 K with 80 scans, a spectral width of 4800 Hz, a relaxation delay of 1 s between scans, and an acquisition time of 3.75 s.

Determination of the Degree of Substitution. The DS, which represents the amount of oleoyl chains per 100 hexuronic acid residues, was calculated by comparing the ratio of methylene protons correlated with carbons 8 and 11 of oleoyl graft ($\delta = 2.0$) to carbons 3−5 of alginate protons ($\delta = 3.3-3.7$) using eq 1:[25]

$$\text{DS}\% = 3A_O/4A_A \times 100 \tag{1}$$

A_O and A_A correspond to the area of the methylene protons on oleoyl grafts and the carbon 3−5 of alginate protons on alginate main chains.

Preparation of Self-Assembled Nanoparticles. OAE nanoparticles were prepared by the probe sonication method.[26] Ten milligrams of OAE was suspended in 10 mL of distilled water at room temperature for 12 h and sonicated using a probe type sonifier (Cole Parmer Co.) at 225 W. The sonication lasted for 3 min to get an optically clear solution using a pulse function (pulse on, 2 s; pulse off, 4 s) in an ice bath at room temperature.

Fluorescence Measurement. Fluorescence measurement was carried out on a fluorescence spectrophotometer (Shimadzu Co., Japan) using pyrene as a fluorescence probe to determine the critical aggregation concentration (CAC).[27] Pyrene was dissolved in ethanol to the concentration of 0.04 mg/mL, and 40 μL of this solution was added into a test tube. The ethanol was evacuated under vacuum in darkness. Four milliliters of OAE nanoparticle solution was added into the test tube. The concentration of OAE solution varied from 1×10^{-4} to 5.0 mg/mL. The mixture was incubated for 3 h in a water bath at 65 °C and shaken in a shaking water bath (GuoHua Co., China) overnight at 20 °C. Pyrene emission spectra were obtained using the fluorescence spectrophotometer. The probe was excited at 343 nm, and the emission spectrum was collected in the range of 360−500 nm at an integration time of 1.0 s. The excitation and emission slit openings were 15 and 1.5 nm, respectively.

Particle Size Distribution and Transmission Electron Microscopy (TEM). The average particle size and size distribution were evaluated by dynamic light scattering (DLS) with a Malvern Zetasizer (Malvern Co., U.K.).[28] Three milliliters of an OAE nanoparticle solution (1 mg/mL) was put into polystyrene latex cells and measured at a detector angle of 90°, a wavelength of 633 nm, a refractive index of 1.33, and a real refractive index of 1.59. Measurements were made at 25 °C.

The morphology of the nanoparticles formed by OAE with the largest DS (OAE^c) was observed by TEM (JEOL Co., Japan).[29] For the following experiment, OAE^c conjugates was used for the smaller CAC and particle size of its self-aggregations. A solution of OAE^c was placed onto a copper grid. After excess fluid was drained at room temperature, the grid was negatively dyed in a 2% phosphotungstic acid. The specimens were air-dried and examined using a transmission electron microscope at an accelerating voltage of 80 kV.

Gastrointestinal Stability of OAE^c Nanoparticles. Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared as described in the United States Pharmacopoeia.[30] The SGF medium contained 0.32% (w/v) pepsin; the pH was 1.2. The SIF medium was composed of 1% (w/v) pancreatin with a pH of 7.5. Ten milliliter nanoparticle solutions were centrifuged at 29200g at 4 °C for 30 min. The pellet, containing the nanoparticles, was then separated from the serum by decantation. Nanoparticles were resuspended in SGF/SIF solution of the same volume as the removed serum by whirlpool mixer.

The size and morphology of OAE^c nanoparticles in SGF/SIF were measured by quasielastic laser light scattering and TEM as described above, after the nanoparticles in SGF/SIF solution were incubated at 37 °C for 0.5 h in SGF and 6 h in SIF, respectively.

The thermodynamic stability of OAE^c nanoparticles in SGF/SIF was carried out on a fluorescence spectrophotometer as the procedure described above with modification. Instead of the OAE^c nanoparticles solution, OAE^c nanoparticles dispersed in SGF/SIF were added into the test tube.

