

Physical and biochemical characterization of chemically-treated pollen shells for potential use in oral delivery of therapeutics

Md Jasim Uddin^a, Sumedha Liyanage^b, Nouredine Abidi^b, and Harvinder Singh Gill^{a,*}

^a *Department of Chemical Engineering, Texas Tech University, Lubbock, TX 79409, USA*

^b *Department of Plant and Soil Science, Texas Tech University, Lubbock, TX 79409, USA*

*Corresponding author:

Dr. Harvinder Singh Gill

Texas Tech University

Department of Chemical Engineering

8th Street and Canton Ave, Mail Stop 3121, Lubbock, TX 79409-3121, USA.

E-mail address: harvinder.gill@ttu.edu

Abstract

Allergen-free pollen shells obtained from natural pollen grains have recently attracted attention as microcapsules for oral therapeutic delivery. We have recently developed a chemical treatment method that enables successful retrieval of hollow pollen shells from diverse species. A comprehensive characterization is critical to characterize the effects of chemical treatment which will not only benchmark the pollen treatment process, but it can also lay the foundation of quality control procedures to check allergen removal efficiency during pollen treatment. Therefore, in this study, we followed the effects of chemical treatment on four different pollen species using electron microscopy, elemental analysis, gel electrophoresis, confocal microscopy, Fourier transform infrared (FTIR) spectroscopy, and thermogravimetric analysis. These analyses revealed that acetone treatment removed lipids from the pollen surface. Phosphoric acid treatment removed proteins and nucleic acids from the pollen core and transformed esters into carboxylic acids. KOH hydrolysis changed carbohydrate composition of the pollen wall. Chemically treated pollen shells exhibited hydroxyl and carboxyl functional groups on their surface. Overall, we propose that confocal microscopy could be used as a rapid scanning technique to visualize the removal of biomolecules, while FTIR combined with gel electrophoresis could be used as a more objective approach for analysis and benchmarking.

Keywords: Fourier transform infrared (FTIR) spectroscopy; microcapsules; microencapsulation; mucosal vaccination; oral drug delivery; physical characterization; thermogravimetric analysis (TGA); vaccine adjuvants

Introduction

Pollen grains are an engineering feat of nature. They are tough microcapsules that enclose the male gametophyte of the plant and safely transport them for plant sexual reproduction.¹ To serve this important role, pollen grains possess high mechanical, thermal, and chemical resilience.² This toughness allows them to withstand climatic and other assaults to protect the fragile gametophyte they carry. Recently, this resilient nature of pollens has been exploited for a wide range of applications. For example, pollens have been used to microencapsulate oral drugs,^{3,4} vaccines,^{5,6} live yeast cells,⁷ food supplements such as fish oil for taste masking,⁸ and a magnetic resonance imaging (MRI) contrast agent.⁹

In nature, pollens appear with high diversity in size, shape, porosity, and surface morphology.¹⁰ Majority of the pollens consist of a thick wall made of two layers: an inner layer called intine, and an outer layer called exine. The intine predominantly consists of carbohydrates such as cellulose and pectin,¹¹ while the exine is composed of sporopollenin, which is a highly resistant biopolymer made of carotenoids, phenylpropanoids, and phenolic compounds.^{2,12} Pollens contain both lipid-based and proteinaceous molecules. The lipids are often found covering the exine surface as a sticky material called pollenkitt or tryphine.¹³ The double-layered pollen wall and the pollenkitt facilitates pollination and protects the gamete from dehydration, hydrolysis, UV radiation, and microbial invasion.¹³ Proteins are largely found in the core of pollens. Some of these proteins are known to cause airway allergy.¹⁴ Thus, before pollens can be utilized as microcapsules, these proteins need to be removed to create vacant space and to avert any potential allergic reaction.

