Outer Eggshell Membrane as Delivery Vehicle for Polysaccharide/Protein Microcapsules Incorporated with Vitamin E

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ABSTRACT: This study investigates the features of a new type of delivery system prepared by combining a natural outer eggshell membrane (OESM) with emulsified microcapsules. The loading efficiency, controlled release properties, and forming mechanisms of the prepared system were studied. The polysaccharide/protein microcapsules incorporated with vitamin E can be attached to highly cross-linked protein fiber networks of OESM. This attachment could be reinforced more than 2-fold using glutaraldehyde as a cross-linking agent. The combined OESM/microcapsule delivery system significantly exhibited better controlled release properties than the microcapsules alone because of the steric blocking effect. Moreover, the OESM delivery system incorporated with microcapsules formed by pectin/protein as wall material showed more resistance against enzymatic attacks because of the formation of compact aggregates promoted by electrostatic effects.

KEYWORDS: outer eggshell membrane, microcapsules, delivery vehicle, controlled release

■ INTRODUCTION

Eggs are composed of an eggshell and eggshell membrane (ESM) on the outside and albumen and yolk on the inside. The membrane adhering inside the eggshell has been the focus of several studies. This membrane, a natural and nontoxic byproduct of eggs, is a double-layered membrane composed of a 50−70 μm thick outer layer and a 15−30 μm thick inner layer.[1] Each layer has its own composition and microstructure. The two layers are discontinuous and discernible, as shown in Figure 1. The outer eggshell membrane (OESM) is mainly composed of highly cross-linked protein fibers and possesses biocompatibility and excellent gas and water permeability,[2] whereas the inner eggshell membrane (IESM) is much more compact. Several studies have shown that ESMs can be used to immobilize and transport active substances, such as proteins, enzymes, and antibodies. Therefore, ESMs can be employed as biovehicles or biosensors.[3−7] Tang[8] successfully immobilized R-phycoerythrin on the ESM surface as a fluorescence probe to determine the level of salbutamol in urine samples. A novel immunosensor constructed using ESM as immobilization platform for goat anti-human immunoglobulin M antibody exhibited remarkable storage stability, permeability, and high biocompatibility.[9] A low-cost and stable biovehicle for screening homocysteine in human plasma was also fabricated by immobilizing D-amino oxidase on ESM with the help of glutaraldehyde.[10] However, the existing delivery systems are mainly based on the whole ESM. The highly cross-linked protein fibers of keratin, collagen, and elastin within OESM are structurally similar to a meshwork of entangled threads. The favorable macroporous structures of OESM are potentially useful as a biologic separator,[11] which was therefore expected to be used to encapsulate and transport active substances.

Controlled release properties are often required for an efficient delivery vehicle. Unfortunately, a simple ESM delivery system does not have such features. Several studies have used microcapsules as another common delivery vehicle because of their ability to stabilize active ingredients by protecting them from adverse environmental conditions and releasing them at a controlled rate.[12,13] The combination of ESM and microcapsules may certainly improve the functions of the simple ESM system. Thus, this combined system has potential application in nutritional, pharmaceutical, and cosmetic industries. However, to our knowledge, no studies have directly investigated this combined system.

The current study fabricates and investigates the properties of the combined OESM/microcapsule delivery system. An emulsified microcapsule was prepared using liposoluble vitamin E (V_E) as core material. This substance has long been used in drug models and common food grade biopolymers, in which it is combined with polysaccharide/protein complex. Whey protein and gelatin (G) were selected because of their nutritional values and wide application in food science. For comparison, two polysaccharides were selected according to their surface charges and other concerned physicochemical properties. The microcapsule loading efficiency on OESM, controlled release properties, and forming mechanisms were also studied.