Vitamin D_3 Loading Capacity and Efficiency of the Nanoparticles. Noncovalent binding was achieved by dropwise addition of a 1 mg/mL solution of the vitamin in absolute ethanol into a 1 mg/mL OAE^c solution, with stirring.[3] The concentration of vitamin ranged from 5 to 20 μg/mL. Then, the solutions were sonicated and centrifuged to separate the unloaded vitamin. The unloaded vitamin which is in the supernatant was extracted by solid phase extraction (SPE) with C$_{18}$ disks (Agilent) based on Technical Note: SPE columns (5982-1165) were conditioned sequentially with 3 mL of methanol and 3 mL of distilled water. Five milliliters of supernatant was loaded onto the columns. The columns
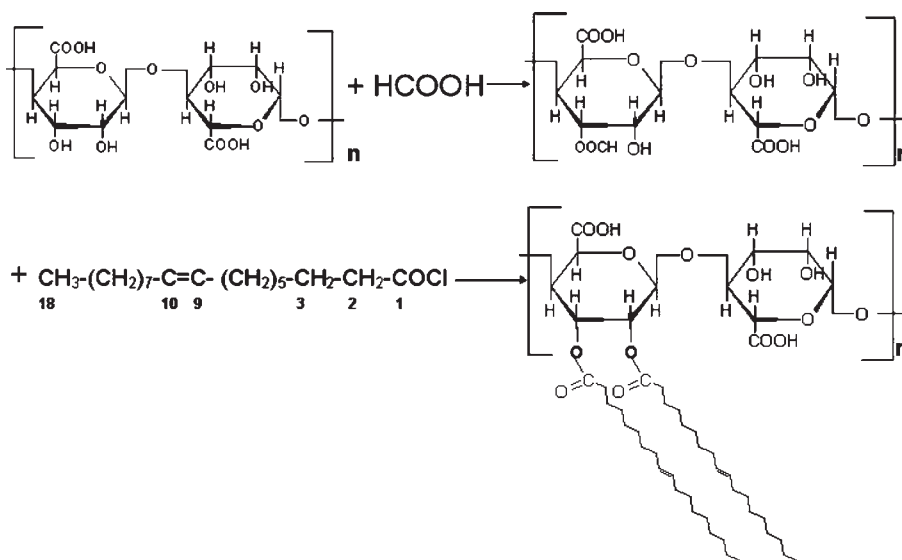
Figure 1. Synthesis scheme of esterification of alginate by octanoyl chloride in the presence of formic acid. First, formic acid highly reacts with alginate, producing formate esters. Second, an ester bond between oleic acid and alginate is formed, and the formate group is removed for its inherent instability.

were then washed with 4 mL of distilled water and eluted with 5 mL of methanol. The procedures mentioned were applied at a speed below 1 mL/min in vacuum.

The vitamin D_3 content in the supernatant was measured by high-performance liquid chromatography (Agilent), using a TC-C18 (2) RP-HPLC column at room temperature and a UV detector at 265 nm. Methanol served as mobile phase. A calibration curve was prepared using vitamin D_3 standard dissolved in methanol at seven concentrations ranging from 5 to 100 μg/mL.

The vitamin D_3 loading capacity (LC) and loading efficiency (LE) of nanoparticles were calculated by using eqs 2 and 3, respectively:[27]

$$LE = \frac{\text{total amount vitamin} - \text{unloaded amount vitamin}}{\text{total amount vitamin}} \times 100\%$$

$$(2)$$

$$LC = \frac{\text{total amount vitamin} - \text{unloaded amount vitamin}}{\text{polymer weight}} \times 100\%$$

$$(3)$$

In Vitro Release Study. Vitamin D_3 loaded nanoparticle in SGF/SIF was achieved as follows: 1 mg/mL solution of the vitamin D_3 in absolute ethanol was added dropwise into 25 mL of OAE^c solution to the final concentration of 20 μg/mL. The solutions were sonicated and centrifuged and then resuspended in 50 mL of SGF/SIF by whirlpool mixer.