A widely used pollen treatment protocol is the one used to treat lycopodium spores (*Lycopodium clavatum*).¹⁵ In this protocol, the spores are exposed sequentially to acetone (4 h reflux), KOH (12 h reflux), and phosphoric acid (7-day reflux) with multiple wash steps in between each treatment. The spores produced through this processing are intact and the residual nitrogen content is almost zero, which is taken as an indicator that the spores have zero to negligible residual protein that could be a source of allergies. While successful for lycopodium spores, this thorough protocol has not been successful in the context of removing allergens from other pollen species. For example, exposure of both sunflower (SF) (*Helianthus annuus*)¹⁶ and short ragweed (*Ambrosia artemisiifolia*) pollens¹⁷ to KOH resulted in the pollens getting embedded in an insoluble matrix from where they could not be recovered. As a result, many variations of the lycopodium treatment protocol have been developed to avoid the formation of the insoluble matrix. The approaches include: i) bypassing the base hydrolysis step altogether and instead directly treating pollens with phosphoric acid,¹⁶ ii) using HCl followed by NaOH,¹⁸ iii) using ionic liquids,¹⁹ and iv) neutralizing NaOH by HCl followed by ethanol reflux.¹⁵ The treatment times and processing temperatures have also been reduced in order to prevent pollens from getting damaged.¹⁶ The nitrogen content, which is taken to be a measure of residual protein in the pollens has either not been measured in many studies,^{3,18,20} or when measured^{15,19,21} was found to be still high with respect to the lycopodium spore treatment protocol.

To overcome the lack of a universally-applicable protocol for producing allergen-free and intact pollens from different species, we have recently developed a rigorous treatment protocol,²² which mirrors the thoroughness of the lycopodium treatment protocol. Using the new protocol, we were able to successfully produce intact and clean common ragweed (RW) (*Ambrosia elatior*) pollens with a low nitrogen content (0.19 % nitrogen). After performing oral vaccination with these allergen-free RW pollens, we confirmed the lack of an allergenic response in mice.⁶ More specifically, analysis of serum antibody from RW vaccinated mice

showed no RW protein-specific IgE antibody, which indicated that the RW pollen treatment process was thorough in removing RW allergenic proteins.

Since pollens of each species are biochemically distinct, the chemical treatment process can affect pollen surface chemistry and can produce characteristically different pollen shells. Hence, besides measuring nitrogen content, more rigorous analytical tests are required to characterize pollen shells. Fourier transform infrared (FTIR) spectroscopy²³⁻²⁵ is a widely used technique to characterize lipids, proteins, carbohydrates, and sporopollenin in natural pollen grains. However, complex and overlapping infrared vibrational bands from biomolecules and residual moisture often mask critical features of protein vibration. Hence, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)²⁶ can be used to complement protein detection. Here we studied the effects of chemical treatment on black alder (BA) (*Alnus glutinosa*), lamb's quarters (LQ) (*Chenopodium album*), RW, and SF pollen. A suite of analytical methods including scanning electron microscopy (SEM), elemental analysis, SDS-PAGE, FTIR spectroscopy, confocal microscopy, and thermogravimetric analysis (TGA) was used to physically evaluate and biochemically analyze the pollens.

Materials and methods

Pollens and chemicals

BA, LQ, RW, and SF pollens were obtained from Pharmallerga (Lisov, Czech Republic). All pollens were stored at 4 °C before chemical processing. All chemicals and materials were purchased from Fisher Scientific (PA, USA) unless otherwise stated. Fluorescein isothiocyanate (FITC)-conjugated ovalbumin (OVA), lane marker reducing sample buffer, and Coomassie Brilliant Blue G-250 dye were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Tris/Glycine/SDS electrophoresis buffer, 4-20% Mini-PROTEAN TGX precast gels for SDS-PAGE, and protein standards (10-250 kD) were obtained from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Common RW extract was obtained from Greer® (Lenoir, NC, USA). Ultrapure Milli-Q water (Direct-Q 3 UV system, MilliporeSigma, MA, USA) with an electrical resistivity of 18.2 MΩ.cm at 25 °C was used in all applications.

Preparation of empty pollen shells

Pollen shells from natural pollens were extracted by a method previously described.⁶ Briefly, 30 g of natural raw pollens were stirred (300 rpm) and refluxed in 350 ml of acetone at 70 °C for 12 h. Solutions were cooled and filtered using a vacuum filtration unit (MilliporeSigma, Billerica, MA, USA) with Whatman filter paper (grade 4). The filtrates were kept at room temperature to evaporate acetone, dried at 60 °C overnight to remove residual acetone, and the wax-like residue obtained after drying was analyzed. The pollens recovered on the filter paper were next subjected to acidolysis by stirring them in 400 ml of 85% (w/w) phosphoric acid at 60 °C for seven days. After phosphoric acid treatment, pollen solutions were cooled, diluted with water, filtered, and extensively washed with hot water, acetone, 2 M HCl, 2 M NaOH, water, acetone, and 70% ethanol successively. After air-drying, pollens were refluxed in 350 ml of 6% (w/v) KOH for 12 h at 80 °C (KOH renewed after 6 h with a fresh solution). Pollen solutions were then cooled, diluted with water, filtered, and extensively washed with hot water, acetone, and 70% ethanol. Finally, pollens were dried at 60 °C for 24 h in an oven. Weights of the final dried pollen were measured. All pollens were stored at room temperature before use in the experiments. Pollen samples after each chemical treatment step were also stored for further analysis.

