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Encapsulation of Oil in Silk Fibroin Biomaterials

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ABSTRACT: Microencapsulation is becoming increasingly important in the food, cosmetics, and medicinal industries due to its potential for stabilization and delivery of volatile and delicate compounds. Novel food-safe techniques for encapsulating oil in silk biomaterials using emulsion-based processes that exploit silk's unique properties (including amphiphilicity, biocompatibility, aqueous and ambient processing, and tunable physical crosslinking behavior) are described. The sonication-induced self-assembly of silk previously applied to hydrogel fabrication replaced the use of the thermal or chemical suspension crosslinking traditionally used to stabilize the aqueous protein phase in emulsions. Stable, physically crosslinked silk micro- and macro-particles loaded with oil or water-soluble dye were produced by aliquoting sonicated silk solutions into an oil bath. Oil micro-droplets emulsified in aqueous silk solutions did not impede the self-assembly of silk into films or hydrogel networks. In O/W/O emulsions, particle morphology and silk permeability to a model lipophilic dye in the interior phase were controllable via processing. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 39990.

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INTRODUCTION

Long used in the pharmaceutical industry to improve drug bioavailability, stabilize drugs against various degradation pathways, minimize side effects or modify drug release kinetics, encapsulation techniques have gained increased attention in other fields, particularly food[1,2] and fragrances.[3,4] Lipids, though useful both as ingredients in food products and as solvents for hydrophobic substances, are generally difficult to disperse in aqueous media and can be susceptible to auto-oxidation.[5] Carotenoids in food products also suffer from susceptibility to degradation, which lowers the final nutritional properties of products.[5] Though sustained presence of volatile fragrances in consumer products is desirable because it is associated with a feeling of pleasantness or cleanliness, the volatility of fragrances prevents persistence over long time frames.[3,4] Flavors can be the most valuable ingredients in food products, but these precious compounds are usually highly volatile and chemically unstable, degrading in the presence of air, light, moisture, and high temperatures.[2,6–10] Techniques to reduce the loss of volatile compounds and to sustain the presence of fragrances in consumer products and protect and stabilize flavors, lipids, and other sensitive food additives would therefore greatly benefit the food and fragrance industries.

Microencapsulation of flavors and fragrances in carrier matrices has potential in the food and cosmetics industries because it can provide protection against degradation, prevent loss of volatile compounds, increase shelf-life and/or allow controlled release.[2] Microencapsulation defines a process in which tiny particles or droplets are surrounded by a protective coating layer, or embedded within an encapsulating matrix or membrane, providing a physical barrier between the incorporated compound and the surrounding environment.[2,5,8,10] Encapsulation protects sensitive fragrances and flavors from deterioration by shielding them from degradative conditions such as oxygen, moisture, temperature, and light.[6,7,9,10] The barrier that the encapsulating material provides can also delay evaporation of volatile compounds,[11] particularly if the compound interacts with the encapsulant material.[5] Microencapsulation can also improve dispersion of oils and oil soluble ingredients (which would otherwise be immiscible) in aqueous environments.[5,9] In addition to stabilizing and protecting encapsulated flavors, fragrances, and oils, encapsulation may also be able to provide controlled release under desired conditions.[5] Controlled release may be defined as a method by which one or more active agents or ingredients are made available at a desired site and

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time and at a specific rate. This precise timing and targeting of release could be used to maximize a given compound's effectiveness and optimize dosage.[3,4,7,12]

Various encapsulation materials and techniques have been previously investigated.[1,2,5,7,12] Requirements for encapsulation materials are summarized in Table I.

Many biopolymers have been used for the microencapsulation of various food ingredients, including natural gums (gum arabic, alginates, and carragenans), proteins (milk or whey proteins, gelatin), maltodextrins with different dextrose equivalences, and waxes and their blends.[5] Proteins are especially attractive encapsulant materials because their physicochemical properties (including amphiphilic character, ability to self-associate and interact with a variety of substances, high molecular weight, and molecular chain flexibility) provide excellent functional properties for encapsulation (including solubility, viscosity, emulsification, and film-formation).[2,5,8,13] During emulsion formation, protein molecules are able to act as emulsifiers by rapidly adsorbing at the newly formed oil–water interface, forming a steric-stabilizing layer.[2,13,14] Proteins also show high binding capacity for many flavor and fragrance compounds.[5,8] Gelatin is one of the most commonly used encapsulation materials, but suffers from serious drawbacks that limit its widespread use. Gelatin is highly viscous even in low concentrations, possesses low solubility in cold water, and glutaraldehyde (the chemical used to cross-link gelatin) is toxic to humans.[11,15] In addition, concerns regarding the safety of animal-derived proteins have increased in response to the recent emergence of diseases such as the prions.[16]

Though many materials have been proposed for encapsulation in food, cosmetic, and medicinal applications, silk fibroin is an especially attractive encapsulant material due to its unique array of chemical and physical properties. Silk fibroin is a biologically derived protein polymer purified from the domesticated silkworm (*Bombyx mori*) cocoons that is FDA-approved, edible,[17,18] non-toxic, and relatively inexpensive.[19] Silk exhibits excellent mechanical properties, biocompatibility,[20–22] and biodegrades to non-toxic products via proteolysis.[23,24] Fibroin has been widely applied to cosmetics, food, and the chemical industry[25] and has recently been investigated as a scaffold for tissue engineering[26,27] and a drug carrier for controlled release.[28–30]

While other encapsulation approaches require processing conditions which can potentially degrade delicate compounds and/or compromise the food safety of the final product, such as exposure to high heat or the use of toxic cross-linking chemicals,[31–35] stable silk biomaterials can be prepared using mild, ambient, and aqueous processing conditions.[28,29] In particular, silk self-assembly into films occurs during drying at ambient conditions of temperature and pressure.[35] and physically cross-linked beta-sheet rich silk hydrogels have been prepared using sonication.[36]

Unlike many biologically derived proteins, silk is inherently stable to changes in temperature, pH and moisture[37,38] and is mechanically robust.[27] Due to its unique block copolymer structure (consisting of large hydrophobic domains and small hydrophilic spacers), silk self-assembles into organized nanoscale crystalline domains (β-sheets) separated by more flexible hydrophilic spacers

Table I. Criteria for Flavor and Fragrance Encapsulation Material[5]

Yield highly stable encapsulated products.
Tunable material properties to ensure appropriate release behavior (rapid vs sustained).
Effectively encapsulate without the use of crosslinking agents.
Soluble in water.
Controllable viscosity in aqueous solution.
High emulsification activity (i.e., prevents lipid separation from the emulsion during dehydration).
Process and materials should be relatively inexpensive.
Formation of fine, dense network during matrix formation and drying.
Safe for use in humans/edible for flavor encapsulations.
Relatively high mechanical strength.

that produce a highly stabilizing environment for incorporated proteins and small molecules.[39] For example, β-carotene is highly sensitive to oxidation after extraction,[40] but can be stabilized by adsorption onto silk fibroin due to the ability of silk to substitute for the proteins that naturally stabilize β-carotene *in vivo*.[41] We have recently reviewed and described the exceptional capacity of silk to stabilize sensitive incorporated compounds, including small molecules and proteins.[42] Previously, a wide range of water-soluble compounds and proteins (including enzymes and growth factors) have been successfully encapsulated in silk biomaterials.[28,29,42] Despite the highly attractive potential applications of oil encapsulation (particularly the stabilization and controlled release of volatile flavors and fragrances) and the unique properties of silk, fabrication approaches for oil-loaded silk biomaterials have not been investigated.

