Chemical processing strategies to obtain sporopollenin exine capsules from multi-compartmental pine pollen

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ABSTRACT

Pine pollen is widely used in traditional Chinese medicine and has been consumed as a food product for thousands of years. Owing to wind pollination, its pollen grains are composed of a sporoplastic central cavity along with two empty air sac compartments. While this architectural configuration is evolutionarily optimized for wind dispersal, such features also lend excellent potential for encapsulating materials, especially in the context of preparing sporopollenin exine capsules (SECs). Herein, we systematically evaluated one-pot acid processing methods in order to generate pine pollen SECs that support compound loading. Morphological properties of the SECs were analysed by scanning electron microscopy (SEM) and dynamic imaging particle analysis (DIPA), and protein removal was evaluated by CHN elemental analysis and confocal laser scanning microscopy (CLSM). It was identified that 5-h acidolysis with 85\% w/v phosphoric acid at 70 °C yielded an optimal balance of high protein removal and preservation of microcapsule architecture, while other processing methods were also feasible with an additional enzymatic step. Importantly, the loading efficiency of the pine pollen SECs was three-times greater than that of natural pine pollen, highlighting their potential for microencapsulation. Taken together, our findings outline a successful strategy to prepare intact pine pollen SECs and demonstrate for the first time that SECs can be prepared from multi-compartmental pollen capsules, opening the door to streamlined processing approaches to utilize pine pollen microcapsules in industrial applications.

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Introduction

There is broad interest in utilizing natural resources to develop functional materials for a wide range of applications in chemistry and materials science [1–4]. Given their diverse range of highly controlled sizes and structures that vary according to the plant species, pollen grains as well as related spores are a promising example of a natural resource that offers many robust options for microencapsulation strategies [5–7]. They possess a large cavity surrounded by an inner structural support layer (the intine) that is composed of cellulose, hemicellulose, and pectin, which is itself surrounded by a rigid outer coating (the exine) that is composed mainly of a biopolymer, sporopollenin [8–10]. As a key structural component that supports pollen grains' natural function to protect genetic material, sporopollenin possesses high physical and chemical resistance, ultraviolet shielding, and antioxidant capability [9,11–14]. Owing to these features, one of the most promising directions to utilize pollen grains for microencapsulation involves the chemical processing of pollen grains to yield sporopollenin exine capsules (SECs), which faithfully preserve the architectural features of the original grains while lacking sporoplastic and intine contents [15–25]. The resulting SECs are largely devoid of...
protein, making them less allergenic and offering more volume for molecular encapsulation [26]. Combined with high natural abundance and renewable production, these advantageous properties make SECs an ideal delivery vehicle for encapsulating therapeutic drugs, nutrients, and microorganisms as well as for other material science applications [26–36].

Pollen processing has mainly focused on single-compartment pollen species, typically ones which function via biotic pollination and hence possess thicker exine walls [37,38]. Early attempts to prepare SECs involved sequential treatments of organic solvents, alkali, and acid in order to remove lipids, proteins, and intine layers [39–42]. One-pot methods aimed at streamlining the process were later introduced and included individual treatments with hydrochloric acid [15,16], acetic acid [19,21], hydrofluoric acid [18], and combinations of aqueous 4-methylmorpholine-N-oxide and succrose [17,20]. Multi-compartmental pollens, however, including saccate pollens that travel by wind dispersal, are generally more fragile due to thinner exine walls and often rupture during harsh chemical treatments. Indeed, these challenges have proven a common theme among thin-walled pollen, and have motivated the search for improved processing strategies [43]. As a result, milder processing methods such as purely enzymatic treatments have been developed [39–42]. However, enzymatic processing is not practically feasible at larger scales because the necessary enzymes are costly and required in relatively high concentrations. Developing robust chemical methods to prepare SECs from multi-compartmental pollen grains would increase the general utility of pollen SECs, especially since these grains are among the most abundant in nature [38].