Pollen shell characterization

To visually study the interior of the pollen shell, pollen samples were manually fractured in the presence of dry ice, mounted on an aluminum stub covered with carbon conductive tape, and coated with gold-palladium (Technics Hummer V sputter coater, Anatech USA, CA, USA). Pollen surface morphology was visualized by a scanning electron microscope (Hitachi S-4300 E/N FESEM, NY, USA). Pollen morphological features were measured from electron micrograph images using ImageJ (National Institutes of Health, Bethesda, MD, USA). Carbon, hydrogen, and nitrogen content in the pollen samples were analyzed using PerkinElmer 2400 Series II CHNS/O Analyzer (PerkinElmer, Inc., Waltham, MA, USA). Protein percentage was calculated from nitrogen analysis by multiplying with a factor of 6.25.⁵ All measurements were performed in triplicate.

SDS-PAGE

Proteins were extracted from raw and treated pollens and analyzed with SDS-PAGE using a method published previously.²⁷ Briefly, dry pollens were first fragmented using a magnetic stir bar (1000 rpm, 3 h, room temperature) in a glass vial, the broken pollens were then stirred in phosphate buffered saline (PBS) (20 mg/ml) for 24 h at 4 °C, and the solutions were filtered with Vivaspin centrifugal concentrators (1000 kD MWCO, Sartorius AG, Goettingen, Germany) at 10,000 × g for 5 min at 4 °C to obtain particle-free protein extracts. Extracts were used immediately for SDS protein analysis or stored at 4 °C for later use. For SDS-PAGE analysis, 25 µl of extract mixed with SDS loading buffer (60 mM Tris-HCl, 1% SDS, 10% glycerol, 20 mM dithiothreitol) was loaded in a well of a 4-20% gradient gel. Proteins separated by SDS-PAGE were visualized by staining the gel with colloidal Coomassie solution (10% v/v of 85% phosphoric acid, 10% w/v of ammonium sulfate, 0.12% w/v Coomassie brilliant blue G250, and 20% v/v of methanol dissolved in ultrapure Milli-Q water sequentially) in a rocking shaker for 2.5 h and de-staining with Milli-Q water overnight.²⁶

OVA encapsulation and confocal laser scanning microscopy

Confocal images were obtained from both natural and chemically treated pollens by mounting them under a glass coverslip (number 1.5) in Vectashield™ mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA), and visualizing with a confocal microscope (Nikon Eclipse Ti-E inverted microscope equipped with C2 confocal scanning head, Nikon Instruments Inc., Melville, NY, USA). Four lasers of 405, 488, 561, and 640 nm were used to excite the pollens with 417-477, 500-550, 570-1000, and 417-477 nm bandpass emission filter, respectively. All images were captured with Nikon Plan Apo VC 100x oil immersion lens (numerical aperture 1.4). Micrographs of individual channels were overlaid in Nikon NIS-Elements AR software (version 4.13.01) to obtain the merged image. Laser power (15 mW power output from the fiber tip) was set to values between 7% and 15%. Photomultiplier tube (PMT) detector gain was adjusted between 70 and 80. Digital offset value was set between -4 and -7 to eliminate background signals. Pinhole size, scan speed, and pixel size were set to 90 µm, 1/16th frame/s, and 512 × 512, respectively.

To study OVA encapsulation, FITC-OVA solution was prepared by dissolving 1 mg of OVA in 1 ml of PBS. Chemically treated pollens were then mixed with FITC-OVA solution (16.7 mg pollen/ml of solution), vortexed for 1 min, and incubated at room temperature under vacuum (25 in. of Hg). After overnight incubation pollens were separated from the solution, the outer liquid was allowed to air-dry for 1-2 min, and then imaged. FITC-OVA loaded pollens were excited with 488 and 561 nm lasers and images were captured as described above.

