Journal of Industrial and Engineering Chemistry 61 (2018) 255–264

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 20 December 2017

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.
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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
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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), monoaperturate 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].

Mass spectrometry

Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry was performed with ground and ethanol extracted pollen and SECs. 5 mg of sample was pulverized using a mortar and pestle, suspended in 0.5 mL ethanol and vortexed for 1 min. 0.7 μL of the suspension was inserted into a MALDI plate and mixed with 1 μL of an alpha-cyano-4-hydroxycinnamic acid matrix solution. Samples were thoroughly dried and analyzed in a Shimadzu Biotech Axima Performance MALDI-TOF system in raster-scanning mode with a power of 80, with 100 profiles per run. The mass-to-charge (m/z) scanning range was set to $0 < m/z < 10{,}000$. All samples were run in triplicate; data were baselined (100) and smoothed (Gaussian: 100) using Shimadzu Maldi M5 software.

Confocal laser scanning microscopy (CLSM)

Defatted cattail pollen grains and SECs were sandwiched between sticky slides (Ibidi, Germany) using Vectashield® mounting medium and scanned using a Carl Zeiss LSM700 (Germany), as described previously [35]. Fluorescence was observed using three spectral fluorescence/reflected detection channels, six laser lines (405/458/488/514/561/633 nm), and a Z1 inverted microscope (Carl Zeiss, Germany). Images were collected at laser excitation lines 405 nm (6.5%), 488 nm (6%) and 561 nm (6%) with DIC in an EC Plan-Neofluar 40 x_1.3 oil objective M27 lens. The fluorescence was collected in photomultiplier tubes equipped with the following emission filters: 416–477, 498–550, 572–620. The laser scan speed was set at 67 s per each phase (1024 x 1024: 84.94 mm^2) and plane mode scanning with a pixel dwell 12.6 μs. The iris was set optimal for the sample conditions and all images were captured at the mid-region of the particle. All other settings were fixed for all samples, and per sample, at least three images were captured and processed using ZEN2008 software (ZEISS, Germany).

Scanning electron microscope analysis (SEM)

SEM imaging was performed using a JSM 5410 (JEOL, Tokyo, Japan). Samples were sputter-coated with a 10 nm-thick gold film using a JFC-1600 instrument (JEOL, Tokyo, Japan) at 20 mA for 60 s. Images were captured at an accelerating voltage of 5 kV at different magnifications and morphological changes were observed. Visual inspection of SEM images containing $n \geq 20$ randomly chosen SEC particles from all processing conditions was used to quantify the morphological changes in cattail pollen SECs from different processing concentrations and PEG concentrations.

Dynamic image particle analysis (DIPA)

Dynamic image particle analysis (DIPA) was conducted using the FlowCam®: Bench top system (FlowCamVS, Fluid Imaging Technologies, Maine, USA). The equipment housed a 200 μm flow cell (FC-200), a 20× magnification lens (Olympus®, Japan) and was controlled by a visual spreadsheet software version 3.4.11. Prior to the start of analysis, the instrument was cleaned by flushing the system with 1 mL deionized water (Millipore, Singapore) at a flow rate of 0.5 mL/min and calibrated using polystyrene microspheres (50 ± 1 μm). Raw pollen, defatted pollen, and processed SECs were mixed with a pre-run volume of 0.5 mL deionised water and primed into the flow cell. Samples were analyzed at a flow rate of 0.1 mL/min and at a camera rate of 10 frames per second, allowing a sampling efficiency of approximately 9%. A minimum of 10,000 particles were analyzed during each run, and measurements were performed in triplicate. Data analysis to obtain morphological statistics including aspect ratio, diameter, and circularity was carried out using 300 highly focused particles. DIPA analysis was used to confirm morphological properties (unfolded and folded) particles within each population by quantitatively assessing circularity, diameter, and aspect ratio.

Results and discussion

SEC process development and characterization

We began this study by developing a scheme to extract protein-free sporopollenin exine capsules (SECs) from cattail pollen (Fig. 1). First, raw pollen is manually sieved and then defatted with warm acetone to remove lipids on the pollen exine and facilitate protein removal [12]. Next, reflux in hot phosphoric acid removes proteinaceous intine contents while leaving the robust