In this regard, pine (Pinus taeda) pollen is noteworthy because it has been widely used in traditional Chinese medicine for thousands of years [44] and its structure has been rigorously studied in the biological sciences [45–48]. Pine pollen has two lightweight, empty air sacs attached to its sporopollenic central cavity that aid in wind pollination and the compartments collectively form a micron-scale size (45–75 μm) tripartite structure [49,50]. These relatively fragile sacci possess higher permeability on account of porous features that are nearly two orders of magnitude larger than those of the central cavity (200 nm vs 4 nm) [51], and understanding how these different permeability barriers might be useful for microencapsulation remains to be explored [5,52,53]. Indeed, while the membrane permeability properties of isolated pine pollen exines have been investigated in the context of fundamental pollen biology [50,51,54], there have been no attempts to develop focused strategies for preparing pine pollen SECs. Addressing this gap would significantly advance efforts to achieve scalable chemical processing methods for obtaining high-quality pine pollen SECs as well as to explore the feasibility of loading the SECs with macromolecules for microencapsulation applications. Indeed, while pine pollen has been shown to have relatively low allergenicity (see, e.g., its low skin sensitivity [55,56] and steric limitations for pulmonary uptake [57]), SEC development could further improve this species’ immunogenic profile [58] as well as increase the scope of applications based on enhanced loading of selected compounds into hollow cavities.

Herein, we conducted the first systematic evaluation of one-pot acidolysis protocols for pine pollen SEC extraction in addition to comprehensive characterization of pine pollen SEC morphology and encapsulation efficiency. Processing times, storage methods, temperatures, and acid type were varied in order to achieve maximum removal of potentially allergenic protein components while preserving microcapsule architecture. In selected cases, additional enzymatic treatment with trypsin was used to further demonstrate the potential of fully optimizing sporoplastic removal. A comprehensive set of experimental techniques was utilized in order to characterize the morphological and chemical properties of processed samples as well as to investigate the loading of macromolecules.

**Experimental**

**Materials**

Defatted *P. taeda* pollen was purchased from Greer Laboratories (Lenoir, NC, USA). Sodium bicarbonate, sodium dodecyl sulphate, sodium chloride, organic solvents (acetone, ethanol), BSA, and FITC-BSA were obtained from Sigma–Aldrich (Singapore). Phosphoric acid (85% w/v) was procured from Merck (Singapore). Hydrochloric acid (37% w/v) and sulphuric acid (95% w/v) were purchased from VWR Chemical (Singapore). Polystyrene microspheres (50 ± 1 μm diameter) and 0.25% Trypsin-EDTA were purchased from Thermo Fischer Scientific (Waltham, MA, USA).

**Extraction of pine sporopollenin exine capsules (SECs)**

**Acidolysis processing**

Defatted pine pollen (2 g) was placed into a round-bottomed perfluoralkoxy (PFA) flask fitted with a glass condenser and refluxed (70°C) in aqueous 85% phosphoric acid solution (15 mL) at a stirring rate of 200 rpm for one hour. Samples were collected by vacuum filtration and washed sequentially with warm distilled water (150 mL × 5), acetone (100 mL × 1), 2 M HCl (100 mL × 1), distilled water (100 mL × 5), acetone (100 mL × 1), and ethanol (100 mL × 2). Washed microcapsules were dried in an oven at 60°C for 6 h, followed by storage at room temperature as either a dry powder or aqueous suspension in water. This protocol was repeated for different batches of the same pollen, using different reflux durations (1 h, 2.5 h, 5 h, 10 h, and 20 h) and samples were collected for analytical characterization. The reflux duration that demonstrated the highest yield of intact pollen microcapsules was re-tested using one or more of the following adjustments: wet storage, as a suspension of SECs in distilled water that is kept at room temperature; lowered reflux temperatures (50°C and 25°C); and different acid reagents (hydrochloric and sulphuric acids).

**Enzymatic treatment with trypsin**

As an extension of the study, one sample (18% w/v hydrochloric acid for 5 h at 70°C) was chosen for additional enzymatic treatment with trypsin. This batch was washed with room temperature distilled water (100 mL × 5) over vacuum filtration and transferred into a 0.25% trypsin-EDTA (15 mL) solution for 24 h incubation at 35°C. Samples were subsequently washed with distilled water (100 mL × 5), transferred into sodium bicarbonate solution (10 g/L) containing sodium dodecyl sulphate (1 g/L), and finally dried for 24 h at room temperature. After drying, the SECs were thoroughly washed with distilled water and divided into wet and dry storage.