Emulsions are defined as mixtures of two immiscible phases (namely, water and oil) with an emulsifier added to stabilize the dispersed droplets.[13] Emulsions are characterized as oil-in-water (O/W) or water-in-oil (W/O) depending on the identities of the dispersed and continuous phases. Multiple emulsions, such as oil-in-water-in-oil (O/W/O) emulsions, may be prepared to contain multiple phases. Previously, protein microspheres have been prepared from water-in-oil emulsions where aqueous protein solutions are dispersed in an oil bath (sometimes stabilized with emulsifiers and/or surfactants), then the proteins are stabilized via suspension crosslinking, either through thermal or chemical treatment.[14,43–45] Imsombut et al.[45] have prepared silk microspheres using this method, with ethyl acetate as the oil phase, Span80 as an oil-soluble emulsifier, and genipin as a crosslinker. However, a process devoid of chemical additives is preferable as they may have toxic side-effects *in vivo* or damage delicate compounds.[44] Proteins droplets in water-in oil emulsions have been successfully converted to microparticles without chemical treatment by heating the oil bath to crosslink the protein matrix.[14,44] However, heating should also be avoided given the volatile nature of many fragrances and flavors.[9,11] In contrast, sonication has been shown to induce the physical crosslinking of silk.[36] This mild process has been successfully applied to encapsulation of stem cells[36] and labile proteins,[46] suggesting it would be similarly well-suited to the encapsulation of delicate,

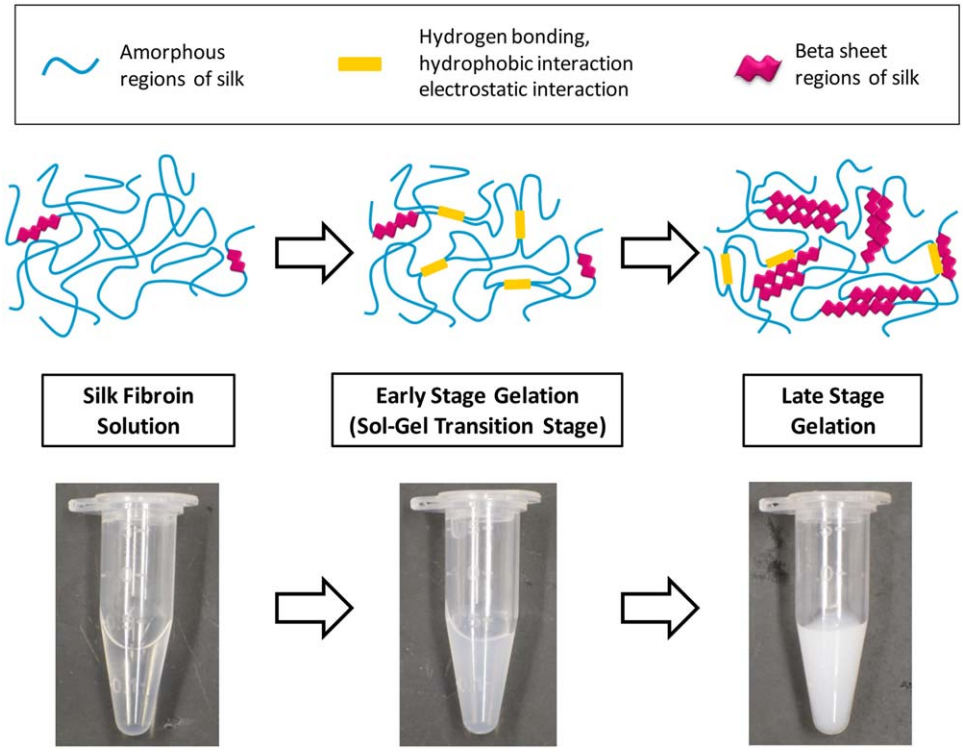

Figure 1. Silk hydrogel formation following sonication represented schematically[36,47] accompanied by representative photographs of silk fibroin in the various stages in the gelation process. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

volatile compounds in oils. In addition, the sonication-induced silk gelation technique also possesses the advantage that the sol–gel transition time can be controlled through the sonication treatment. As a result, the silk remains in the solution state for tunable timeframes and compounds can be mixed into the silk solution prior to the final onset of gelation (Figure 1).

Our objective was to exploit the mild processing options available for silk biomaterials (i.e., sonication-induced physical crosslinks, avoiding the use of cross-linking chemicals or heating) to produce encapsulation systems for flavors, fragrances, oils, and oil-soluble compounds. For this study, we encapsulated sunflower oil as a model lipid, to represent not only lipids alone (which can potentially benefit from stabilization effects of encapsulation), but also to model possible use of lipids as solvents in which hydrophobic substances such as volatile aromatic compounds (flavors and fragrances) and lipophilic vitamins and drugs could be solubilized for storage and delivery.[5]

EXPERIMENTAL

Materials

Cocoons of *Bombyx mori* silkworm silk were purchased from Tajima Shoji Co. (Sumiyoshicho, Naka-ku, Yokohama, Japan). Sunflower oil, doxorubicin, and Oil Red O were purchased from Sigma Aldrich (St. Louis, MO). Limonene was provided by Firmenich (Newark, New Jersey).

Silk Solution and Materials Preparation

Silk fibroin solution was prepared from *B. mori* cocoons as we have previously described.[48] Briefly, cocoons were boiled for either 30 or 60 min in a solution of 0.02*M* Na_2CO_3 and rinsed, then dried at ambient conditions overnight. The dried fibroin was solubilized in a 9.3*M* aqueous LiBr solution at 60°C for 2–4 h, yielding a 20% (w/v) solution. LiBr was then removed from the silk by dialyzing the solution against distilled water for 2.5 days using Slide-a-Lyzer dialysis cassettes (MWCO 3,500, Pierce Thermo Scientific, Rockford, IL). Silk fibroin concentration was determined by evaporating water from a solution sample of known volume and massing using an analytical balance. Silk solutions were stored at 4–7°C before use.