In vitro release studies were carried out at 37 °C with agitation (100 rpm).[31] The vitamin release properties in SGF and SIF were carried out for 3 and 7 h, respectively. The stability of vitamin D_3 in distilled water at 37 °C was also investigated by the same procedure. At regulated intervals, 1 mL samples were withdrawn. An equivalent volume of the fresh buffer was replaced each time after the sampling. The amount of vitamin D_3 in pellet was assayed as follows: 1 mL samples were centrifuged and resuspended in 1 mL of distilled water. A volume of 0.6 mL of KOH (5% w/v) was added to dissociate the nanoparticles. Thereafter, 0.3 mL of 1% pyrogallol in ethanol solutions was subsequently added. The tubes were flushed with nitrogen, capped, and then left to stir slowly in the dark for 12 h at room temperature.[3] The concentration of vitamin D_3 was extracted and assayed by HPLC as described above.

Statistical Analyses. The assays were performed in triplicate on separate occasions. Results were expressed as the mean value ± standard deviation.

■ RESULTS AND DISCUSSION

Synthesis and Characteristics of OAE. Introduction of ester groups into polysaccharides has received considerable attention because of their potential application in food and nonfood sectors. Particularly, polysaccharide esters present hydrophobic and thermoplastic properties, but these polysaccharide esters were usually synthesized by methods involving toxic solvent. Aburto has reported the solvent-free preparation of long-chain esters of starch using fatty acid chloride.[24] Using this method, we successfully synthesized OAE, which was suitable for use as a nutraceutical delivery system. The reaction includes two stages, and the processes are shown in Figure 1: first, formic acid highly reacts with hydroxyl groups of alginate, producing formate esters. This initial reaction reduces the number of hydrogen bonds and renders the remaining hydroxyl group more accessible to the fatty acid chloride. Second, the formation of an ester bond between carboxyl of oleic acid and hydroxyl of alginate acts as a thermodynamic force. The inherent instability of the formate group enables its removal at the end of the reaction, giving rise to pure OAE.

Formation of an ester linkage between alginate and oleoyl was confirmed by FT-IR and ^{1}H NMR spectra. The FT-IR spectra of alginate and OAE, which are shown in Figure 2, suggest the presence of an ester bond and oleoyl group. The reduced peaks at 1612 cm^{-1} representing asymmetric stretching of carboxyl groups and increased peaks at 1736 cm^{-1} representing C=O stretching vibration showed the presence of the C=O component of an ester bond.[32] The increased peaks at 1027 and 1460 cm^{-1} exhibited the presence of oleoyl group. The peak at 1027 cm^{-1} was assigned to the stretching vibration of C—C,[33] and the peak at 1460 cm^{-1} was assigned to a deformation vibration of CH$_2$.[34] Also, the ^{1}H NMR spectra shown in Figure 3 further demonstrate the presence of an oleoyl group to the alginate. The band at 3.3−3.7 indicated the presence of carbons 3−5 of the native alginate.[27] New peaks and increased peaks showed the presence of new functional groups
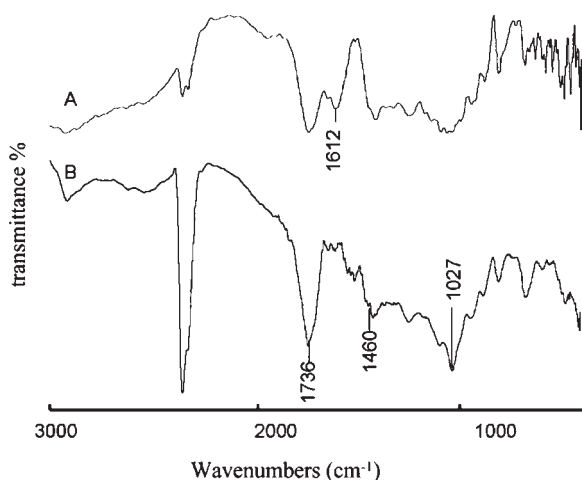
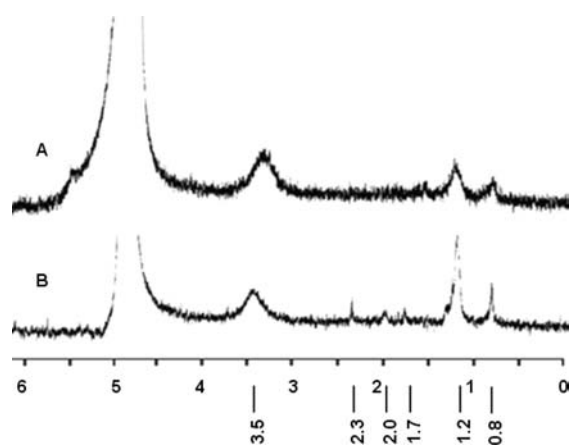**Figure 2.** FT-IR spectra of (A) alginate and (B) OAE.