**Micromeritic evaluation by dynamic imaging particle analysis (DIPA)**

DIPA was performed using a FlowCam® benchtop system (FlowCamVS, Fluid Imaging Technologies, Maine, USA) equipped with a 200 μm width flow cell (FC-200) and 20× magnification lens (Olympus®, Japan). The flow cell was visually inspected and cleaned with ethanol prior to each sample run. Polystyrene microspheres (50 ± 1 μm diameter) served to calibrate the microscope focus, and a representative histogram was plotted to verify the proper operation under the defined measurement settings. Defatted natural pine pollen and processed SECs (at 2 mg/mL concentration) were sonicated in a water bath for 10 min and filtered through 100 μm diameter filter meshes prior to experiment. The samples were then manually added into the flow cell via
a pump-controlled syringe and analysed at a flow rate of 0.1 mL/min. A minimum of 10,000 particles was scanned and three separate measurements were performed. Data analysis was carried out using 300 well-focused particles.

**Surface morphology evaluation by scanning electron microscopy (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 both interior (cross-sectional) and exterior morphological changes were observed.

**Elemental CHN analysis**

A VarioEL III elemental analyser (Elementar, Hanau, Germany) provided CHN analysis to determine the amount of residual protein. All samples were dried at 60 °C for 1 h before being combusted in excess oxygen at high temperature to release compositional carbon, hydrogen, and nitrogen. Protein content was calculated using percent nitrogen with a 6.25 multiplication-factor that corresponds to the protein content in pine pollen SECs, in accordance to recommendations from the Association of Analytical Communities [59]. All measurements were conducted in triplicate.

**Confocal laser scanning microscopy analysis (CLSM)**

Defatted pine pollen, processed SECs, and fluorescein isothiocyanate-bovine serum albumin (FITC-BSA)-loaded SECs were mounted on sticky slides with Vectashield® and scanned via confocal laser scanning microscopy (Carl Zeiss LSM700, Germany), as previously described [60]. Laser excitation lines were set to 405 nm, 488 nm, and 561 nm at a scan speed of 67 s per phase. Images were collected with differential interference contrast at 405 nm (6.5%), 488 nm (6%) and 561 nm (6%) using enhanced-contrast (EC) Plan-Neofluar 100× and 1500 × 1.3 oil objective M27 lenses. The fluorescence emission was collected in photomultiplier tubes equipped with different filters (416–477 nm, 498–5 nm, and 572–620 nm) and analysed by using the ZESS 2008 software package.

**Encapsulation of bovine serum albumin (BSA)**

BSA protein loading was achieved by using vacuum loading protocols [54,61]. Unprocessed pollen and select batches of dry-stored processed SECs (85% phosphoric acid for 5 h at 70 °C, and 18% hydrochloric acid for 5 h at 70 °C, with additional 24 h trypsin treatment) were suspended in 0.5 mL of 50 mg/mL aqueous BSA solution within polypropylene tubes and mixed via vortexing (IKA, Staufen, Germany) for 1 min. The mixture was then subjected to vacuum treatment at 0.006 mbar for 2 h. The tubes were collected and the loaded particles were washed by centrifugation with 1 mL of water at 12,000 rpm for 3 min and then freeze-dried overnight. Blank batches of untreated pollen and SECs were prepared similarly without BSA loading. In addition, FITC-BSA was encapsulated in the same way and imaged via CLSM in order to observe the molecular localization of loaded components within SEC particles. Following preparation, BSA-loaded SECs (10 mg) were crushed using a mortar and pestle for 5 min to expel encapsulated BSA [62]. The crushed powder was mixed with phosphate-buffer saline (pH 7.4) (2 mL), vortexed for 5 min, and centrifuged at 15,000 rpm for 5 min. The supernatant was filtered using a 0.45 μm diameter polyethersulfone (PES) syringe filter (Agilent, CA, USA). Absorbance values were measured at 280 nm by using a Boecos220 spectrophotometer (Hamburg, Germany) along with appropriate controls (SEC blank and BSA standards at different concentrations), and the amount of BSA in the SECs was calculated by the following equation:

\[
\text{% BSA loading} = \frac{\text{Weight of BSA}}{\text{Weight of BSA} - \text{loaded SECs}} \times 100\%
\]

