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All-Natural Oil-Filled Microcapsules from Water-Insoluble Proteins

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The formation and characterization of a novel class of all-natural digestible microcapsules containing a liquid lipid core encapsulated by a water-insoluble protein shell with tunable thickness is demonstrated. As an example of a water-insoluble protein, zein is used—the protein of corn—which is an attractive biomaterial from a sustainable source. The microcapsules are prepared by a direct and simple method, based on the precipitation of protein from the continuous phase of an oil-in-(water/ethanol) emulsion onto the oil droplets without the need of any surfactant. The shell thickness can be controlled by the amount of precipitated protein. An in vitro digestion assay is performed to study the lipid hydrolysis and biodegradability. The rate of lipid hydrolysis and release of fatty acids are highly dependent on the protein shell thickness. All-natural edible microcapsules with controlled degradation under gastrointestinal conditions can enable new applications for oral delivery systems. They may further be used as a model system for controlled release studies of lipophilic compounds and could promote the sustainable use of underutilized water insoluble proteins as functional biomaterials.

1. Introduction

Microcapsules play an important role in the formulation, delivery and controlled release of functional materials in medicine,[1] biotechnology,[2] cosmetics,[3] food,[4] agriculture[5] and

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in advanced applications such as heat transfer[6] and self-healing materials.[7] Liquid-core microcapsules are usually made by in situ reactions, using particle or surfactant stabilized emulsions as a template to produce polymeric or inorganic shells through interfacial polymerization,[8] self-assembly,[9] coacervation by evaporation of a good solvent,[10] or chemical condensation processes.[11] Capsules can also be prepared by solidification of the middle phase, which becomes the shell, of a water-in-oil-in-water double emulsion template.[12] Many of these synthesis routes, however, may suffer from lack of biocompatibility due to the chemical modification or presence of surfactants, which may limit their use in oral delivery applications. When pharmaceutical or edible products are required, further restrictions on synthesis and materials apply. Since encapsulation of oil and oil-soluble ingredients is of particular interest to current food,[13] drug[14] and personal care industries, the formation of well-defined biodegradable, biocompatible and edible oil-filled core-shell microcapsules is in high demand. Furthermore, microcapsules whose release location can be influenced through the rate of digestion of the shell can be convenient delivery systems with potential therapeutic and nutritional value.

The use of natural biopolymers from natural sustainable sources[15] for new, advanced materials is a rapidly developing field. Their use for designing core-shell structures is attractive for encapsulation and controlled release,[16] and proteins in particular are highly desirable materials for microencapsulation. However, most efforts have focused on the more naturally ubiquitous water-soluble proteins. Their use as shells relies on their affinity for (usually reversible) adsorption at the oil-water interface as mono-, bi- or a few of layers, which limits their use as sufficiently thick shells. In order to be used in the predominantly aqueous environment, they often require physical (e.g. complex coacervation)[17] or chemical cross-linking (e.g. enzymatic[18] or non-enzymatic[19] cross linking) or coupling with synthetic polymers[20] to prevent their dissolution or control their biodegradability. While these approaches are being widely explored, the resulting particles often suffer from pH sensitivity and loss of food grade status.

Water insoluble proteins, such as zein, a prolamin found in corn, have unique solubility features that make them a potential

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candidate for designing biocompatible and biodegradable delivery systems for functional ingredients.[21] Characterized by its high proline content and its almost equal amount of hydrophilic and hydrophobic amino acid residues,[22] zein is water insoluble.[23] However, once in binary solutions of water and a lower aliphatic alcohol, such as ethanol, it becomes soluble. The solubility of zein in water-ethanol mixtures is known[22] from earlier attempts to commercialize it as a coating material for drug tablets and foods.[24] Its precipitation can be controlled by several parameters such as temperature,[25,26] protein concentration[27] and the presence of stabilizers such as surface active molecules and polymers.[28–30] In the past, various zein-based colloidal particles have been reported for nutraceutical[29] and drug delivery,[31] for emulsion stabilization,[32] design of colloidal pigments[33] and composite particles comprising encapsulated oil in the protein matrix.[13,26]

Herein, we demonstrate the synthesis of structurally well-defined oil-core zein-shell microcapsules using a one-step heterogeneous precipitation process. Due to the core-shell morphology, large amounts of oil can be encapsulated in a controlled and reproducible fashion, while shell thickness is easily controlled by the amount of precipitated zein at certain oil volume fraction. Finally, we illustrate the effect of shell thickness on biodegradability and lipid hydrolysis with an in vitro digestion assay.