FTIR spectroscopy

FTIR spectra of both intact and manually fractured pollen samples were recorded under environmentally controlled conditions as specified below using an FTIR instrument (Spotlight 400, PerkinElmer, Inc., Waltham, MA, USA) equipped with a Universal Attenuated Total Reflectance (UATR) accessory. The UATR-FTIR was also equipped with a ZnSe-Diamond crystal composite (single bounce) that allows collection of FTIR spectra directly on a sample without any special preparation. The “pressure arm” of the instrument was used to apply a constant pressure (monitored by PerkinElmer Spectrum version 10.5 software) to the pollen samples positioned on top of the ZnSe-Diamond crystal to ensure a good contact between the sample and the incident IR beam, thereby minimizing loss of the IR beam. All FTIR spectra were collected at a spectral resolution of 4 cm^{-1} , with 32 co-added scans over the range from 4000 to 650 cm^{-1} . A background scan of the clean ZnSe-Diamond crystal was acquired before scanning the samples. The PerkinElmer Spectrum was used to perform spectra normalization and baseline corrections.

TGA

TGA of pollen samples was performed using the Pyris 1 TGA equipped with a 20-sample auto-sampler (PerkinElmer Shelton, CT, USA). Thermograms were recorded between $37\text{ }^{\circ}\text{C}$ and $600\text{ }^{\circ}\text{C}$ with a heating rate of $10\text{ }^{\circ}\text{C}/\text{min}$ in a flow of nitrogen at $20\text{ ml}/\text{min}$. Pollen samples were conditioned in a laboratory maintained at $65 \pm 2\%$ relative humidity and $21 \pm 1\text{ }^{\circ}\text{C}$ for at least 48 h. Then, sub-samples (between 1.5 mg and 2 mg) were placed in the sample pan. The Pyris software was used to calculate the first derivatives of the thermograms (DTG) and to calculate the percent weight loss for each sample.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). A two-tailed t-test was used to analyze data from elemental analysis.

Results

Morphological characteristics of pollen shells after chemical treatment

Each pollen species has a unique size, shape, and ornamentation on its exine surface, which can get damaged during chemical treatment. Thus, we used SEM to examine pollen species before (raw) and after chemical treatment. Raw BA pollens ($26\text{--}27\text{ }\mu\text{m}$ in diameter) are oblate in shape and highly monodisperse in size (Figure 1A). They contain 4-5 apertures that are sealed with an operculum (Figure 1B). Each aperture is oblong-shaped with a major axis and a minor axis measuring about $3.6\text{ }\mu\text{m}$ and $2.4\text{ }\mu\text{m}$, respectively (Figure 1C). Internal view of the raw BA pollen shows cellular materials that are naturally found inside the pollen core (Figure 1D). After chemical treatment, BA pollen remained intact, monodisperse ($22\text{--}23\text{ }\mu\text{m}$ in diameter), and their morphology did not change (Figure 1E). However, chemical treatment opened the apertures and caused them to shrink along the minor axis to half their original length (Figure 1F-G). After treatment BA showed an empty and a clean core (Figure 1H).

Raw LQ pollens are spherical in shape with a diameter of about $25\text{--}26\text{ }\mu\text{m}$ and are monodisperse in size (Figure 1I-J). Their outer surface has approximately 70-80 operculum-sealed apertures each about $1.6\text{--}1.8\text{ }\mu\text{m}$ in diameter (Figure 1J). The entire exine surface is

further covered with spinules (raised features) and puncta (depressions, 40-50 nm in diameter) (Figure 1K). Similar to BA, natural LQ also contains cellular material in their core (Figure 1L). After chemical treatment, LQ pollens did not show any structural damage (Figure 1M-N), the operculum got removed from some of the apertures (Figure 1N-O), and a clean core was obtained (Figure 1P).

Natural RW pollens are spherical, monodisperse, and about 20-22 μm in diameter (Figure 2A-B). The aperture in the raw RW is sealed as shown in Figure 2C, and it also contains cellular material in the core (Figure 2D). After treatment, RW pollens remained intact and retained their morphology and monodisperse (15-18 μm in diameter) nature (Figure 2E-F). Distinct short conical spines measuring approximately 1.5 μm in height are evident in Figure 2F. The apertures opened up (Figure 2G) after treatment and a clean core was obtained (Figure 2H). As seen in BA, the aperture of RW also shrank after treatment.