Figure 3. ^{1}H NMR spectra of (A) alginate and (B) OAE in pyridine.

Table 1. Characterization of OAE Samples under Different Conditions

OAE[a]	DS(%)	CAC (mg/mL)	mean particle size (nm)
OAE[a]	0.84	0.38	559.3 ± 9.5
OAE[b]	2.60	0.29	336.5 ± 6.2
OAE[c]	3.85	0.25	305.3 ± 6.3
OAE[SGF]		0.21	257.7 ± 21.8
OAE[SIF]		0.58	757.1 ± 29.7

[a] OAE[a], 8 mL of oleoyl chloride reacted with 1 g of alginate at 50 °C; OAE[b], 16 mL of oleoyl chloride reacted with 1 g of alginate at 30 °C; OAE[c], 16 mL of oleoyl chloride reacted with 1 g of alginate at 50 °C; OAE[SGF], OAE nanoparticles in SGF; OAE[SIF], OAE nanoparticles in SIF.

linked to alginate. The proton assignment of the oleoyl graft is as follows (Figure 3B): $\delta_{2.3}$ = CH$_2$ (carbon 2 of oleoyl graft); $\delta_{2.0}$ = CH$_2$ (carbons 8 and 11 of oleoyl graft); $\delta_{1.7}$ = CH$_2$ (carbon 3 of oleoyl graft); $\delta_{1.2}$ = CH$_2$ (carbons 4−7 and 12−17 of oleoyl graft); $\delta_{0.8}$ = CH$_3$ (carbon 18 of oleoyl graft).[35] The data of DS calculated from ^{1}H NMR spectra are shown in Table 1.