2. Results and Discussion

2.1. Mechanism of Formation and Microcapsule Characterization

The schematic representation of the process used for the preparation of the microcapsules is shown in **Figure 1**. The oil is added and emulsified in the zein-ethanol-water solution under constant mixing, a rapid process, which takes a few seconds. While we continue the intense mixing, water is slowly added to decrease the solubility of zein, a comparatively slow process which takes tens of minutes. As the solubility of zein in the ethanol-water phase decreases, zein starts to precipitate on the droplet surface forming a shell around the oil core. Thus, the mechanism of shell formation is based on nucleation of zein at the oil interface and growth by zein precipitation from the bulk water-ethanol phase.

In the absence of oil, addition of water to the initial zein solution results in homogeneous precipitation and the
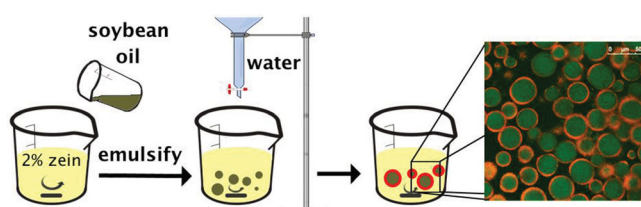

Figure 1. Schematic illustration of the formation of the oil-core zein-shell microcapsules: emulsification of the oil in a 2% or 3% w/v zein solution in 60% v/v aqueous ethanol and continuous mixing thereafter, during which slow water addition decreases the solubility of zein. The zein precipitates on the droplet surface forming a shell around the oil core as shown schematically and at a confocal image.
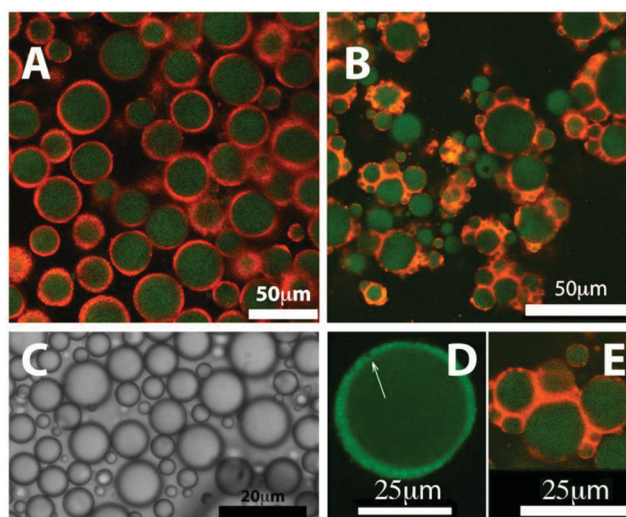

Figure 2. Confocal (A,B; D,E) and light microscopy (C) images of microcapsules in water made from 2% initial zein solution and different O/Z ratios at the final stage after the addition of water and ethanol evaporation. (A) Nile blue stained thick shell capsules with O/Z ratio 1.75. Green corresponds to the oil environment, red to the zein environment. (B) Nile blue stained aggregated capsules formed with O/Z 7.8. (C) Light microscopy image of the capsules shown in (A). (D) Nile Red stained microcapsule. Arrow points to a possibly oil-pocket inclusion. (E) Detailed image of an O/Z 7.8 aggregated capsule with thin zein shells.

formation of solid particles of pure zein. In the presence of oil, an emulsion is formed and as a result zein preferentially nucleates at the oil-(ethanol/water) interface. If there is sufficient zein, a complete shell around the oil droplets is formed, which in turn grows thicker due to further deposition of zein from the bulk (**Figure 2**). If there is insufficient zein available, encapsulation of all the oil droplets by a complete shell is impossible. We do not find significant amounts (i.e. not visible in confocal microscopy) of pure zein particles alongside the oil-covered zein microcapsules, which in principle can be formed due to secondary nucleation and growth in the bulk phase. The effect of the rate of water addition was not investigated further.

