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ARTICLE

Nanoscalar Structures of Spray-Dried Zein Microcapsules and in Vitro Release Kinetics of the Encapsulated Lysozyme As Affected by Formulations

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Sustained release of antimicrobials may be a viable solution to enhance the bioavailability during the shelf life of food products. In this work, spray-drying was used to encapsulate a model antimicrobial of lysozyme in corn zein. The effects of zein/lysozyme (20:1 to 4:1) and zein/thymol (1:0 to 4:1) ratios on the microstructures of microcapsules and in vitro release profiles of the encapsulated lysozyme were investigated. In all cases, less lysozyme was released at higher pH, resulting from stronger molecular attraction between zein and lysozyme. Nanoscalar matrix structures of microcapsules were correlated with release characteristics of the encapsulated lysozyme. At intermediate zein/lysozyme (10:1) and zein/thymol (50:1) ratios, microcapsules had a continuous matrix structure and showed sustained release (11.1−65.3%) of lysozyme at pH 6 over 49 days. This work may be developed into practical food grade delivery systems of antimicrobials.

KEYWORDS: Lysozyme; corn zein; release kinetics; microstructure; thymol; loading level; spray-drying

INTRODUCTION

Sustained release of drugs is a well-recognized research area to enhance the bioavailability of drugs that could potentially lose bioactivity in physiological fluids due to decomposition/denaturation or binding with nontargeted sites. Delivery systems have been researched for numerous processes, carrier materials, and drugs, as extensively reviewed (*1*−*4*). Many materials and solvents used in drug delivery may not be directly applicable for food production due to toxicity, cost, and availability concerns. In addition, many existing processes may not be cost-effective or scalable for food production.

Nevertheless, advances in drug delivery have attracted interest in the food science community because of the similarities in research problems. Many papers have demonstrated enhanced efficacy of antimicrobials incorporated in edible or nonedible films that can be used to wrap solid food products (*5*−*8*). Liposomes (*9*−*12*) and surfactant micelles/microemulsions (*13*−*15*) have been studied as particulate delivery systems that could be potentially incorporated in food products. However, much work is needed to develop particulate delivery systems that use scalable, low-cost processes and generally recognized as safe (GRAS) and affordable food ingredients.

Food biopolymers may be suitable carrier materials of food antimicrobials because of their low cost, abundance, and sustainability, in addition to known physicochemical properties. Development of food biopolymeric delivery systems may also utilize knowledge of drug delivery systems in which synthetic and naturally existing or naturally derived biopolymers are used as carrier materials. For example, several studies used synthetic polymers as carrier materials to produce particulate delivery systems with sustained release of antimicrobials. Lysozyme was encapsulated in microcapsules of a polymer blend of poly(ethylene glycol) and poly (butylene terephthalate) using a multiple emulsion process, and the encapsulated lysozyme was observed to follow zero-order release kinetics (*16*). In another study, nisin was encapsulated in poly(L-lactide) using a supercritical antisolvent technique (SAS), and the release of encapsulated nisin from nanocapsules lasted over 45 days (*17*). In addition, spray-drying was used to encapsulate drugs in synthetic polymers of poly(lactide-*co*-glycolide) and poly(D,L-lactide), and the encapsulated drugs were released in vitro over 30 days for some formulations (*18*−*25*).

We are interested in using zein (prolamines extracted from maize kernels) as a food grade biopolymer carrier of antimicrobials because zein is insoluble in conventional food beverage solvent conditions. Antimicrobial delivery systems based on zein thus may maintain structural stabilities when incorporated in beverages that have long shelf life. In addition, zein is one of a few categories of food biopolymers that are soluble in aqueous alcohol. Aqueous ethanol may be used as a solvent to dissolve both lipophilic bioactive compounds and the carrier zein for subsequent preparation into particulate delivery systems using appropriate processes. Zein has been used in many studies to prepare films (*26*, *27*) and particles (*28*, *29*) using processes other than spray-drying. In one study, thymol, a naturally occurring plant essential oil with antimicrobial properties, was incorporated in zein nanoparticles precipitated by shearing a stock solution of zein and thymol (in aqueous ethanol) into deionized water (*30*). In two

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recent studies (*31*, *32*), thymol was incorporated in zein films at a thymol concentration of 5−35% of the zein mass. The microstructures of zein films were affected by the addition of thymol, indicating thymol may be used to manipulate the matrix structure of zein capsules.

In an earlier study (*33*), we encapsulated lysozyme in zein microcapsules using the SAS by spraying a solution of zein and lysozyme (in 90% aqueous ethanol) into supercritical CO_2 at 40 °C and 10 MPa. Lysozyme was gradually released in vitro from microcapsules over >40 days when the phosphate buffers were at pH 2−8. Our work suggested that zein may be a potential carrier material for manufacturing GRAS antimicrobial delivery systems. Furthermore, low-temperature encapsulation in supercritical CO_2 may be particularly suitable for heat- and oxidatively unstable lipophilic bioactive compounds. However, the SAS currently is costly and faces a scalability issue for food production.

In a separate study (*34*), we partially purified lysozyme from hen egg white by extraction using aqueous ethanol, and the extract was used to dissolve zein for spray-drying. No sustained release of lysozyme was observed from capsules produced with extracts adjusted to 60−90% ethanol, corresponding to porous internal structures of microcapsules. After the addition of thymol at a zein/thymol ratio of 50:1 (in 90% ethanol), sustained release of lysozyme was observed, especially at pH 8 from microcapsules with a homogeneous matrix structure. Our findings suggested the possibility of using spray-drying to manufacture GRAS delivery systems of food antimicrobials.