**Results and discussion**

**Process development and analytical characterization**

Phosphoric acid (H₃PO₄) is widely used in consumer products, generally-recognized-as-safe, and affordable, and has consequently proven an attractive solvent for SEC processing [11,22,59]. Pollen SEC extraction using phosphoric acidolysis has been conventionally performed at temperatures up to 180 °C for durations as long as one week (168 h) [26,32–35,52,63]. While such protocols may suit SEC extraction from single-compartment, thick-walled pollen grains and spores such as Lycopodium clavatum, they can cause significant damage to the tripartite microstructure of P. taeda pollen grains, including the fragile air sacs [51]. In recent work, our group has successfully established streamlined acidolysis protocols to extract SECs from Healianthus annuus (sunflower) [64], L. clavatum (moss) [61] and Zea mays (corn) [43] using highly efficient acidolysis protocols that require much shorter time intervals. Building on these efforts with single-compartment pollen grains and in light of the challenges associated with multi-compartment pine pollen, the following experimental and analytical characterization strategies were aimed at identifying suitable processing strategies to extract pine pollen SECs.

**Processing scheme**

As presented in Fig. 1, we systematically tested one-pot phosphoric acid processing across a variety of durations, temperatures, and storage methods based on the following SEC extraction process: natural pine pollen was first defatted with diethyl ether, refluxed with acid under the appropriate conditions, and then washed and dried. Extracted pine SECs were stored in either dry (conventional) or aqueous wet conditions (to mitigate potential collapse of the thin-walled pollen), and characterized using various analytical techniques in order to evaluate the degree of structural preservation as well as the removal of sporoplastic and protein contents. From these experiments, optimal conditions were identified and then extended to strong acids and, in some cases, the treatment protocols included an additional enzymatic processing step [15,16]. A detailed description of all processing conditions is provided in Table 1.

**Evaluation of SEC structural preservation**

Depending on the extraction process, the structural integrity of the resulting SECs will vary and the morphological structure of SEC particles can be analysed at the single-particle level by high-throughput DIPA measurements [61]. We divided the pine pollen SECs into three groups for classification: “intact,” “fractured,” and “collapsed.” Representative examples of particles that were assigned to each group are presented in Fig. 2. Preserved particles closely resemble unprocessed pine pollen, with both air sacs attached to the central cavity in a tripartite microstructure and show no significant breaks or cracks. However, they may have minor deviations in shape, average diameter, or increases in surface roughness, as compared to the untreated case. Fractured...
SECs have clearly visible holes, cracks, or missing sections in one or more compartment, but at least one uncompromised, fully enclosed compartment remaining based on the visual inspection. Examples of fractured SECs may include a complete central cavity that has damaged or missing air sacs, or an intact air sac that is attached to other damaged/missing compartments. Collapsed SECs appear shrivelled and flat and have very little inner volume for loading. These classifications provide a starting point to aid the development of optimized processing strategies.

Effects of processing conditions on particle intactness

Following this characterization strategy, we initially examined the effects of processing duration, storage method, and reflux temperature on SEC particle morphology, and the results are summarized in Table 2. Before processing, the natural pollen grains were 99% intact with only trace amounts of collapsed and fractured particles. The first parameter that was then tested was the time scale of pollen grain processing in 85% phosphoric acid at 70°C, followed by conventional dry storage. After 1 h of processing, 45% of particles were intact while 32% were collapsed. With increasing processing time, the percentage of intact particles increased to ~60% while the percentage of collapsed particles decreased to approximately 10% and 2% after 5 h and 20 h, respectively. This decrease in the fraction of collapsed particles at longer processing times supports past accounts of pine pollen grains undergoing rapid collapse due to acid shock resulting from phosphate ion exchange between membranes [32]. At the same time, processing times of 10 h or longer led to a large number of fractured particles, reaching around 40%. Optimal results in this test series were obtained with processing in 85% phosphoric acid at 70°C for 5 h, with ~60% intact particles, as well as ~30% fractured and ~10% collapsed.