Keeping the initial concentration of zein in ethanol/water low (2% and 3% w/v), we mix several parts of the zein solution with one part soybean oil. We report the data as the mass ratio of the oil-to-zein (O/Z). As expected, without oil, solid zein particles are formed. Increasing the O/Z ratio to 0.6 results in the formation of oil-filled particles with core-shell morphology and small amount of zein particles formed from the excess zein available for precipitation. Further increase of the O/Z to 1.75 (Figure 2A, C, D) results in only oil-core zein-shell microcapsules with a well-defined spherical shape. A larger increase to O/Z 3.01–4.5 results in core-shell spheres with a thinner shell. Further increase of O/Z to 5.27–7.8 not only leads to thinner shells, but results in a change in morphology from spheres to aggregated structures of intact oil-filled spheres, presumably because the oil droplets have a small separation due to the increased oil volume fraction and come into contact during mixing. Increasing the O/Z to 9, leads to failure of encapsulation of all the oil which in turn phase separates.
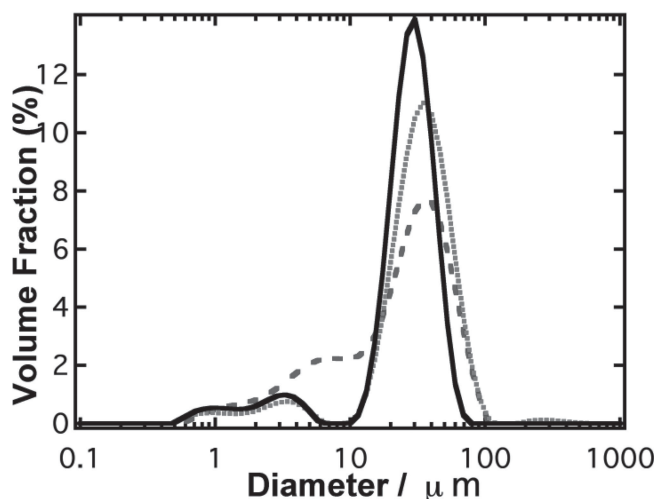
Figure 3. Volume averaged size distribution of the microcapsules in water. Oil-to-zein ratios: 1.75: solid, 4.5: dashed, 7.8: dotted. The O/Z 1.75 curve corresponds to an initial 3% zein mixture, while the other two to a 2%. For identical preparation conditions, the particle size distribution is similar.

2.2. Microcapsule Size

The microcapsules that successfully encapsulate oil for the O/Z range 1.75 to 4.5 are spherical, as defined by the templating action of the droplets, but due to the preparation method polydisperse (**Figure 3**). The particle size distribution is similar among different O/Z ratios since the size distribution is dictated by the emulsifying conditions.

The average hydrodynamic diameter peaks at 30–40 μm with the majority of the capsules sized between 20–100 μm. In all cases we observe the presence of a fraction of smaller capsules. Those can be the candidates for the oil inclusions observed in the zein shells of larger capsules (see Figure 2D and Movie S1).

2.3. Shell Characterization

In this work, shell thickness is controlled via the O/Z ratio during the precipitation process. Since the average oil droplet size is mostly determined by the mixing conditions, increasing the amount of precipitated protein increases the shell thickness if no secondary precipitation occurs in the bulk. In principle, the shell thickness could also be controlled by changing the average oil droplet size during mixing while keeping the zein amount constant, an option not explored in this work. **Table 1** summarizes the results of the observations regarding the microcapsules and their shell at various O/Z ratios as determined from direct imaging of the microcapsules using confocal microscopy.

The microstructure of the zein shells was revealed under confocal microscopy

Table 1. Summary of the microcapsule shell structure and overall morphology.