■ MATERIALS AND METHODS

Chemicals. The eggs were purchased from DQY Agricultural Technology Co. Ltd. (Beijing, China) in the local market. The whey protein isolate (WPI, 97%, w/w) was purchased from Davisco Foods International (Eden Prairie, MN, USA). α-Tocopherol (V_E), pepsin (1:60000; from crystallized and lyophilized porcine stomach mucosa),
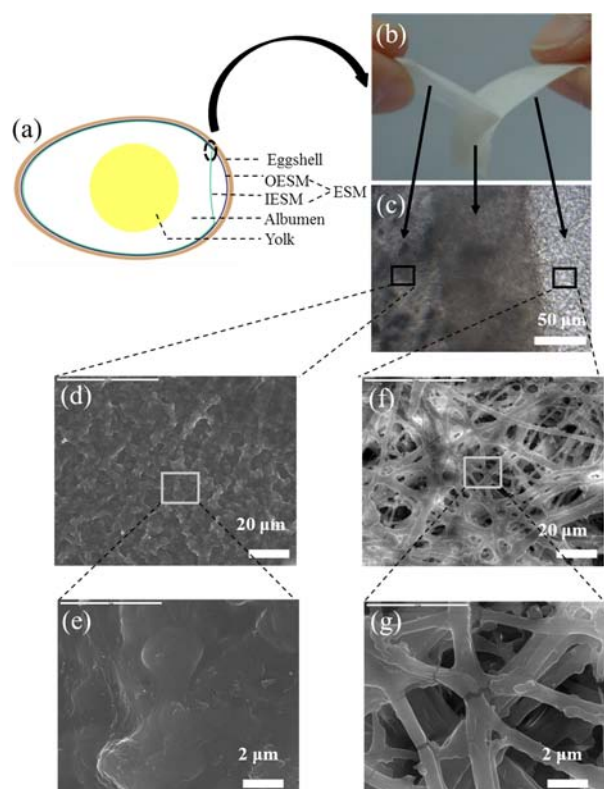
Figure 1. Micrographs of ESM: (a) schematic of an egg; (b) peeling procedure of the OESM; (c) separated OESM and IESM; (d, e) SEM micrograph of IESM and enlargement; (f, g) SEM micrographs of OESM and enlargement.

pancreatin 8X (from hog pancreas), fluorescent dye fluorescein isothiocyanate (FITC), and Nile red were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Gelatin (G), gum arabic (GA), low-methoxyl pectin (LMP), and soybean oil were of food grade and provided by a local market (Beijing, China). Polyglycerol poly-ricinoleate 90 (PGPR) was provided by Danisco (China). Sodium hydroxide (NaOH), hydrochloric acid (HCl), glutaraldehyde, monosodium phosphate, disodium hydrogen phosphate, sodium chloride, and hexane were all of analytical grade (Beijing Chemical Factory).

Preparation of OESM. The OESM was carefully peeled from fresh eggshell and separated from the inner layer at the blunt end where the air cell was found. The OESM was then cleaned with deionized water to remove the albumen completely. The obtained OESM, with the size of 3 cm × 0.5 cm, was stored in deionized water at 4 °C.

Scanning Electron Microscopy (SEM). The morphology of the OESM surface was examined using SEM (Hitachi S-4500, Japan). Membranes were mounted on aluminum stubs using glue paste and carbon paint.

Measurement of Zeta Potential and Particle Size. Poly-saccharide and protein powders were dissolved in deionized water using selected concentrations at 6% (w/w) and 1.25% (w/w), respectively. The obtained OESM was ground to powder in the atmosphere of liquid nitrogen. The powder (0.50 ± 0.01 g) was dispersed in 50 mL of deionized water, stirred for about 2 h, and ultrasonicated for 5 min. The solutions were maintained at room temperature for 12 h for complete hydration. The pH of the solutions was adjusted from 3.0 to 9.0 using 1 mol/L HCl or 1 mol/L NaOH, respectively. The zeta potentials and particle sizes of the solutions were measured with a Delsa-Nano particle analyzer (Beckman Coulter Inc., Fullerton, CA, USA). The refractive index of the medium (water) was 1.333, and the temperature was 25 °C.

Preparation of Microcapsules. V_E (5 g) and PGPR (0.8 g) were dissolved in 10 g of soybean oil and added into a 50 g aqueous solution

of protein (pH 3.5). The mixture was pre-emulsified with an Ultra Turrax homogenizer (Ultra Turrax, model FA25, Fluko, Germany) at 10000 rpm for 5 min. The emulsion was then slowly added into a 150 g aqueous solution of polysaccharide (pH 3.5), homogenized at 10000 rpm for 5 min, and then passed through a high-pressure homogenizer (AH100D; ATS Engineering Inc., Brampton, Canada) at 100 bar for 5 min.

Measurement of Encapsulation Efficiency (EE). A 0.5 mL emulsion microcapsule was dispersed and washed in 1.5 mL of hexane. The amount of extracted V_E was determined by ultraviolet (UV) absorbance at 285 nm using a standard curve (UVmini-1240 spectrophotometer; Shimadzu, Japan)[14] and calculated as

$$EE\,(\%) = \frac{M_0 - M_1}{M_0} \times 100 \tag{1}$$

where M_1 is the amount of V_E extracted from hexane and M_0 is the actual amount of V_E added initially.

