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Encapsulation of Volatile Compounds in Silk Microparticles

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Abstract

Various techniques have been employed to entrap fragrant oils within microcapsules or microparticles in the food, pharmaceutical, and chemical industries for improved stability and delivery. In the present work we describe the use of silk protein microparticles for encapsulating fragrant oils using ambient processing conditions to form an all-natural biocompatible matrix. These microparticles are stabilized via physical crosslinking, requiring no chemical agents, and are prepared with aqueous and ambient processing conditions using polyvinyl alcohol-silk emulsions. The particles were loaded with fragrant oils via direct immersion of the silk particles within an oil bath. The oil-containing microparticles were coated using alternating silk and polyethylene oxide layers to control the release of the oil from the microspheres. Particle morphology and size, oil loading capacity, release rates as well as silk-oil interactions and coating treatments were characterized. Thermal analysis demonstrated that the silk coatings can be tuned to alter both retention and release profiles of the encapsulated fragrance. These oil containing particles demonstrate the ability to adsorb and controllably release oils, suggesting a range of potential applications including cosmetic and fragrance utility.

Keywords

silk fibroin; microencapsulation; coatings; stabilization; emulsion; controlled release

Introduction

Fragrances are been linked to many aspects of everyday life[1]; depending on the nature of the scent a fragrance can spark emotion [2,3], induce feelings of relaxation and stress reduction[2], improve alertness[4] or enhance memory[5]. Maintaining the appropriate intensity level of fragrances in commercial products is desirable for both product function and consumer satisfaction. However due to the delicate nature and high volatility of these compounds, the sustained presence of fragrances in consumer products is challenging. The volatility of fragrance molecules is usually due to the low molecular weight and the presence of functional groups, such as hydroxides, aldehydes and ketones[6]. These chemical groups

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readily react with other compounds and are sensitive to environmental factors including light, oxygen, temperature, and humidity[7]. Degradation of fragrances diminishes the scent and its associated benefits, and can also increase flammability and generate by-products that may be allergenic[6, 8-10].

To address challenges related to the long-term controlled release of fragrances and to increase product stability, encapsulation techniques have been employed to entrap fragrant oils within microcapsules or microparticles in the print, food, pharmaceutical, and chemical industries [11-15]. The microparticle format increases product stability while expanding versatility in applications and the ability for long-term use when compared to the volatile liquids. Techniques including spray drying, melt extrusion, coacervation, and aqueous emulsions have been used to generate fragrance-containing microparticles [1617]. Although promising these processes can reach elevated temperature, require toxic crosslinking agents or produce low levels of product incorporation. [16, 1816, 17, 19]. Aqueous emulsions are typically composed of two or more oil-water components. The ease and safety of aqueous emulsions to form fragrance-containing particles makes this approach attractive for fragrance encapsulation, however, its use has been limited because of low encapsulation and retention capabilities [17].

Silk is a highly versatile protein utilized in the textile, cosmetic, and chemical industries. Recently, it has also been developed for use in a broader range of biomaterials and regenerative medicine needs[20-22], as well as for cell encapsulation[23, 24] and controlled drug delivery systems[25]. Silk is a natural polymer product; a relatively inexpensive, biocompatible, biodegradable, and non-toxic FDA-approved protein derived from the *Bombxi mori* silkworm cocoon[26-28]. Unlike other biological proteins, with minimal processing silk can be transformed into various material formats, including but not limited to sponges, films, gels, fibers, mats, coatings and microspheres[29, 30]. The silk protein consists of a block copolymer structure composed of large hydrophobic domains and smaller hydrophilic spacers, as well as hydrophilic chain ends; thus an amphiphilic polymer. This unique structure allows silk to self-assemble into crystallized β-sheets.

These crystalline regions, which are physical crosslinks to exclude water, increase stability, result in water insoluble silk materials, and impart mechanical strength[31]. Temperature, water vapor, alcohols, salt, pH and mechanical stimulation can be used to induce tunable physical cross-links (the (β-sheet crystals), thereby providing a versatile and ambient set of process controls to regulate this feature[21, 32, 33]. For example, by exploiting the sonication process silk can be used as the aqueous phase of an oil-water-oil emulsion, with controllable gelation for the incorporation of volatile fragrances and to modulate their stability and release[34, 35].

The aim of the present study was to increase the stability and retention of volatile fragrances via encapsulation in silk microspheres. The use of silk aqueous solution allows the final materials to be all natural, biocompatible and controllable in terms of properties, while avoiding the use of heat and chemical cross-linkers known to be detrimental to fragrances. A novel silk/polyethylene oxide coating was developed, and fragrance retention and release from coated microspheres studied.

