JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Cellular Uptake and Transport of Zein Nanoparticles: Effects of Sodium Caseinate

Yangchao Luo,[†] Zi Teng,[†] Thomas T. Y. Wang,[‡] and Qin Wang*[,†]

[†]0112 Skinner Building, Department of Nutrition and Food Science, University of Maryland, College Park, Maryland 20742, United States

[‡]Diet, Genomics and Immunology Laboratory, Beltsville Human Nutrition Research Center, Agricultural Research Service (ARS), U.S. Department of Agriculture (USDA), Beltsville, Maryland 20705, United States

ABSTRACT: Cellular evaluation of zein nanoparticles has not been studied systematically due to their poor redispersibility. Caseinate (CAS)-stabilized zein nanoparticles have been recently developed with better redispersibility in salt solutions. In this study, zein−CAS nanoparticles were prepared with different zein/CAS mass ratios. The prepared nanoparticles demonstrated good stabilities to maintain particle size (120−140 nm) in cell culture medium and HBSS buffer at 37 °C. The nanoparticles showed no cytotoxicity for Caco-2 cells for 72 h. CAS not only significantly enhanced cell uptake of zein nanoparticles in a concentration- and time-dependent manner but also remarkably improved epithelial transport through Caco-2 cell monolayer. The cell uptake of zein−CAS nanoparticles indicated an energy-dependent endocytosis process as evidenced by cell uptake under blocking conditions, that is, 4 °C, sodium azide, and colchicine. Fluorescent microscopy clearly showed the internalization of zein−CAS nanoparticles. This study may shed some light on the cellular evaluations of hydrophobic protein nanoparticles.

KEYWORDS: *zein, sodium caseinate, nanoparticles, cell uptake, Caco-2 monolayer, coumarin 6*

INTRODUCTION

Natural polymers derived from foods are the ideal materials for oral delivery of nutrients and drugs. In general, they are biocompatible and biodegradable and less toxic, compared with other synthetic and semisynthetic materials. Protein-based biopolymers are of particular interest in the design of colloidal drug delivery systems, due to their high nutritional values, abundant renewable sources, high drug binding capacity, and significant uptake into targeted cells.[1−3] Moreover, protein-based nanocarriers can be simply fabricated and easily scaled up during manufacture.[4] Zein is a maize prolamine protein with two-thirds lipophilic and one-third hydrophilic amino acid residues, leading to its unique aqueous alcohol solubility and film-forming properties.[5] Due to the poor solubility in water and the imbalanced essential amino acid profile, zein is not used directly for human consumption. Becaue zein dissolves only in 60−85% aqueous ethanol/isopropanol binary solutions, zein micro/nanoparticles are easily prepared by liquid−liquid phase separation method by shearing zein solution into distilled water.[6] In recent years, zein has become a popular plant protein to develop delivery systems for encapsulation and controlled release of hydrophobic drugs/nutraceuticals, including invermectin,[7] fluorouracil,[8] vitamins,[9,10] riboflavin,[11] and antimicrobials.[12,13] Moreover, zein has also been proven as a promising biomaterial for coating polysaccharide nanoparticles,[14] tissue engineering,[15,16] and bioactive packaging.[17,18]

It has become clearer that cellular evaluation of polymeric delivery systems plays a critical role in the exploration of their potential cytotoxicity and efficacy and in the understanding of specific mechanisms in defined biological conditions. For delivery of nutrients, although most delivery systems are prepared with biodegradable and biocompatible polymeric materials, it is not unusual to encounter toxicity or side effects of nanoparticles made from generally recognized as safe (GRAS) biomaterials when the in vitro cellular evaluation is performed.[19−21] Although extensive studies have been done to investigate the fabrication parameters of zein nanoparticles and encapsulation applications of different nutrients/drugs, in vivo and in vitro evaluations of the efficacy and toxicity of zein nanoparticles are limited. A recent study reported that zein nanoparticle suspensions accumulated in liver and prolonged blood residence time of encapsulated drug by intravenous injection.[8] In our previous trials, after zein nanoparticles were lyophilized, the obtained powders were unable to be redispersed in water or buffer solutions, and even diluting zein nanoparticle dispersion (before freeze-drying) into different aqueous buffers would also destabilize zein nanoparticles. Recently, Patel and colleagues prepared sodium caseinate-stabilized zein colloidal nanoparticles, which retained good stability against a wide range ionic strength (15 mM−1.5 M NaCl) and good redispersibility after drying.[22] Zein−caseinate-based nanoparticles have been recently studied to encapsulate thymol for improving redispersibility and enhancing biological[23] and antimicrobial activities.[24,25] Sodium caseinate is a water-soluble salt of casein, a set of milk proteins consisting of several components (α_{s1}-, α_{s2}-, β-, and κ-casein) with various portions of both hydrophobic and hydrophilic groups. Casein-based formulations have been widely used as emulsifiers and stabilizers[26] and as drug delivery systems[27] in food/pharmaceutical applications.

Recently, to investigate cellular uptake of zein-based delivery systems, some new fabrication methods or modifications were

Received: May 19, 2013
Revised: July 12, 2013
Accepted: July 16, 2013
Published: July 16, 2013

developed to prepare novel zein nanoparticles. Xu and coworkers fabricated hollow zein nanoparticles with the presence of sodium carbonate as core materials.[28] The hollow zein nanoparticles with an average diameter of 65 nm were capable of loading hydrophobic drugs and penetrating into the cell cytoplasm. In another study, methoxy poly(ethylene glycol) (mPEG) was conjugated to zein to synthesize novel zein micelles to reduce hydrophobicity and avoid macrophage uptake.[29] The prepared mPEG−zein micelles were able to encapsulate hydrophobic drugs and significantly increase cell uptake relative to free drug in drug-resistant NCI/ADR-RES cancer cells.