As the sporopollenin exine walls of pine pollen are relatively thin and hence fragile, we next explored whether a wet storage environment that provides structural stability [43] could reduce the percentage of collapsed particles and improve the overall yield. To explore this option, the particles were processed in 85% phosphoric acid at 70°C, followed by wet storage. In this case, the percentage of intact particles increased to 78% while the percentage of collapsed particles decreased to 0.5%. These findings support that wet storage facilitates particle intactness by avoiding the structural collapse/buckling that occurs with dehydrated pollen microcapsules [65–68], although dry storage would likely increase durability and shelf-life of the SEC particles for industrial applications. Similar results were also obtained with processing in 42% phosphoric acid, supporting that the high acid concentration is suitable for SEC production. Based on this optimized condition (5 h phosphoric acid, wet storage), the effect of temperature was next investigated and the number of intact particles increased at lower temperature, reaching 93% intact particles when processed at 25°C. Hence, good control over the processing steps could be achieved as indicated by retention of pine pollen exine morphology in the SECs.

For comparison, we used a similar standard protocol (5 h at 70°C) to test two strong acids, including hydrochloric (HCl) and
sulphuric (H₂SO₄) acids. Processing in 18% hydrochloric acid successfully yielded 92% intact and 7% fractured particles, while processing in 27% hydrochloric acid was less optimal, resulting in 83% intact and 17% fractured particles. On the other hand, processing in sulphuric acid yielded 77% intact and 23% fractured particles. As these two strong acids are known to dissolve cellulosic intine materials but not proteaceous materials [69], we also explored the feasibility of adding an additional processing step with trypsin protease (as described in Ref. [51]) to the 18% hydrochloric acid protocol and identified that the number of intact particles decreased to ~75%. Collectively, the findings demonstrate that both weak and strong acids that are widely used in SEC extraction protocols successfully work with pine pollen grains, and that the fragile nature of this thin-walled pollen species is an important factor for optimizing the processing conditions.

Based on the data collected, the initial evidence suggests that a large percentage of intact particles can be obtained with single-pot phosphoric acid processing or a combination of strong acid (e.g.,

![Fig. 2](image-url) Representative optical micrographs of processed SECs with different morphological states. Based on visual inspection of the optical micrographs, individual particles were classified as (A) intact (preserved tripartite microstructure with no ostensible breaks or cracks), (B) fractured (cracked or missing portions, with at least one fully preserved compartment), or (C) collapsed (significantly shrivelled with low encapsulation volume).

<table>
<thead>
<tr>
<th>Processing condition</th>
<th>Intact (%)</th>
<th>Fractured (%)</th>
<th>Collapsed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unprocessed</td>
<td>99.0 ± 0.3</td>
<td>0.8 ± 0.2</td>
<td>0.1 ± 0.2</td>
</tr>
<tr>
<td>85% H₃PO₄ at 70°C for 1 h (dry)</td>
<td>44.8 ± 6.6</td>
<td>23.2 ± 6.0</td>
<td>31.8 ± 4.2</td>
</tr>
<tr>
<td>85% H₃PO₄ at 70°C for 2.5 h (dry)</td>
<td>56.6 ± 5.8</td>
<td>241 ± 2.4</td>
<td>18.6 ± 2.6</td>
</tr>
<tr>
<td>85% H₃PO₄ at 70°C for 5 h (dry)</td>
<td>60.6 ± 0.8</td>
<td>298 ± 1.9</td>
<td>9.4 ± 2.3</td>
</tr>
<tr>
<td>85% H₃PO₄ at 70°C for 10 h (dry)</td>
<td>55.2 ± 8.7</td>
<td>412 ± 9.2</td>
<td>3.7 ± 0.5</td>
</tr>
<tr>
<td>85% H₃PO₄ at 70°C for 20 h (dry)</td>
<td>55.2 ± 3.8</td>
<td>432 ± 4.1</td>
<td>3.6 ± 0.6</td>
</tr>
<tr>
<td>85% H₃PO₄ at 70°C for 5 h (wet)</td>
<td>80.6 ± 4.2</td>
<td>187 ± 4.3</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td>42% H₃PO₄ at 70°C for 5 h (wet)</td>
<td>76.1 ± 8.7</td>
<td>23.3 ± 8.7</td>
<td>0.5 ± 0.5</td>
</tr>
<tr>
<td>85% H₃PO₄ at 50°C for 5 h (wet)</td>
<td>82.0 ± 3.6</td>
<td>18.0 ± 3.6</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>85% H₃PO₄ at 25°C for 5 h (wet)</td>
<td>93.3 ± 3.2</td>
<td>6.7 ± 3.2</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>18% HCl at 50°C for 5 h (wet)</td>
<td>77.1 ± 6.4</td>
<td>22.8 ± 6.4</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>27% HCl at 25°C for 5 h (wet)</td>
<td>92.2 ± 2.0</td>
<td>7.2 ± 2.1</td>
<td>0.1 ± 0.2</td>
</tr>
<tr>
<td>25% H₂SO₄ at 50°C for 5 h (wet)</td>
<td>83.0 ± 3.7</td>
<td>17.0 ± 3.7</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