Material and Methods

Materials

B. mori silkworm cocoons were supplied by Tajima Shoji Co (Yokohama, Japan). Sodium carbonate, lithium bromide, poly(ethylene oxide) (PEO), polyvinyl alcohol (PVA), and Corning transwells were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Slide-a-Lyzer dialysis cassettes (MWCO 3,500) were purchased from Pierce, Inc. (Rockford, IL). Limonene was provided by Firmenich (Plainsboro, New Jersey)

Solution preparation

B. mori silk cocoons were boiled in 0.02M aqueous sodium carbonate for either 10, 30 or 60 minutes to extract sericin and isolate the silk fibroin protein as we have previously described [36]. Isolated silk fibroin was then rinsed three times in deionized water and allowed to dry for 24 h. Dried silk was dissolved in 9.3M LiBr at 60°C for 3 h, and the resulting 20% w/v solution was dialyzed against deionized water for three days to remove salts. The final concentration of aqueous silk fibroin ranged from 6.0-8.0 wt. %, which was calculated by weighing the remaining solid after drying.

Silk microsphere formation

5% (w/v) PVA solution was added to silk solution described above (5% w/v) at a volumetric ratio of (4:1). PVA-silk mixture was placed on a stir plate for three hours at room temperature, resulting in the aggregation and subsequent precipitation of silk microparticles. Stirred mixtures were cast to thin films and allowed to air overnight. The PVA films were dissolved in 40mL of deionized water, leaving behind silk microparticles. The silk particles were rinsed three times in 40 mL of deionized water, collected via centrifugation and stored dry until needed.

Fragrance incorporation

Fragrances were incorporated within the microspheres via passive diffusion, by soaking dry silk microspheres in excess fragrance under constant gentle agitation. Incorporation times varied from 1 hour to 24 hours depending on the experiment. Microparticles were harvested via centrifugation to form a pellet and all excess fragrance aspirated.

Scanning electron microscopy

Scanning electron microscopy (SEM) was used to observe the microparticles. Silk alone and silk-fragrances loaded microspheres were aired dried for 24hours prior to imaging. Environmental scanning electron microscopy was used to avoid the necessity of sample sputter coating, allowing for visualization of surface coatings (Carl Zeiss Inc., model Supra 55VP).

Thermogravimetic analysis

Thermogravimetric analysis (TGA) (TA Instruments Q500, New Castle, DE) was used to measure weight changes in the microparticles. For rapid estimates of microparticle composition the TGA was heated from 23° C to 50°C at a rate of 5°C/min. To distinguish

surface fragrance from encapsulated fragrance samples a 240 minute incubation at 50°C was performed prior to continued heating.

Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy (FTIR) analysis of the silk films was performed with a Jasco FT/IR-6200 spectrometer (Easton, MD), equipped with a multiple reflection, horizontal MIRacle attenuated total reflectance (ATR) attachment (ZnSe crystal, from Pike Tech., Madison, WI). For each measurement, 128 scans were performed each with nominal resolution of 4 cm^{-1}. To identify secondary structures in the protein samples peak positions of the amide I region (1595-1705 cm^{-1}) were determined based on the absorption spectra. Fourier self-deconvolution and curve fitting were performed using Jasco Spectra-Manager Analysis Software (Jasco, Easton MD), as we have previously described[32].

Results and Discussion

Incorporation of fragrances in preformed silk microparticles

A well-established method to form silk-based microparticles for fragrance encapsulation involving the emulsion of silk and polyvinyl alcohol (PVA) was studied. Unlike particles prepared from traditional O/W/O, those with PVA were not formed directly in the presence of the fragrance, but were first formed separately and loaded post-preparation with the desired compound.

To incorporate fragrances the hollow microparticles were incubated in solutions of the fragrances. The semi-rigid, porous network of the microparticles[37] dictated that the fragrance occupy the void spaces and thus a high degree of swelling was not expected, even for fully saturated particles. Microparticles were formed using 30 minute process times and fragrance was passively taken up without noticeable swelling even after 24 hours of soaking (Figure 1). The time for complete fragrance uptake was determined by varying microparticle incubation time and analysis of fragrance content by TGA. Figure 1C-D shows TGA thermographs of microparticles immersed for 1 or 24 hours in limonene oil. For both times the limonene fraction was about 85-90% (wt.), indicating that 1 hour was sufficient for microparticle saturation. Similar incorporation fractions were found for the other process times and fragrances studied, with total fragrance incorporation after 1 hour ranging from 80-90% (data not shown). We hypothesize that the hydrophobic nature of the silk protein and the high surface area of these domains in the assembled silk particles is responsible for the uptake and high loading capacity of the materials. Given the process time did not affect fragrance uptake, 10 minute processed silk microparticles with 1 hour of fragrance loading were used in all of the following experiments.