In the present study, the zein−caseinate nanoparticles were prepared by liquid−liquid phase separation, and their stabilities were studied in different cell culture media and buffer solutions. The cytotoxicity and cell uptake of zein−caseinate nanoparticles were evaluated with Caco-2 cells. The Caco-2 cell monolayer was further used to study the epithelial transport of zein−caseinate nanoparticles. Possible mechanisms of cell uptake and transport were also discussed.

MATERIALS AND METHODS

Materials. Zein sample with a minimum protein content of 97% was provided by Showa Sangyo (Tokyo, Japan). Sodium caseinate (CAS), coumarin 6 of 98% purity, 8-anilino-1-naphthalenesulfonate (ANS), and Hanks' balanced salt solution (HBSS) were purchased from Sigma-Aldrich. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), 100× nonessential amino acids, 100× penicillin and streptomycin, 0.25% trypsin (w/v) with EDTA, 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), phosphate buffer saline (PBS), 4′,6-diamidino-2-phenylindole (DAPI), and methylthiazolyldiphenyltetrazolium bromide (MTT) were all purchased from Life Technologies Corp. Transwell permeable polycarbonate inserts (0.4 μm) and 96- and 12-well cell culture plates were obtained from Corning Inc. Dimethyl sulfoxide (DMSO) of molecular biology grade was purchased from Sigma-Aldrich. All other reagents were of analytical grade and purchased from Sigma-Aldrich.

Preparation of Nanoparticles. Zein nanoparticles were prepared by liquid−liquid phase separation as described in previous literature.[9] Briefly, zein (10 mg/mL) was first dissolved in 70% ethanol, and CAS was dissolved in distilled water as stock solution. Five milliliters of zein solution was rapidly poured into 12.5 mL of CAS solution with concentrations of 2, 4, and 8 mg/mL, to give three different zein/CAS mass ratios, that is, 1:0.5, 1:1, and 1:2, designated samples A, B, and C, respectively. Subsequently, the zein−CAS nanoparticles were hardened by removing ethanol under a nitrogen stream and then freeze-dried for 48 h to obtain dry powders.

Scanning Electron Microscopy (SEM). Morphological structures of nanoparticles were observed using a scanning electron microscope (Hitachi SU-70, Pleasanton, CA, USA). Samples were first cast-dried on an aluminum pan and then cut into proper shapes before being adhered to conductive carbon tapes (Electron Microscopy Sciences, Fort Washington, PA, USA). Subsequently, the samples were coated with gold and then observed under a scanning electron microscope. Representative SEM images were reported.

Particle Size and Zeta Potential. The freshly prepared nanoparticles were subject to particle size and zeta potential measurements. The particle size was measured as the hydrodynamic diameter by a dynamic light scattering instrument equipped with a 35 mM HeNe laser beam at a wavelength of 637 nm (DLS, BI-200SM, Brookhaven Instruments Corp., Holtsville, NY, USA). The zeta potential was calculated from electrophoretic mobility, determined by a laser Doppler velocimeter (Zetasizer Nano ZS90, Malvern, UK).

Surface Hydrophobicity. The surface hydrophobicity was determined using hydrophobicity fluorescence probe ANS, modified from our previous study.[30] Briefly, the freeze-dried nanoparticle powders were dispersed in 0.01 M PBS (pH 7.0) at 1 mg/mL as stock suspension except for zein nanoparticles, which were prepared at 1 mg/mL without adding CAS, and then diluted with PBS directly without freeze-drying. The stock suspension of each sample was diluted with PBS to obtain concentrations ranging from 20 to 100 μg/mL. The diluted samples (150 μL) were added into a 96-well plate, and then an equal volume of ANS (50 μg/mL dissolved in PBS) was added. The plate was incubated for 30 min at 37 °C. The fluorescence intensity was measured with excitation at 355 nm and emission at 460 nm by a multilabel microplate reader (Victor X3, PerkinElmer 2030). The initial slope of fluorescent intensity versus nanoparticle concentration (calculated by linear regression analysis) was used as an index for surface hydrophobicity. The regression line with $R^2 > 0.995$ was obtained.

Stability of Nanoparticles. The freeze-dried nanoparticles were redispersed in water, DMEM, and HBSS, at a concentration of 1 mg/mL for stability tests. The nanoparticles in different conditions were then incubated at 37 °C for 4 h, and the particle size was measured as a function of time.

Cell Culture. Caco-2 cells were generously provided by Dr. Liangli (Lucy) Yu, Department of Nutrition and Food Science, University of Maryland, College Park, MD, USA. Caco-2 cells were cultivated in DMEM supplemented with 10% FBS and 1% penicillin−streptomycin at 37 °C in a humidified environment with 5% CO_2. The medium was changed every other day, and the cells were subcultured after reaching 80−90% confluence. Caco-2 cells between passages 10 and 15 were used in this study.

Cytotoxicity of Nanoparticles. The freeze-dried nanoparticle powders were dissolved in DMEM at different concentrations, that is, 0.2, 0.5, and 1.0 mg/mL. Caco-2 cells were seeded in 96-well microplates at a seeding density of 2×10^4 cells/well. Cells were incubated for 24 h to allow cell attachment. Then, cells were treated with DMEM containing different samples. Each treatment had six replicates. At designated time intervals (24, 48, and 72 h), the medium was removed and the cells were washed with PBS three times to remove free nanoparticles. Then, 100 μL of cell culture medium containing MTT (10 μL, 5 mg/mL in PBS) was added into each well. After incubation for 4 h, the culture solution was carefully aspirated, and residue was left in the wells. Subsequently, 100 μL of DMSO was added to each well to solubilize the formazan crystals formed. The absorbance at 550 nm was measured by a multilabel microplate reader (Victor X3, PerkinElmer 2030). The cell viability was calculated by the absorbance percentage of nanoparticle treated cells versus blank cells (treated with DMEM only).

Cell Uptake of Nanoparticles. To investigate the cell uptake of nanoparticles, coumarin 6, as a fluorescent marker, was encapsulated into the nanoparticles, and both quantitative and qualitative studies were carried out according to previous literature.[31] The zein solution containing 0.02% coumarin 6 (dissolved in 80% ethanol solution) was used in the preparation of fluorescent nanoparticles with all other conditions remaining the same. The cell uptake experiments, both quantitative and qualitative studies, were carried out as described below.