Formation of Self-Aggregated Nanoparticles. At low concentrations, the I_{372}/I_{383} ratios, which remain nearly unchanged, were close to the value of 1.83. However, the I_{372}/I_{383} ratios decreased with the increase of OAE concentration (data not shown). The I_{372}/I_{383} ratios of OAE samples of 3 mg/mL with DS 0.84, 2.60, and 3.85% decreased to 1.585, 1.578, and 1.548, respectively. Moreover, the larger DS, the faster the I_{372}/I_{383} ratio decreases. The decrease in this ratio could be used to evidence hydrophobic microdomain formation after the formation of self-aggregations. The observed decrease demonstrated the ability of these copolymers to organize in solution.[36] The CAC values of the OAE conjugates, which were determined from the crossover point in the low concentration ranges, were 0.38, 0.29, and 0.25 mg/mL (Table 1). Hence, we could suggest that the increase of DS might facilitate the formation of nanoparticles, and the reason may be that hydrophobic interactions between fatty acid alkyl chains participate in a self-assembled network organization.[37]
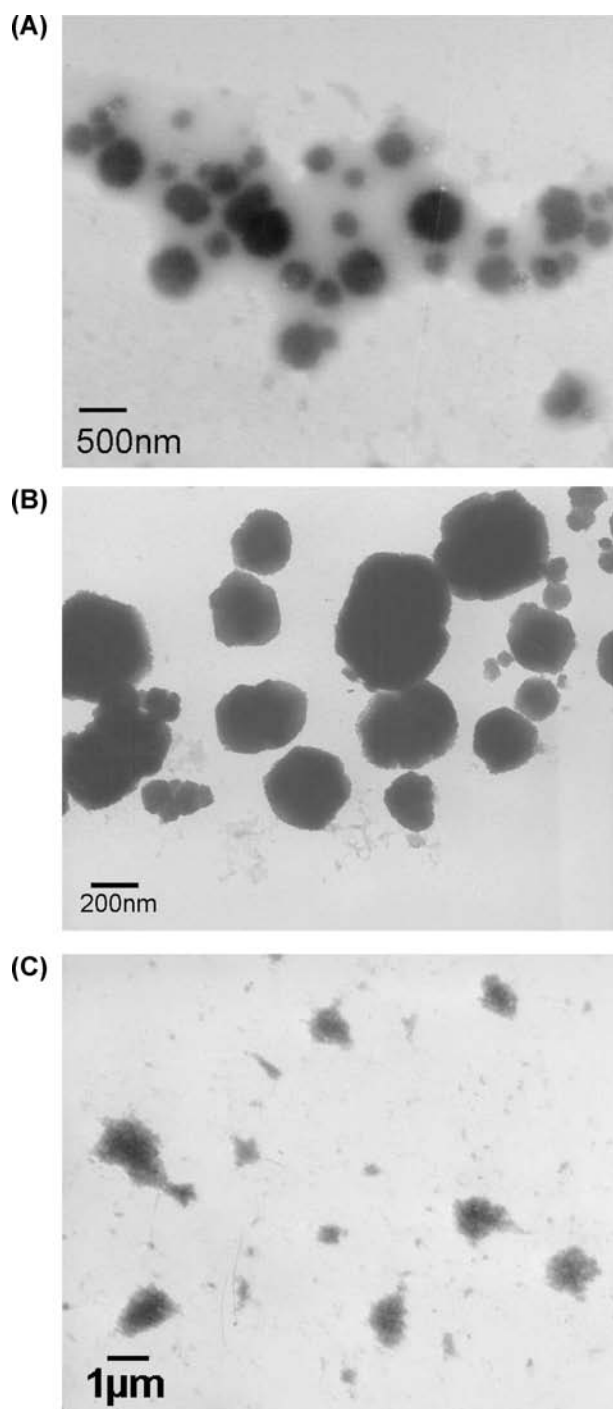
Size and Morphology of Nanoparticles. The prepared nanoparticles were of a unimodal particle size distribution (data not shown). The mean hydrodynamic diameters of the particles were 559.3 ± 9.5, 336.5 ± 6.2, and 305.3 ± 6.3 nm (Table 1). The size of self-aggregates decreased as the DS increased, indicating formation of denser hydrophobic cores in high DS sample. The reason may be that the stronger intra- and intermolecular hydrophobic interaction between oleoyl grafts causes the hydrophobic chains to self-associate into denser aggregates.

The TEM image of OAE[c] self-aggregates shown in Figure 4B indicated that the prepared nanoparticles had spherical shape and good structural integrity with the size of about 200−400 nm, which matched with the result of DLS.

Stability of Nanoparticles in SGF and SIF. The stability of OAE nanoparticles in SGF/SIF was studied by TEM and DLS measurements. As shown in Figure 4A,C, the OAE[c] self-aggregates retained their structural integrity in both SGF and SIF. However, the nanoparticles had small spherical shape in SGF and big irregular shape in SIF. The same results were obtained by DLS determination; for example, the mean sizes of nanoparticles formed by OAE[c] were 257.7 ± 21.8 and 757.1 ± 29.7 nm in SGF and SIF, respectively (Table 1). It may be that acid-insoluble alginate led to condensed structures of nanoparticles in SGF. Contrarily, the alginate is soluble in alkali, which leads to the formation of soluble complexes with quite loose spatial structures. Similar results were also observed by CAC determination. CAC is an important parameter to characterize the thermodynamic stability of polymeric nanoparticles,[38] the CAC values of OAE[c] in SGF and SIF were 0.21 and 0.58 mg/mL, respectively. Moreover, in contrast with distilled water, the CAC values of polymeric nanoparticles in SGF decreased, indicating higher thermodynamic stability, but increased in SIF, indicating lower thermodynamic stability.[37]

Vitamin D$_3$ Loading Capacity and Loading Efficiency. Nanoparticles of OAE[c] were successfully used to solubilize hydrophobic vitamin D$_3$ molecules in aqueous solution. As the concentration of vitamin D$_3$ increased, the loading capacity increased from 0.33 ± 0.02 to $0.91 \pm 0.03\%$, whereas the loading efficiency decreased from 67.6 ± 2.76 to $45.8 \pm 1.55\%$ as shown in Table 2.