Silk Film Casting

Silk films were cast as previously described.[35] Briefly, silk solution was aliquoted into Teflon coated molds or patterned molds, then dried overnight at ambient conditions. Oil-loaded silk films were prepared by sonicating oil into silk solution of the desired concentration at various volumetric ratios of oil : silk using a Branson Digital Sonifier 450 at 10–15% amplitude for 5 s, then aliquoting and casting as described.

Sonication-Induced Silk Gelation

Sonication-induced gelation was carried out as previously described.[36] Briefly, silk solution of the desired concentration and prepared with the degumming duration of interest was sonicated using a Branson Digital Sonifier 450 at 10–15% amplitude for varied duration (the various conditions of silk concentration, degumming duration, and sonication amplitude and duration are specified throughout the results section).

Silk Hydrogel Macroparticle Preparation

Aqueous stock solution of water soluble compounds (either food coloring or the chemotherapy drug doxorubicin) was

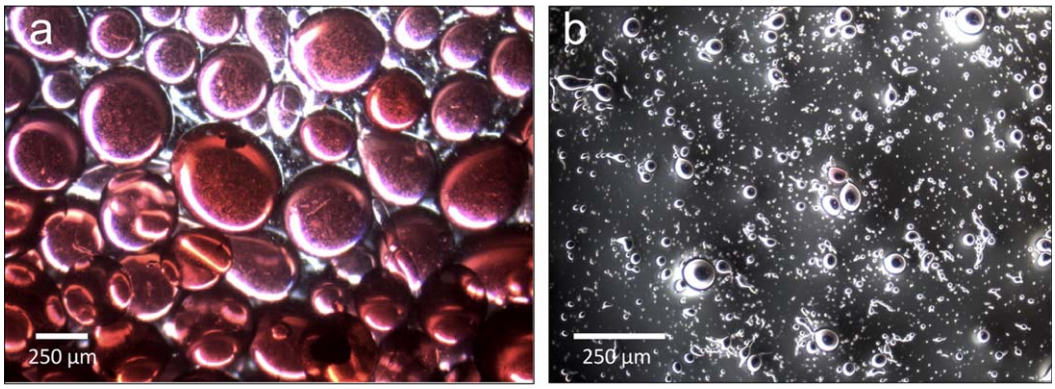

Figure 2. Emulsions of sunflower oil containing Oil Red O mixed with 7% (w/v) aqueous silk solution in a 1 : 3 (v/v) ratio of oil : silk, mixed with inversion (~10 min) (A) prior to sonication and (B) after gentle sonication (10% amplitude for 5 s). Scale bars = 250 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

added to the silk solution just after sonication (sol–gel transition had been initiated, but the silk was still in the solution state) and the solutions were mixed by inversion. Immediately after mixing, the solution was added dropwise to a sunflower oil bath in a petri dish. The petri dish was covered and incubated overnight at ambient conditions of temperature and pressure to allow completion of the sol–gel transition. After gelation completed, macroscale silk particles were removed from the oil bath with a mesh screen. To investigate the effects of water removal by drying, isolated silk hydrogel particles were incubated in uncovered petri dishes overnight at ambient conditions of temperature and pressure.

Oil Loaded Silk Particle Fabrication: Silk Hydrogel and Oil Emulsion Preparation

Emulsions were prepared either by manually mixing oil and sonicated silk solutions (gentle mixing for ~10 min) or by sonication of the silk and oil mixture with a Branson Digital Sonifier 450. In cases where sonication was not used to also prepare the emulsion (i.e., in cases where emulsions were prepared by mixing) but was still required for crosslinking of the silk, the silk solution was sonicated for the desired duration and amplitude prior to mixing with oil. Duration and amplitude of sonication (ranging from 5 to 45 s and 10% to 15%, respectively), concentration and degumming duration of the silk (ranging from 2% to 7% and 30 to 60 min, respectively) and ratio of silk to oil all varied, and these parameters are specified throughout the results section for each prototype carrier presented.

Oil Red O Loading and Measurement of Absorbance at 518 nm

Oil red O (an oil-specific dye used in histology) was used as a model to represent oil soluble compounds and investigate relative silk capsule diffusivity during overnight incubation. To estimate permeability of silk capsules prepared with various silk concentrations and degumming durations, double emulsions were prepared by adding Oil Red O labeled sunflower oil to silk solution that had been sonicated for varied durations and amplitudes using a Branson Digital Sonifier 450. Sonication initiated crosslinking of the silk, and Oil Red O labeled sunflower oil was dispersed into the aqueous silk solution phase by mixing (gentle shaking, ~10 min). This solution was then added to a second continuous sunflower oil bath, transferred to petri dishes, covered with petri dish lids and incubated overnight at ambient conditions of temperature and pressure. After incubation, the oil bath containing the Oil Red O labeled oil in silk microparticles was transferred to a 50 mL Falcon tube, centrifuged (5000 RPM, 1 min) to separate the particles from the oil bath, and loss of Oil Red O to the exterior continuous oil phases was estimated by measuring absorbance at 518 nm with UV–Vis spectroscopy. O/W/O emulsions with distilled water and unsonicated silk solution as the aqueous phase were also prepared for comparison.

Thermogravimetric Analysis

Thermogravimetric analysis (TGA; TA Instruments Q500) was used to measure weight changes of silk films assembled from 1% w/v silk fibroin solutions. TGA curves were obtained under nitrogen atmosphere with a gas flow of 50 mL/min. Analysis was first performed by heating the sample from 25C to 600°C at a rate of 2°C/min. Silk film weight loss was recorded as a function of temperature. Onset temperature of degradation was determined using points just off the baseline.

RESULTS AND DISCUSSION

Emulsions of Oil in Silk Solution (O/W Emulsions)

Manual mixing (gentle shaking for ~10 min) of an Oil Red O-loaded sunflower oil solution mixed with silk solution produces stable emulsions of the oil in water (O/W) type [Figure 2(A)]. Emulsions of sunflower oil in silk were prepared with silk concentrations of 2, 4, and 6% (w/v) and volumetric ratios of oil to silk of 1 : 1, 1 : 2, and 1 : 4 and no phase separation was observed for any of the oil in silk emulsions after 48 h stored at 4°C, compared to near total phase separation of 1 : 1, 1 : 2, and 1 : 4 mixtures of sunflower oil and distilled water.