Quantitative Study. Caco-2 cells were seeded in 96-well black plates (BD Falcon, NJ, USA) and incubated until a confluent monolayer was formed. Then, the cell culture medium was replaced with transport buffer (HBSS supplemented with 25 mM HEPES, pH 7.4) prewarmed at 37 °C for equilibration for 20 min. Cell uptake was initiated by incubating cells with 200 μL of different samples dissolved in HBSS at various concentrations (0.2, 0.5, 1.0 mg/mL) for 0.5−4 h. At designated time intervals, the cell monolayer was washed with PBS three times to remove free nanoparticles. Cells were then treated with 100 μL of lysis buffer (0.5% Triton X-100 in 0.2 M NaOH solution) to allow permeabilization of cell membrane and expose the internalized nanoparticles. The fluorescent intensity was then measured with excitation at 485 nm and emission at 535 nm, using a multilabel microplate reader (Victor X3, PerkinElmer 2030). Uptake was expressed as the percentage of fluorescent intensity in cells versus the original intensity present in feed medium.[32]

Inhibition Study. To evaluate the specific mechanism of nanoparticles involved in the cell uptake process, cell uptake studies were then performed under three different blocking conditions.[33] For the first condition, Caco-2 cells were incubated with nanoparticles at 4 °C for 4 h. For the other two conditions, Caco-2 cells were preincubated with the

Table 1. Characterization of Zein−Caseinate Nanoparticles[a]

sample[b]	particle size (nm)	PDI[c]	count rates (kcps)	zeta potential (mV)
A	146.7 ± 2.7b	0.19 ± 0.02a	368.5 ± 10.3b	−47.4 ± 2.5b
B	133.9 ± 1.5a	0.26 ± 0.01ab	383.2 ± 7.0bc	−48.6 ± 4.1b
C	131.0 ± 1.1a	0.21 ± 0.02ab	405.5 ± 8.8c	−54.9 ± 3.1a
D	166.9 ± 0.9c	0.28 ± 0.03c	227.9 ± 14.5a	−13.2 ± 0.3c

[a]Values having different letters in the same column are significantly different ($P < 0.05$). [b]A, nanoparticles with zein/caseinate mass ratio of 1:0.5; B, nanoparticles with zein/caseinate mass ratio of 1:1; C, nanoparticles with zein/caseinate mass ratio of 1:2; D, zein control nanoparticles, without caseinate. [c]Polydispersity index.

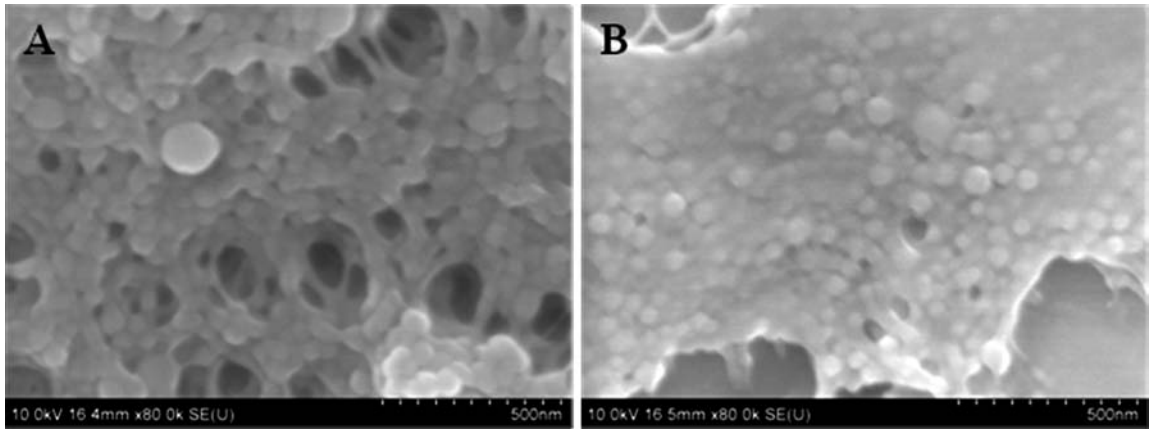

Figure 1. Scanning electron microscopy (SEM) images of zein (A) and zein−caseinate nanoparticles (B) at a zein/CAS mass ratio of 1:1.

metabolic inhibitor sodium azide (10 μM) and endocytosis inhibitor colchicines (5 μg/mL) for 30 min, respectively, and then cells were incubated with nanoparticles for 4 h at 37 °C for cell uptake experiment. The control was cells incubated without inhibitors. The results were expressed as the inhibition percentage versus control.

Qualitative Study. Caco-2 cells were seeded on Lab-Teks chambered cover glasses (Nalge Nunc International, Naperville, IL, USA) and incubated until cells were about 80% confluence. Prior to the uptake test, the growth medium was replaced with transport medium, HBSS buffered with 25 mM HEPES (pH 7.4), and equilibrated for 20 min at 37 °C. Then, the nanoparticles dissolved in transport medium (1 mg/mL) were added into the wells and incubated for 0.5−4 h. In the end, cells were washed three times with cold PBS to remove free nanoparticles. Cells were then fixed by 70% ethanol for 15 min under −20 °C, after which the nuclei were stained by DAPI. Green fluorescent protein (GFP) channel was used for green fluorescence of coumarin 6, and DAPI channel was used for blue fluorescence of DAPI. Fluorescent images were taken after deconvolution using AxioVision release 4.7.2.0 coupled to a Zeiss Axio Observer Z1m fluorescence microscope (Zeiss, Thornwood, NY, USA).