Like other pollens, raw SF pollens are also monodispersed, but are spheroidal in shape with a diameter of approximately 40 μm (Figure 2I-J) along the major axis. Their exine surface is decorated with spines longer than RW (approximately 5.6 μm long) (Figure 2J) and have a sealed aperture (Figure 2K). Natural SF also contained cellular material inside their core (Figure 2L). After treatment, SF pollens remained intact and retained their morphology and monodisperse nature (Figure 2M-N), the apertures opened up (Figure 2O), and a clean core was obtained (Figure 2P). Similar to BA and RW, SF pollen reduced in size (31-32 μm) after treatment. Both RW and SF pollens show a laminated exine wall with distinct ectexine (outer exine) and endexine (inner exine) wall.

Protein analysis

Although electron microscopic analysis showed that the pollen core was clean, we further analyzed the pollens for the presence of proteins through elemental analysis and SDS-PAGE. Elemental analysis indicates that the percent of carbon in pollen increased significantly ($p \leq 0.0001$) after chemical treatment (Figure 3A), the hydrogen content remained similar (in BA and RW) or decreased slightly (in LQ and SF) (Figure 3B), and the protein content (nitrogen content $\times 6.25$) dramatically decreased to about 2% or lower from 21 to 28% (Figure 3C).

Since elemental analysis indicated the presence of trace amounts of nitrogen-containing compounds in the treated pollens, we extracted and compared proteins from both raw and treated pollens. Raw pollen contained proteins between 10 and 100 kD as shown in Figure 3D (lane 2, 4, 6, and 8). In contrast, chemically treated pollen did not show any detectable protein band (Figure 3D: lane 3, 5, 7, and 9), suggesting that treated pollens are protein-free. It is to be noted that equal masses of raw and treated pollens were compared in this analysis. Since treated pollens are lighter, this group contained approximately threefold more pollen shells per unit mass than raw pollen.

OVA encapsulation into hollow pollen shells

We saw that after chemical treatment the apertures in the pollens got opened. These openings were sufficiently large that they could serve as entryways to fill the shells with therapeutic molecules. Therefore, we used FITC-OVA as a model protein drug to evaluate the potential of loading of these pollen shells. We found that the hollow interior of the pollens could be filled with molecules by simply mixing dry pollens with an aqueous solution of the molecule and applying mild vacuum (Figure 4 and S-1, in the Supporting Information). For comparison purposes, raw and treated pollens are also shown (Figure 4). It can be seen that before treatment, pollen

shells and their cores exhibit intrinsic fluorescence at various wavelengths of light (Figure 4 and S-2, in the Supporting Information). In contrast, after treatment, the pollen core lost its fluorescence, which indicates the removal of material from the pollen cores (Figure 4 and S-3, in the Supporting Information). The pollen walls of all species retained a strong autofluorescence even after chemical treatment.

FTIR analysis of raw pollens

To gain an understanding of how pollens of different species differ in their chemical composition, we characterized and compared FTIR spectra of all four untreated (raw) species in intact and broken form. Each raw pollen showed a distinct FTIR spectrum (Figure 5: manually broken, Figure S-4: intact, in the Supporting Information). Common functional groups observed from their spectroscopic analysis are listed in Table 1.

The broad peak centered at about 3290 cm^{-1} in raw pollens (Figure 5 and S-4, in the Supporting Information) is associated with O-H stretching in alcohol groups of pectin, cellulose or in phenolic groups of lignin.^{25,28} Adsorbed water also contributes to this vibration. The sharp peaks at 2925 and 2854 cm^{-1} in raw pollens are associated with out-of-phase CH_2 stretching and in-phase CH_2 stretching, respectively,^{25,29,30} which can be attributed to lipids, proteins or carbohydrates. Raw BA and LQ pollens also showed vibration at 3012 cm^{-1} which may arise from $=\text{C-H}$ stretching in unsaturated fatty acids or lipids.²⁸ A sharp peak in the range $1743\text{--}1734\text{ cm}^{-1}$ in all raw pollens is due to C=O stretching in esterified pectin and lipids.^{25,28,30} Our commercially sourced non-defatted raw pollens showed this distinct absorption, although defatted raw pollens that are also commercially available may lack this peak. Other lipid-associated vibrations were 1462 (CH_3 asymmetric bending),³¹ 1378 (CH_3 symmetric bending), 1231 (C-O stretching),³² 1169 (CO-O-C asymmetric stretching), and 720 cm^{-1} (CH_2 rocking).³¹