In this work, spray-drying was studied further as a commercially practical process to microencapsulate lysozyme in zein to achieve sustained release of the antimicrobial. To simplify sample preparation and improve sample consistency, purified lysozyme was used in this study to investigate the effect of formulation on the matrix microstructures of spray-dried microcapsules and release kinetics of the encapsulated lysozyme. Specifically, two formulation variables were investigated: ratios of zein/thymol (1:0 to 4:1 at a fixed zein/lysozyme ratio of 10:1) and zein/lysozyme (20:1, 10:1, and 4:1 at a fixed zein/thymol ratio of 50:1).

MATERIALS AND METHODS

Materials. Ethanol (200 proof) and purified corn zein were obtained from Acros Organics (Morris Plains, NJ). Thymol, lyophilized, purified hen egg white lysozyme (product no. L-6876), and powdered *Micrococcus lysodeikticus* were purchased from Sigma-Aldrich (St. Louis, MO). Other chemicals were products of Fisher Scientific (Pittsburgh, PA).

Protocol of Encapsulation. Zein, thymol, and lysozyme were dissolved in 60 mL of 90% (v/v) aqueous ethanol according to the formulations in **Table 1**. The solution was then spray-dried with a B-290 mini spray-dryer (Büchi Labortechnik AG, Flawil, Switzerland). The inlet and outlet temperatures were set at 80 and 50−55 °C, respectively, and a feed rate of 6.67 mL/min was used for all samples. The spray-dried powders were collected and stored in a −20 °C freezer.

Particle Yield and Encapsulation Efficiency. The encapsulation efficiency was defined as the ratio of total lysozyme units in the collected product to those in the feed before spray-drying (eq 1). Because it was difficult to collect powders sticking to the drying chamber and a small amount of mass (**Table 1**) was processed, significant errors of evaluating encapsulation efficiency may result from sample collection. An additional parameter of particle yield was used to compare the ratio of collected powder mass to that of the nonsolvent mass in the feed before spray-drying (eq 2).

$$\text{encapsulation efficiency } \% = 100\% \times \frac{\text{total lysozyme units in the collected product}}{\text{total lysozyme units in the feed}} \quad (1)$$

The total number of lysozyme units in the collected product was determined by multiplying the mass of the collected product by the lysozyme units per unit mass of product, which was determined after dissolving 27 mg of a powdered product in 1.5 mL of 55% aqueous ethanol.

$$\text{particle yield } \% = 100\% \times \frac{\text{mass of collected product}}{\text{nonsolvent mass in the feed}} \quad (2)$$

Total Solids Contents of Spray-Dried Microcapsules. Total solids contents of spray-dried microcapsules were measured following AOAC Method 925.09 (*35*) with slight modification in the sample mass. Vacuum-drying was performed at 100 °C for 5 h in a vacuum oven (Isotemp model 280A, Fisher Scientific, Pittsburgh, PA) with 625 mmHg of underpressure. Weight changes before and after incubation were used to estimate total solids contents of the samples.

In Vitro Release Kinetics. To evaluate in vitro release kinetics of lysozyme, 66 mM potassium phosphate buffers were prepared and adjusted to pH 2−8 with 1 N potassium hydroxide or 1 N hydrochloric acid. Microcapsules were suspended at a solids concentration of 27 mg per 1.5 mL buffer in polystyrene microcentrifuge tubes, attached to an end-to-end shaker (Laboratory Industries Inc., Berkeley, CA) operated at room temperature. After centrifugation at 5000*g* for 5 min (model MiniSpin Personal, Eppendorf, Westbury, NY), 1.0 mL of the supernatant was sampled for determination of lysozyme activity. The remainder of the sample was added with 1.0 mL of the corresponding fresh buffer, and particles were resuspended and mixed for continued release studies. Release tests were stopped when the

Table 1. Summary of the Particle Yield[a], Total Solids Content, Encapsulation Efficiency[b], and Lysozyme Specific Activity Ratio Percent[c] for Microcapsules Produced with Different Formulations

	compositions						
sample	zein (% w/v)	zein/lysozyme ratio	zein/thymol ratio	particle yield (%)	total solids (%)	encapsulation efficiency (%)	lysozyme specific activity ratio (%)
A	1.67	10:1	1:0	48.7	98.11	48.2	101.0
B	1.67	10:1	100:1	40.9	97.89	40.5	101.1
C	1.67	10:1	50:1	46.1	97.86	49.1	93.8
D	1.67	10:1	10:1	47.8	94.62	44.2	108.1
E	1.67	10:1	4:1	42.6	89.42	37.0	115.1
F	1.67	20:1	50:1	34.1	97.80	42.1	81.0
G	1.67	4:1	50:1	32.7	97.92	35.4	92.4

[a] Particle yield is defined in eq 2. [b] Encapsulation efficiency is defined in eq 1. [c] Lysozyme specific activity ratio percent is defined as the ratio, in percentage, of lysozyme units per unit of nonsolvent mass after and before spray-drying.

sample lysozyme concentration fell below the sensitivity limit of the assay method. The total amount of lysozyme in unit mass of powders, that is, 100% release, was estimated for the sample prepared with 27 mg of powders dissolved in 15 mL of 55% ethanol. When 100% release into the aqueous buffers used in this study is evaluated, the solubility of lysozyme needs to be examined. Lysozyme is less soluble at higher pH, and the solubility limit of lysozyme is about 55 mg/mL at pH 8 (*36*). The maximum possible concentration of lysozyme (3.6 mg/mL at a zein/lysozyme ratio of 4:1) used in our release studies is thus much lower than the solubility limit.