Transport of Nanoparticles via Caco-2 Cell Monolayer. The maintenance of Caco-2 cell monolayer followed the reported protocol.[34] Briefly, Caco-2 cells were seeded on the tissue culture-treated polycarbonate filter (diameter = 12 mm, growth area = 1.1 cm^2) in Costar Transwell 12 wells/plate (Corning Costar Corp., NY) at a seeding density of 3×10^5 cells/cm^2. The medium was replaced every 48 h for the first 6 days and every 24 h thereafter. The cultures were used for the transport experiments after 21−29 days. The transepithelial electrical resistance (TEER) values of the Caco-2 cells monolayer were monitored using EVEO2 with an Edohm chamber (World Precision Instruments, Sarasota, FL, USA). Once the TEER values reached 600−800 Ω/cm^2, the monolayer was used for transport study. To initiate the transport experiments, the culture media in the donor and receiving compartments were carefully aspirated, and the cells were rinsed twice with prewarmed transport medium (HBSS supplemented with 25 mM HEPES, pH 7.4). Following a 30 min equilibration with the transport medium at 37 °C, the cells were incubated with nanoparticles for 2 h for transport study. The TEER value of the monolayer was also monitored at designated time points during the transport study. The transport studies were performed in both directions as follows. For apical-to-basolateral transport (a−b), 1.2 mL of transport medium was added in the basolateral part and 0.4 mL of nanoparticles (1 mg/mL) dissolved in HBSS−HEPES solution was added into the apical part. At designated time intervals, 0.6 mL of basolateral transport medium was withdrawn for fluorescent intensity measurement and replenished with an equal volume of fresh transport medium. For basolateral-to-apical transport (b−a), 1.2 mL of nanoparticles dissolved in HBSS−HEPES solution (1 mg/mL) was added in the basolateral part and 0.4 mL of transport medium was added into the apical part. At designated time intervals, 0.2 mL of apical transport medium was withdrawn for fluorescent intensity measurement and replenished with an equal volume of fresh transport medium. To determine the coumarin 6 recovery rate, at the end of the experiment, the transport media from both apical and basolateral compartments were collected for fluorescent intensity measurement to determine the concentration of coumarin 6. The apparent permeability coefficient (P_{app}) was calculated as

$$P_{app} = \frac{\partial Q}{AC_0 \partial t}$$

where $\partial Q/\partial t$ is the permeability rate, A is the surface area of the membrane filter, and C_0 is the initial concentration in the donor compartment (apical compartment in a−b transport study, basolateral compartment in b−a transport study)

Statistical Analysis. The experiments of nanoparticle characterization were conducted in triplicate, and the experiments of cellular evaluation were carried out in at least six replicates. All of the data were expressed as the mean ± standard error. Experimental statistics were performed using SAS software (version 9.2, SAS Institute Inc., Cary, NC, USA). The analysis of variance (ANOVA) Tukey's multiple-comparison tests were performed to compare the significant difference. The significance level (P) was set at 0.05.

RESULTS AND DISCUSSION

Characterization of Zein−CAS Nanoparticles. Table 1 shows particle size, polydispersity index (PDI), count rates, and zeta potential of zein−CAS and zein control nanoparticles.

dx.doi.org/10.1021/jf402198r | J. Agric. Food Chem. 2013, 61, 7621−7629

Particle size is an important characteristic for polymeric drug delivery vehicles, indicating the capability for efficient drug delivery.[33] The particle size for all formulations was between 130 and 170 nm, and more CAS in the formulation resulted in significantly smaller particle size. The zein control nanoparticles without CAS had the largest particle size of 166.9 nm, which decreased to 131.0 nm for nanoparticles C. The PDI for all formulations was within 0.3, indicating narrow distribution of particle size. The count rate was the cumulative light pulses of the sample over the duration of the DLS experiment, an indirect indicator to estimate the total number of particles.[35,36] Zein nanoparticles had the smallest count rate, whereas the count rate of zein−CAS nanoparticles was greatly increased. More CAS in the formulation resulted in a greater count rate. This observation demonstrated that CAS interacted with zein protein and facilitated the formation of zein nanoparticles, partly due to the strong repulsive forces provided by the high surface charge of CAS. Zeta potential is another important characteristic for polymeric nanoparticles, indicating the stability of colloidal suspension. A zeta potential of ±30 mV is a well-established borderline for colloidal stability. Formulations A, B, and C possessed good stability with zeta potential from −47.4 to −54.9 mV, whereas the zeta potential of zein nanoparticles was only −13.2 mV. The low zeta potential resulted in poor colloidal stability of zein nanoparticles, which were observed to aggregate during storage. Both zein−CAS and zein nanoparticles had spherical shape with uniform particle size. Similarly to our previous study,[9] zein nanoparticles produced by a low-energy liquid−liquid phase separation were clumped and connected to each other (Figure 1A), whereas zein−CAS nanoparticles formed a thin film during cast-drying with nanoparticles uniformly distributed on the film (Figure 1B).

The surface hydrophobicity of nanoparticles and CAS control were also characterized as shown in Figure 2. Zein control nanoparticles (D) showed a high surface hydrophobicity, which may explain the aggregation caused by strong hydrophobic interactions when dispersed in cell culture medium and buffer solutions of high ionic strength. As a sodium salt form of casein, CAS has been known to have excellent water-dispersible and hydrophilic properties, as indicated by its low surface hydrophobicity. Compared with zein nanoparticles, a significant reduction ($P < 0.05$) in surface hydrophobicity was observed for zein−CAS nanoparticles, and greater mass ratio of CAS resulted in higher reduction. The reduction in surface hydrophobicity confers improved water dispersibility to the zein−CAS nanoparticles.