Nanoparticles with a hydrophobic core and a hydrophilic shell are suitable for trapping hydrophobic substances through hydrophobic interactions, such as adriamycin taxol and fluorescent probes.[26,28] Vitamin D$_3$ may interact with hydrophobic domains of nanoparticles through oleoyl group interactions. The higher the concentration of vitamin D$_3$, the more vitamin D$_3$ loaded to nanoparticles. However, with more vitamin D$_3$ binding to hydrophobic domains, fewer vitamin D$_3$ binding sites are left and the loading efficiency decreases.
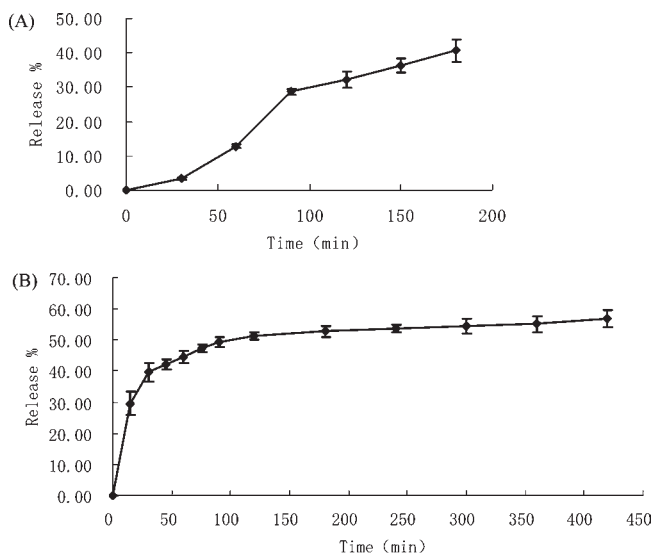
Figure 4. TEM of nanoparticles in (A) SGF for 0.5 h, (B) distilled water, and (C) SIF for 6 h.

Table 2. Influence of Vitamin D$_3$ Concentration on Loading Efficiency and Loading Capacity

vitamin D$_3$ concn (μg/mL)	loading capacity (%)	loading efficiency (%)
5	0.33 ± 0.02	67.6 ± 2.76
10	0.48 ± 0.03	48.5 ± 3.27
20	0.91 ± 0.03	45.8 ± 1.55

Figure 5. Release profiles of vitamin D$_3$ from OAE nanoparticles: (A) in SGF; (B) in SIF.

In Vitro Vitamin D$_3$ Release Study. In the simulated gastric pH (1.2) <5% of the vitamin D$_3$ was released from nanoparticles in the first 0.5 h, and the vitamin D$_3$ released from OAE[c] nanoparticles in 3 h was about 40% as shown in Figure 5A. In SIF, 39.7% of the vitamin D$_3$ was released in the first 30 min from OAE[c] nanoparticles, and then the release rate slowed significantly as shown in Figure 5B. Vitamin D$_3$ was released steadily with increasing time, and 56.9% of the encapsulated vitamin D$_3$ was released after 7 h of the test.

Vitamin D$_3$ was sightly released from OAE[c] nanoparticles at pH 1.2. This may be due to the fact that the alginate layer, being acid resistant, acts as a diffusion barrier. However, there was a burst release of vitamin D$_3$ early in SIF, and vitamin D$_3$ was released slightly and steadily with increasing time. When the nanoparticles were suspended in alkali medium, the alginate dissolves and the nanoparticles abruptly swell to release vitamin D$_3$ attached on the shell of the nanoparticles. The vitamin D$_3$ attached on the core of the nanoparticles could be steadily and slowly released into SIF. The result indicates that the nanoparticles could realize sustained release in gastrointestinal fluid.

In summary, a hydrophobic alginate derivative was prepared by modification of alginate by acid chloride reaction using oleoyl chloride without organic solvents. It self-assembles into stable and compact nanoparticles in aqueous medium, and nanoparticles retained their structural integrity in both SGF and SIF. The nanoparticles can be used as carriers for liposoluble nutraceuticals such as vitamin D$_3$ with controlled release in gastrointestinal fluid.

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