Immobilization of Microcapsules on OESM. Five grams of OESM was placed into sealed tubes with 1, 2, 3, 4, 5, 10, 15, and 20% (w/w) glutaraldehyde solution (5 mL) at a stirring speed of 100 rpm and a temperature of 25 °C. After 60 min, the OESM was rinsed with, and then immersed in, deionized water for 30 min to remove the excess glutaraldehyde. A 5 mL emulsion microcapsule was then mixed with the OESM attached with glutaraldehyde, and the mixture was stirred at 100 rpm and 25 °C. After 60 min, the combined OESM/microcapsule system was rinsed with 10 mL of deionized water several times alternately.

Confocal Scanning Laser Microscopy (CLSM). The combined OESM/microcapsule system was stained with Nile red (fluorescent lipid dye) and FITC (fluorescent polysaccharide and protein dye) for observation with CLSM (Nikon, model EZ-C1; Japan). Ar ion (488 nm) and He−Ne (543 nm) lasers were used as excitation sources.

Study on the Controlled Release Properties. The release rate of V_E was determined by incubating accurate amounts of emulsified microcapsules and combined OESM/microcapsule system in sealed tubes with 10 mL of release medium at a stirring speed of 100 rpm and a temperature of 37 °C. Four release media were used: (a) HCl solution (pH 1.2); (b) simulated gastric condition (pH 1.2) with 0.1% (w/v) pepsin; (c) phosphate-buffered saline (pH 7.4); and (d) simulated intestinal condition (pH 7.4) with 1.0% (w/v) pancreatin. The tubes containing different release media were withdrawn at either 0.5 or 1 h intervals. The release of V_E was detected by UV absorbance at 285 nm using a standard curve and calculated as

$$R\,(\%) = \frac{G_1}{G_0} \times 100 \tag{2}$$

where G_1 is the released amount of V_E in the media and G_0 is the actual amount of V_E initially added.

Diffusion Model Analysis. The diffusion of compounds in polymeric network microstructure was generally governed by two main simultaneous events: a Brownian motion described by Fick's second law and a polymer relaxation driven by stress dissipation provoked by penetrant. The latter can be described using a first-order kinetic equation. A combined model of the above mechanisms was proposed by Flores et al.:[15]

$$\frac{M_t}{M^{eq}} = X \times \left\{ 1 - \frac{8}{\pi^2} \times \sum_{n=0}^{\infty} \frac{1}{(2n+1)^2} \times \exp[-(2n+1)^2 \times k_F \times t] \right\}$$
$$+ (1 - X) \times [1 - \exp(-k_R \times t)] \tag{3}$$

M_t is the total mass released from the polymeric network at time t, M^{eq} is the mass released at equilibrium state, D is the diffusion coefficient, k_F is the Fickian diffusion rate constant ($k_F = D \times (\pi^2/l^2)$), k_R is the polymer relaxation rate constant ($k_R = 1/\tau$), l is the effective diffusion distance, τ is the relaxation time associated to polymer relaxation, and X is the fraction of Brownian contribution. The value of X varied between 0 and 1. $X = 1$ referred to Brownian diffusion, whereas $X = 0$ referred to anomalous diffusion. In the investigated conditions, eq 3

can be simplified to eq 4 using the first term of the Taylor series because the difference between one term or more terms was minor:[16]

$$\frac{M_t}{M^{eq}} = X \times \left[1 - \frac{8}{\pi^2}\exp(-k_F \times t)\right] + (1 - X)$$
$$\times \left[1 - \exp(-k_R \times t)\right] \tag{4}$$

Statistical Analyses. All experiments were performed in triplicate. Statistical data were analyzed using Origin 8.0 and SPSS 16.0. Statistics were performed on a completely randomized design using the General Linear Models procedure with one-way analyses of variance. Duncan's multiple-range test ($p < 0.05$) was used to detect differences among the mean values.