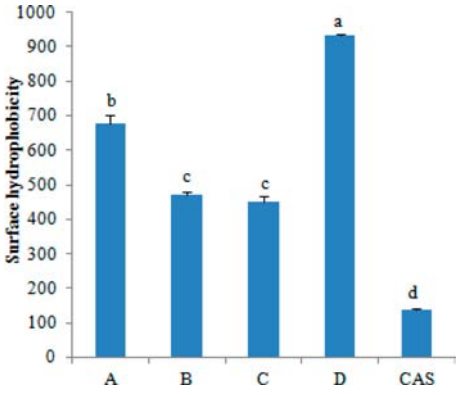

Figure 2. Surface hydrophobicity of nanoparticles and caseinate control. A, nanoparticles of zein/caseinate 1:0.5; B, nanoparticles of zein/caseinate 1:1; C, nanoparticles of zein/caseinate 1:2; D, zein control nanoparticles; CAS, sodium caseinate. Values having different letters are significantly different ($P < 0.05$).

Stability of Nanoparticles. To accurately perform the cellular evaluation, the nanoparticles should be stable and able to maintain particle size during incubation at 37 °C in culture media and buffer solutions. Therefore, it is of great significance to evaluate the redispersibilities and stabilities of nanoparticles prior to cellular evaluation. DMEM is a culture medium for mammalian cell lines, and HBSS−HEPES is a buffer solution generally used for cell uptake and monolayer transport study. The results of nanoparticle stabilities are shown in Figure 3. The freeze-dried powder in all formulations possessed good redispersibility in three solutions (1 mg/mL). The opaque nanosuspensions without large aggregates were easily obtained after gentle vortex. When the samples were incubated in water and HBSS−HEPES solution at 37 °C during the first hour, all nanoparticles remained in similar particle sizes before freeze-

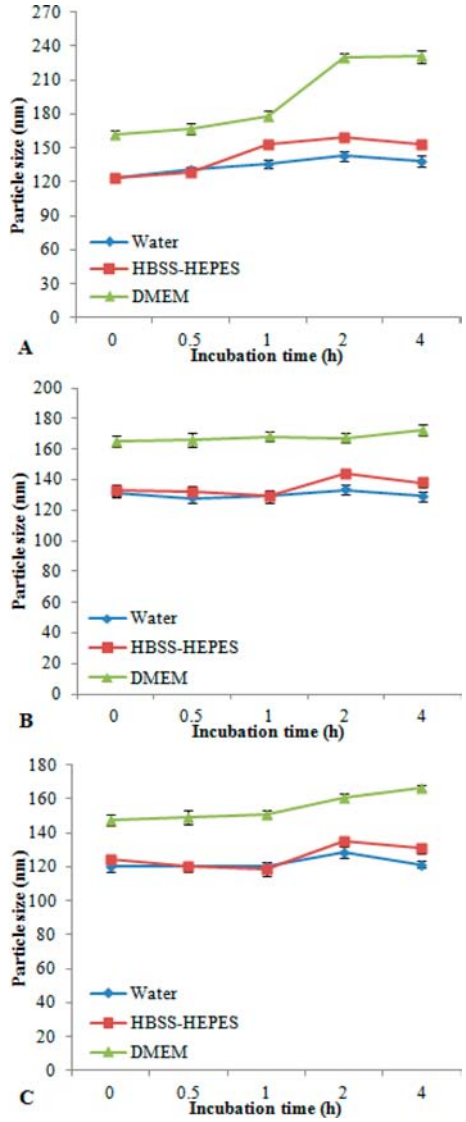

Figure 3. Particle sizes in different culture media: (A) nanoparticles of zein/caseinate 1:0.5; (B) nanoparticles of zein/caseinate 1:1; (C) nanoparticles of zein/caseinate 1:2.

dx.doi.org/10.1021/jf402198r | J. Agric. Food Chem. 2013, 61, 7621−7629

drying, that is, 120−150 nm. After that, the particle size increased slightly by 5−10 nm. Redispersing nanoparticles in DMEM resulted in an augment of particle size of all formulations. The particle sizes were 150−160 nm in DMEM before the 37 °C incubation and increased to 225 nm for the A nanoparticles, which contained the lowest amount of CAS. For the B and C nanoparticles, only a slight increase by 5−10 nm was observed. The greater particle size of dispersion in DMEM may be due to its high glucose content, which may interact with protein complex nanoparticles to form larger particles. Besides, the stronger ionic strength in DMEM was also partly attributed to the aggregation of nanoparticles. Better stabilities of particle size were observed in the nanoparticle formulations with more CAS content.

It has been shown in previous literature that a minimum zein/CAS ratio of 1:0.3 was required to avoid large aggregates during preparation and to provide good stability in phosphate buffer.[22] In our study, a zein/CAS ratio of 1:0.5 was sufficient to provide good stability for three tested media at 37 °C for 4 h, and only a slight increase of particle size was observed even after 48 h (data not shown). Therefore, they were all considered as suitable to incubate with cells while maintaining stable particle size. As an amphiphilic stabilizer, CAS was able to adsorb to the surface of zein nanoparticles due to strong hydrophobic interactions. Additionally, CAS also carries a large portion of negatively charged groups, which provide electrosteric stabilization of zein nanoparticles against aggregation.

Cytotoxicity of Nanoparticles. The cell viability of Caco-2 cells was tested for each nanoparticle formulation at three concentrations, 0.2, 0.5, 1.0 mg/mL. Figure 4 demonstrated that all CAS stabilized nanoparticles showed no toxic effect on cellular viability at all concentrations, up to three days of incubation.

Cell Uptake. To investigate the cell uptake of zein nanoparticles, coumarin 6 was selected as a fluorescent marker. Coumarin 6 is one of the most common and suitable fluorescent markers to study cell uptake of nanoparticles.[37,38] It has relatively high fluorescent intensity and interacts with polymeric nanoparticles via strong hydrophobic interactions. It has also been reported that free coumarin 6 cannot be directly internalized by Caco-2 cells.[39] Therefore, the fluorescence detected represents the uptake of coumarin 6-encapsulated nanoparticles.