O/Z ratio	Shell thickness ± stand. deviation [μm]	Particle structure
0	no shell	solid spheres
0.60	not full encapsulation	solid/core-shell
1.75	4 ± 0.7	core-shell
3.01–4.5	3 ± 0.7	core-shell
5.27–7.8	1.5 ± 0.6	aggregates of core-shell particles
9	failure to encapsulate	phase separation

and scanning electron microscopy (SEM). From the confocal imaging (Figure 2D) it is evident that the shells contain oil in form of spherical inclusions, which could be the smallest particles becoming incorporated in the shell. The SEM imaging of the air-dried microcapsules (**Figure 4**A) shows that they are indeed spherical with a rough and oil-filled porous shell. Upon drying, the capsules are driven together by the capillary forces, but the shells remain intact indicating they can withstand mild forces. For the air dried capsules of Figure 4A, the oil is within the intact core of the capsules. What appears as capillary bridges could be oil that has permeated the dried porous shells due to its lowered viscosity because of heating due to the beam. Features in the order of 0.1–1 μm in the porous shell microstructure were revealed after oil removal (Figure 4B–D). The formation of such porous structures has also been observed in the preparation of other polymeric core-shell microspheres.[34]

A simple calculation can be done to demonstrate the effect of the zein concentration and the O/Z ratio on the shell thickness, which is measured from the confocal images (**Figure 5**). Experimentally known are the values of O/Z mass ratio, the average (hydrodynamic) oil drop radius R and the shell thickness δR
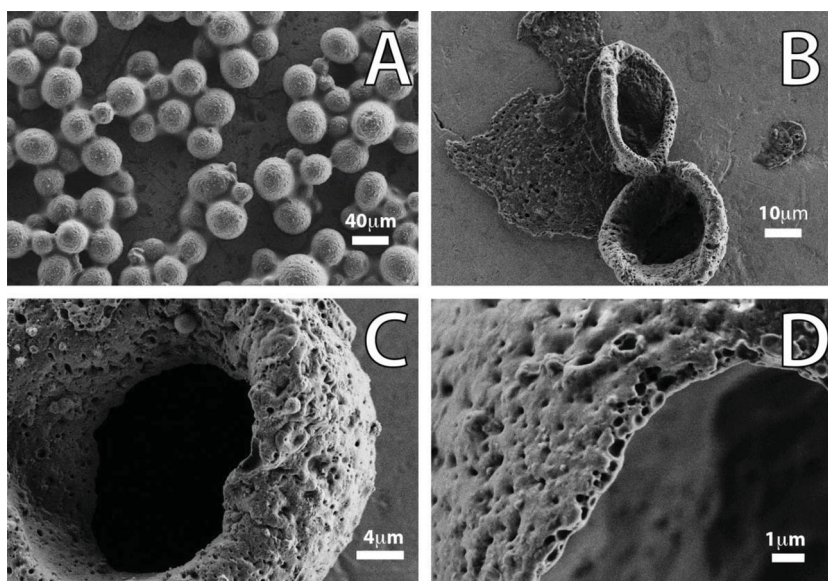

Figure 4. SEM images of the microcapsules. (A) Their size corresponds well to the one measured by light scattering. Dried in ambient conditions, they remain intact. (B–D) Their shell with inclusions 0.1–1 μm is evident. Images obtained after oil removal.
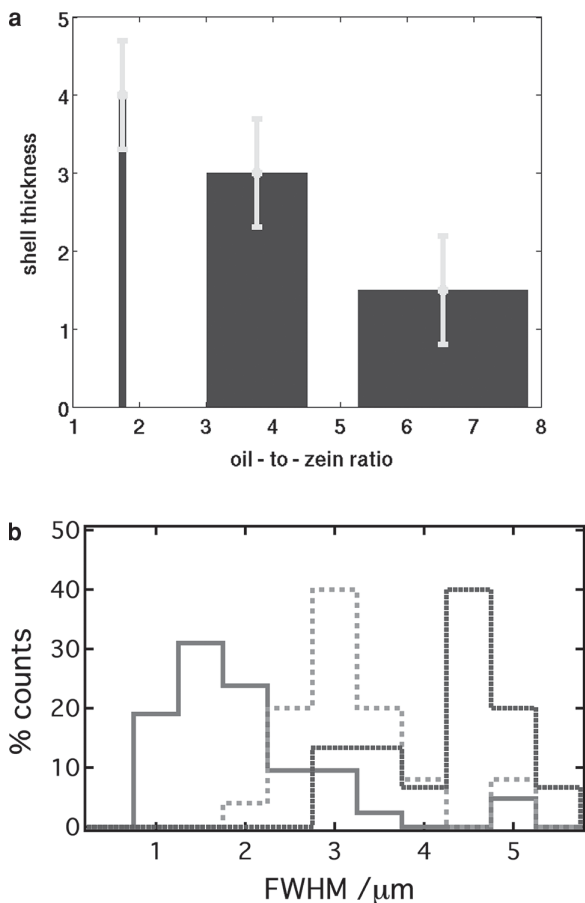
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Figure 5. Results from measuring the shell thickness from the confocal images (a) Average shell thickness δR as a function of the O/Z ratio. (b) Distributions of the shell thicknesses for the different types of particles: O/Z: 7.8 solid, 4.5 dashed, 1.75 dotted.