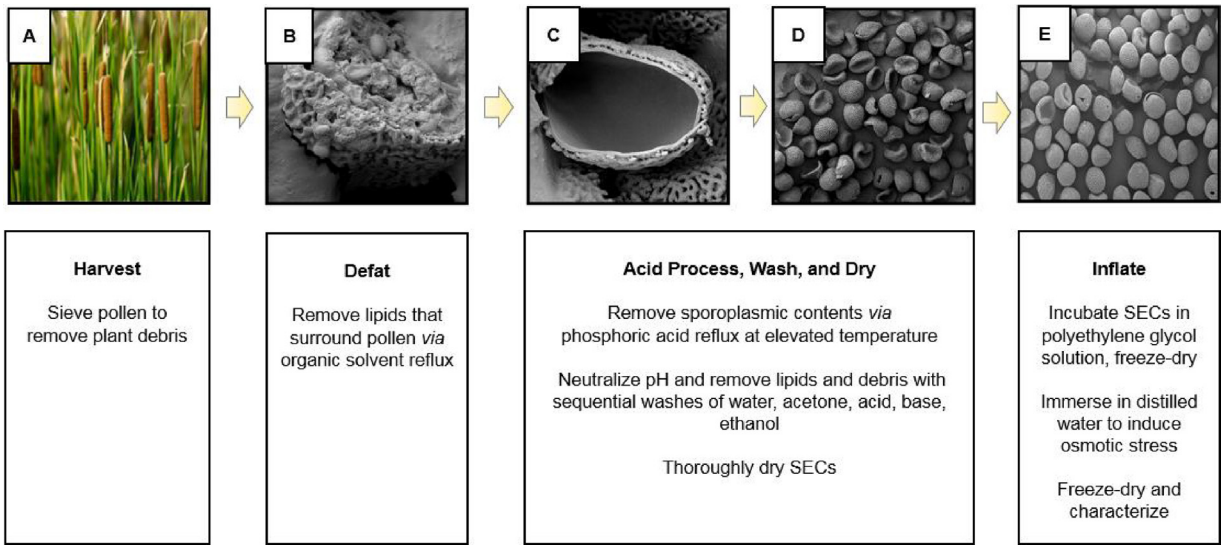

Fig. 1. Process to extract SECs from cattail pollen grains: (A) harvesting of raw cattail (*Tyhphae angustfolia*) pollen. (B) Defatting with organic solvent. (C,D) Acid processing, washing, and drying to remove sporoplasmic materials. (E) Reformatting treatment with polyethylene glycol (PEG) solution, freeze-drying, and dilution to induce negative osmotic pressure.

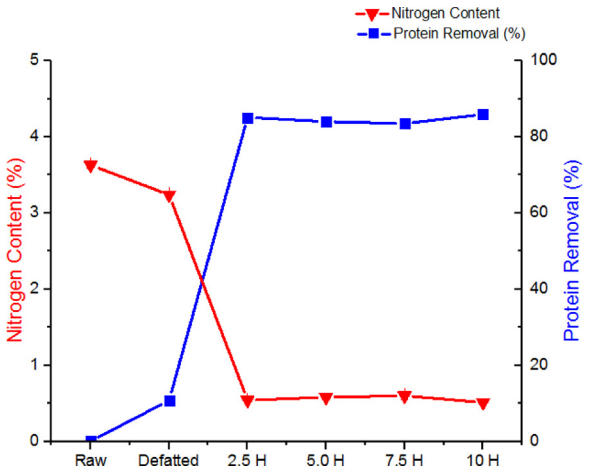

Fig. 2. Protein content of unprocessed and processed cattail pollen grains determined by elemental (CHN) analysis. Acid processing for 2.5 h or longer removed approximately 85% of proteinaceous content from raw (natural) cattail pollen grains. The data represented above is the average of triplicate measurements with standard deviations indicated by error bars.

sporopollenin exine intact. To optimize this process, phosphoric acid processing was explored over different durations (2.5 h, 5 h, 7.5 h, 10 h) to determine the most efficient reflux time, as has been previously described [6–8,19]. Finally, microcapsule collapse was addressed by incubating SECs in polyethylene glycol (PEG) solutions and pure water to induce osmotic stress, followed by lyophilization to preserve inflated microcapsule structures.

Evaluation of protein removal

Phosphoric acid is commonly used in the food industry to produce hydrolyzed vegetable protein and has become a preferred reagent for pollen protein removal [6,13,19,36]. Similarly to vegetable proteins undergoing industrial hydrolysis, pollen proteins that undergo short-duration, medium-heat, concentrated acid processing are also broken down into smaller peptide fragments under 10 kDa in weight [37]. Protein removal is achieved as heat and agitation diffuses hydrolyzed protein fragments out from SECs, where they are highly concentrated, into the surrounding acid solution, where they are less concentrated, *via* micro-pores present throughout the exine shell [8].

Protein removal can be evaluated by elemental (CHN) analysis because the quantity of protein within plant material is proportional to its nitrogen content [34]. For all samples processed with phosphoric acid for 2.5 h or longer, an average 87% reduction in nitrogen content was observed (Fig. 2). This suggests that the majority of pollen protein was successfully removed from SECs for all processing conditions. Although small amounts of nitrogen remained, these are indicative not of native pollen proteins but of hydrolyzed protein fragments and therefore should pose no allergenic concern. To test this assumption, matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry was performed, as mass spectrometry is routinely used to identify allergenic proteins in pollen [38]. In such studies, enzymatic digestion of pollen protein produces smaller peptides that are then analyzed *via* mass spectrometry over a narrow range ($200 < m/z < 2500$) of mass-to-charge ratios [39–41]. Phosphoric acid-hydrolysis of proteins is a cheaper, albeit less specific, means of protein degradation; samples in this study were therefore scrutinized over a broader range ($0 < m/z < 10{,}000$) of mass-to-

