Journal of Industrial and Engineering Chemistry xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Journal of Industrial and Engineering Chemistry

journal homepage: www.elsevier.com/locate/jiec

Preserving the inflated structure of lyophilized sporopollenin exine capsules with polyethylene glycol osmolyte

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ARTICLE INFO

Article history:
Received 10 September 2017
Received in revised form 19 November 2017
Accepted 11 December 2017
Available online xxx

Keywords:
Pollen inflation
Osmotic stress
Sporopollenin exine capsule
Microencapsulation
Hollow microcapsules

ABSTRACT

Extracted from natural pollen grains, sporopollenin exine capsules (SECs) are robust, chemically inert biopolymer shells that posess highly uniform size and shape characteristics and that can be utilized as hollow microcapsules for drug delivery applications. However, it is challenging to extract fully functional SECs from many pollen species because pollen grains often collapse, causing the loss of architectural features, loading volume, and bulk uniformity. Herein, we demonstrate that polyethylene glycol (PEG) osmolyte solutions can help preserve the native architectural features of extracted SECs, yielding inflated microcapsules of high uniformity that persist even after subsequent lyophilization. Optimal conditions were first identified to extract SECs from cattail (*Typhae angustfolia*) pollen *via* phosphoric acid processing after which successful protein removal was confirmed by elemental (CHN), mass spectrometry (MALDI-TOF), and confocal laser canning microscopy (CLSM) analyses. The shape of SECs was then assessed by scanning electron microscopy (SEM) and dynamic image particle analysis (DIPA). While acid-processed SECs experienced high degrees of structural collapse, incubation in 2.5% or higher PEG solutions significantly improved preservation of spherical SEC shape by inducing inflation within the microcapsules. A theoretical model of PEG-induced osmotic pressure effects was used to interpret the experimental data, and the results show excellent agreement with the known mechanical properties of pollen exine walls. Taken together, these findings demonstrate that PEG osmolyte is a useful additive for preserving particle shape in lyophilized SEC formulations, opening the door to broadly applicable strategies for stabilizing the structure of hollow microcapsules.

Introduction

Sporopollenin exine capsules (SECs) are microscale biopolymer shells derived from natural pollen grains following processes to remove cytoplasmic materials [1–3]. Owing to their high physicochemical resistance, monodisperse size distributions, and natural abundance, SECs have been increasingly explored as alternatives to synthetic microencapsulants for a variety of food science and drug delivery applications [4–6]. The basic principle underlying SEC-based microencapsulation is to use acids, bases, or enzymatic processing techniques to remove allergenic proteins from within the pollen grain and to isolate the outer exine shell, made of a robust biopolymer known as sporopollenin, for the protection and delivery of desired compounds. To date, small molecules, oils, enzymes, and live cells have been successfully encapsulated into and released from SECs made from diverse plant species [7–10].

During processing, however, large fractions of SECs have been observed to enter an irreversible “collapsed” physical state marked by diminished size and architectural features [6,11,12]. SECs that were originally turgid and round as raw pollen frequently become flattened or crumpled after processing, exerting detrimental effects upon payload capacity, core material protection, and release rate [13,14]. Collapsed SECs also lose exterior

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https://doi.org/10.1016/j.jiec.2017.12.023
1226-086X/

ornamentation and particle homogeneity, which are necessary parameters for functionalizing particle surfaces and for tuning the bulk properties of particulate mixtures [15–18]. The uncontrolled occurrence of SEC collapse as a result of conventional processing is thus an outstanding issue facing the advancement of SEC applications. As of yet, no robust solutions have been proposed.

In recent work, our group found that aqueous storage preserves the shape of thin-walled SECs, suggesting that desiccation is partly responsible for the collapse of processed pollen walls [19]. Nonetheless, the need for flexible processing approaches in this developing field requires a method to produce uniformly inflated SECs that are compatible with dry-storage, *i.e.*, that can be freeze-dried without collapsing and stored as a free-flowing powder. As found throughout the scientific literature, the problem is common among hollow microcapsules in general and overcoming this challenge would have broad implications for numerous applications, including drug delivery, microencapsulation, and taste-masking. We hypothesized that osmolyte-induced pressure gradients, which have been successfully used to control particle shape across various length scales [20], could restore the shape of SECs by reversing particle collapse. Indeed, aqueous polymeric osmolytes are known to exert osmotic pressures across porous membranes and this has been exploited to inflate micron-scale entities such as protoplasts and hollow synthetic microcapsules [21,22]. One particularly useful polymeric osmolyte is polyethylene glycol (PEG), a safe and water-soluble polymer that can create high osmotic pressures and that induces water potentials across porous plant cell walls [23]. Considering as well its frequent use in the food and pharmaceutical industries, we chose to investigate PEG for its ability to inflate collapsed SECs as part of our goal to optimize pollen-based microcapsules for food and drug delivery-based loading applications.