as measured from confocal images. Assuming the oil is in the core and the zein is present only in the shell, we can write the mass of zein in the shell

$$m_{zein} = \rho_{zein}^{shell} V^{shell} = \rho_{zein}^{shell} 4\pi R^2 \delta R$$

and the oil-to-zein mass ratio

$$\frac{m_{oil}}{m_{zein}} = \left(\frac{O}{Z}\right) = \frac{\rho_{oil}}{3\rho_{shell}}\frac{R}{\delta R}$$

From the above equation, assuming a fixed shell density, the $R/\delta R$ fraction can be estimated. However, the estimated value, if one assumes that the overall shell density is fixed, gives $\delta R^{(4.5)}/\delta R^{(1.75)} \approx 0.5$ This value barely agrees with the experimental data $\delta R^{(4.5)}/\delta R^{(1.75)} = \frac{3}{4} \pm \frac{3}{4}\sqrt{\left(\frac{(0.7)}{3}\right)^2 + \left(\frac{(0.7)}{3}\right)^2} = 0.75 \pm 0.2$. As we have noted, increased O/Z leads not only to thinner shells, but to shells with fewer oil droplets inclusions. The explanation for the discrepancy between the prediction and the measured values for the shell thickness may be due to the reduced shell effective density by the included oil droplets and not just due to higher amount of zein available for building thicker shells.

2.4. Particle Stability

Left without stirring, the microcapsules cream within minutes due to their lower density dominated by the oil core and their large size. However the close packing in the cream does not affect their stability and after two years in storage, little free oil is seen on the surface and the dense creamed layer can be easily re-suspended by gentle mixing (see Figure S2). Responsible for the enhanced stability are not just the hard shells, but also the electrostatic interactions. In our case, the large size of the non-Brownian capsules, which cream during the measurement, prevents a simple zeta potential measurement by skewing the electrophoretic mobility. However, the existence of the Coulomb repulsion is measurable and has been reported[30,32] for pure zein colloidal particles. The zeta potential of the pure zein colloidal particles is approximately 40 mV at pH 5, indicating their electrostatic stability. We expect protonation of the zein side groups to take place, as there is a change of the pH of the original aqueous alcohol mixture from 5.1 to 3.8, well below 6.5 the isoelectric point of zein. The presence of surface charge prevents sticking of the particles when concentrated, as in the case of creaming.

2.5. Lipid Hydrolysis and Biodegradability

Lipids, oils such as triacylglycerides (TAGs) in soybean or fish oil, and numerous oil-soluble bioactive molecules (e.g. oil soluble vitamins) provide health benefits and it is desirable to deliver them in a controlled and targeted fashion in the body, as well as to incorporate them in aqueous formulations. Therefore, we would like to control the modulation of the rate of hydrolysis of the TAGs and release of the free fatty acids (FAs), and in general of lipophilic compounds,[35] which can have very complex phase behavior, while, retaining the structural properties of a product.[36] Zein is known for its slow digestibility by trypsin and pepsin,[13b] a property that makes it an appropriate choice as a degradable biomaterial for a shell. Modulating the shell thickness around the lipid droplets therefore can be used to affect the hydrolysis rate of TAG and the formation of free FAs.