Prior to sonication, an emulsion of sunflower oil containing Oil Red O mixed with 7% (w/v) aqueous silk solution in a 1 : 3 (v/v) ratio of oil : silk exhibited an average droplet diameter of 419.5 ± 126.9 μm. Gentle sonication (10% amplitude for 5 s)of the O/W emulsions reduced the average oil particle diameter to less than 25 μm (a sample of 200 particles in the image in Figure 2(B) measured with ImageJ exhibited an average diameter of 24.6 ± 11.4 μm, but the large number of particles less than

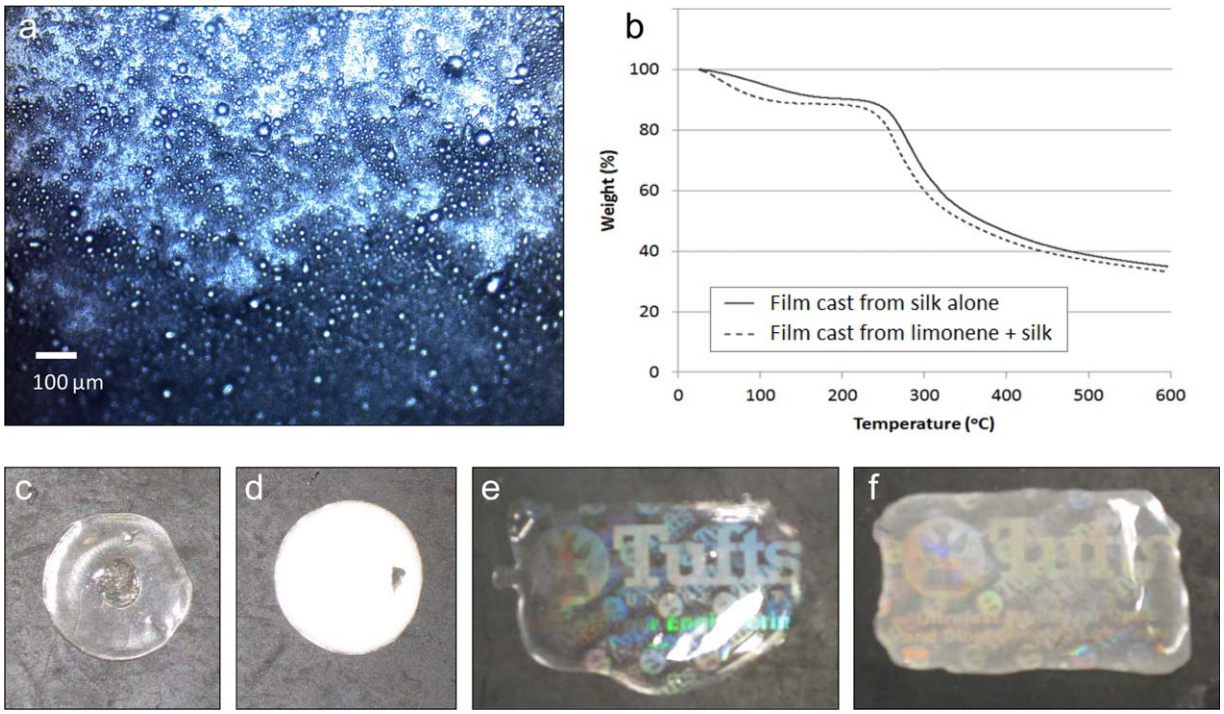

Figure 3. Casting oil-loaded silk films: (A) Emulsion of limonene in silk solution; (B) TGA thermograms of silk films prepared from silk alone and limonene emulsions in silk solution. Silk films prepared from(C) silk solution alone and (D) limonene emulsion (1 : 3 oil : silk; silk is 6% (w/v) prepared with a 30-min degumming time) cast using the same circular, Teflon-lined molds; Patterned silk films prepared from (E) silk solution alone and (F) oil emulsion (1 : 20 oil in silk; silk is 3% (w/v) prepared with a 45-min degumming time) cast using the same hologram-patterned mold. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

10 µm in diameter were not included in this average as they could not be accurately measured using ImageJ). Emulsions prepared by sonication of sunflower oil doped with oil red O and limonene in silk are shown in Figures 2(B) and 3(A), respectively.

The microscale oil droplets produced by sonication are stabilized when silk protein is present in the continuous aqueous phase, and are maintained during self-assembly of silk films during drying [Figure 3(C–F)] and self-assembly of silk hydrogel networks [Figure 4(B)] following sonication, as expected based on the literature on proteins acting as emulsion stabilizers.[2,13] Following dispersal of oil into the silk solution via sonication [Figure 3(A)], this stable emulsion can be treated as silk solution and can be cast into films as previously described,[38] including rapidly dissolving films,[49] compound-loaded films for biosensors and diagnostics,[42] and sustained release films for drug-delivery.[35,50] TGA analysis revealed a slight decrease in thermostability of the silk films loaded with microparticles of oil compared with silk alone [Figure 3(B)]: the onset temperature of degradation for the silk film alone is 214°C, while the onset temperature of degradation for the limonene loaded silk film is 191°C. However, self-assembly of the silk into films takes place on both Teflon coated molds [Figure 3(C,D)] and patterned molds [Figure 3(E,F)], even when the silk solution contained microparticles of oil. The presence of micron-scale oil droplets in the silk films renders the films opaque rather than transparent, with greater final film opaqueness resulting from higher oil content in the solution [Figure 3(C–F)]. Once the films were self-assembled by drying overnight at ambient conditions of temperature and pressure, re-dissolution upon exposure to distilled water and phosphate buffered saline was confirmed (data not shown). Though further evaluation of the release kinetics is needed, this suggests that if films prepared from emulsions of oil in aqueous silk solution receive no further treatment post-drying, the silk network will re-solubilize upon exposure to aqueous media, releasing the incorporated oil microparticles. Alternately, the films can be treated by water-annealing to increase beta-sheet content in the silk network and render the films water insoluble, as has previously been described for films cast from silk alone.[51]

After confirming that emulsions of oil are stable in aqueous silk solutions (O/W emulsion) and do not interfere with silk matrix assembly, we next evaluated a gentle, aqueous process to produce stable silk particles in oil baths, so that these two components could ultimately be integrated into O/W/O emulsions for microencapsulation. As previously described, sonication induces physical crosslinking of silk over tunable timeframes.[36] As a result of this controllable delay between the initiation of the sol–gel transition and the final onset of gelation, sonicated silk still in the solution state aliquoted into oil baths or suspended in self-stabilizing water-in-oil emulsions will complete physical crosslinking without heating or chemical treatment (unlike other emulsion-based processes for preparation of protein microspheres). Stable, physically crosslinked silk macroscale spherical particles were produced by sonicating a 6–7%, 30 min degumming time, silk solution for ~30–45 s at an amplitude of