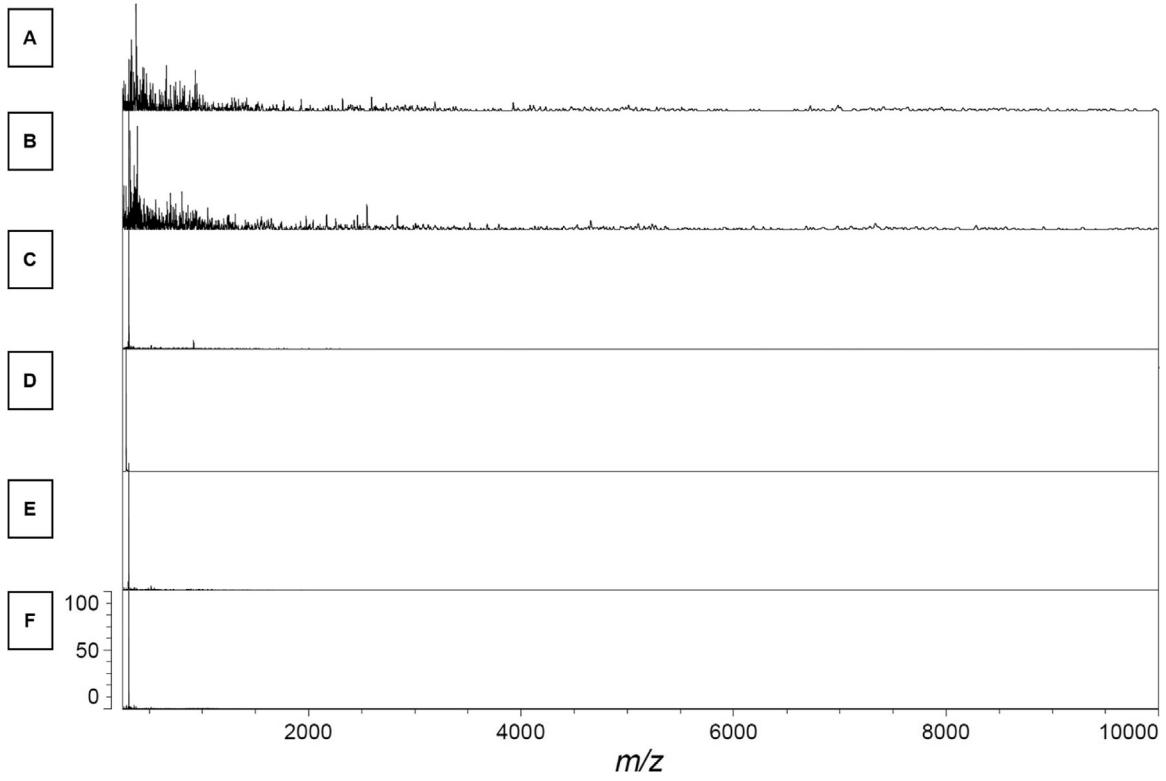

Fig. 3. Protein content analysis by MALDI-TOF for cattail pollen and sporopollenin exine capsules (SECs) based on sample grinding and ethanol extraction: (A) raw cattail pollen, (B) defatted cattail pollen, and SECs from cattail pollen processed in 85% (w/v) phosphoric acid for (C) 2.5 h, (D) 5 h, (E) 7.5 h, and (F) 10 h. All acid-processing conditions led to nearly complete removal of biomacromolecules under 10,000 Da.