Quantitative Study. The cell uptake of zein nanoparticles was studied in Caco-2 cells as a function of nanoparticle concentration and incubation time, as shown in Figure 5. Among three formulations, nanoparticles A showed the minimal uptake by Caco-2 cells. The cell uptake of all nanoparticles increased gradually with incubation time. During the first hour, the uptake increased to 10% for all nanoparticles, but afterward the uptake increase rate of nanoparticles A (Figure 5A) was significantly slower than that of nanoparticles B (Figure 5B) and C (Figure 5C). The uptake of nanoparticles was also concentration-dependent, showing that the nanoparticles of higher concentration had a significantly higher uptake ($P < 0.05$). The cumulative uptake of nanoparticles A was 16.0% for the concentration of 1 mg/mL, whereas it reached 37.2 and 39.8% for nanoparticles B and C, respectively.

On the basis of our results, CAS enhanced cell uptake of zein nanoparticles in a concentration- and time-dependent manner. CAS is a common emulsifier and stabilizer used to produce stable nanoemulsions or nanoparticles to increase the solubility and diffusivity of hydrophobic nutrients. For instance, CAS has been used to produce the nanodispersion of a poorly soluble carotenoid pigment, and it was found that CAS enhanced the

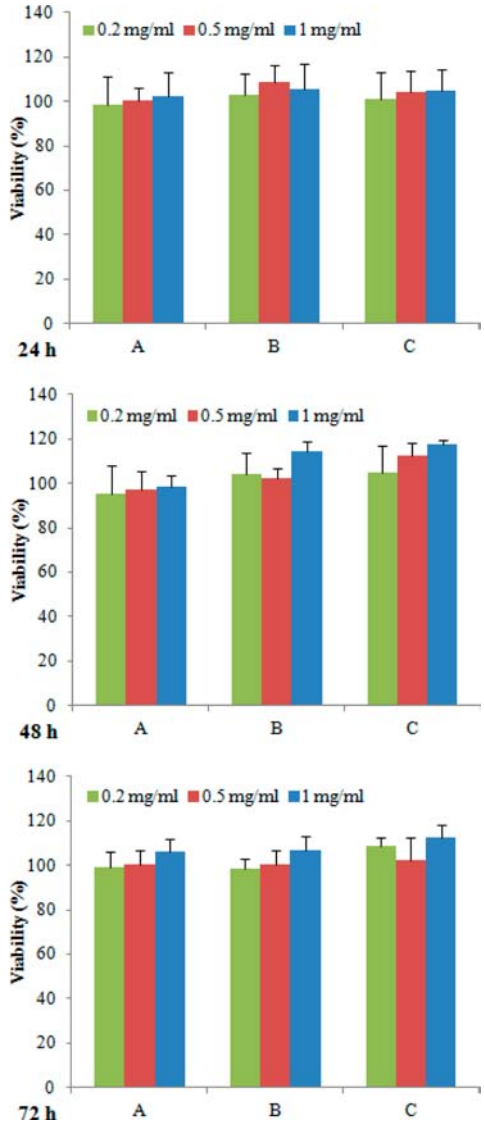

Figure 4. Cell cytotoxicity of nanoparticles. A, nanoparticles of zein/caseinate 1:0.5; B, nanoparticles of zein/caseinate 1:1; C, nanoparticles of zein/caseinate 1:2.

diffusivity of the nanoparticles and facilitated cell uptake by human colonic epithelial cells.[40] Another recent study pointed out that the dietary proteins, especially CAS and zein, significantly improved the cell uptake of heme iron by Caco-2 cells,[41] suggesting that certain peptides from zein and CAS were necessary to activate uptake mechanisms in the enterocyte. Another possible mechanism of the enhanced uptake effect of CAS on zein nanoparticles may be that adsorption of CAS on zein surface could lower the interfacial tension and hence improve the interactions between zein nanoparticles and cell membranes. The higher ratio of CAS in nanoparticles resulted in higher surface charge. The surface carboxyl groups have also been reported to play an important role in the cell uptake process, and the higher surface charge resulted in a higher cell uptake of nanoparticles.[33,42]

Inhibition Study. The cell uptake of polymeric nanoparticles with particle size <150 nm has been previously characterized as endocytotic internalization.[43] To investigate the possible mechanisms involved in cell uptake of zein nanoparticles, three blocking conditions against endocytosis were tested on Caco-2

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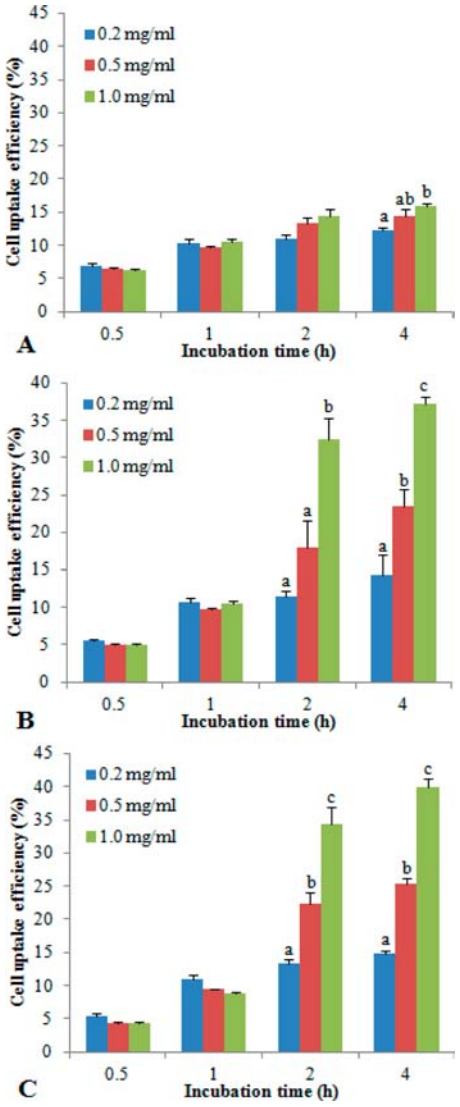

Figure 5. Cell uptake of zein nanoparticles at different concentrations: (A) nanoparticles of zein/caseinate 1:0.5; (B) nanoparticles of zein/caseinate 1:1; (C) nanoparticles of zein/caseinate 1:2. Values having different letters within the same time point are significantly different.