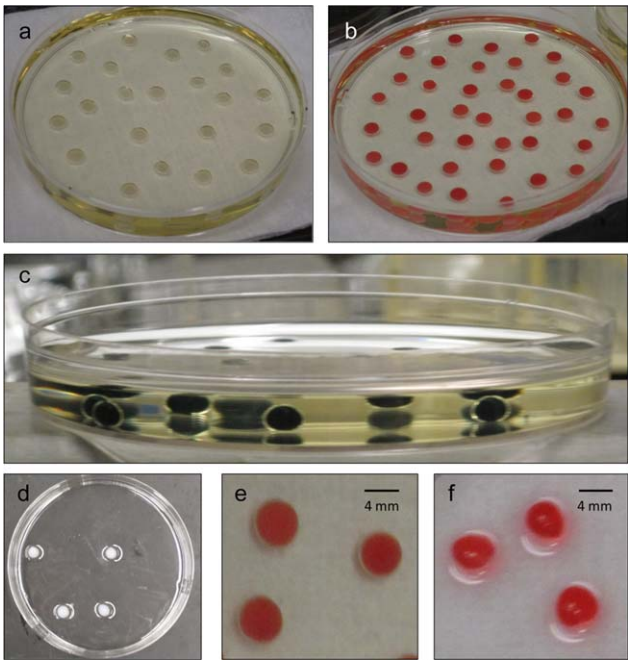

Figure 4. (A) Sonicated silk solution is held in spherical droplets in a sunflower oil bath (silk has not completed transition to hydrogel state, as evidenced by the slight translucence of the particles). (B) Sonicated silk solution containing a dispersion of Oil Red O loaded oil microdroplets held in spherical droplets in a sunflower oil bath. (C) Side view of sonicated silk solution containing green food coloring for ease of visualization. (D) Hydrogel silk spheres prepared from sonicated silk alone allowed to complete crosslinking in a sunflower oil bath retain their shape after removal from the oil bath. (E) Oil loaded silk hydrogel microspheres prior to dehydration (silk matrix is soft hydrogel) (F) oil loaded silk spheres characterized by a firmer, denser silk encapsulation matrix resulting from dehydration of the silk hydrogel network with overnight drying at ambient conditions. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

15%, mixing in solutions of distilled water containing model water-soluble small molecule compounds (doxorubicin or food coloring) and aliquoting the sonicated silk-drug mixture into a sunflower oil bath. In the oil bath, the aqueous silk droplets are held in a spherical conformation until gelation completes [Figure 4(C)]. Figure 4(A) shows sonicated silk solution in the oil bath prior to the completion of gelation and Figure 4(D) shows the same silk droplets after overnight incubation in the oil bath: once crosslinking of the silk network is complete, the silk droplets transition from translucent [Figure 4(A)] to opaque and retain their spherical shape when removed from the oil bath [Figure 4(D)]. How rapidly the silk transitions from the translucent solution state to the opaque crosslinking hydrogel state depends on the kinetics of the sol-gel transition, which is dependent on both the properties of the silk and the intensity of the sonication (duration and amplitude). Sonication-induced emulsion of Oil Red O loaded sunflower oil into silk prior to adding silk dropwise into the oil bath [Figure 4(B)] produces crosslinked silk spherical particles with fine, microscale oil particles suspended throughout, resulting in a red coloration of the final silk macroparticle [Figure 4(E)]. Dehydration of physically crosslinked silk macroparticles by drying overnight at ambient conditions compresses the silk network into a smaller, dense, pellet-like particles [oil-loaded in Figure 4(F) and water-soluble dye loaded in Figure 5(B)].

By its nature, this extrusion-like process is characterized by precise control of particle size and compound loading due to the pipetting of controlled volumes of known composition into the oil bath. Figure 5(A) shows silk hydrogel macroparticles produced by pipetting sonicated silk solution (loaded with doxorubicin post-sonication) in various volume-size droplets (from 100 μL down to 1 μL) into the sunflower oil bath. Microparticles produced by pipetting 10 or 50 μL of sonicated silk solution (loaded with food coloring post-sonication) and the denser, firmer, smaller particles that result when the hydrogel macroparticles are dehydrated overnight at ambient conditions are shown in Figure 5(B). The average diameter of silk hydrogel microspheres prepared from 10 μL of sonicated silk solution loaded with dye was 2.8 ± 0.2 mm prior to drying, and decreased to 1.9 ± 0.3 mm after drying. The average diameter of silk hydrogel microspheres prepared from 50 μL of sonicated silk solution loaded with dye was 4.6 ± 0.1 mm prior to during, and decreased to 2.3 ± 0.1 mm after drying. Drying the hydrogels at ambient conditions collapses the hydrated crosslinked

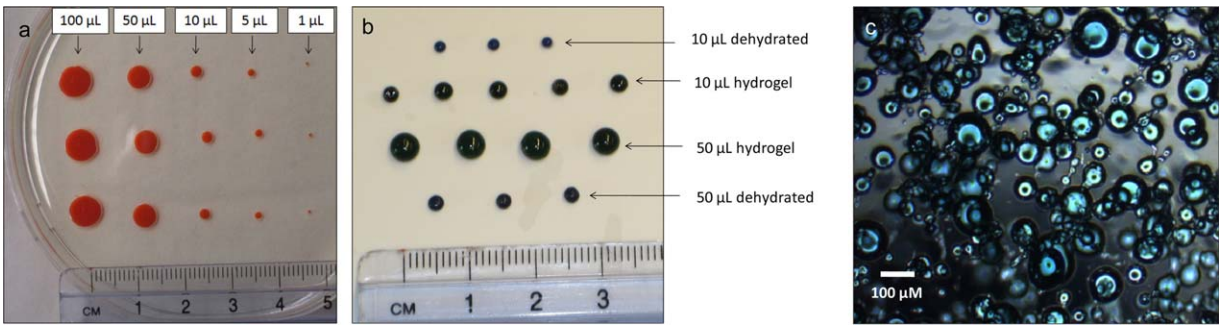

Figure 5. (A) Silk hydrogel macroparticles loaded with doxorubicin prepared by pipetting controlled volumes into sunflower oil bath. (B) Silk hydrogel macroparticles loaded with green food coloring prepared by pipetting controlled volumes (10 or 50 μL) into sunflower oil bath and dehydrated silk macroparticles prepared by drying silk hydrogel macroparticles. (C) Silk microspheres prepared by sonication of silk into a sunflower oil bath (W/O emulsion) (silk contains 1 : 100 volumetric ratio blue food coloring for visualization). Scale bar = 100 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