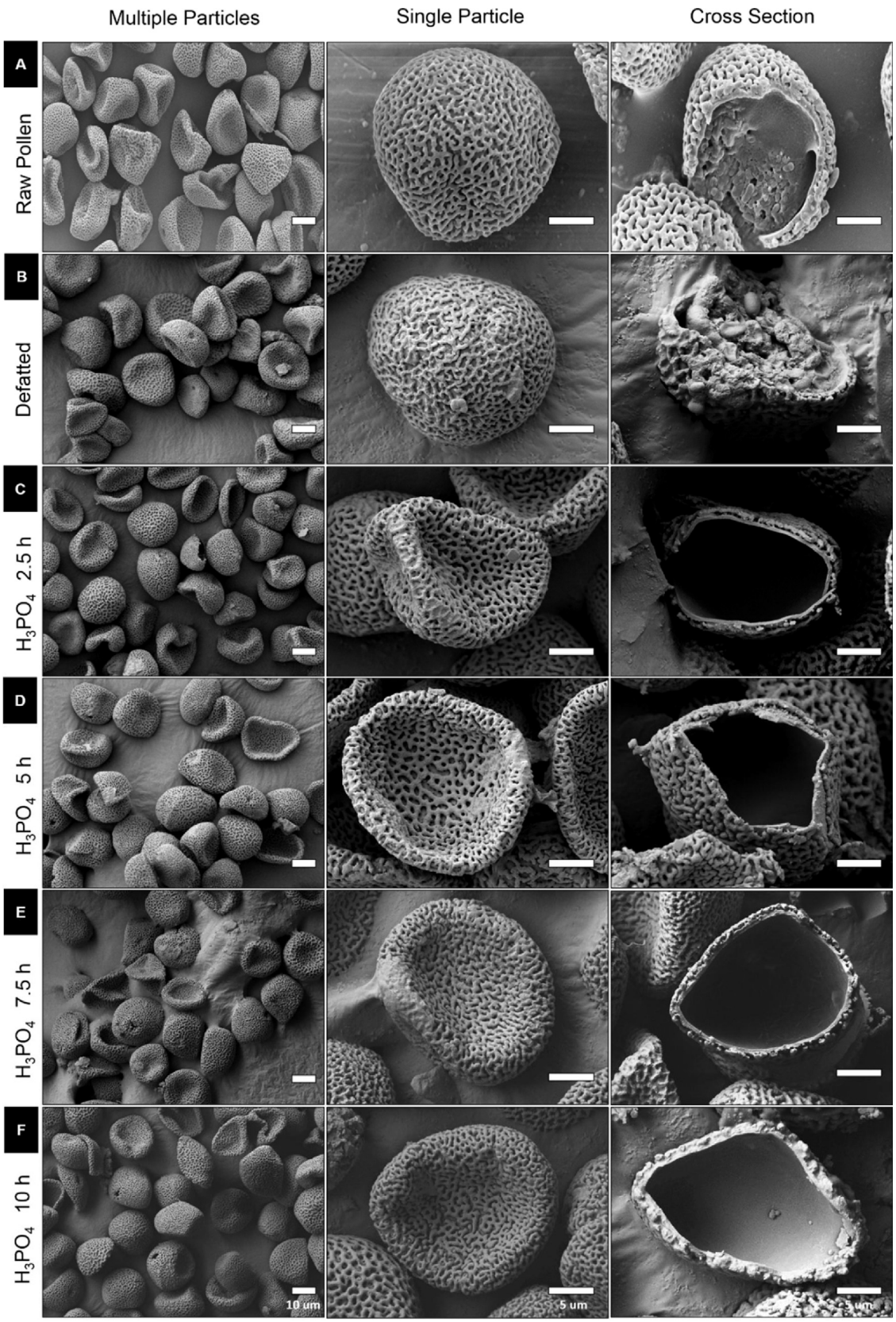

Fig. 4. SEM images of (A) raw cattail pollen (B) defatted cattail pollen and (C–F) defatted cattail pollen processed with 85% (w/v) phosphoric acid. Samples were convection dried at 60 °C for 8 h after processing. The majority of all acid-processed microcapsules lack inner sporoplasmic contents, corroborating protein removal. Morphologically, large fractions of SECs appear collapsed regardless of acid processing duration.

charge ratios to account for a potentially wider variety of hydrolyzed protein fragments. Indeed, analysis of raw and defatted cattail pollen indicated multiple peaks under m/z = 10,000 that correspond to the presence of large peptide molecules (Fig. 3). In contrast, acid-processed SECs had very few peaks within these ranges, supporting elemental (CHN) observations that protein was successfully removed. Taken together, these observations suggest that processing cattail pollen with 85% (w/v) phosphoric acid at 70 °C for 2.5 h or longer effectively clears protein from cattail pollen SECs.

Evaluation of microcapsule structure

Scanning electron microscopy (SEM) was used to check chemical observations of protein removal along with changes in SEC morphology due to phosphoric acidolysis (Fig. 4). Immediately noticeable are the stark differences in sporoplasmic content between raw and defatted *vs* acid-processed SECs. Unlike raw and defatted pollen grains that exhibit thick proteinaceous aggregates within the central cavity, processed SECs are devoid of inner content. As for microcapsule shape, SEM micrographs reveal raw and defatted cattail pollen grains that are symmetric and mildly folded due to natural pollen harmomegathy. The harmomegathic effect is a defensive mechanism inherent to many raw pollen species in which cellular volume is reduced in response to the low internal osmotic pressure of a desiccated state [11]. Harmomegathic folding differs from structural collapse in that it is limited in severity by inner mechanical support provided by interior protein constituents and in that it is reversible with hydration. Acid-processed cattail pollen grains, in contrast, appear flattened and crumpled, consistent with reports of SEC collapse. The shriveled cattail pollen SECs indicate a more severe structural change than that of raw or defatted pollen grains, suggesting that protein content helped to stabilize SEC shape before it was removed. Unlike harmomegathically folded raw pollen grains, the collapse observed among processed SECs cannot be reversed with hydration alone [42]. Overall, SEM observations suggest that acid-processed SECs from all processing durations (2.5 h, 5.0 h, 7.5 h, and 10 h) are protein-free, but that the majority of them have collapsed shapes.

The 2.5 h processed SECs were chosen for osmotic inflation studies due to their satisfactory protein removal (comparable to that of longer durations) and similar morphological characteristics (highly collapsed). Prior to PEG-treatment, these SECs were scrutinized using confocal laser scanning microscopy (CLSM) to ensure protein removal and provide detailed structural information. Natural pollen grains auto-fluoresce due to the presence of carotenoids and phenolic compounds present in the sporopollenin biopolymer and intine compounds [43]. Fluorescence level is therefore an optical indicator of biomaterial content in the pollen shell and capsule interior that can visually confirm particle morphology and the removal of sporoplasmic proteins. Raw cattail pollen and 2.5 h processed SECs show marked difference in fluorescence emission: whereas raw pollen grains exhibit high fluorescence intensity across all laser wavelengths in the capsule interior, SECs treated with phosphoric acid 85% (w/v) for 2.5 h show no interior fluorescence (Fig. 5). Together with CHN, MALDI-TOF, and SEM data, the CLSM evidence strongly suggests that acidolysis treatment at the shortest duration successfully removes sporoplasmic materials and, as was observed even in longer processing durations, results in large proportions of collapsed SEC particles.

Evaluation of osmoticpressure-induced inflation

Collapsed SECs lack uniformity and interior volume for biomacromolecular loading and hinder the development of industrial scale pollen-based microencapsulation applications. To address this problem, we sought to transform collapsed SECs into uniform, turgid capsules that display maximum volume for molecular loading. Polyethylene glycol (PEG) is a common industrial additive that is used, among other purposes, to stabilize the texture of powders; it is also safe for use in food, cosmetic, and medical applications and therefore of broad interest to the field. PEG is a strong osmolyte that induces osmotic pressure gradients across semi-porous membranes. With this in mind, SECs in this study were subjected to an osmotic-stress inducing "inflation treatment" consisting of suspension in PEG solution, freeze-drying, rinsing in pure water, and an additional freeze-drying (Fig. 6). Collapsed particles were first incubated in warm aqueous PEG solution under agitation to induce PEG uptake, and then freeze-dried to leave only crystallized PEG within and around the microcapsule. PEG-treated SECs were then soaked in pure water to dissolve PEG within the capsule and to form an osmotic pressure