■ RESULTS AND DISCUSSION

Zeta Potential and Particle Size of Pure Components. Figure 2a shows the comparison between the variations of the
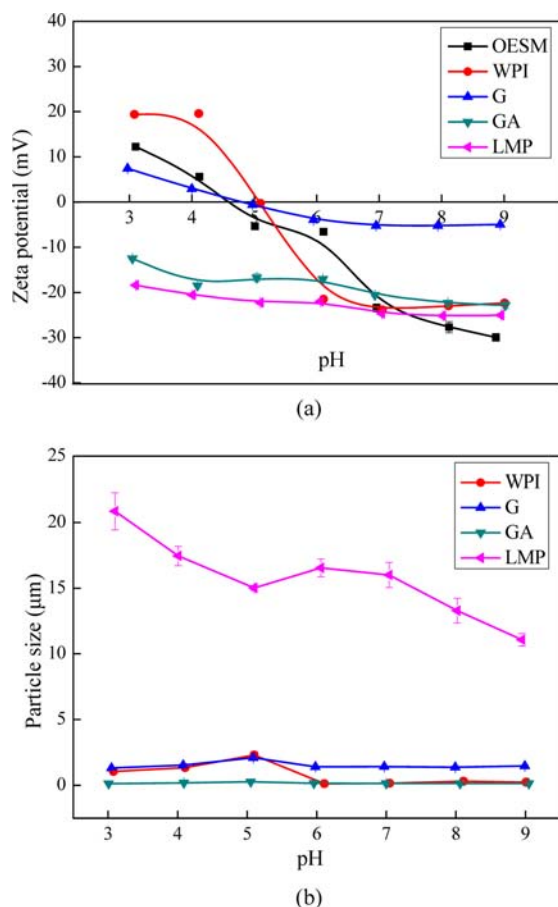

Figure 2. Variations of the zeta potential (a) and particle size (b) of different solutions of polysaccharide, protein, and OESM versus pH.

zeta potential values of the OESM, different polysaccharides, and proteins versus pH. The isoelectric point (pI) values of OESM, WPI, and G were 4.6, 5.2, and 4.9, respectively, indicating that OSEM was as amphoteric as WPI and G molecules. The zeta potential of the polysaccharides, such as GA and LMP, were both negative and had only a minor variation versus the pH, and the values of the former were lower than those of the latter. The particle size variations of the polysaccharides and proteins versus pH are shown in Figure 2b. The proteins aggregated slightly when the pH was close to the pI because of the charge neutralization.[17,18] For the polysaccharides, particularly for the larger and more charged

LMP particles, the size decreased with increasing pH because of the intermolecular electrostatic repulsion.

Zeta Potential of Polysaccharide/Protein Mixtures. The electrostatic attraction between polysaccharides and proteins at acidic environment can be used to prepare solid complexes.[19,20] The variations of the zeta potential of the different complexes with a polysaccharide/protein mass ratio of 1:5 at pH 3.5 are shown in Figure 3, wherein the charge
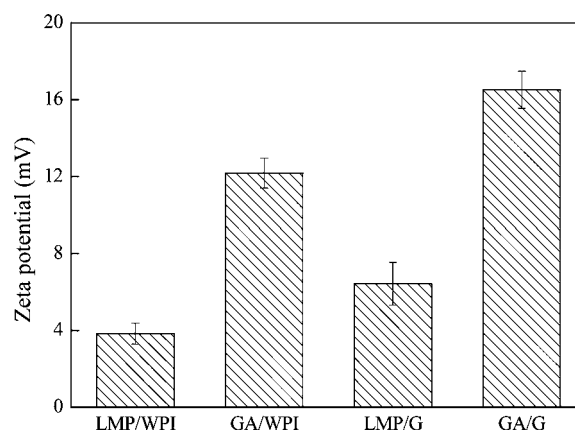

Figure 3. Variations of the zeta potential of different complexes of polysaccharide/protein.

neutralizations between the negative polysaccharides and the positive proteins occurred as expected. Given that the charge amount of LMP was higher than that of GA, the zeta potential of the LMP/protein mixture for a given protein, WPI, or G was lower than that of the GA/protein mixture. Surprisingly, for a given polysaccharide, LMP, or GA, the zeta potential decrease of the polysaccharide/WPI mixture was larger than that of the polysaccharide/G mixture. The steric conformation of the molecules can also significantly affect the neutralization process.[21,22] The abnormal lower zeta potential of the polysaccharides/WPI indicated that WPI chains were more twisted than G,[23] thereby allowing WPI to more easily neutralize the charges of the polysaccharides, whereas the relatively rigid straight G chains had no such ability. Oil droplets had no essential effect on the electrostatic interaction. Thus, the features of the zeta potential of the polysaccharide/protein mixture emulsion containing oil shown in Figure 4 were almost the same as those in Figure 3.