Cumulative lysozyme release after the *i*th sampling at time t_i was calculated according to eq 3, detailed elsewhere (*34*).

$$R_{t_i}(\%) = \frac{\sum_{n=1}^{i-1} a_n + 1.5a_i}{U_0} \times 100\% \quad (3)$$

a_i is the volumetric concentration of lysozyme (units/mL) in the microcentrifuge tubes at the *i*th sampling, and U_0 is the total units of lysozyme in 27 mg of microcapsules, that is, theoretical 100% release.

Estimation of Lysozyme Activity. Lysozyme activity was determined according to a method of Sigma-Aldrich (St. Louis, MO) for product HEW lysozyme (catalog no. L6876), using *M. lysodeikticus* as the test microorganism. One unit of lysozyme is defined as an absorbance reduction rate of 0.001 per minute at 450 nm using a 66 mM potassium phosphate buffer at 25 °C and pH 6.24. The UV−vis spectrophotometer (model Biomate 5, Thermo Electron Corp., Woburn, MA) had a cuvette cell with a thermal jacket supplied with a recirculating water stream maintained at 25 °C. Each sample was tested in triplicate.

Scanning Electron Microscopy (SEM). Both inner and outer structures of microparticles were imaged using a surface scanning electron microscope (LEO Electron Microscopy, Oberkochen, Germany). To observe surface morphology, powdered samples were loosely glued onto a black adhesive tape mounted on a stainless steel stub. To observe internal structures of particles, a razor blade was used to fracture particles before observations with the microscope, as used by Lee and Rosenberg (*37*). Samples were sputter-coated with a gold layer of ~5 nm thickness before imaging.

Statistical Analysis. Lysozyme activities of all samples were measured three times, and averages and 95% confidence intervals from the three measurement replicates were reported. Statistical differences were used to compare particle yields and encapsulation efficiencies from various formulations using a least-significant difference ($P < 0.05$) mean separation method (LSD). The analysis of variance for comparing the means of particle yields and encapsulation efficiencies was performed using the *t* test at a significance level of 0.05.

RESULTS AND DISCUSSION

Total Solids Content, Particle Yield, and Encapsulation Efficiency. Total solids contents (**Table 1**) of spray-dried microcapsules were >97.5% for samples with a zein/thymol ratio of 50:1 or higher (samplex A, B, C, F, and G), indicating the drying conditions successfully remove the majority of solvent—90% ethanol. At a zein/thymol ratio of 10:1 (sample D), the total solids content decreased to 94.62% after vacuum-drying. The total solids content for sample E with a zein/thymol ratio of 4:1 further decreased to 89.42%. Thymol has a boiling point of 227 °C, a melting point of 50 °C, and vapor pressures of 931 and 333 Pa at 100 and 80 °C, respectively. Conversely, water and ethanol have vapor pressures of 101,291 and 210,870 Pa at 100 °C and 47,371 and 101,890 Pa at 80 °C, respectively (*38*). Therefore, the loss of thymol during short-time spray-drying at 80 °C may have been minimal as indicated by similar particle yields observed for all samples (discussed below). In contrast, during long-time vacuum-drying (100 °C for 5 h), the volatility of thymol may have resulted in lower total solids contents measured for the spray-dried samples D and E that had theoretical thymol concentrations of 8.3 and 15.4% w/w (**Table 1**), respectively. Other samples had a thymol content of 4.3% w/w or less.

Particle yields and encapsulation efficiencies of the samples prepared from various formulations are listed in **Table 1**. Both particle yields and encapsulation efficiencies were ca. 35−50%. There was no significant difference in particle yield or encapsulation efficiency among the samples. There was also no difference between the means of particle yields and encapsulation efficiencies ($P > 0.50$).

Low encapsulation efficiencies may result from low particle yields or losses of lysozyme activity after spray-drying. Although lysozyme generally has a good thermal stability in aqueous solutions (*39*), the stability of lysozyme activity in aqueous ethanol at the spray-drying conditions has not been quantified. To further examine whether low encapsulation efficiencies were due to low particle yields but not losses of lysozyme activity after spray-drying, ratios of lysozyme units per unit mass, analogous to the specific activity of enzymes (units per unit mass proteins), after and before spray-drying are presented in **Table 1**. Specific activity ratios were >80% for all samples and >100% for four of seven samples. As analyzed above, the significant vaporization of thymol during spray-drying is not expected. Furthermore, because the other two nonsolvent compounds in the solution used in spray-drying, that is, lysozyme and zein, are polymers, the proportional precipitation of nonsolvent compounds is expected in the atomized solution droplets during drying. Therefore, similar lysozyme mass percentages with respect to nonsolvent mass are expected for each sample before and after spray-drying. Because lysozyme activity was used to calculate parameters in **Table 1** and the average specific activity ratio of all samples is 98.9%, most lysozyme activities were maintained after spray-drying, and low encapsulation efficiencies were indeed due to low particle yields.