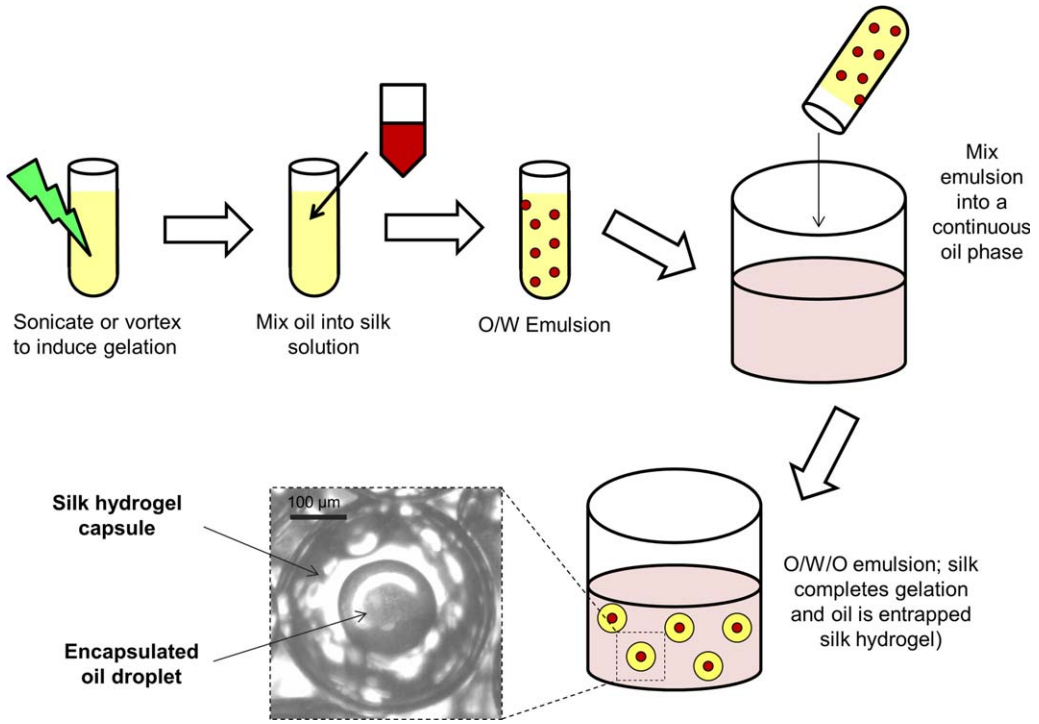

Figure 6. Schematic of silk microparticle preparation using O/W/O emulsions containing sonicated aqueous silk fibroin solution as the encapsulating water phase. Once sonicated, silk begins transitioning to the physically crosslinked water-insoluble hydrogel state, but remains in solution state for controllable durations dependent on the silk properties and sonication parameters. In the solution state, oil can be emulsified in the silk solution, and the O/W emulsion can be further emulsified in a continuous oil phase. In the continuous oil phase, the oil-encapsulating silk droplets are held in a spherical conformation until crosslinking completes, at which point the silk becomes a stable, water-insoluble hydrogel encapsulation matrix for the oil. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

silk network into a denser, more compact crosslinked silk network: following drying, the 10 and 50 μL microspheres lost 68.8 and 87.5% of their volume, respectively. The volume change resulting from dehydration will depend on the concentration of silk and volume of the initial hydrogel sphere, but further work is needed to elucidate this relationship. Smaller silk microparticles (average volume less than 1 μL) were produced by dispersing silk into oil in a W/O emulsion using sonication [Figure 5(C)]. We anticipate microfluidics could be used to produce even smaller, more tightly controlled silk particles using the described approach (silk sonication followed by dropwise addition to an oil bath), as has been described for other biomaterial microparticles.[52–54]

In addition to varying size and loading, these physically crosslinked silk particles can be further manipulated through post-crosslinking treatments: they can be (1) maintained in a rubbery, hydrated gelled state, (2) dehydrated to produce dense, hardened matrices [Figures 4(F) and 5(B)] or (3) freeze-dried to produce dry, porous, sponge-like material.[55] These different spherical silk particles (all produced using gentle, food-safe processes) span a wide range of material properties and sizes, suitable for a diverse array of potential applications.

Oil-Encapsulating Silk Microparticles Derived from O/W/O Emulsions

Based on stabilization of emulsified micron-scale oil droplets in aqueous silk solution and sonicated silk's formation of macro-scale hydrogel particles in oil baths, microparticles were prepared with a double emulsion of the type $O_1/W/O_2$ where O_1 is the oil of interest to encapsulate (here sunflower oil loaded with Oil Red O), W is a sonicated aqueous silk solution and O_2 is a sunflower oil bath. The silk solution comprising the water phase is sonicated such that it remains in the solution phase long enough to perform the double emulsion, then completes crosslinking, thereby encapsulating the interior oil phase (schematic representation of this process shown in Figure 6). The silk also acts as a natural emulsion stabilizer, preventing the interior oil phase (loaded with a compound of interest) from separating and leeching the compound of interest into the continuous oil phase. Morphology or O/W/O emulsions prepared from sonicated silk of varied silk composition and sonication treatment was examined with light microscopy, and diffusivity of the silk encapsulating matrices was evaluated by measuring absorbance at 518 nm of the external oil bath (an indicator Oil Red O leeching into the continuous oil phase).

O/W/O emulsions prepared with 60-min degumming time regenerated silk fibroin solution are shown in Figure 7. Using the higher concentration, aqueous silk solution in the water phase (6% w/v) produces a dispersion of oil droplets suspended throughout the silk sphere [this encapsulation configuration is termed a microsphere, also called a matrix system; [12]Figure 7(A)]. Use of a lower concentration aqueous silk solution (3% w/v) to prepare the emulsions results in a microcapsule