Encapsulation Efficiency. Figure 5 shows the EE of V_E (EEV$_E$) of the emulsified microcapsules formed with different polysaccharide/protein mixtures as wall materials. EEV$_E$ can be improved dramatically with increasing protein fraction. Protein molecules can be adsorbed directly on the surface of oil droplets as the first layer with its hydrophobic part through hydrophobic interactions, whereas polysaccharide can associate with protein through electrostatic attraction, hydrogen bonds, or van der Waals forces.[22] When the surface of oil droplets was occupied entirely by protein, the excess protein molecule minimally improved the EEV$_E$, resulting in a plateau. Moreover, the EEV$_E$ of the LMP/WPI and GA/WPI systems increased relatively faster than that of the LMP/G and GA/G systems, indicating that the emulsifying and binding ability of WPI with oil was stronger than that G and the association and entrapping capacity of polysaccharide/WPI was higher.[24,25] The microcapsules with a high EEV$_E$ at the polysaccharide/protein ratio of 1:5 were used in our subsequent measurements.
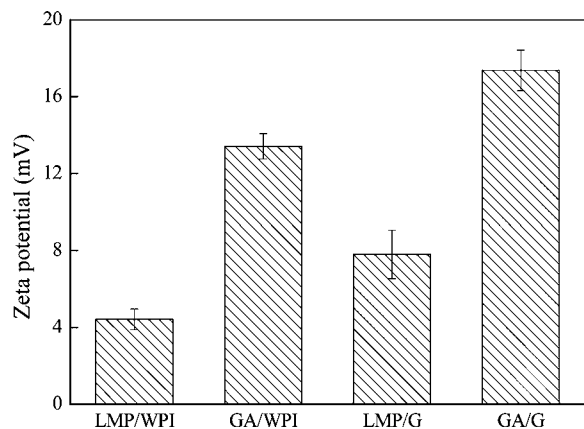
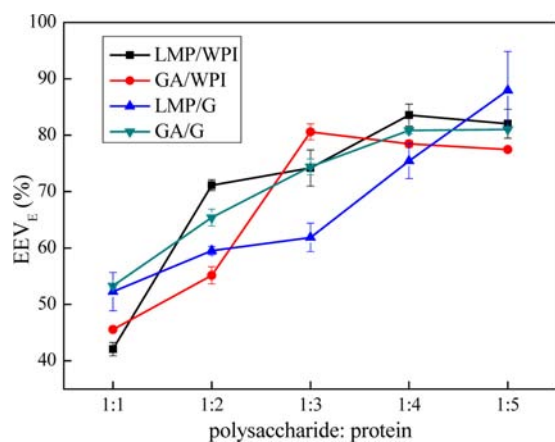**Figure 4.** Variations of the zeta potential of different polysaccharide/protein emulsions containing oil.

Figure 5. Variations of the EEV_E of the emulsified microcapsules versus polysaccharide/protein ratio.

Effect of Glutaraldehyde. At pH 3.5, the OESM was positively charged and the emulsified microcapsules can associate with these protein fibers through electrostatic attraction, hydrogen bonds, and van der Waals forces. Figure 6 shows the EEV_E of the combined OESM/microcapsule system versus the glutaraldehyde concentration. Glutaraldehyde is a pH-independent cross-linking agent.[26] The aldehyde
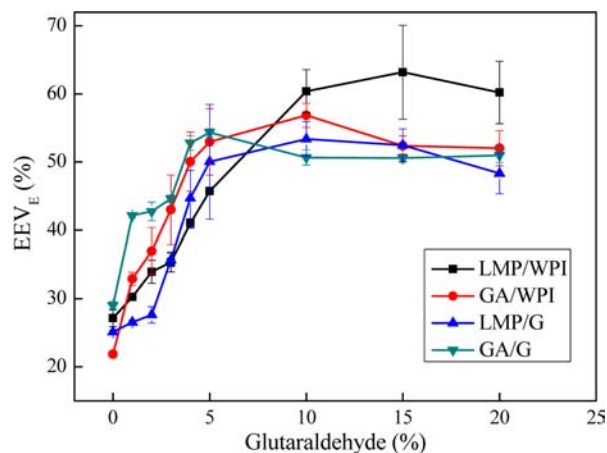

Figure 6. Variations of the EEV_E on OESM versus glutaraldehyde concentration.

groups of glutaraldehyde can react irreversibly with the ε-amine groups of lysine or the hydroxylysine residues of protein.[27,28] One aldehyde group of glutaraldehyde can anchor on the surface of the OESM, and the other can connect with the wall materials of the microcapsules. Without this substance, the values of EEV_E were only about 22−29%. By contrast, EEV_E can increase by >40% with 5% glutaraldehyde and reach a plateau in the present experimental conditions. β-Lactoglobulin, a major component of WPI, is a protein rich in lysine. This protein aided the WPI-containing system, particularly the LMP/WPI system, to have a better EEV_E than that of the G systems when the glutaraldehyde concentration was >10%. For comparison, the systems containing 10% glutaraldehyde were used in the subsequent measurements.