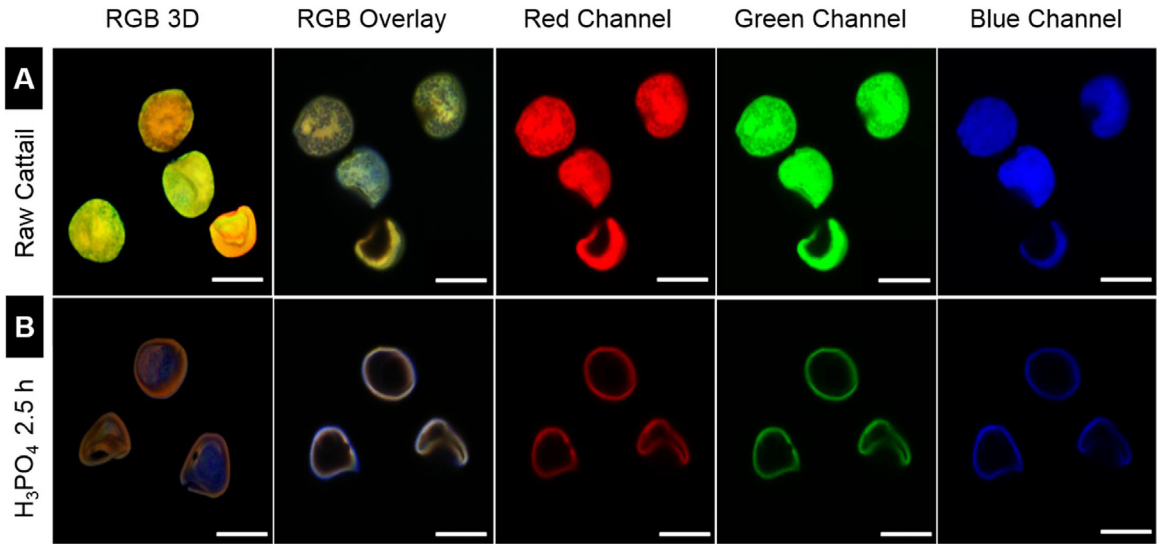

Fig. 5. Confocal laser scanning microscopy (CLSM) analysis of raw cattail pollen grains before and after acidolysis treatment. (A) Raw natural cattail pollen grains, and (B) SECs extracted from 2.5 h reflux with 85% (w/v) phosphoric acid. The colored autofluorescence corresponds to intine compounds that are naturally present in pollen or SECs. Sporoplasmic contents disappear after just 2.5 h of acid processing. Scale bars are 20 μm.

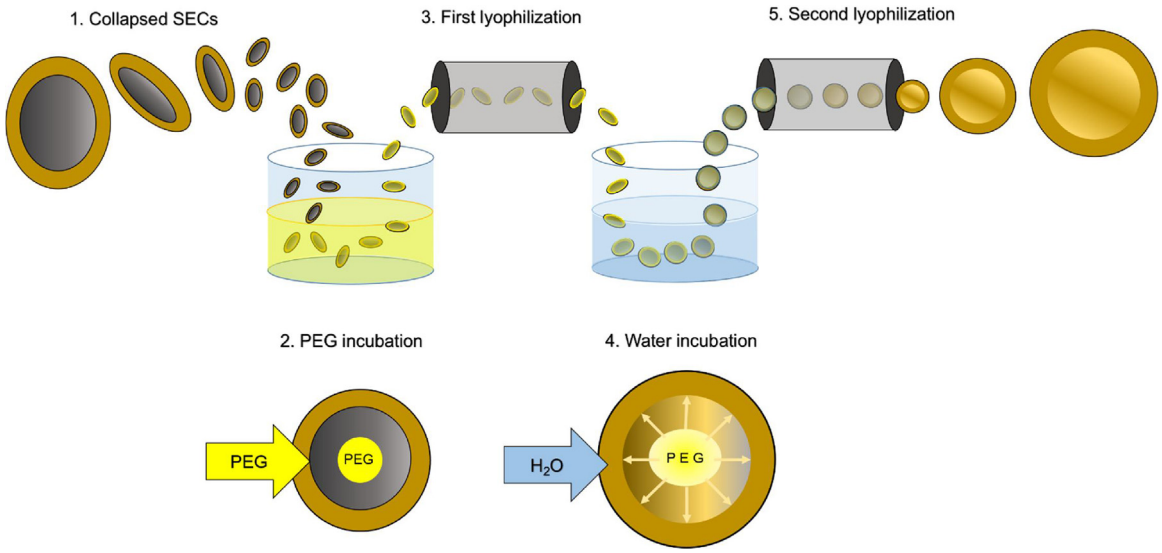

Fig. 6. Schematic for PEG osmotic inflation protocol. (1) Collapsed SECs are placed into PEG solution. (2) Aqueous PEG enters SECs. (3) Lyophilization removes water and concentrates PEG within SECs. (4) Incubation in pure water forms a PEG-water concentration gradient that creates negative osmotic pressure and forces SEC inflation. (5) A second lyophilization step preserves inflated SECs in dry state.

gradient due to high concentrations of osmolyte within the SEC cavity. As the concentration of PEG within the capsule exceeds the concentration of PEG in surrounding solution, the ensuing gradient was predicted to force expansion within the SEC cavity as water enters the semipermeable pollen capsule. Finally, all SECs were removed from solution and, containing residual PEG on the exine surface, freeze-dried once more to preserve the inflated state.