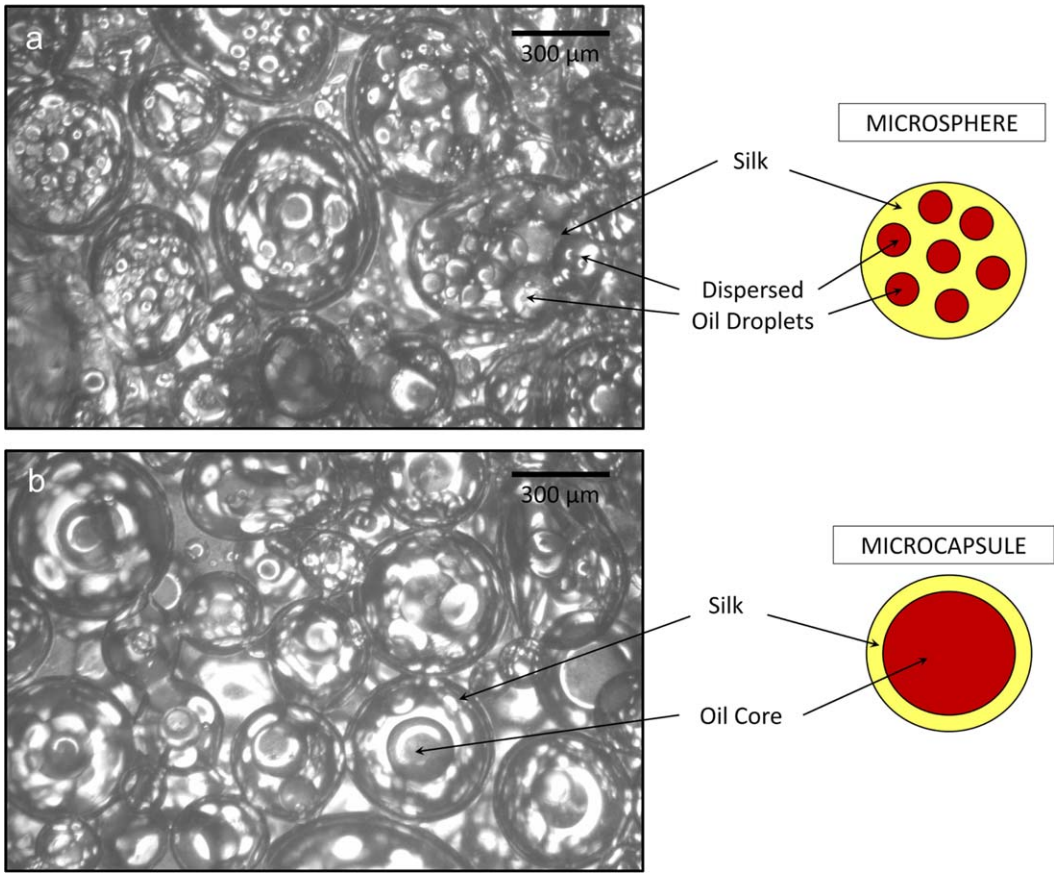

Figure 7. Microparticles prepared using O/W/O emulsions with 60-min degumming time regenerated silk fibroin solution. (A) O/W/O emulsion prepared with 6% (w/v) (higher concentration), 60-min degumming time silk sonicated at an amplitude of 15% for 45 s (B) O/W/O emulsion prepared with 3% (w/v) (lower concentration), 60-min degumming time silk sonicated at an amplitude of 15% for 30 s. Scale bars = 300 µm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

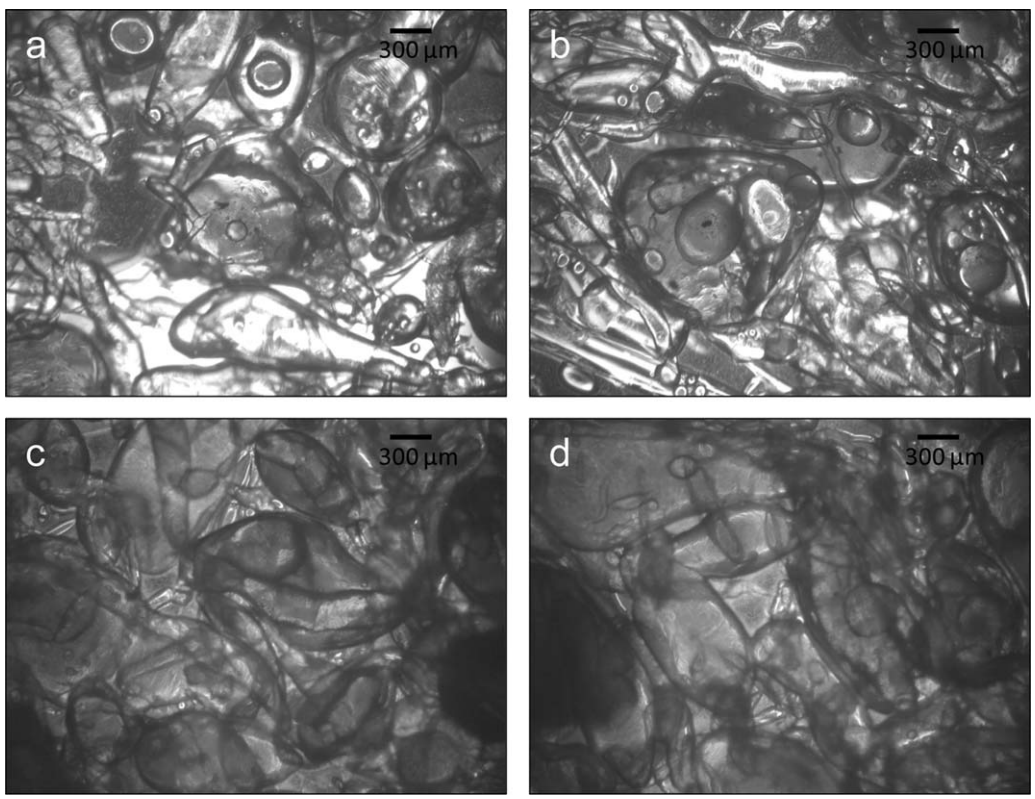

Figure 8. Microparticles prepared using O/W/O emulsions with 6% (w/v) 30-min degumming time regenerated silk fibroin solution treated with different sonication parameters: silk sonicated at an amplitude of 10% for 15 s (A,B) and silk sonicated at an amplitude of 15% for 15 s (C,D)