CLSM Micrographs. The CLSM micrographs of different combined OESM/microcapsule delivery systems are shown in Figure 7. The insets in these micrographs were the enlarged
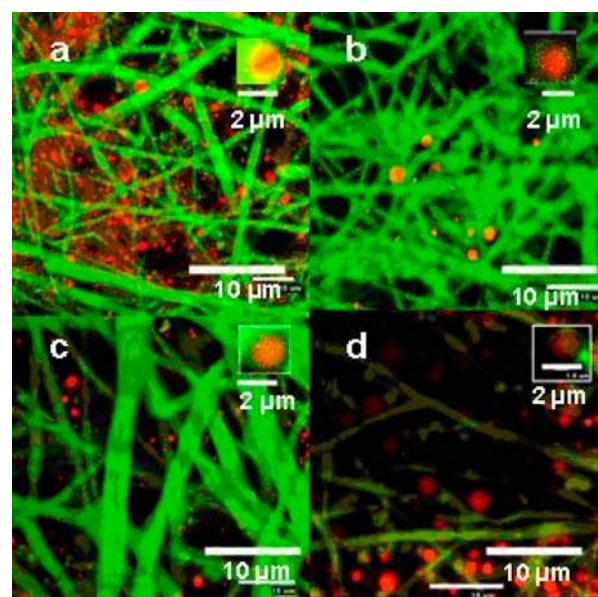

Figure 7. CLSM micrographs of different combined OESM/microcapsule systems: (a) LMP/WPI; (b) GA/WPI; (c) LMP/G; (d) GA/G.

images of each typical emulsified microcapsule. The V_E oil was stained with Nile red, and the proteins and polysaccharides were stained with green FITC. The diameter of the OESM fibers varied between 1 and 5 μm, and the network microstructure had a high porosity, as shown in Figure 7. These observations were in accordance with previous studies.[2,29] A large amount of spherical microcapsules was attached to OESM fibers and dispersed in the cavities of the network. The oil cores of the microcapsules were wrapped with a biopolymer shell, and the microcapsule sizes ranged from 2 to 3 μm.

Controlled Release Properties. The V_E release properties of the free microcapsules and the combined OESM/microcapsule system in different release media are compared in Figures 8 and 9, respectively. The fitting results of eq 4 are shown in Tables 1 and 2.