Microstructures of Capsules Prepared at Different Zein/Thymol Ratios. The surface morphologies of particles prepared from formulations with different zein/thymol ratios are shown in **Figure 1**. Wrinkled particles varied in size. Particles prepared with a zein/thymol ratio of 50:1 appeared to be the biggest, whereas those prepared with a ratio of 10:1 were the smallest. However, quantitative comparisons of particle sizes and distributions as affected by formulations were not the focus of this work and are not discussed further.

The internal matrix structures of microcapsules are presented in **Figure 2**. Without thymol (sample A in **Table 1**), the matrix was composed of heterogeneously sized particles mostly smaller than 100 nm (**Figure 2A**). The nanoparticles were also observed when the zein/thymol ratio was 100:1 (**Figure 2B**). When the zein/thymol ratio was 50:1, the matrix structure became more homogeneous (**Figure 2C**). When the zein/thymol ratio was decreased to 10:1 and 4:1, nanoparticles were also observed (**Figure 2D**,**E**) but were smaller and more uniform than those in **Figure 2A**,**B**.

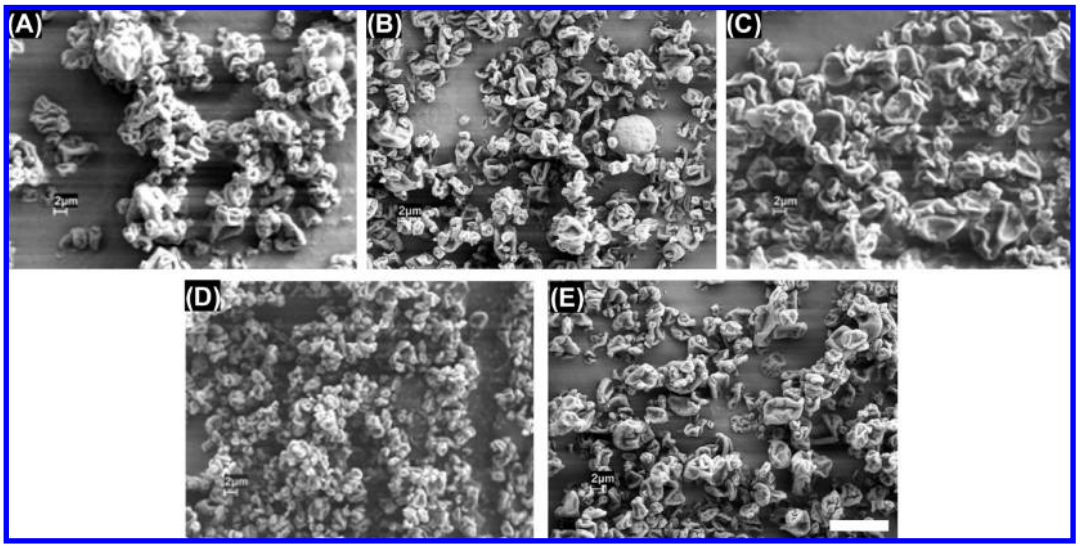

Figure 1. SEM images of spray-dried microcapsules produced with the same 10:1 zein/ lysozyme ratio but with zein/thymol ratios of (**A**) 1:0, (**B**) 100:1, (**C**) 50:1, (**D**) 10:1, and (**E**) 4:1. Scale bar = 10 μm.

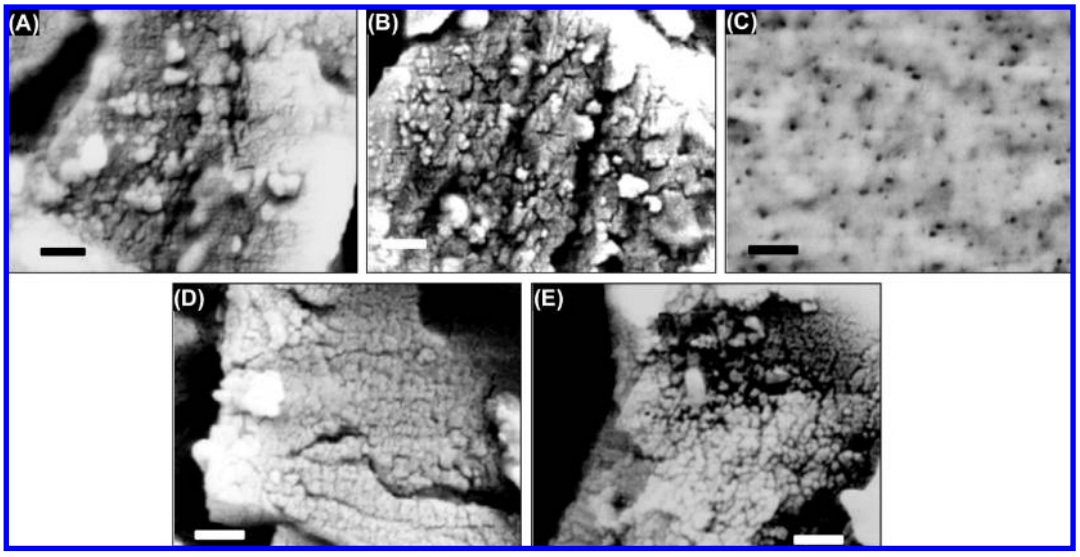

Figure 2. SEM images of matrix structures of spray-dried microcapsules produced with the same 10:1 zein/lysozyme ratio but with zein/thymol ratios of (**A**) 1:0, (**B**) 100:1, (**C**) 50:1, (**D**) 10:1, and (**E**) 4:1. Microcapsules were fractured before exposure of internal structures for imaging. Scale bars = 200 nm.