Fragrance retention in silk microparticles using silk coatings

Hydrated barriers have been demonstrated to alter the rate of compound release from silk, hyaluronic acid, gelatin, and alginate systems[38-42]. To stabilize the encapsulated fragrances, to increase retention and to prolong release rates, the microparticles were coated with layers of silk.

Fragrance loaded microparticles were placed within a ~8 μm pore size filter. The membrane held the particles stationary while small quantities of coating solutions were passed over them. The small pores allowed liquid to flow through but prevented the particles above 8 μm to pass through the mesh. Each coating was composed of a polyethylene oxide (PEO) layer surrounded by a silk film. Particles were coated with 1, 3 or 5 layers. Coated particles maintained spherical shape, membrane flaking indicative of silk film deposition and showed minimal less aggregation. Figure 2B-D depicts SEMs of these particles. Figure 2E and Table 1 summarize the TGA findings, demonstrating that even one hydrated coating was sufficient to retain up to 3% of the encapsulated fragrance even after the 240 minute incubation at 50°C.

The particles with three coatings did not show an improvement in fragrance protection when compared to the control samples. The control samples consisted of microparticles that had not been exposed to fragrance prior to coating. The observed lack of improvement could be due to a number of factors including poor initial encapsulation, fragrance loss during coating, poor layer deposition or incomplete silk crystallization. Particles coated with five layers showed increased fragrance retention up to 2% (Table 1). The silk/PEO combination was effective at maintaining the encapsulated fragrance, even at elevated temperature. The use of the silk to encapsulate limonene provides a fully biodegradable, biocompatible encapsulation system which demonstrates the potential for limonene stabilization at elevated temperatures[6].

Although the PEO is highly viscous and functions as a good water retention barrier, the silk coating is crucial for protection of the encapsulated compound. PEO coatings without a silk layer quickly dispersed when submerged in an aqueous environment. Additionally, PEO alone was not sufficient to prevent water evaporation when subjected to heating. The silk layer limited diffusion of PEO and prevented rapid water loss. These two factors help to maintain hydration around the microparticles and prevent premature loss of fragrance.

Tuning silk coatings for altered release

To delay the release of the fragrant oil, a single silk-PEO coating was applied to fragrance loaded microparticles. The layer was treated to induce β–sheet structure in the silk coating. The fragrance-containing spheres that were not exposed to layer treatments showed 30% β-sheet (Figure 3A). It is likely that the β-sheet content measured in the non-treated spheres was induced by the escaping fragrance oil. Generally, crystallinity increased with exposure to treatments known to induce conformational changes in the silk protein secondary structure, such as water annealing or exposure to alcohol. The highest crystalline content was observed in the spheres exposed to ethanol, with these spheres displaying up to 36% β-sheet.

Release of fragrance from the treated particles was determined using TGA. Figure 3B shows normalized release of fragrance from microspheres exposed to various coating treatments. The spheres with non-treated coatings consisted of 10% of weight by fragrance. This fragrance was released in a first order profile corresponding to the increase in temperature. The 1.5 hour water anneal treatment demonstrated the same retention and release profile as the non-treated spheres, which was not surprising given that they displayed the same β-sheet

content as the controls (Figure 3A),. The spheres exposed to 6 hours of annealing displayed 7% fragrance retention, which is slightly lower than the control. Samples exposed to 12 hours of annealing demonstrated higher fragrance retention, 12%, with the same first order release profile as with the other water annealed samples. Ethanol treatment showed 10% fragrance retention which was comparable to the control and samples exposed to 6.5 hours or less of watering. The release profile of the fragrance from these spheres was different, displaying a zero order release with respect to increasing temperature. β-sheet content could play a role in this release, establishing a more densely packed coating which would slow fragrance escape. In treatments in which the overall β-sheet content was the same, specifically for ethanol and 12 hour water annealing, processing differences could account for the observed differences in oil content and release profiles.

The water annealed spheres were annealed in a dry state which could limit exposure to water vapor and thus produce heterogeneously crystalized coatings. Spheres that undergo ethanol treatment were suspended in ethanol, which ensured exposure of the entire coated surface to the β-sheet inducing solvent. This more homogenous crystalline coating could explain the zero order release observed, while the heterogeneous coating formed in the water annealing process could account for the larger initial burst release and the observed variability in fragrance retention with respect to total annealing time.