To evaluate effects of PEG treatment, we devised qualitative and quantitative parameters to categorize collapsed and inflated particles. First, scanning electron microscopy (SEM) micrographs were visually inspected to provide qualitative shape definitions: “collapsed” particles were defined as those appearing severely aspheroidal with large surface depressions, and “inflated” particles were defined as those appearing nearly spheroidal with few or no surface depressions. Next, quantitative definitions of structure were defined based on aspect ratio, which is a ratio of a particle’s length to its width and derived from dynamic imaging particle analysis (DIPA) measurements. Because cattail particles are naturally spheroidal, their aspect ratios approach unity as they inflate. Collapsed particles, on the other hand, are reduced in one or more dimensions and therefore have lower aspect ratios, *i.e.*, larger differences between length and width. By assigning aspect ratio (AR) boundaries, quantitative definitions were also established: “collapsed” particles were defined as those whose AR is less than 0.85, and “inflated” particles were defined as those whose AR exceeds 0.85. Examples of representative SECs that conform to the given definitions of “collapsed” and “inflated” particles are shown in Fig. 7. Taken together, SEM and DIPA observations were used to

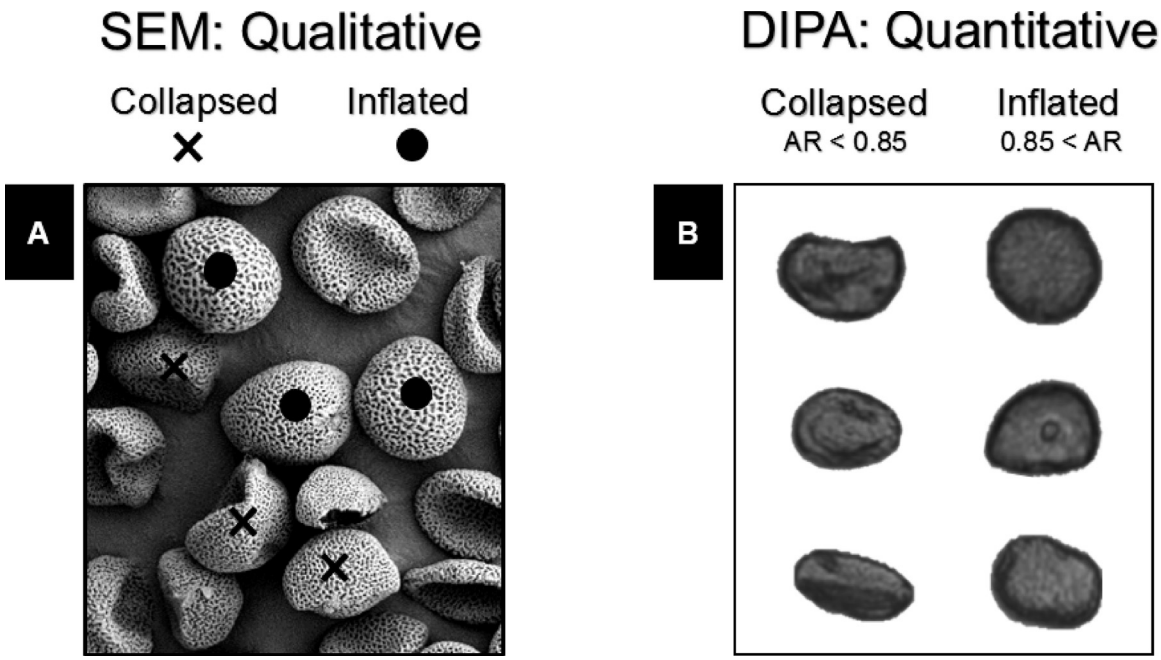

Fig. 7. Qualitative and quantitative methods for determining SEC structural morphology using representative SECs. (A) Visual inspection of SEM micrographs enables qualitative particle categorization that is based on perceived roundness and presence of surface depressions. (B) DIPA analysis enables quantitative particle categorization based on algorithmically determined aspect ratios (AR) that correlate with diameter and turgidity.