We compared the hydrolysis rate of TAG of a surfactant-stabilized 1% soybean oil emulsion prepared at the same emulsification conditions, with two types of core-shell microcapsules: the ones with O/Z 1.75 (shell 4 ± 0.7 μm) and O/Z 7.8 (shell 1.5 ± 0.6 μm). In the control sample, the lipase action starts immediately to hydrolyze the TAG at a much higher initial rate as compared to the microcapsules (**Figure 6**) as there is very little protection.

However, in the core-shell case, the rate-limiting step is the full or partial zein digestion by the proteases pepsin and trypsin, during the gastric and intestinal phases respectively, which eventually allows access of the lipase to the oil phase. The zein shell effectively acts as a protective barrier for the oil phase and the shell thickness can be used to tune the mechanism of digestion. For the O/Z 7.8 thinner shell capsules, the release of FA initially starts slowly. At the gastric phase, the capsules appear morphologically identical to the original capsules (**Figure 7**A). However, pepsin may have weakened the shell. After the addition of bile salts and pH increase (Figure 7B), it appears that the weakened shell breaks, creating access to the
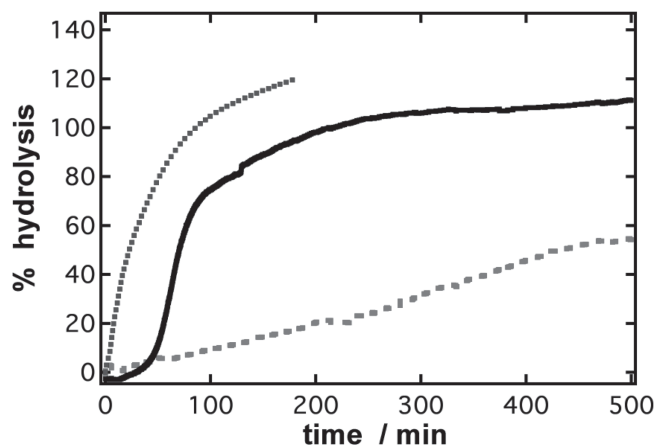
Figure 6. Dotted curve: Degree of TAG hydrolysis for 1% soybean oil surfactant stabilized oil-in-water emulsion. Solid curve: microcapsules with O/Z 7.8 (thinner shells). Dashed curve: microcapsules with O/Z 1.75 (thicker shells). 100% hydrolysis corresponds to two free fatty acids released from one triacylglyceride, while above 100% the hydrolysis of monoglycerides is measured.

oil. Thus the reaction accelerates to the same rate as the surfactant-stabilized oil emulsion. In the case of O/Z 1.75 capsules that have thicker shells, the digestion appears linear in time. An intriguing behavior indeed, suggesting a different mechanism of FA release. The lack of an abrupt increase in the hydrolysis rate indicates that the shells are not broken but rather remain semi-permeable for the enzymes and bile via their porous structure and oil that permeates them. The reaction continues with a steady slow rate, perhaps limited by diffusion. To validate our hypothesis, we look at the capsule morphology during the gastric and intestinal phases of the digestion for capsules with O/Z 7.8 and 4.5.

From a microscopic point of view, the low pH gastric phase does not affect particle morphology: the particles remain charged and stable for all O/Z ratios. The shells appear intact and the oil still encapsulated (Figure 7a,c,e), however the pepsin may have partially weakened the shells as they appear deformed in the beginning of the intestinal phase. The thicker shells (O/Z 1.75) show less deformation and more oil is stored in the shell compared to the thinner ones (O/Z 4.5). Note that in the control experiment, a pH increase with addition of bile salts or proteases in the original microcapsule dispersion in water results in no significant breaking of the microcapsules or oil release. The confocal data suggest that the presence of bile, which contains powerful bio-surfactants (i.e. bile acids), and pepsin action have degraded the shells enough to be softer and easily breakable. Zein is known to solubilize in the presence of strong surfactants,[37] a property that can be used to tune the release. The encapsulation and release of model bioactive oil-soluble compounds may be studied in a precise and quantitative manner, but falls outside the scope of this paper.