Release of small molecules from silk film constructs similar to the applied coatings display profiles comprising of an initial steady-state diffusional release and followed by complete matrix degradation and complete product recovery[43]. The retarded release of model molecules from films with higher crystallinity was attributed to increased domain interconnectivity in the silk structure which in turn increases the molecules resonance time[43]. A similar phenomenon was observed here, with fragrance release being delayed from the spheres with highly crystalized coatings. The silks hydrophobic nature may also cause adsorption of the oils within the sphere as well as in the film coatings, delaying fragrance release. This interaction is facilitated by the mismatch in hydrophobicity between the encapsulated oil and first PEO layers. The hydrated PEO helps retain fragrance and allow the application of additional silk coatings.

Conclusions

A tunable biocompatible method of producing silk microparticles for encapsulation of volatile compounds as well as soluble molecules was developed for fragrance and flavor delivery. A novel fully biocompatible silk coating method was described which can be applied to delivery systems. The encapsulated microparticles were prepared without the use of toxic crosslinkers, or exposure to high temperature, as is common for other encapsulation methods. Hydrated silk coatings prevented fragrance escape from encapsulated microparticles. Release profiles were tuned by controllably altering the crystallinity (β-sheet content) of the coating layer.

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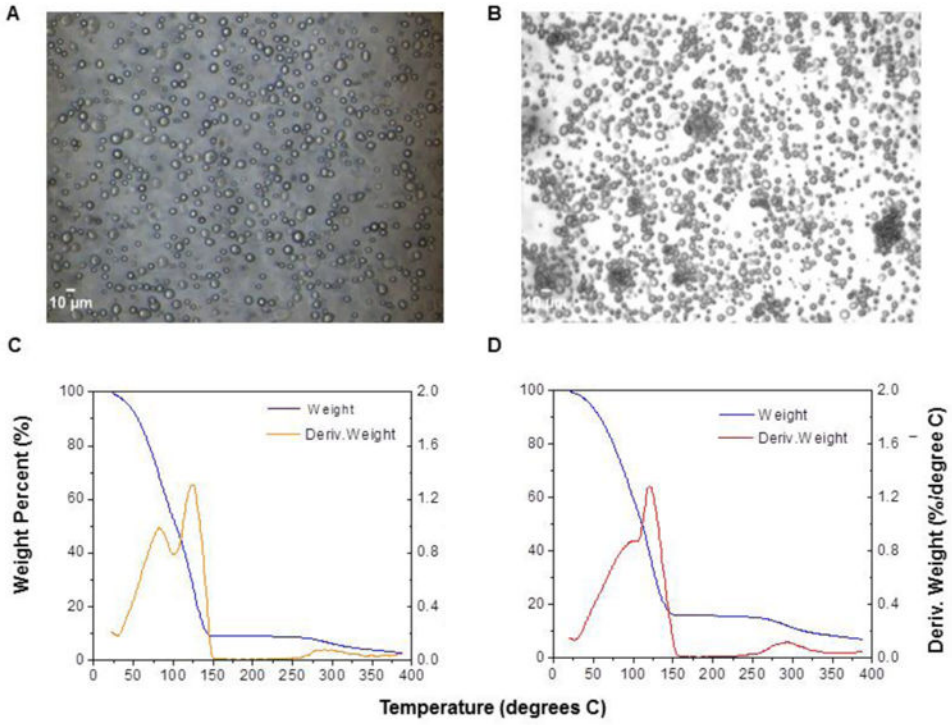

Figure 1.

Microparticles formed using PVA/silk emulsions (A) before and (B) 24 hours post-immersion in limonene. TGA thermographs for silk microparticles immersed in limonene for (C) one hour and (D) 24 hours were used to estimate fragrance content. Scale bars =10 um