The values of X were generally lower at pH 1.2 or 7.4 without digestive enzymes than those with enzymes, as shown in Tables 1 and 2. The low values of X indicated relatively complete network microstructures of the systems, whereas
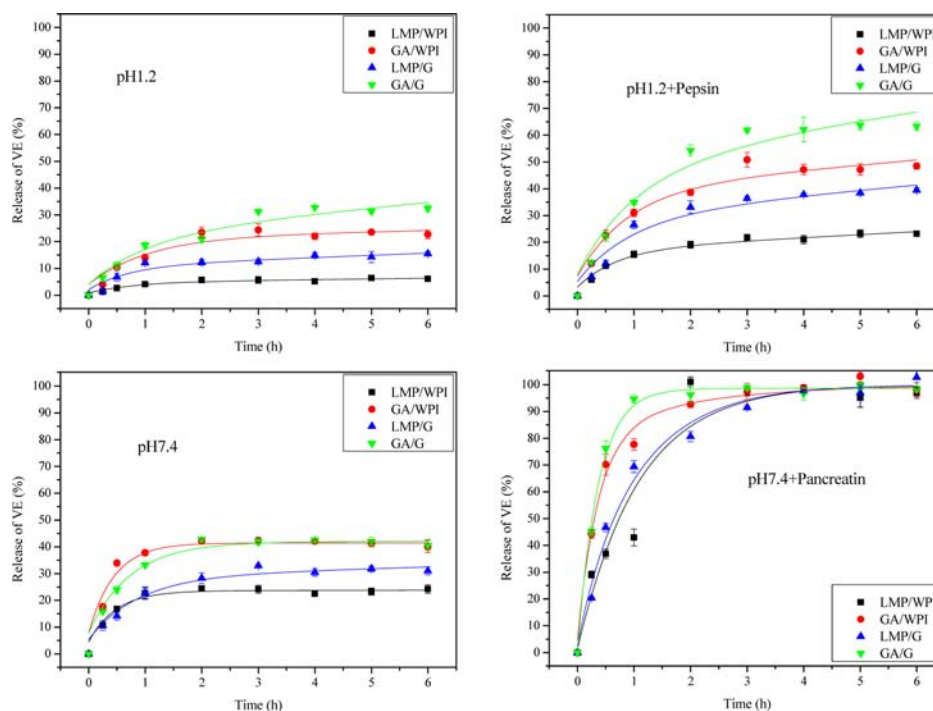
Figure 8. Release profiles of the emulsified microcapsules in simulated gastric and intestinal fluids.
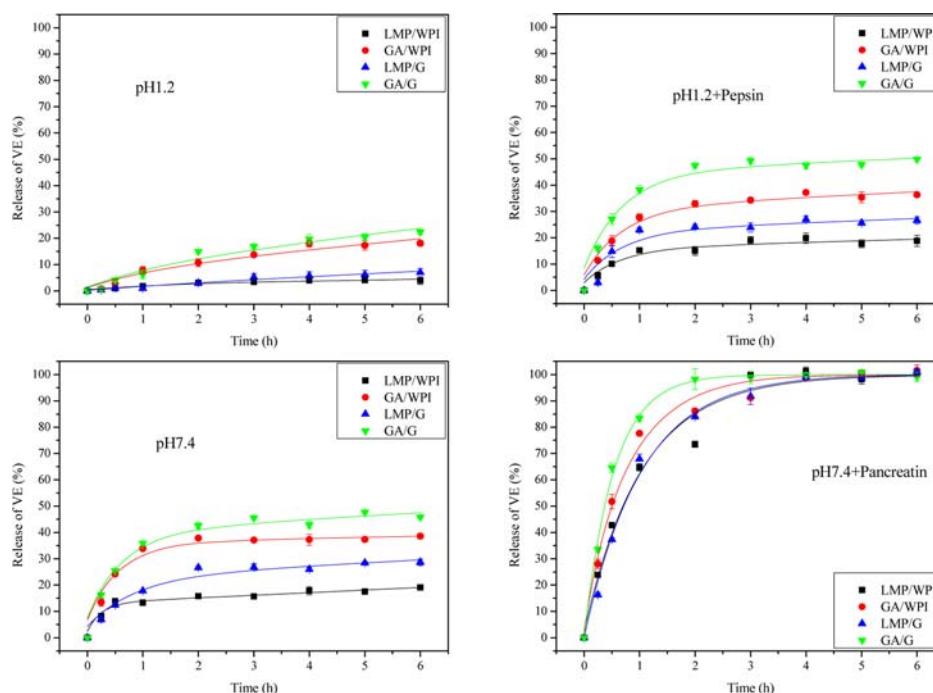

Figure 9. Release profiles of the combined OESM/microcapsule system in simulated gastric and intestinal fluids.

higher values indicated damage to the microstructure from enzymatic attacks. The values of k_F related to the Brownian behaviors of released V_E were roughly in the same order of magnitude regardless of the environmental pH or the presence of enzymes. The values of k_F were 1.4 ± 0.5 h^{-1} for free microcapsules and 1.3 ± 0.4 h^{-1} for combined OESM/microcapsule system. By contrast, the change of k_R related to the diffusion of V_E in the polymer network was relatively large because of the amount of damage to the microstructure and the nature of the wall materials.

At pH 1.2 and 7.4 without digestive enzymes, the V_E release increased and reached a plateau in 1−2 h. The release of V_E at pH 1.2 was always lower than that at pH 7.4 for both the pure microcapsules and the combined system. The higher V_E release at pH 7.4 was mainly attributed to the loose shell structure of the wall materials caused by the intermolecular electrostatic repulsions between the negatively charged polysaccharide and protein molecules. This assumption was consistent with the fitting results that the values of X and k_R for pH 1.2 were lower than those for pH 7.4.

Table 1. Results of Fitting the Linear Superposition Model to the Experimental Data of the V_E Release from the Emulsified Microcapsules

release medium	sample	R^2	X	k_F (h^{-1})	k_R (h^{-1})
pH 1.2	LMP/WPI	0.944	0.048	1.251	0.003
	GA/WPI	0.920	0.213	0.959	0.006
	LMP/G	0.916	0.106	1.468	0.010
	GA/G	0.953	0.211	0.984	0.032
pH 1.2 + pepsin	LMP/WPI	0.970	0.170	1.453	0.015
	GA/WPI	0.954	0.386	1.134	0.037
	LMP/G	0.949	0.284	1.087	0.034
	GA/G	0.961	0.423	1.053	0.102
pH 7.4	LMP/WPI	0.949	0.225	2.272	0.004
	GA/WPI	0.882	0.369	1.869	0.008
	LMP/G	0.958	0.286	1.155	0.009
	GA/G	0.958	0.415	1.374	0.002
pH 7.4 + pancreatin	LMP/WPI	0.923	0.718	0.786	0.785
	GA/WPI	0.966	0.821	2.251	0.554
	LMP/G	0.966	0.740	0.973	0.624
	GA/G	0.957	0.912	2.410	0.805