cells, as shown in Figure 6. Cell uptake of all nanoparticles was significantly inhibited at 4 °C, with the inhibition percentage around 85%. Sodium azide and colchicine also significantly inhibited cell uptake of nanoparticles, although the inhibition percentage was not as high as those under 4 °C condition. The remarkable inhibition of cell uptake at 4 °C was due to the low metabolic activity and poor membrane fluidity, and the 10−15% cell uptake may be attributed to physical adhesion or binding of nanoparticles to the cell membrane.[33,37] Sodium azide is a metabolic inhibitor that blocks cellular ATP synthesis. The decrease of cell uptake, but not the complete inhibition, of nanoparticles could be attributed to the utilization of exogenous ATP by cells.[44] The inhibition by 4 °C condition and sodium azide suggested that the cell uptake of zein nanoparticles was through active endocytosis, an energy-dependent process. During endocytotic internalization, it has been reported that actin polymerization and rearrangement were required in the clathrin- and caveolae-mediated process.[33,45] Therefore, the actin polymerization inhibitor, colchicine, was also tested and observed to reduce cell uptake nanoparticles. This indicated

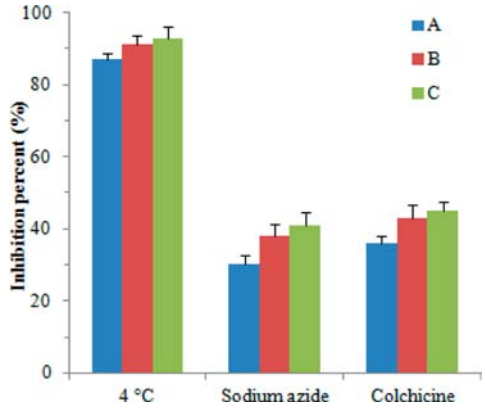

Figure 6. Cell uptake inhibition percentage of zein nanoparticles under different blocking conditions. A, nanoparticles of zein/caseinate 1:0.5; B, nanoparticles of zein/caseinate 1:1; C, nanoparticles of zein/caseinate 1:2.

that clathrin- or caveolae-mediated endocytosis was involved in the cell uptake process of zein−CAS nanoparticles. Nevertheless, Liu and co-workers prepared hollow casein nanospheres by the method of acrylic acid polymerization and investigated their cell uptake using four different cell lines, that is, LoVo, Hela, L929, and Jurkat cells.[46] Their study demonstrated that the highly negative charged casein nanospheres entered cells via a temperature- or energy-independent mechanism as well as in a nonendocytosis fashion. In our study, CAS was adsorbed onto zein nanoparticles surface and provided a strong negative surface charge, which in turn promoted the uptake of zein nanoparticles by Caco-2 cells. The detailed mechanisms involved in the entry route and pathway of cell uptake of zein nanoparticles require further investigation.

Qualitative Study. The qualitative study was carried out using a fluorescent microscope to visualize the cell uptake of nanoparticles. As shown in Figure 7, the zein−CAS nanoparticles were clearly internalized into Caco-2 cells, strongly supporting the aforementioned quantitative measurement of cell uptake. Zein−CAS nanoparticles were found throughout the cytoplasm surrounding the nucleus. The fluorescence intensity was different in three nanoparticles, showing the strongest intensity in nanoparticles C, which had the highest content of CAS. This observation was consistent with the quantitative study and confirmed that CAS improved the cell uptake of zein nanoparticles. Negligible fluorescence was detected in free coumarin 6 control (figure not shown), which was consistent with a previous report that coumarin 6 cannot be directly internalized by Caco-2 cells.[32] Therefore, the coumarin 6 detected was due to the internalization of nanoparticles.

Transport Study. On the basis of the cell uptake study, nanoparticles at a concentration of 1 mg/mL exhibited the highest uptake efficiency, and hence this concentration was selected for permeability study using Caco-2 cell monolayer. Both a−b and b−a directional transport were evaluated for three nanoparticle formulations, as shown in Figure 8. For a−b transport, the P_{app} values for nanoparticles A and B were 0.24×10^{-6} and 0.25×10^{-6} cm/s, respectively. However, the P_{app} of nanoparticles C was remarkably increased to 0.32×10^{-6} cm/s. Similar trends were observed for b−a directional transport. The higher permeability of the b−a direction compared with the a−b direction may be, in part, due to the tighter junction of the Caco-2 cell monolayer in the apical membrane than in basolateral membrane, as also observed in other literature.[47,48]