Proteins in all raw pollens were characterized by amide I vibrational band at $1668\text{--}1654\text{ cm}^{-1}$ that corresponds to C=O stretching^{30,33} and amide II band at $1548\text{--}1544\text{ cm}^{-1}$ due to N-H bending and C-N stretching.^{30,33} However, protein vibrations are complicated by the overlapping aromatic ring vibration ($1660\text{--}1500\text{ cm}^{-1}$)³⁴ and adsorbed water vibration ($1640\text{--}1600\text{ cm}^{-1}$)²⁵ in the same spectral region. The strong vibration at 1626 cm^{-1} in raw LQ pollen corresponds to C=C bond²⁵ which was absent in other raw pollens while the vibration 1606 cm^{-1} appears as a shoulder in raw LQ. The bands at 1606 , 1515 , 1169 , and 833 cm^{-1} can be associated with the vibration of aromatic rings in the sporopollenin.^{30,31,35,36} It has been shown that ferulic acid and p-coumaric acid act as building blocks of sporopollenin in pollens and moss spores.^{37,38} These phenylpropanoid acids are the likely contributors to the phenyl ring vibrations.²⁹ The strong vibration at 1435 cm^{-1} was due to C-H bending vibration in sporopollenin, lipids, proteins, and carbohydrates.^{25,32,36} The vibration at 1415 cm^{-1} present only in LQ could be due to symmetric stretches of carboxylate ($-\text{COO}^-$).³⁹ The vibration at 1378 cm^{-1} was present in all raw pollens except LQ pollen where it appears as a shoulder. This could be attributed to CH_3 bending in cellulose, hemicelluloses, and lipids.^{29,40} The vibration in the range $1334\text{--}1322\text{ cm}^{-1}$ was due to C-C and C-O skeletal vibrations in cellulose.²⁵ The peak 1282 cm^{-1} could be assigned to C-O-C vibration in pectin.³⁴ Besides lipids and phospholipids, the vibration $1236\text{--}1228\text{ cm}^{-1}$ may also arise from phosphate group (PO_2^-) of nucleotides.⁴¹ The band 1169 cm^{-1} can be attributed to the vibration of pyranose ring (C-O-C) in cellulose³² and sporopollenin.³¹

Apart from the common vibrational spectra at 988 cm^{-1} due to C-O in cellulose,⁴² all pollens showed distinct carbohydrate vibrations. The specific vibrations were at 1101 , 1043 , and 925 cm^{-1} for BA; 1101 and 1013 cm^{-1} for LQ; 1034 cm^{-1} for RW; and 1073 and 1027 cm^{-1} for SF. These vibrations were attributed to C-O stretching in carbohydrates (arabinogalactan,

amylose),^{24,40}, secondary alcohols of pectin³⁴ or cellulose.²⁴ Thus, carbohydrate 'fingerprint' region ($1200\text{--}900\text{ cm}^{-1}$) was particularly unique for each of the studied pollen species.

FTIR analysis to study the effect of different chemical treatments on RW pollen

To obtain a better understanding of what each chemical step does to the pollens, we selected RW pollen and characterized it after each chemical step. RW FTIR spectra significantly changed after each chemical treatment step (Figure 6). The vibration 1743 cm^{-1} (C=O stretching in lipids) decreased after acetone treatment while all other peaks remained similar. This suggests that acetone treatment removed lipid materials from RW surface without significantly affecting the proteins. To confirm this, we further analyzed the wax-like extract obtained after acetone treatment (Figure S-5, in the Supporting Information), in which lipid peaks (2922 , 2853 , 1732 , 1465 , 1377 , 1171 , 1070 , 975 , and 721 cm^{-1}) appeared to be the prominent vibrations.

Commercially available proteins extracted from RW (Greer®) were also analyzed for comparison (Figure S-6, in the Supporting Information). As expected, the Greer® RW protein extract showed protein vibrations of 1639 and 1539 cm^{-1} , however, the corresponding protein vibrations in the raw RW pollen (Figure 5 and S-4, in the Supporting Information) and after acetone treatment (Figure 6) were shifted. This shift could be due to the interactions of protein vibrations with vibrations of other pollen-constituents.