3. Conclusions

We demonstrate a one-step synthesis of all natural, edible oil-core zein-shell microcapsules using a plant-derived water
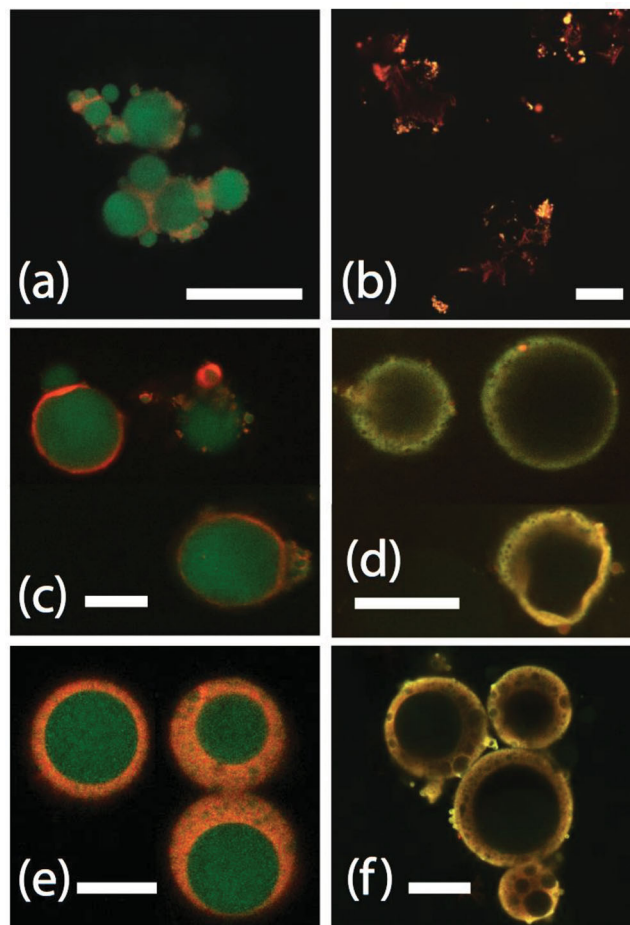

Figure 7. Behavior of the microcapsules with O/Z 7.8 (a,b), O/Z 4.5 (c,d) and O/Z 1.75 (e-f) during the gastric (left panels) and the beginning of the intestinal phases (right panels). All capsules appear intact at the low pH gastric phase, but a different behavior is observed in the beginning of the intestinal phase, a few minutes after bile addition and before pancreatin addition. The thin shells appear to break and release their contents. The thicker shells appear to have been deformed and while most of the captured oil is released, oil has largely permeated the shell from where is it slowly released. In (b, d and f) note the superimposed zein (red) and oil (green) indicating the oil still trapped in the shell. Scale bars 25 μm.

insoluble food-grade protein as shell material. We successfully do so without the need for chemical additives, surfactants, cross-linkers or any non-biocompatible materials. This is partly due to the choice of single step synthesis and partly due to the unusual properties of the zein protein. The shell is formed by direct protein precipitation on the oil-water interface and grows as the system is driven deeper in the precipitation region of the phase diagram by water addition, which acts as the antisolvent. The shell thickness can be controlled by the choice of mass ratios of zein and oil in the initial preparation as presented. Using an in vitro digestion method, we first demonstrate that the capsules pass intact from the gastric phase of the digestion and eventually the oil gets digested at the intestinal phase. We also demonstrate that a small difference in the shell thickness (1.5 versus 3 μm) strongly affects the hydrolysis rate of TAG and the release of free FAs due to a different mechanism of access to oil.

A specific advantage of this system is its natural origin and edible nature, which is critical for a variety of industrial applications as well as for the advancement of technologies based on sustainable biomaterials. Possible extensions of the presented zein-coated microcapsules study could be the quantification of the release profiles of oil soluble compounds, their test application for encapsulation and delivery, the use of other water insoluble proteins which have similar properties such as gliadin, and the creation of monodisperse microcapsules with the use of microfluidics.[38] Possible extension to smaller nanoscale core-shell particles can be made via the use of surfactant-stabilized nanoemulsions. Furthermore, the ability to design all natural edible microcapsules with controlled degradation in gastrointestinal conditions opens new opportunities for them as oral delivery systems.

4. Experimental Section

Materials: Zein from corn (Z3625), soybean oil (S7381) and acetone were obtained from Sigma Aldrich. Ethanol was obtained from VWR BDH Chemicals. Ultrapure water (MilliQ, Millipore was used in all experiments.