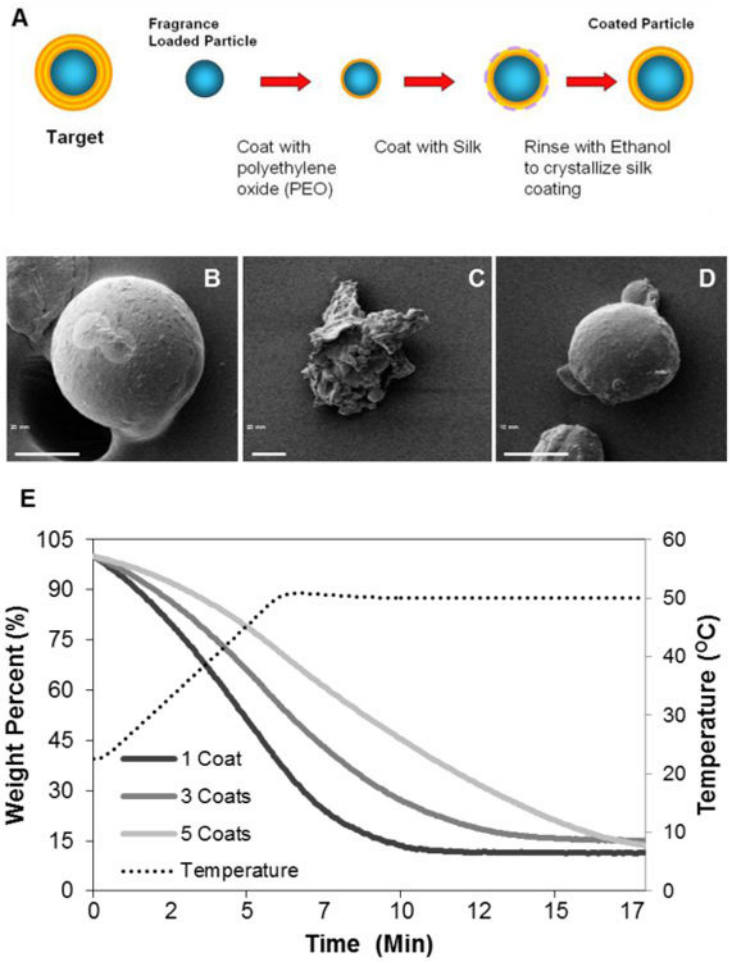

Figure 2.

(A) Schematic of PEO/silk coated particles. SEM images of microparticles with (B) one, (C) two, or (D) three coatings. TGA thermograph (E) of limonene encapsulated microparticles layered with one, three or five coatings of PEO/silk. Scale bars = 20 μm (B,C) Scale bar = 10 μm (D)

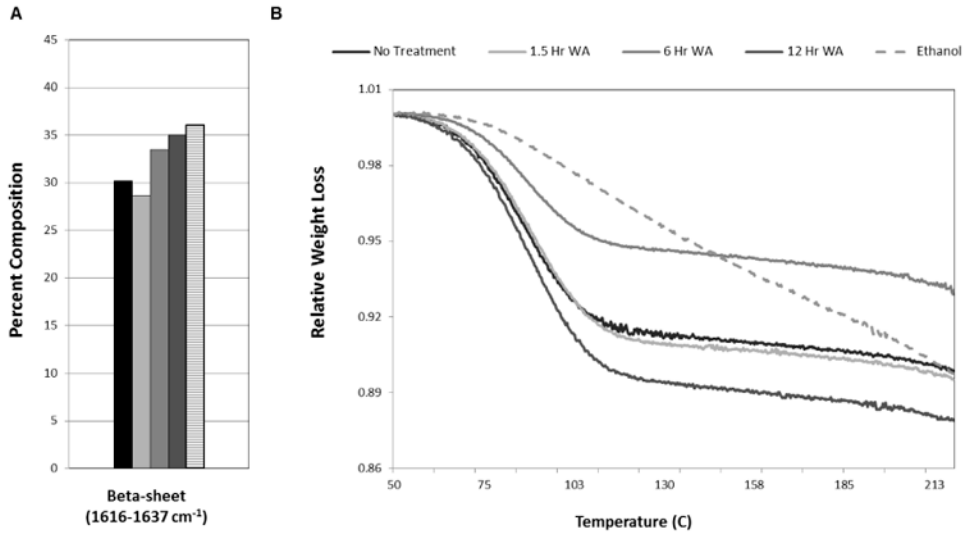

Figure 3.

Beta sheet content (A) and TGA micrograph of phenethyl alcohol release (B) from silk microspheres coated with one silk-PEO layer and treated with either 1.5, 6, 12 hr water annealing or ethanol exposure.

Table 1

Percent weight loss from silk and silk-limonene-containing microparticles with one, three or five PEO/silk coatings. TGA temperature increased stepwise at a rate of 5°C/min and maintained isothermally for at 50°C for 240 minutes. Mean +/- SEM)

Applied Coating	Uncoated microspheres	1 Coating	3 Coatings	5 Coatings
Fragrance Released	14.1 +/- 1.6%	11.6 +/- 0.44%	14.6 +/- 0.73%	12.7 +/- 1.5%