Table 2: Effect of different processing conditions on the morphological properties of processed SEC samples. The number of intact, fractured, and collapsed particles are expressed as percentages from data collected for >300 particles.
hydrochloric acid) processing followed by enzymatic treatment with a protease enzyme.

**Micromeritic properties**

In addition to assessing the structural integrity of chemically processed samples, DIPA analysis was conducted in order to determine the number-weighted average particle diameter of each sample, as presented in spline curve fit histograms in Fig. 3. While unprocessed pollen grains had an average diameter of 62 μm, the average diameter shrank to around 56 μm for samples treated with 85% (70 °C) phosphoric acidolysis for 2.5 h or shorter durations. With longer processing times, the average particle diameters once again approached the values for unprocessed pollen grains (Fig. 3A), and the average diameter of particles processed for 5 h duration or longer was around 59 μm. Furthermore, the average diameters of particles treated with 85% or 42% phosphoric acid were similar, as were the samples processed at different temperatures, once again demonstrating that the high acid concentration and high temperature are suitable for the processing step (Fig. 3B, C). The strong acid treatments also did not affect the particle size (Fig. 3D). Taken together, the data reinforce that an optimal processing time (in the range of 5–10 h, and defined to be 5 h for our experiments) overcomes initial particle collapse due to acid shock while avoiding excessive particle damage due to fracturing.

**Morphological investigation**

Representative SEM micrographs of pine pollen grains after phosphoric acid acidolysis for different processing times are presented in Fig. 4. The cross-sectional image of the unprocessed pine pollen grains reveals the presence of pollen constituents inside the large central inner cavity (Fig. 4A). As discussed above, the majority of SEC particles appeared to collapse after acidolysis for a period of 1 h and some were still present after 2.5 h (Fig. 4B, C). However, at longer processing times, the fraction of collapsed particles decreased and the 5 h time point again showed an optimal balance of intact particles (Fig. 4D–F).

In addition, the SEM micrographs for pine pollen SECs prepared using strong acids are presented in Fig. 5. In all three tested cases, the SECs appear largely intact as expected. While most particles were observed to be intact, the fraction of damaged particles were generally more fractured than collapsed. Indeed, cross-section images were obtained from highly damaged particles and, in these cases, proteinaceous components were still visible in the central cavity. These aspects are further discussed below in the context of elemental analysis and highlight that phosphoric acid is advantageous for removing sporoplasmic contents in general, while strong acids require an additional processing step with a proteolytic enzyme to aid SEC production.

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**Fig. 3.** Size histograms of processed SEC particles. (A) Effect of processing time in 85% phosphoric acid at 70 °C. (B) Effect of phosphoric acid concentration. The time and temperature were fixed at 5 h and at 70 °C, respectively. (C) Effect of processing temperature for 5 h processing in 85% phosphoric acid. (D) Alternative processing strategies with strong acids (5 h at 70 °C). Data is collected from >300 individual particles per sample.
Fig. 4. SEM micrographs of (A) unprocessed pine pollen and (B–F) SECS processed with 85% phosphoric acid at 70°C for varying durations of processing time.
Fig. 5. SEM micrographs of SECs processed with different strong acids for 5 h at 70°C.