Table 2. Results of Fitting the Linear Superposition Model to Experimental Data of the V_E Release from the Combined OESM/Microcapsule System

release medium	sample	R^2	X	k_F (h^{-1})	k_R (h^{-1})
pH 1.2	LMP/WPI	0.956	0.025	0.794	0.003
	GA/WPI	0.955	0.069	0.837	0.025
	LMP/G	0.966	0.013	0.593	0.011
	GA/G	0.960	0.076	0.811	0.032
pH 1.2 + pepsin	LMP/WPI	0.941	0.159	1.511	0.007
	GA/WPI	0.964	0.309	1.446	0.017
	LMP/G	0.899	0.224	1.462	0.011
	GA/G	0.960	0.445	1.443	0.018
pH 7.4	LMP/WPI	0.882	0.131	1.750	0.012
	GA/WPI	0.953	0.358	1.762	0.007
	LMP/G	0.957	0.227	1.167	0.015
	GA/G	0.963	0.393	1.617	0.024
pH 7.4 + pancreatin	LMP/WPI	0.967	0.693	0.806	0.806
	GA/WPI	0.969	0.752	1.382	0.602
	LMP/G	0.963	0.709	1.073	0.636
	GA/G	0.963	0.873	1.614	0.964

The addition of pepsin or pancreatin can greatly accelerate the release of V_E in both systems because of enzymolysis. However, at pH 1.2 with pepsin, the protein shell can be reinforced by adsorbing a second negative polysaccharide layer through the electrostatic attraction. The aggregates associated by these biomacromolecules can form a barrier that can effectively block the penetration of digestive enzymes,[11,12] whereas in the presence of pancreatin, V_E release dramatically increased in the first 1 h and then, gradually, until a maximum of nearly 100% was attained after 3 h. The relatively higher values of X and k_R indicated that the network microstructures were seriously damaged by enzymolysis and more V_E oil was

released. These data clearly indicated that the prepared microcapsules also had the ability to retard core release in simulated gastric conditions and undergo enteric liberation of the core in simulated intestinal conditions, similar to other polysaccharide/protein complexes.[21,30] In addition, the release of V_E from the combined OESM/microcapsule system was slower than that from free microcapsules because of the blocking effect of the highly cross-linked network of OESM. The network of OESM can well hinder the migration of the oil core from microcapsules to the release medium, which hampers the release of V_E. The hindrance of diffusion via polymer obstruction was similar to that observed in the cellulose/chitin blend membranes.[31] Notably, the microcapsules and combined system formed by LMP/protein as wall material exhibited better controlled release properties than those of GA/protein. These results were consistent with the zeta potential measurements. Higher association capacity between LMP and protein resulted in a thicker shell of microcapsules and a slower release of the core.

Therefore, the OESM and microcapsules can both serve as efficient delivery vehicles for active ingredients because of their cavity microstructure. The polysaccharide/protein microcapsules incorporated with V_E were adsorbed on OESM covalently using glutaraldehyde as a cross-linking agent to improve the functions of the delivery systems, such as encapsulation effect, controlled release ability, and applications. Both the free microcapsules and combined OESM/microcapsule system retarded V_E release in simulated gastric conditions and underwent enteric liberation in simulated intestinal conditions. However, the combined OESM/microcapsule system exhibited a more remarkable effect on controlled release property because of its steric blocking effect. Compared with GA, the combined system formed by pectin/protein as wall material of the microcapsules showed better encapsulation efficiency and more resistance against enzymatic attacks. This phenomenon may be attributed to the higher association capacity and compact aggregate shell formed between pectin and proteins.

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Notes
The authors declare no competing financial interest.

■ ABBREVIATIONS USED

CLSM, confocal scanning laser microscopy; EE, encapsulation efficiency; ESM, eggshell membrane; FITC, fluorescein isothiocyanate; G, gelatin; GA, gum arabic; IESM, inner eggshell membrane; LMP, low-methoxyl pectin; OESM, outer eggshell membrane; PGPR, polyglycerol polyricinoleate 90; pI,

isoelectric point; SEM, scanning electron microscopy; V_E, vitamin E; WPI, whey protein isolation

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