On the basis of the information from circular dichroic spectroscopy, zein in 70% aqueous methanol was described as a distorted cylinder with "nine adjacent, topologically antiparallel helices joined by glutamine-rich turns or loops" (*40*). A later study based on small-angle X-ray scattering indicated the conformation of "an elongated prism-like shape with an approximate axial ratio of 6:1", with a length of approximately 13 nm in 70% aqueous ethanol (*41*). On the other hand, lysozyme has a hydrodynamic radius of approximately 1.9 nm (*42*). The SEM images thus indicate that (1) nanoparticles observed from the matrix microstructures may have formed from molecules of zein, lysozyme, and thymol and (2) thymol affected the microstructure formation in the atomized droplets during spray drying. SEM, however, does not enable identification of the compounds corresponding to the observed microstructures.

The effect of thymol concentration on the microstructure of microcapsules formed during spray-drying may be interpreted analogously by observations of polymer properties as affected by plasticizer concentrations. Plasticizers are usually used as additives to decrease glass transition temperatures of polymers for improved mechanical properties of polymeric materials (*43*). However, antiplastization effects have been observed at low plasticizer concentrations for synthetic polyolefins (*44*, *45*) and naturally occurring polymers such as starch (*46*). One hypothesis to interpret antiplasticization effects was first made by Ghersa (*47*) and later supported by several studies that used nuclear magnetic resonance to elucidate interactions between polymers and plasticizers (*48*−*50*). At low concentrations, plasticizers serve as "cross-linkers," in addition to the conventional function of manipulating polymer crystallization. The most homogeneous microstructure of microcapsules prepared with a zein/thymol ratio of 50:1 (in the range of 1:0 to 4:1) indicates that thymol may have functioned as a plasticizer during spray-drying of zein solutions and the antiplasticization effect may be the case at low thymol concentrations. However, future work such as using nuclear magnetic resonance to study the interactions between thymol and zein will be needed to illustrate the role of thymol during microstructure formation of zein capsules.