Fig. 6. Protein content and protein removal efficiency for processed SEC samples as determined by CHN analysis. The removal efficiency is determined based on the protein content of the processed samples relative to an unprocessed sample.
Assessment of protein removal

In addition to retaining morphological structure, a key requirement of SEC production is the removal of potentially allergenic proteins from the pollen microcapsules. To verify protein removal, CHN elemental analysis based on high-temperature combustion was conducted on untreated and treated pine pollen samples, and the percentage of protein removal was determined based on the amount of nitrogen remaining in the samples. The key measurement principle behind this approach is that it is known that proteins are the only major component of pollen grains which contain nitrogen, and hence measuring the nitrogen content of SEC particles provides an indication of how much protein was removed as a result of chemical processing [52]. The percentage of sporoplasmic protein removal was determined by the following equation:

\[
\text{%Sporoplasmic removal} = \frac{\%N_{\text{Unprocessed}} - \%N_{\text{Processed}}}{\%N_{\text{Unprocessed}}} \times 100\%
\]

As presented in Fig. 6, unprocessed pine pollen was determined to have a protein content of around 11%, which agrees well with literature values for other pollen species tested in our group (8–32% depending on species) and this control sample provided the reference value for 0% protein removal. While 1 h treatment with 85% phosphoric acid at 70 °C led to a 70% reduction in protein content, longer treatments with 85% phosphoric acid were more effective, yielding around 85–89% removal of protein content. This efficiency agrees well with SECs prepared from other pollen species. When using 85% phosphoric acid at 70 °C, treatment times of 2.5 h or longer were equivalent in their utility for protein removal. By contrast, treatment with 85% phosphoric acid at lower temperatures was less effective at removing proteins, achieving removal efficiencies around 75%. Likewise, 5 h treatment with 42% phosphoric acid at 70 °C also had poor removal efficiency, around 63%.

Taken together, these data support that 5 h treatment with 85% phosphoric acid at 70 °C was particularly effective at removing protein and, in line with the morphological analysis, this processing condition was selected from among the one-pot options as the optimal strategy for preparing SECs for microencapsulation.

On the other hand, treatments with strong acids were less effective at removing protein. In all cases, one-pot treatment of pine pollen grains with strong acids (5 h treatment at 70 °C) led to protein removal efficiencies around 57–65%. These values are consistent with the previous observations that indicate that strong acids are typically less effective at cleaning SECs. To improve protein removal, trypsin was added to the hydrochloric

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**Fig. 7.** CLSM images of unprocessed pine pollen and processed SEC samples without and with loaded BSA. The processing conditions were either 85% phosphoric acid for 5 h at 70 °C, or 18% hydrochloric acid for 5 h at 70 °C followed by trypsin treatment. Left column: cross-section of pollen or SECs before protein loading. The blue autofluorescence corresponds to compounds naturally present in pollen or SECs. The other columns present 3D reconstructions of BSA-loaded pollen or SECs, for which the dual-channel CLSM images show compounds naturally present in pollen or SECs (blue) and loaded FITC-labeled BSA (green). All scale bars are 20 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
acid-treated pollen sample and this two-step protocol had a protein removal efficiency around 93%. As a control, it was also identified that trypsin alone was ineffective for removing protein. Hence, sequential treatments with hydrochloric acid and trypsin were selected as the two-step method of choice for preparing SECs for further exploration for microencapsulation.

Evaluation of loading efficiency

Fig. 7 presents confocal laser scanning microscopy (CLSM) images characterizing the loading properties of unprocessed and processed pollen grains. As discussed above, two SEC samples were selected for this evaluation based on one-pot treatment (85% phosphoric acid for 5 h at 70 °C) and two-step treatment (18% hydrochloric acid for 5 h at 70 °C, followed by trypsin incubation), respectively. The CLSM approach is useful because the pollen exine wall as well as its sporoplastic contents are known to autofluoresce across a wide range of excitation wavelengths, hence providing a visual means to assess structural integrity as well as qualitatively verify protein removal [35,61].