To this end, we extracted SECs from cattail pollen (*Typhae angustfolia)* for the first time. Cattail pollen is prized for its nutritional and hemostatic properties and has been consumed for hundreds of years as both a supplementary food source and as a traditional Chinese medicine [24–27]. Recent studies on its medical application report anti-atherosclerotic, anti-atherogenic, and anti-inflammatory effects that have sparked interest in exploration of its pharmacology [28–30]. Furthermore, cattail pollen grains are small (less than 20 μm diameter), mono-aperturate spheroids whose shape can be mathematically modeled in accordance with previous studies on exine wall rigidity [31,32]. Herein, we therefore report the production of uniformly inflated cattail pollen SECs produced *via* an optimized acid-processing and PEG-based osmotic pressure inflation treatment. Chemical characterization was performed with elemental (CHN), matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF), and confocal laser scanning microscopic (CLSM) analyses, and morphological characterization was done using scanning electron microscopic (SEM) and dynamic imaging particle analyses (DIPA). Finally, the observed osmolyte-induced inflation phenomenon was mathematically modeled to provide a translatable study for other microcapsule models.

Experimental

Pollen and processing material

Raw *T. angustifolia* pollen grains were purchased from a local traditional Chinese medicine (TCM) store (Wong Yiu Nam TCM supplies, Chinatown, Singapore). Metal sieves with 1 mm pores were purchase from Fairprice Pte Ltd. (Singapore). Perfluoroalkoxy Polymer (PFA) flasks were procured from Vitlab (Grossostheim, Germany). Acetone and ethyl alcohol (95% and 99.9%, respectively) were purchased from VWR International (Singapore). Phosphoric acid (85% w/v) was procured from Merck (Singapore). Polyethylene glycol (PEG) chains with a monomer weight of 4000 Da were purchased from Sigma (Singapore) and, according to the mushroom model, have a Flory radius of 5.2 nm (Ref. [33]). Polystyrene microspheres (50 ± 1 μm) for DIPA calibration were purchased from Thermo Scientific (CA, USA).

Extraction of T. angustfolia *sporopollenin exine capsules (SECs)*

Raw, natural cattail pollen grains were manually sieved to remove plant debris, leaving a fine cattail pollen powder. From this starting material, cattail SEC extraction was executed in four sequential steps. (1) Defatting: raw cattail pollen grains (10 g) were suspended in acetone (100 mL) in a round bottomed PFA RB flask fitted with a glass condenser and then placed into a 45 °C water bath to under gentle magnetic stirring (180–200 rpm) for 30 min. The suspension was vacuum filtered and the drained pollen grains were washed thoroughly with warm acetone. The resulting defatted pollen grains were dried in a convection oven (60 °C) for 12 h. (2) Acid processing: defatted cattail pollen grains were segmented into four 2 g batches, each of which was then placed into a PFA RB flask containing 85% (w/v) phosphoric acid (15 mL). The mixture was vortexed for 2 min to ensure a homogenous suspension, fitted with glass condenser, and placed into a 70 °C water bath to reflux for different durations (2.5, 5, 7.5, or 10 h) under magnetic stirring (180–200 rpm). At the end of the specified duration, flasks were removed from reflux and allowed to cool to room temperature. The phosphoric acid suspension was vigorously stirred with 150 mL deionized water and then vacuum filtered, with SECs collected in a clean 250 mL beaker filled with 150 mL of fresh deionized water. Vacuum filtration and washing with deionized water was repeated 5–7 times for each batch until each litmus testing indicated pH 6. (3) Washing: after acidolysis, SECs were collected in a 250 mL clean beaker and a 15-step sequential washing procedure using hot solvents was performed: acetone (2×100 mL), 2 M hydrochloric acid (100 mL), 2 M sodium hydroxide, water (5×100 mL), acetone (100 mL), ethanol (2×100 mL), and again water (3×100 mL). For each washing step, SECs were vigorously stirred in a beaker with the solvent to ensure maximal contact with the solvent before solvents were removed by vacuum filtration. Washed SEC capsules were spread out over a large petri dish and dried in a vacuum oven at 60 °C in the presence of silica desiccant for 12 h. (4) Polyethylene glycol treatment: washed, dried cattail SECs were suspended in varying concentrations (0.125%, 1.25%, 2.5%, 5%, 10%) of polyethylene glycol (PEG) solution (10 mL) and vortexed to obtain a homogenous suspension. PEG suspensions were stir-incubated (150 rpm, 37 °C) for 12 h and then centrifuged at 4500 rpm for 8 min to remove PEG supernatant. Wet SECs were lyophilized for 12 h. SECs were then suspended in distilled water (10 mL) for 4 h to induce PEG uptake and vacuum filtered once more to remove the water and residual PEG. SECs underwent additional lyophilisation for 12 h and were finally stored in a desiccating cabinet until further characterization.

Elemental analysis

CHN elemental analysis was conducted using a calibrated VarioEL III elemental analyzer (Elementar, Hanau, Germany). Prior to conducting elemental analysis in triplicate, all samples were dried at 60 °C for a minimum of 1 h. The samples were subjected to complete combustion under high temperature and excess oxygen, producing carbon, hydrogen and nitrogen in the gaseous state. The final protein concentration of both processed and unprocessed capsules was subsequently measured using the percentage of nitrogen with a conversion factor of 6.25 [34].

Please cite this article in press as: M.K. Corliss, et al., Preserving the inflated structure of lyophilized sporopollenin exine capsules with polyethylene glycol osmolyte, J. Ind. Eng. Chem. (2017), https://doi.org/10.1016/j.jiec.2017.12.023