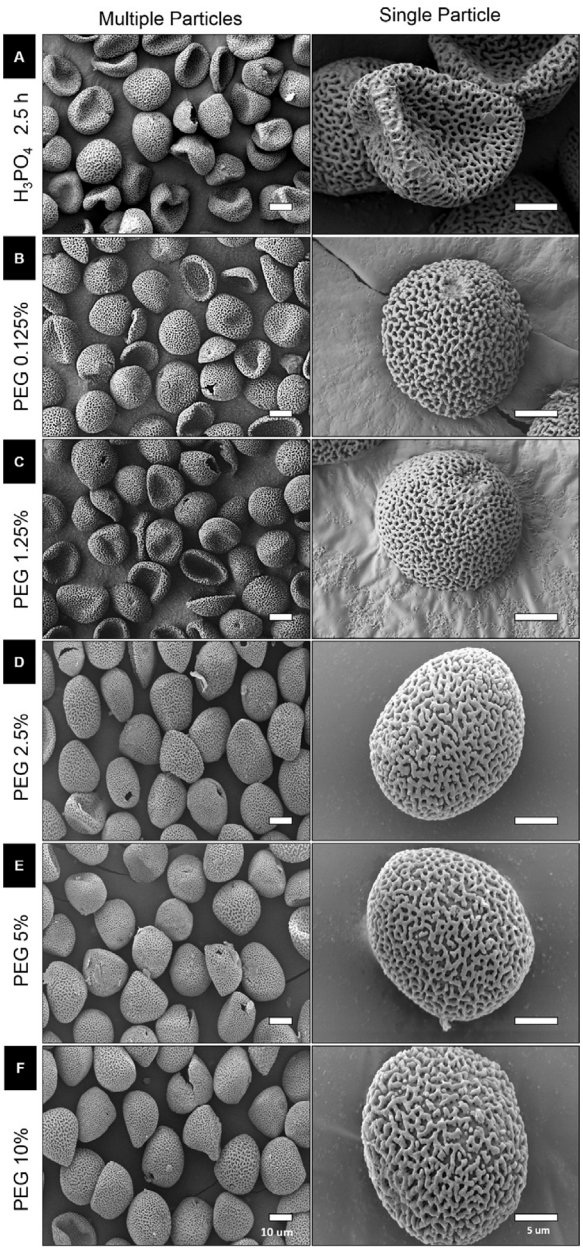

Fig. 8. SEM images of processed cattail pollen grains under reflux with 85% (w/v) phosphoric acid for 2.5 h that have been subsequently treated with (A) 0%, (B) 0.125%, (C) 1.25%, (D) 2.5%, (E) 5%, and (F) 10% (wt%) PEG solution. Steady increases in inflation accompany increasing PEG concentrations up to 2.5% PEG.

evaluate the effects of PEG inflation treatment on microcapsule shape.

SEM micrographs of 2.5 h acid-processed SECs were compared with SECs incubated in progressively increasing concentrations of PEG solution (Fig. 8). The PEG-treated SECs steadily recovered their native spheroidal shape when exposed to increasing PEG concentrations (0%, 0.125%, 1.25%, and 2.5%), suggesting that osmolyte-induced inflation was taking place. Most processed SECs untreated by PEG solution (0%) appear collapsed and misshapen; at 0.125% and 1.25% PEG concentrations, particles appear slightly inflated as evidenced by decreases in surface depressions; at PEG concentrations of 2.5%, particles appear fully inflated as evidenced by a distinct lack of surface depressions and recovery of the native spheroidal particle shape. PEG concentrations beyond 2.5% showed no additional inflation, with SECs incubated in 5.0% and 10% PEG solution appearing similarly turgid and spheroidal. These observations indicate that osmotic inflation was maximized at PEG concentrations of 2.5%.

The amount of inflated particles due to PEG treatment was estimated from both qualitative SEM observations and quantitative DIPA observations. Visual inspection of SEM micrographs (sample size n = 20 or greater) suggested that the number of inflated particles rose fourfold, from 15% in untreated SECs to 60% in SECs treated with PEG concentrations above 2.5% (Fig. 9). At the same time, quantitative DIPA observations of larger data sets (sample size n = 1000) indicated a twofold increase in the amount of inflated particles, from 30% to 60% as by the number of SECs whose aspect ratios exceeded 0.85. Finally, DIPA measurements indicated significant increases in mean particle aspect ratios for treated populations, from 0.78 in untreated particles to 0.86 for those treated with PEG solutions of 2.5% or higher. These data suggest that PEG-inflated SECs have *at least* twice as many uniformly inflated particles as do untreated cattail pollen SECs. Taken together, our findings support the notion that PEG osmolyte effectively inflates lyophilized cattail pollen SECs that suffered from collapse due to processing.

Mechanistic model of microcapsule inflation

To explain our experimental observations, a mathematical model was proposed to investigate the mechanism of microcapsule inflation. Previous investigations have reported that the relationship between PEG concentration and osmotic pressure is not linear but increases according to a second degree polynomial [21]. In our experiment, cattail SEC inflation due to PEG loading was observed to set in above a PEG mass concentration in water of about $\rho = 2.5\,\mathrm{kg\,m^{-3}}$. Here we argue that this effect can be related to the osmotic pressure induced by the PEG molecules inside the SEC. We estimate the corresponding osmotic pressure as [44]:

$$P = cNk_BT, \tag{1}$$

where $c = \rho N_A/M_W$ is the PEG number concentration, $N = M_W/M_M$, is the number of monomers in the PEG, $M_W = 4.0\,\mathrm{kg\,mol^{-1}}$ is the molecular weight of the PEG, $M_M = 0.044\,\mathrm{kg\,mol^{-1}}$ is the molecular weight of one PEG monomer and N_A is Avogadro's number. It is assumed that the osmotic energy PV induces inflation of the material if it exceeds the flexural rigidity D of the cattail exine [45]:

$$D = \frac{h^3E}{12(1-\nu^2)}, \tag{2}$$

where $V = (4\pi/3)a^3$ is the volume of the capsule, $a = 10^{-5}\,\mathrm{m}$, is the radius of the capsule, $h = 10^{-6}\,\mathrm{m}$ is the exine thickness, E is the exine Young's modulus and $\nu = 0.5$ is Poisson's ratio of the exine, which is assumed incompressible. Solving $PV = D$ gives $E = 5\,\mathrm{GPa}$. The product of this Young's modulus and the exine thickness $Eh = 5 \times 10^3\,\mathrm{N/m}$ is within a factor of three of the value found for ragweed pollen using a micro-manipulation technique $Eh = 2 \times 10^3\,\mathrm{N/m}$ [32]. Given the approximate nature of the analysis, a factor of three difference is remarkably small and this is therefore indicative that the model correctly captures the general mechanism, *i.e.*, osmotic inflation. The differences may be attributed to (i) the different structural and chemical properties of the two exine materials, *i.e.*, cattail versus ragweed, as well as to (ii) the assumptions in our model. For instance Eq. (2) is valid for plate bending, while the inflation of cattail involves complex patterns on a curved surface, giving a larger proportionality constant (than 1/12) on the r.h.s. of Eq. (2). Secondly, the concentration c in Eq. (1) is assumed constant and equal to the concentration during loading, while the actual value of c is smaller due to diffusional loss, since SECs are dissolved in pure water during inflation. Taking these