Cross-sectional images of the unloaded natural pollen and two SEC samples confirm that the processing steps effectively removed sporoplastic contents from inside the exine capsules, whereas an extensive quantity of autofluorescent contents is visible inside the natural pollen sample. FITC-labeled BSA protein was next loaded by vacuum methods into the samples as previously described, so that the protein could be visualized by CLSM. Under equivalent image settings, cross-sectional slices were collected and reconstructed to form a three-dimensional representation of the individual particles. It was observed that the fluorescence intensity of loaded protein was typically greater for SEC samples than for natural pine pollen grains, suggesting that the SECs have higher loading efficiencies than natural pollen. In particular, the loading inside the central cavity appeared to be greater for SECs versus the natural pine pollen. Co-localization of the fluorescence signals from the pollen exine wall (blue channel) and the loaded protein (green channel) further supports that the protein is encapsulated within the particles in all three cases.

To verify protein loading, absorbance measurements were also conducted in order to quantitatively determine the loading efficiency of the different samples. The vacuum loading method was utilized, and the loaded samples were extensively washed before measurement. The natural pine pollen had a loading efficiency of 7.9 ± 1.5%, which is comparable to other pollen species. This low efficiency is likely attributed to the presence of sporoplastic contents in the central cavity. By contrast, the loading efficiency of the SECs was around three-times greater, with values in the range of 23–26% that demonstrate excellent loading capacity. The phosphoric acid-treated SECs had a loading efficiency of 23.0 ± 2.6%, whereas the hydrochloric acid-treated SECs (with additional trypsin treatment) had a loading efficiency of 26.5 ± 3.7%. These findings strongly support the CLSM results, and demonstrate that pine pollen SECs can be prepared, which are morphologically intact, devoid of protein contents, and capable of efficient loading. The increased loading of the SECs can be explained by the removal of sporoplastic contents (greater available loading volume per particle and a higher number of particles per unit mass) as well as dissolution of the intine layer, which increases access to the nanoscale channels facilitating protein encapsulation. Of note, while loading appeared to be greater in the air sacs for the natural pine pollen grains, the loading appeared to be greater in the central cavity for the SECs. This difference in loading properties indicates that chemical processing affects the molecular permeability of one or both cavity types. In particular, these findings support that the permeability of the central cavity in natural pine pollen grains is largely controlled by the molecular properties of the intine layer and that chemical processing removes this intine layer [70]. As demonstrated in this work, systematic investigation of chemical processing strategies identified that 85% phosphoric acid for 5 h at 70 °C is optimal while other strategies are also possible.

Conclusion

While pine pollen is among the most widely used pollen species in industrial settings, the development of pine pollen SECs for microencapsulation applications has remained unexplored. Herein, we addressed this gap by conducting a systematic investigation aimed at identifying optimal chemical processing strategies to extract pine pollen SECs. Several processing parameters were tested, including acid type, processing duration, temperature, and storage method, and the results were evaluated by characterizing the morphological properties of resulting SECs as well as the efficiency of protein removal. Based on these characterization efforts, it was observed that one-pot acidolysis with 85% phosphoric acid for 5 h at 70 °C was the optimal condition on account of preserving the multi-compartment capsule architecture and successfully removing proteins. It was also possible to prepare SECs by utilizing hydrochloric acid together with subsequent enzymatic treatment, although hydrochloric acid or other strong acids alone were insufficient to effectively remove proteins. The resulting SECs demonstrated three-times greater loading efficiencies than the natural pollen grains, and CLSM imaging demonstrated that the higher loading efficiency primarily arises from greater encapsulation within the central cavity. This finding directly supports that removal of sporoplastic contents facilitates higher loading efficiencies of the SECs, as compared to the unprocessed pollen grains. In summary, these results outline a successful processing strategy to prepare SECs from multi-compartmental pollen capsules and demonstrate that the resulting capsules can be loaded with high efficiency. Given the wide range of applications for pine pollen grains, there is excellent potential for applying these processing strategies to utilize pine pollen SECs in real-life applications such as drug delivery and taste-masking.

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