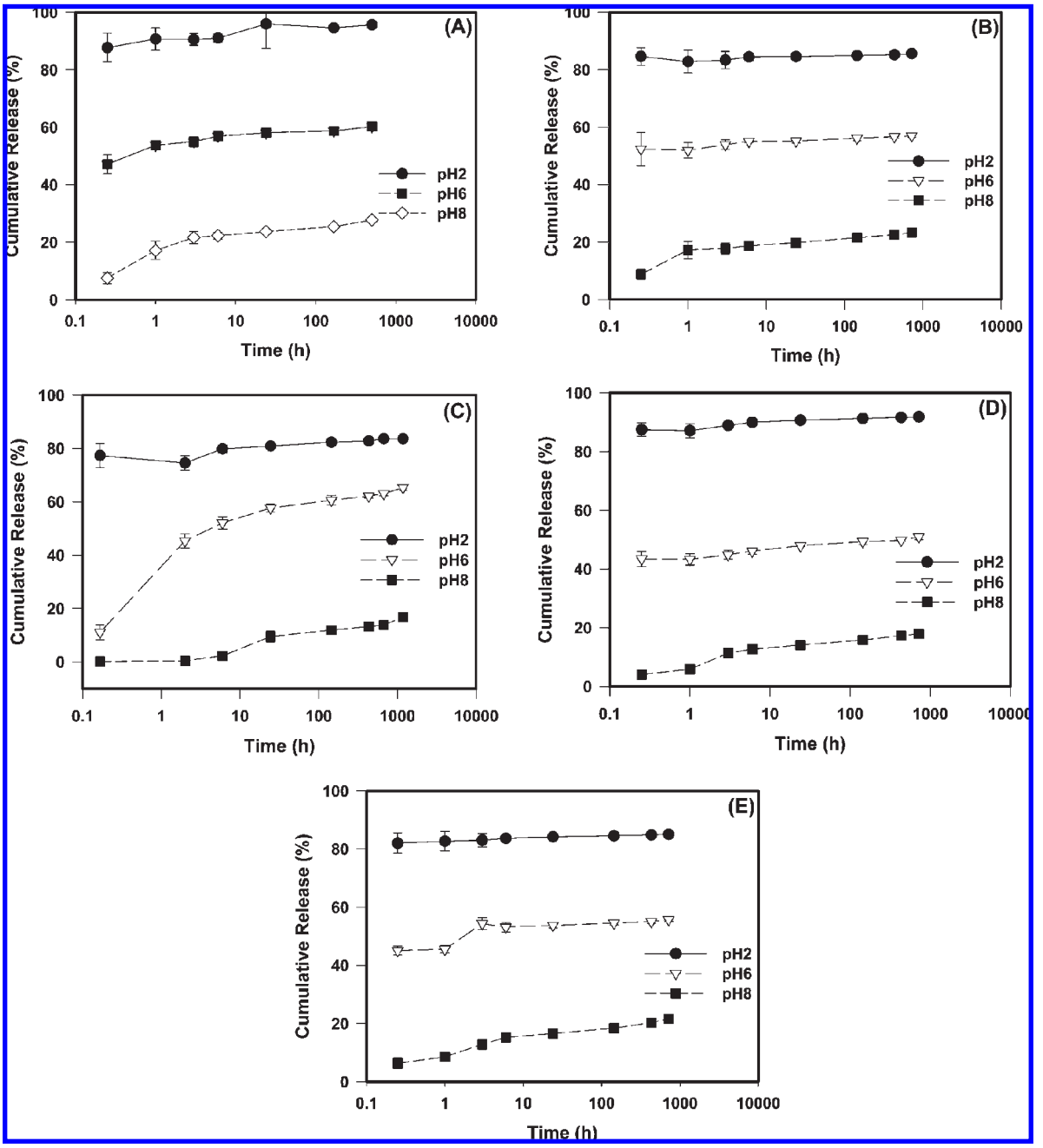

Figure 3. Release kinetics of lysozyme from spray-dried microcapsules produced with the same 10:1 zein/lysozyme ratio but with zein/thymol ratios of (**A**) 1:0, (**B**) 100:1, (**C**) 50:1, (**D**) 10:1, and (**E**) 4:1. Error bars are 95% confidence intervals from three measurement replicates.

It was also interesting to note that the matrix microstructure (composed of nanoparticles) in **Figure 2A** was drastically different from the continuous sponge-like matrix (with no identifiable particles) of zein microcapsules produced from a similar formulation but using the SAS (*33*). The microstructural difference may result from different evaporation rates of 90% ethanol and precipitation kinetics of polymers during drying of atomized solutions in hot air for the case of spray-drying and in supercritical CO_2 for the case of SAS. The evaporation rate of 90% ethanol in supercritical CO_2 is much faster than that in hot air due to the very low interfacial tension in supercritical CO_2 (*51*), which results in fast polymer precipitation in supercritical CO_2, which is a very poor solvent for polymers (*52*).

In Vitro Release Kinetics of Lysozyme from Microcapsules Prepared at Different Zein/Thymol Ratios. Figure 3 shows release kinetics of lysozyme from microcapsules prepared from formulations with different zein/thymol ratios. All samples had more lysozyme released at lower pH. For the sample without thymol, 87.7% lysozyme was released at pH 2 after 20 min and cumulative release increased to 95.6% after 504 h or 21 days (**Figure 3A**). At pH 6, the cumulative release increased from 47.1% after 20 min to 60.2% after 21 days. At pH 8, the release was more sustained; cumulative release increased from 7.6% after 20 min to 27.8% after 21 days.

At a zein/thymol ratio of 100:1, no sustained release was observed at pH 2 or 6 (**Figure 3B**), whereas the cumulative release increased from 8.8% after 20 min to 23.3% after 30 days at pH 8. When the zein/thymol ratio was lowered to 50:1, the release characteristics at pH 2 and 8 (**Figure 3C**) were similar to those in **Figure 3A**,**B**, except the amount of lysozyme released was relatively smaller at the same incubation time. At pH 6, however, a constant increase in

cumulative release of lysozyme was observed: 11.1% after 20 min and 65.3% after 49 days. When the zein/thymol ratio was decreased further to 10:1 and 4:1, the release profiles (**Figure 3D**,**E**) were similar to those in **Figure 3A**,**B**.

The effect of pH on release characteristics was similarly observed from our previous study in which lysozyme was encapsulated in zein microcapsules produced by the SAS (*33*). Molecular interactions between lysozyme and carrier polymer zein were discussed for hydrophobic interactions and electrostatic interactions. Zein is water-insoluble (hydrophobic), and lysozyme is more hydrophobic at a pH closer to the isoelectric point (p*I*) of lysozyme, 10.5−11.0 (*53*). Hydrophobic interactions therefore are stronger at higher pH between 2 and 8. When electrostatic interactions are considered, lysozyme is net positively charged below the p*I*, at pH 2−8, whereas zein has an isoelectric point of 6.8 (*54*) and is net positively charged at pH 2−6 and negatively charged at pH 8. The electrostatic interactions between zein and lysozyme are thus more repulsive (both polymers are more positively charged) at lower pH between 2 and 6. At pH 8, the electrostatic interactions become attractive because the two polymers are oppositely charged. The overall molecular interactions between zein and lysozyme are therefore more repulsive at lower pH, which may have contributed to a larger amount of released lysozyme shown in **Figure 3**.

Lysozyme has a molecular weight of 14.4 kDa (*55*) and a hydrodynamic radius of approximately 1.9 nm (*42*). When lysozyme diffuses out of the microcapsule matrices shown in **Figure 2**, the mass transfer resistance may arise from two factors: molecular (attractive or repulsive) interactions between zein and lysozyme and matrix microstructure. At pH 6, slightly below the p*I* of zein, the attraction between lysozyme and zein may not be very strong: weak electrostatic repulsion (because zein is weakly positively charged) and weak attractive hydrophobic interactions (because lysozyme may not be very hydrophobic at pH well below the p*I*). When the microstructure of capsule matrix was homogeneous (**Figure 2C**, zein/lysozyme = 50: 1), the mass transfer resistance due to the diffusion of lysozyme out of the capsule matrix may be very significant and result in gradual release of lysozyme in **Figure 3C**. Conversely, for samples prepared from other formulations, more heterogeneous capsule microstructures may have enabled the quicker diffusion of lysozyme through the matrix of nanoparticles shown in **Figure 2**. When the electrostatic interactions between zein and lysozyme were strongly repulsive at pH 2, quick lysozyme release was observed regardless of the differences in capsule microstructures. At pH 8, the molecular attraction between lysozyme and zein became strong and may have contributed to mass transfer resistance much more than microstructures, corresponding to similar release kinetics for all samples.