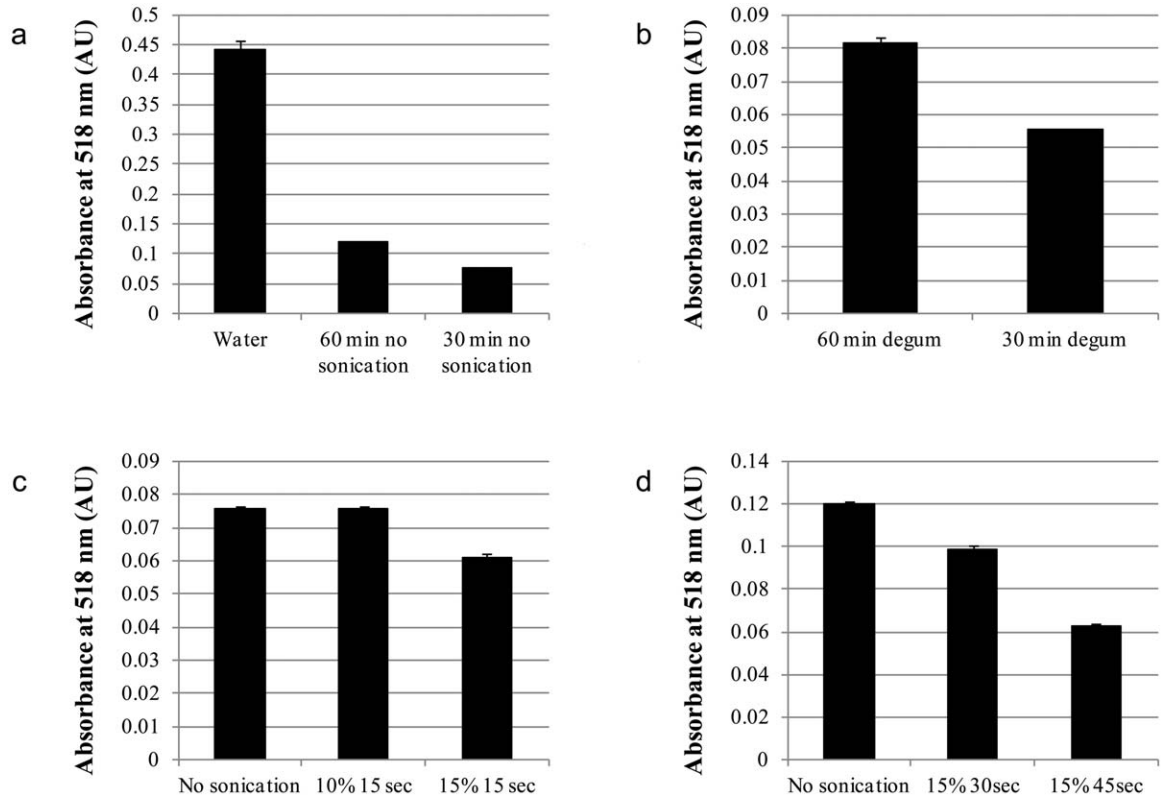

Figure 9. Absorbance at 518 nm (relative Oil Red O diffusion from internal oil capsule to external oil phase/sunflower oil bath). (A) No sonication. (B) Silk solution of concentration of 3% (w/v) sonicated at 15% amplitude for 30 s, degumming duration of the silk is varied: 30 min or 60 min. (C) Silk solution of concentration of 6% (w/v) prepared using a 30 minute degumming duration exposed to varied sonication: no sonication, sonication at 10% amplitude for 15 s, or sonication at 15% amplitude for 15 s. (D) Silk solution of concentration of 6% (w/v) prepared using a 60-min degumming duration exposed to varied sonication: no sonication, sonication at 15% amplitude for 30 s, or sonication at 15% amplitude for 45 s.

configuration (also called a reservoir system[12]), where one large oil droplet surrounded by a silk capsule is incorporated in each individual particle. This demonstrates that the concentration of the silk impacts the morphology of the oil-encapsulating microparticle: the increased viscosity and increased protein concentration of the 6% (w/v) silk might be preventing individual droplets from coalescing into a single core droplet observed with the 3% (w/v) silk W/O/W emulsions.

Recently, we have demonstrated that silk solution viscosity decreases with increasing degumming duration, likely due to the decrease in average fragment size produced by increased exposure to heat and alkalinity.[56] Because increased sonication intensity accelerates the gelation process,[36] increased sonication amplitude and duration are also expected to increase solution viscosity. The viscosity of the silk solution impacts particle morphology and silk's permeability as an encapsulant material. Representative images of O/W/O emulsions produced using 6% (w/v) silk prepared using a 30-min degumming time are shown in Figure 8. Compared with the lower viscosity, shorter fragment size 60-min degumming time silk emulsions, the particles are less spherical and oil encapsulation appears less regular. When sonication intensity increases [10% for 15 s in Figure 8(A,B), compared to 15% for 15 s in Figure 8(C,D)], resulting particles are even more elongated and irregular. The shorter degumming time combined with the increased sonication intensity may cause premature crosslinking, preventing the silk in the emulsion from incorporating an interior oil droplet or adopting a spherical conformation.

During the preparation of microcapsules, material composition and diffusivity of the encapsulating matrix material determine the retention degree of core compounds.[5] At higher solution viscosities, absorbance at 518 nm (an indicator of the Oil Red O content) of the external oil phase (i.e., the sunflower oil bath) decreases, suggesting the permeability of the silk capsule to the Oil Red O in the internal oil phase and consequent "loss" of compound loaded in the internal phase decreases as the viscosity of the silk solution in the double emulsion increases. Compared with an aqueous phase of plain distilled water, unsonicated silk also reduces loss to the external oil phase [Figure 9(A)]. When silk concentration is held constant and sonication treatment is held constant, Oil Red O loss to the external phase decreases with decreasing degumming time/increasing silk solution viscosity [Figure 9(B)]. Similarly, when silk solution concentration and degumming time are held constant [6% (w/v), 30-min degumming time in Figure 9(C); 6% (w/v), 60-min degumming time in Figure 9(D)], and sonication intensity increases (amplitude or duration or both), Oil Red O loss generally decreases [with the exception of 6% (w/v) 30-min degumming time silk exhibiting no change in Oil Red O loss for unsonicated silk solution compared with silk solution sonicated for 15 s at an amplitude of 15%, possibly because this sonication treatment does not

significantly increase viscosity]. Individual averages are reported in Supporting Information Table S1.

The sunflower oil bath comprising the continuous, external oil phase in O/W/O emulsions prepared with distilled water containing no silk as the water phase exhibited the highest absorbance at 518 nm (0.442 ± 0.014), indicating the greatest loss of Oil Red O from the internal oil capsule into the continuous oil phase. The continuous oil phases in O/W/O emulsions with unsonicated aqueous silk fibroin solution prepared using a 60- and 30-min degumming time as the water phase had absorbance values at 518 nm of 0.12 ± 0.001 and 0.076 ± 0.001, respectively. The presence of silk in the water phase reduces Oil Red O leeching into the oil phase, and the increase in viscosity/average fragment length of the silk solution prepared using the shorter degumming time further increases compound retention in the interior oil core [Figure 9(A)]. In addition to silk processing parameters, Oil Red O retention in the interior oil core is also controlled by sonication treatment and concentration (w/v) of the silk solution in the water phase [Figure 9(B–D, Supporting Information Table S1). In addition to the observation that silk encapsulation provides a barrier to Oil Red O diffusion into the external oil phase, morphology of the silk O/W/O emulsions suggests that the silk in the aqueous layer assembles into a capsule around the interior oil phase: puckering and wrinkling of the silk "skin" are apparent (Supporting Information Figure S1).

CONCLUSIONS

Gentle, food-safe, aqueous methods for preparing oil-encapsulating spherical silk-based delivery systems with diameters ranging from less than 300 μm to 4 mm are described which may have a potential application in food, nutritional, consumer, and pharmaceutical products particularly where protection, stabilization, and controlled release are required. The processes described here are carried out at ambient conditions of temperature and pressure and produce stable emulsions without secondary emulsifiers or chemical crosslinking agents. In addition to having useful applications in food, cosmetics, consumer products, and medicine, a stable dispersion of oil throughout a protein network may be more physiologically representative than a simple protein hydrogel in modeling tissues with high lipid content, such as the brain. Future work will include evaluation of the compound loading, controlled release, and stabilization capabilities of silk biomaterials containing lipids and lipid-soluble compounds of interest, including flavors, fragrances, and lipophilic drugs.

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