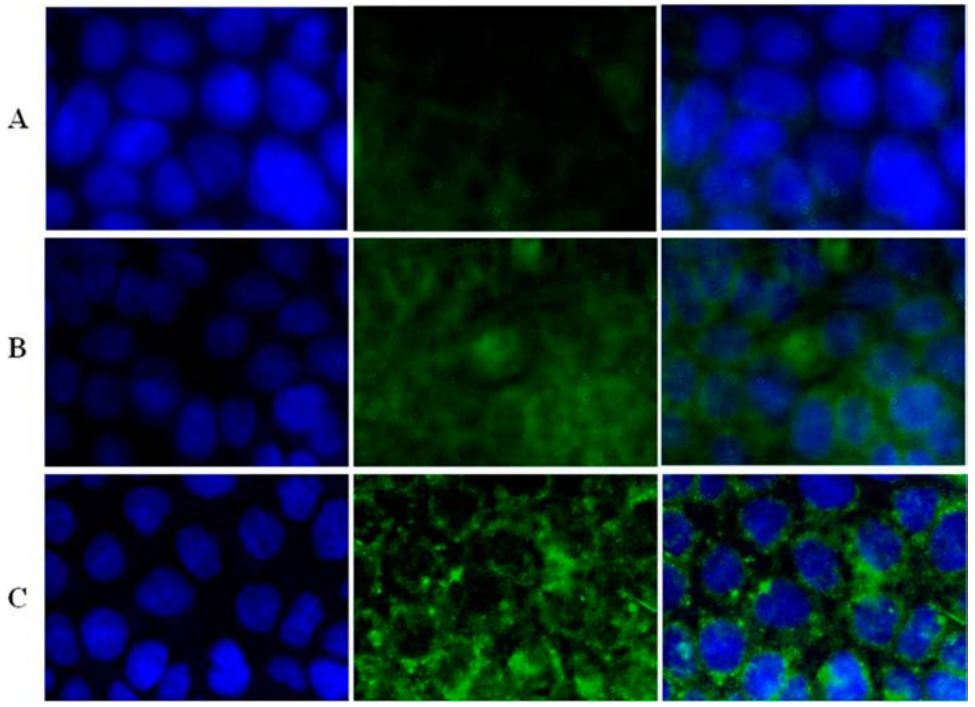

Figure 7. Fluorescent microscopic images for Caco-2 cells after 4 h of incubation with zein−CAS nanoparticles: (A) nanoparticles of zein/caseinate 1:0.5; (B) nanoparticles of zein/caseinate 1:1; (C) nanoparticles of zein/caseinate 1:2. The first column shows cell nuclei stained DAPI (blue); the second column shows cytoplasm filled with coumarin 6-encapsulated nanoparticles (green); the third column is the merged images.

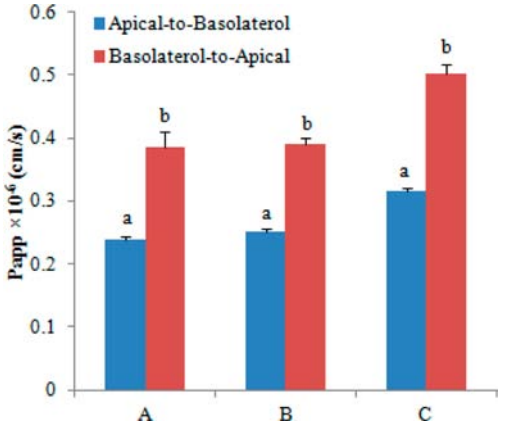

Figure 8. Transport of nanoparticles through Caco-2 cell monolayer. A, nanoparticles of zein/caseinate 1:0.5; B, nanoparticles of zein/caseinate 1:1; C, nanoparticles of zein/caseinate 1:2. Values having different letters within one sample are significantly different.

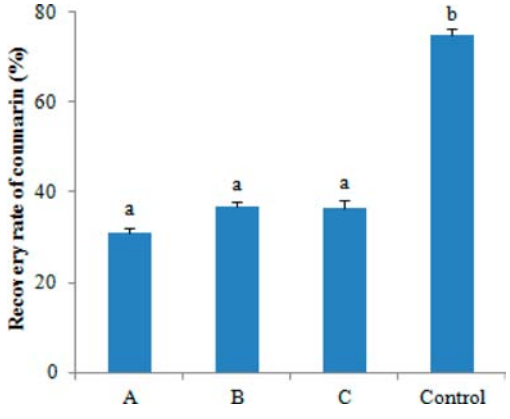

Figure 9. Recovery rate of coumarin 6 from Caco-2 cell monolayer transport study (apical to basolateral). A, nanoparticles of zein/caseinate 1:0.5; B, nanoparticles of zein/caseinate 1:1; C, nanoparticles of zein/caseinate 1:2; Control, free coumarin 6 compound dissolved in DMSO and diluted with HBSS. Values having different letters are significantly different.

To further investigate the transepithelial transport of zein−CAS nanoparticles, the mass balance was checked after the transport study. As shown in Figure 9, the coumarin 6 control showed a 75% recovery rate, indicating the mass balance of free coumarin was reasonable. However, for the coumarin 6 encapsulated in nanoparticles, the recovery rates were much lower, 31, 36, and 36% for nanoparticles A, B, and C, respectively. This observation revealed that some of the zein−CAS nanoparticles were accumulated in cells, suggesting that the transcellular transport might be involved in cell uptake of zein−CAS nanoparticles. Furthermore, the TEER value was monitored throughout the transport study. The TEER values were not altered significantly in all nanoparticle formulations (data not shown). Because the Caco-2 cell monolayers were characterized to carry negative surface charges due to glycoproteins on their surfaces, the negatively charged nanoparticles may only have limited capability to open tight junctions of the monolayers. Hafner and co-workers also reported that negatively charged lecithin nanoparticles had the least effect on tight junction openings compared with chitosan-coated nanoparticles, which possess positive surface charges.[49]

Conclusions. CAS-stabilized zein nanoparticles were prepared with different zein/CAS mass ratios. The effects of CAS on cytotoxicity, cell uptake, and epithelial transport of zein nanoparticles were investigated with Caco-2 cells. Three formulations showed similar good stabilities to maintain particle size in cell culture medium and HBSS buffer at 37 °C. Zein−CAS nanoparticles showed no cytotoxicity for 72 h. The CAS content

in zein nanoparticles favored cell uptake in a concentration- and time-dependent manner. The best cell uptake efficiency of nanoparticles was achieved with a zein/CAS mass ratio of 1:2 at 1 mg/mL, showing 40% cell uptake efficiency within 4 h. The cell uptake of nanoparticles was also visualized by fluorescent microscopy, suggesting that zein nanoparticles were seen throughout the cytoplasm surrounding the nucleus. The uptake of zein−CAS nanoparticles by Caco-2 cells exhibited an energy-dependent endocytosis. The detailed mechanisms involved in the entry route and pathway of cell uptake of zein nanoparticles require further investigation.

AUTHOR INFORMATION

Corresponding Author

*(Q.W.) Phone: (301) 405-8421. Fax: (301) 314-3313. E-mail: wangqin@umd.edu.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We appreciate support from the Maryland NanoCenter at the University of Maryland. We also acknowledge help and valuable advice from Haiqiu Huang in cell culture experiments and help from Caroline Rocourt in fluorescence microscope operations.

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