Because lysozyme is part of the matrix structures shown in **Figure 2**, release of lysozyme from microcapsules will likely change the matrix microstructures during the release studies. Zein is usually insoluble in aqueous buffers at pH < 11 (*56*). Hurtado-López and Murdan (*29*) evaluated the stability of zein microcapsules in aqueous buffers at pH 2, 5, and 7.4 during incubation at 37 °C for 7 days. The authors observed no change in primary structures of zein and turbidity of suspensions and concluded no or limited dissolution/erosion of microcapsules at the studied conditions. However, the authors did not study the matrix microstructural changes during incubation, which may be an interesting topic for our future work to better correlate release kinetics with matrix microstructures of capsules.

The release profile of lysozyme in **Figure 3A** (sample A) was different from the sustained release of lysozyme from zein microcapsules produced using the SAS that showed 71−100, 28−84, and 11−51% release after incubation from 30 min to 28 days at pH 2, 6, and 8, respectively (*33*). As discussed above, microstructures of capsules prepared from spray-drying (aggregated nanoparticles) and those prepared from SAS (continuous sponge-like matrix without identifiable particles) were drastically different and may have resulted in different characteristics of release profiles—less sustained for the former versus more sustained for the latter.

Microstructures of Capsules Prepared at Different Lysozyme Loading Levels. Two additional loading levels of lysozyme were studied for zein/lysozyme ratios of 20:1 and 4:1 at the same zein/thymol ratio of 50: 1 (samples F and G in **Table 1**). The size and appearance of the two samples (**Figure 4**) were similar to those of the sample with a zein/lysozyme ratio of 10:1 (**Figure 1C**). The matrix structures of samples F and G are presented in **Figure 5**. At a zein/lysozyme ratio of 20:1, the capsule matrix (**Figure 5A**) did not have identifiable particles, similar to that in **Figure 2C**, but showed some "cracks." When the zein/lysozyme ratio was 4:1, the capsule matrix was composed of nanoparticles smaller than ca. 10−20 nm (**Figure 5B**). Both samples F and G had a less homogeneous matrix structure than did sample C, which had an intermediate lysozyme loading level.

Because lysozyme is water-soluble and zein is water-insoluble, the distinct thermodynamic difference of the two polymers may result in spinodal decomposition at certain lysozyme loading levels, resulting in lysozyme-rich and

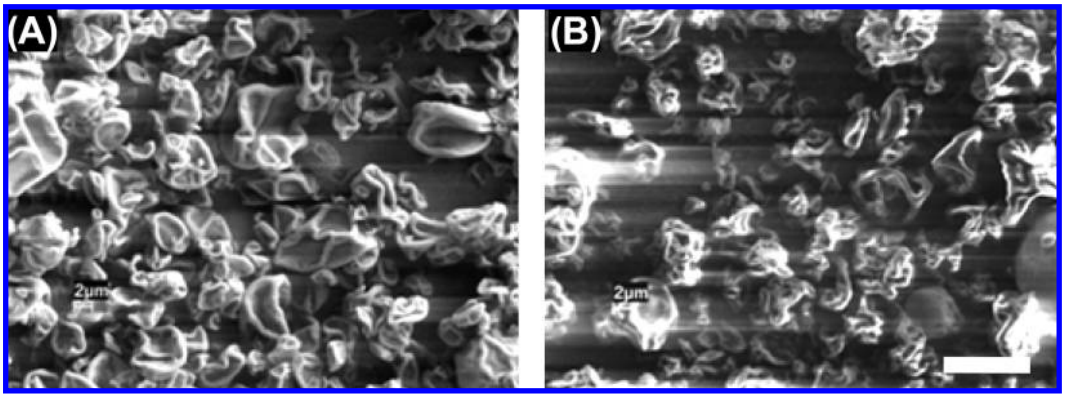

Figure 4. SEM images of spray-dried microcapsules produced with the same 50:1 zein/thymol ratio but with zein/lysozyme ratios of (**A**) 20:1 and (**B**) 4:1. Scale bar = 10 μm.

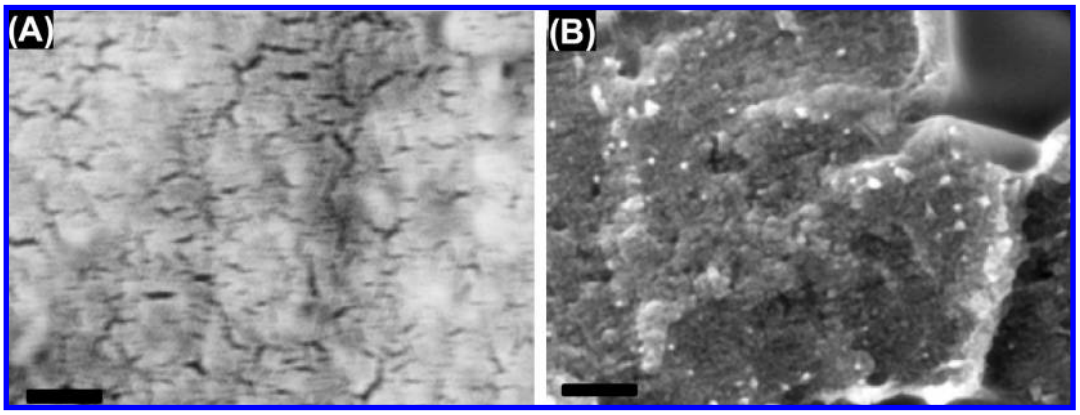

Figure 5. SEM images of matrix structures of spray-dried microcapsules produced with the same 50:1 zein/thymol ratio but with zein/lysozyme ratios of (**A**) 20:1 and (**B**) 4:1. Microcapsules were fractured before exposure of internal structures for imaging. Scale bars = 200 nm.