Phosphoric acid and KOH treatment of RW significantly affected their pollen chemistry as is evident from the vibrations between 1700 and 900 cm^{-1} . The vibrations 1548 and 1231 cm^{-1} in phosphoric acid treated RW completely disappeared suggesting that acid treatment removed all the proteins and nucleic acids from the pollen shell. Notably, three new peaks emerged at 1700 , 1579 , and 1410 cm^{-1} that can be attributed to the C=O stretching in carboxylic acids,³⁰ asymmetric stretching of carboxylate anions (COO^-),⁴³ and symmetric stretching of carboxylate anions (COO^-),⁴³ respectively. The vibration at 1658 cm^{-1} is due to free (COO^-) from pectin.⁴⁴ Acid treatment also removed cellulose as evident from the disappearance of the vibration at 1031 cm^{-1} (C–O–C stretching). KOH treatment affected the carbohydrate region. A new vibration emerged at 972 cm^{-1} which is likely due to C–O vibration in carbohydrates.⁴²

FTIR analysis of BA, LQ, and SF pollens after chemical treatment

To investigate the effects of chemical treatment on BA, LQ, and SF pollens we also analyzed their FTIR spectra (both intact and manually broken) after the last treatment step (KOH step) (Figure 7: manually broken, Figure S-7: intact, in the Supporting Information). The FTIR spectra of all the four treated pollens were very similar in the region 4000 to about 1700 cm^{-1} , however, unique signatures were seen in the region lower than around 1700 cm^{-1} . The broad O–H peak associated with pectin, cellulose, and adsorbed water shifted to the higher wavenumber and centered at about 3375 cm^{-1} in all the treated pollens. Removal of lipids from all pollen species was apparent from the concurrent disappearance of the vibrations around 3012 and 1743 cm^{-1} . Similar to RW, amide II vibrational band in treated BA, LQ, and SF pollens was also absent, suggesting that proteins get removed after chemical treatment. The vibration around 1700 cm^{-1} due to the carboxylic acid group was less distinctive in treated BA and LQ pollens as compared to RW and SF. This suggests that RW and SF contain carboxylic acids functional groups on their surface while BA and LQ have few to none.

The prominent phenyl ring vibrations ($1515\text{--}1500$, 1170 , and 833 cm^{-1}) were preserved after treatment. Furthermore, the strong vibration at 1070 cm^{-1} may be due to amylose²⁴ and arabinogalactan,⁴⁰ while the 1100 cm^{-1} vibration may be due to cellulose.²⁴ The vibration that

remained at 1435 and 1375 cm^{-1} could also arise from carbohydrates while the vibrations at 1575 and 1407 cm^{-1} can be attributed to the carboxylate anions (COO^-).³⁹ Thus, the FTIR spectra show that treated pollens contain aromatic ring bearing compounds (sporopollenin), carboxylic acids functional groups, and carbohydrates (pectin, amylose, and arabinogalactan).

Thermal degradation behavior of raw and treated pollens

In order to compare the thermal characteristics of the pollens, we studied pyrolysis of pollen in an inert nitrogen environment. Both raw and treated pollens showed a decrease in pollen mass when heated up to 600 °C (Figure 8). Untreated BA, LQ, RW, and SF pollen lost approximately 82, 74, 76, and 74% of their initial mass, respectively. In contrast, after chemical treatment, BA, LQ, RW, and SF pollen lost about 73, 59, 43, and 67 % of their initial mass, respectively.