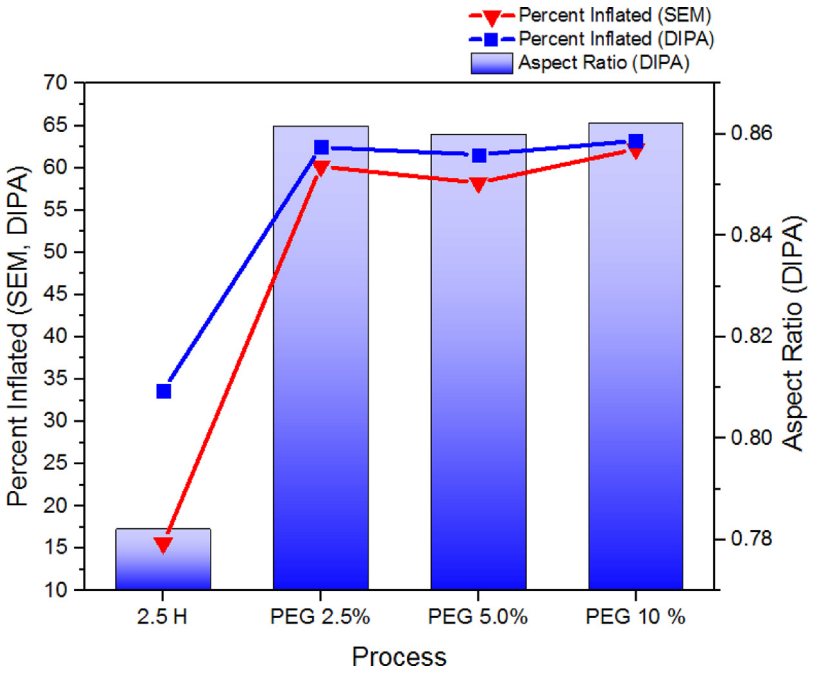

Fig. 9. Morphological evaluation of SECs at fixed acid concentration (85% w/v) processed for 2.5 h without or with incubation step in 2.5, 5.0, and 10% PEG solutions based on qualitative and quantitative analyses from scanning electron microscopy (SEM) and dynamic imaging particle analysis (DIPA), respectively. Inflated particle counts from duplicate analysis of close-up SEM micrographs (red triangles; $n > 20$ highly focused particles) and from triplicate analysis of DIPA population statistics (blue squares; $n = 1000$ focused particles) show that the number of inflated particles increases by at least two-fold with incubation in PEG solution of 2.5% or higher. DIPA analysis also indicated 10% increases in mean aspect ratio (blue bars). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

effects into account would give a smaller *E*, *i.e.*, an even better agreement with the ragweed data. From the above we infer that cattail inflation induced by PEG is due to osmotic pressure, and Eqs. (1) and (2) may serve as a design tool for this purpose. Along this line, a wide range of osmolytes exist in nature, including carbohydrates, amino acids, and methylamines [46], and could be further explored in future works.

Our experimental and mechanistic observations agree that osmotic pressure, induced by an aqueous polymeric solution, inflates and stabilizes sporopollenin microcapsules. We conclude that 2.5% PEG treatment is optimal, because it uses the lowest concentration necessary to cause significant improvements inflated particle count and aspect ratio recovery. PEG treatment improved the morphology of 2.5 h processed SECs up to a point (2.5%), after which no significant gains in morphology were observed. Therefore, this analysis concludes that osmotic pressure treatment using 2.5% or higher PEG solution is suitable for recovering the architectural features of processed cattail SECs.

Conclusions

The efficiency of SEC extraction has been limited by the uncontrolled occurrence of collapsed particles. The fundamental issue of collapsed particles is the lack uniformity, loading volume, and native architecture needed for advancing microencapsulation applications. SEC processing requires the removal of inner protein constituents prior to active loading, and it is after this protein removal phase when particles exhibit a lack of stability that leads to these defects. Whether these defects can be reversed has been the central question of this study. The present study reports the first extraction of sporopollenin exine capsules (SECs) from cattail pollen grains and addresses the issue of SEC collapse *via* a polyethylene-glycol osmolyte-based inflation treatment. Specifically, the systematic investigations herein provide a streamlined procedure for converting raw cattail pollen grains into protein-free SECs that are structurally inflated by osmotic stress from aqueous polyethylene glycol (PEG) solution. We conclude that acetone defatting, 2.5 h reflux in 85% (w/v) phosphoric acid, and sequential washing of raw cattail pollen grains produced clean cattail pollen SECs devoid of cytoplasmic materials, and that additional incubation in aqueous PEG solution (2.5% or higher), followed by rinsing and freeze drying, increased the proportion of inflated cattail SECs from two-to-fourfold, depending on the boundaries used to classify collapsed particles. Furthermore, these reformatted capsules retain their structure even when lyophilized, which facilitates flexible processing approaches for industrial applications. Looking forward, the encapsulation and release studies of active compounds from PEG inflated SECs such as are reported here may tighten the gaps between fundamental pollen-based microencapsulation studies and targeted applications. Taken together, our results suggest that adding PEG osmolyte treatment to lyophilized SEC formulations can dramatically improve the morphology of SECs obtained from raw pollen starting material, opening the door for improved processing strategies for SEC-based microencapsulation applications.

Acknowledgements

This work was supported by the National Research Foundation (NRF-NRFF2011-01) and the Competitive Research Programme (NRF-CRP10-2012-07) of the National Research Foundation of Singapore (NRF), and by the Creative Materials Discovery Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT, and Future Planning (2016M3D1A1024098).

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