lysozyme-depleted microphases in atomized droplets during spray-drying. Future work such as confocal laser scanning microscopy using compounds individually tagged with fluorescent dyes may increase our understanding of the distribution of compounds in the capsule matrices to fundamentally elucidate microstructure formation mechanisms.

In Vitro Release Kinetics of Lysozyme from Capsules Prepared at Different Lysozyme Loading Levels. For the sample with a zein/lysozyme ratio of 20:1, a smaller amount of lysozyme release at lower pH was also observed (**Figure 6A**). Gradual releases of 82.7−93.4, 68.6−79.8, and 58.6−74.9% were observed in 1176 h or 49 days when the phosphate buffer was at pH 2, 6, or 8, respectively. Higher percentages of lysozyme were released than the sample C (**Figure 6A** vs **Figure 3C**), possibly due to the cracks in the matrix structure (**Figure 5A** vs **Figure 2C**). At a zein/lysozyme ratio of 4:1 (sample G, **Figure 6B**), gradual releases of 84.5−93.1, 51.7−77.1, and 8.0−51.1% were observed in 672 h or 28 days when the phosphate buffer was at pH 2, 6, or 8, respectively. When compared to the sample with a zein/lysozyme ratio of 10:1 (sample C), the release at pH 6 was much higher at the first sampling (51.7% for sample G vs 11.1% for sample C) and was less sustained (25.4% increase in 28 days for sample G vs 44.2% increase in 49 days for sample C) for sample G. When release profiles of samples C and G at pH 8 were compared, sample G had more lysozyme released. Besides the microstructural difference, a higher loading level in sample G may correspond to an overall weaker attraction at pH 6 and 8 by the carrier polymer zein because of a higher mass percentage of lysozyme in microcapsules, which may have enabled more complete release of lysozyme

In conclusion, our work has demonstrated some interesting observations when thymol was used at different proportions to the carrier polymer zein. Nanoscalar microstructures of zein capsules changed dramatically with the thymol concentration, corresponding to different release profiles of the encapsulated lysozyme. In addition, molecular interactions between the carrier material and the encapsulated antimicrobial at different pH values were important to the release characteristics of lysozyme. Stronger repulsive forces between zein and lysozyme resulted in quicker and relatively more complete release at pH 2 than at pH 6 and 8. With moderate attractive forces between zein and lysozyme, sustained release of lysozyme was possible with an appropriate mass transfer resistance provided by the capsule matrix. Furthermore, the nanoscalar microstructure of capsule matrices was also affected by the loading levels of lysozyme that

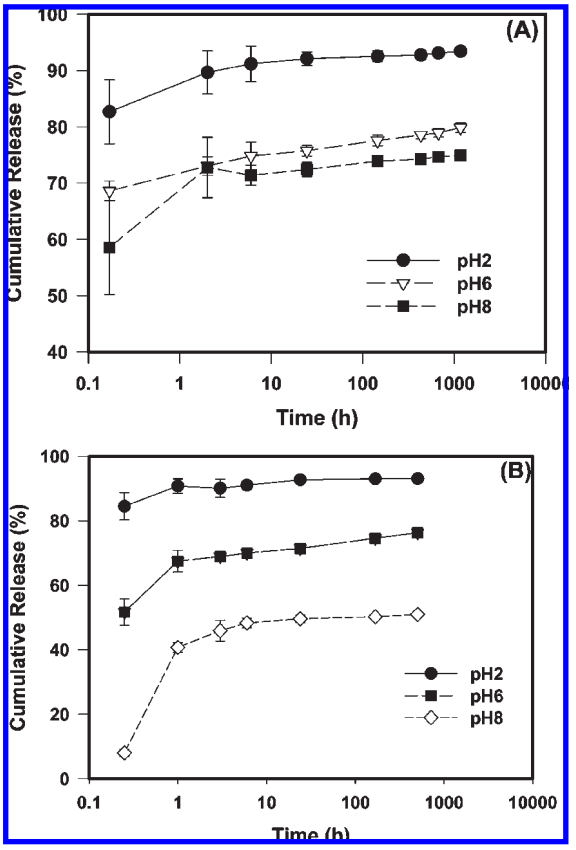

Figure 6. Release kinetics of lysozyme from spray-dried microcapsules produced with the same 50:1 zein/thymol ratio but with zein/lysozyme ratios of (**A**) 20:1 and (**B**) 4:1. Error bars are 95% confidence intervals from three measurement replicates.

corresponded to different release profiles. Sustained release of antimicrobials over long periods of time may be important to enhance the efficacy of antimicrobials against pathogenic and spoilage microorganisms, important to food safety and quality (especially for extending shelf life). The materials used in this work are all GRAS, and spray-drying is a practical process. Our approach may be further developed into a commercially feasible, low-cost, and food grade antimicrobial delivery system.

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