Particle Synthesis: Stock solutions of 2% w/v and 3% w/v zein in 60% v/v aqueous ethanol were prepared. Depending on the targeted shell thickness, 5 to 10 parts in weight of the stock solution were mixed with 1 part soybean oil. The mixture was emulsified with a Silverson Laboratory L4RT-A mixer at 4500 rpm. Since no surfactant was added, constant stirring was necessary to prevent the oil drops from coalescing. In the meanwhile, water was added drop-wise from a separation funnel at an approximate rate of 25 mg s^{-1} for our samples of 150–200 g. The temperature of the emulsion was controlled with a water bath to 40 ± 2 °C for the 2 hours of the synthesis. When the volume of the added water reaches twice the initial volume, the point on the zein-water-ethanol phase diagram has moved from the solution phase, deep into the precipitation phase both due to the addition of water and the partial evaporation of ethanol. At that point, we stop the process and evaporate any excess ethanol in a rotary evaporator.

Static Light Scattering: The particle size distribution was determined by static light scattering using a MasterSizer (Malvern Instruments Ltd.) on dilute particle suspensions in water at 25 °C. Each particle was assumed to be a solid sphere in water with an effective refractive index of 1.5. We assumed that zein and soybean oil have similar refractive index, which was confirmed by holographic video microscopy.

Confocal Laser Scanning Microscopy: For fluorescent imaging, we added Nile Blue or Nile Red (10^{-3} parts of 1% fluorophore in water solution). Fluorescent images of the particles were obtained on an inverted laser-scanning confocal microscope (Leica TCS-SP5 with the DMI6000) with excitations at 488 nm and 633 nm probing the Nile Blue local environment of oil and protein, respectively. For the measurement and comparison of the shell thickness, we select the typical sized particles in the range of 20–50 μm in the confocal images and we fit their intensity profile with a Gaussian. We report the full-width half maximum (FWHM) of the Gaussians as the thickness.

Scanning Electron Microscopy (SEM): Particle morphology was determined by field-emission SEM using the Merlin SEM by Carl Zeiss. Two preparations were made: for the first, microcapsules from the aqueous suspension were let to dry at ambient temperatures. For the second, microcapsules in water were dried at 50 °C, then redispersed in pure acetone which is not a good solvent for zein, were sonicated and washed to promote breakage and removal of the oil. The dispersion was let to dry at ambient temperature.

In vitro Degradation Rate: The rate of degradation of the microcapsules in biological conditions was investigated using an in vitro hydrolysis assay for triacylglyceride (TAG) conversion to free fatty acids

(FAs), simulating the in body gastric and intestinal phases of human digestion. For the gastric phase, the particulate aqueous solution (3 mL) was added to saline (18.5 mL with composition 150 mM NaCl, 5 mM KCl, 5mM CaCl$_2$) along with CaCl$_2$ (6 mL of 100 mM) and pepsin (2 mL of 30 mg L^{-1}) dissolved in 0.1 M HCl at 37 °C. The pH was brought to 1.3 by HCl addition followed by incubation and mixing for 15 min. For the intestinal step, the rate of FA release was measured by the pH-stat method using the DL 21/25 titrator (Mettler Toledo). We manually adjusted the pH near 7, added 0.5 g bile salt in 4.5 mL buffer (40 mM NaCl, 20 mM CaCl$_2$, 5 mM Tris) and started the pre-titration to pH 7.5 using 0.1 M NaOH as the titrant. The hydrolysis was started by the addition of pancreatin from porcine pancreas (30 mg, Sigma, P8096). For the calculations of the degree of hydrolysis, we used 872.5 Da as the molecular weight of the soybean TAG and assumed that two free FAs are produced per one TAG molecule. To correct for the amount of titrant used to deprotonate zein side groups in the absence of oil, we repeated the digestion experiment with dispersed zein flakes and solid zein colloidal particles in water and used this as the baseline. A 1% soybean oil-in-water emulsion stabilized with a non-ionic surfactant (Tween 20) hydrolyzed under the same conditions was used as a reference for comparison.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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