To better comprehend the loss of material during chemical treatment, first derivatives were obtained from the respective thermograms (DTG). Raw pollens (before treatment) showed several decomposition stages (Figure 8). The first decomposition step occurred at 50-58 °C, which accounts for 4.5-5.3% of the mass loss, and is likely due to the evaporation of adsorbed water from raw pollens. Other decomposition steps were at 223-238 °C, 280-282 °C, 321-335 °C, and 450-463 °C that could be due to the degradation of materials from the pollen core as well as the pollen wall. The treated pollens showed a significantly different thermal decomposition profile. Two sharp peaks and one broad peak appeared in their DTG. At 41-45 °C approximately 3.4-8.7% of the mass was lost, which can be attributed to water loss. This suggests a relatively weaker adsorption of water in treated pollens as compared to raw pollens. A broad decomposition was seen between 250 and 380 °C, which accounted for 8-22.5% of the mass loss. This loss is likely due to pectin and cellulose degradation⁴⁵ since the temperatures for the maximum rate of decomposition for pectin and cellulose are 234 °C⁴⁶ and 355 °C,⁴⁵ respectively. The sharp decomposition at 441-452 °C could be due to partial depolymerization and decomposition of the sporopollenin wall. The absence of decomposition at 223-238 °C and 280-282 °C in treated pollen suggests that these decompositions are likely related to the cellular materials that were present in the raw pollen core, but absent in the treated pollens.

Discussion

We have recently developed a chemical treatment process by which pollens of different species can be thoroughly cleaned to remove their lipids, proteins, and cytoplasmic contents, and the resulting clean shells can be recovered in an intact form.²² In this study, our objective was to obtain a deeper insight into the chemical treatment process. To achieve this objective, we selected four different pollens, namely, RW, SF, LQ, and BA, and characterized them with different techniques.

Electron microscopic analysis showed that chemically treated pollen shells preserved their unique morphological characteristics and the pollen walls remained intact. However, some changes were observed. For example, SF pollens attained a more spherical shape after treatment as opposed to their natural spheroidal shape as raw pollens. Moreover, apertures of BA and RW pollens were reduced in size after they got opened due to the chemical treatment. BA, RW, and SF pollen shells reduced in size after chemical treatment. Similar changes in pollen size after different chemical treatments have been observed previously.^{16,47} Thus, an important conclusion valid for all the four pollen species is that the chemical treatment is able to preserve the structural integrity and opens their apertures.

After chemical treatment, the protein content reduced to about 2% or less for all pollen species. Pollen protein content is typically estimated from the total nitrogen content using a nitrogen to protein conversion factor. However, one limitation of this method is to accurately identify the conversion factor, which can vary from 5.18 to 6.38 among animal-based (milk, beef, chicken, egg) or plant-based (flour, rice, bean, nut) protein sources.⁴⁸ For pollens, there is no established conversion factor available.⁴⁹ Nonetheless, a factor of 6.25 has been typically used for pollens. Another limitation of the nitrogen to protein conversion approach is that it can overestimate the protein content. This is because the method assumes that all measured nitrogen comes from proteins, however, the nitrogen can also emanate from non-protein compounds such as alkaloids.^{49,50} Thus, protein percentage obtained from nitrogen analysis is a cautious overestimate of the actual amount of protein. Obtaining protein-free pollen shells is critical because different pollen proteins have been implicated as major allergens.^{14,51} Thus, besides nitrogen elemental analysis we used other complementary techniques to measure proteins. Both gel electrophoresis and FTIR analysis confirmed the removal of proteins from pollen shells.

Chemical treatment not only removes the potentially allergenic proteinaceous material, but it also produces an empty core. This was visually confirmed using electron and confocal microscopy. Furthermore, chemical treatment also opens the apertures and creates entrances through which the pollen core can be filled. Using FITC-OVA solution and confocal imaging we found that cores of all the different pollen species could be filled with the protein solution.

Pollens in their raw state and after chemical treatment have previously been shown to possess distinct autofluorescence at different excitation wavelengths.^{16,52} We similarly observed this natural fluorescence in raw and treated pollens of all four species. We have observed strong autofluorescence in the core of defatted SF pollen under all excitations including 488 nm (Figure S-8, in the Supporting Information). This observation contradicts a previous report that showed defatted SF pollens do not have green autofluorescence in their core or their wall (488 nm).⁵³ In this study, not only do we see green autofluorescence from the core of defatted SF, but we also see it in its exine wall. This intrinsic autofluorescence of the pollen wall can be beneficial to locate pollens in different experimental procedures. For example, we recently used confocal microscopy in conjunction with the pollen autofluorescence property to locate treated club moss spores⁵ and RW pollens²⁷ in the mouse intestinal wall after oral gavage.

It was interesting to see that endexine of RW and SF survived after phosphoric acid treatment. The presence of SF endexine after acid hydrolysis is also supported by a previous report.¹⁶ Interestingly, two distinct exine walls of RW pollen were also visible after chemical treatment in confocal micrographs. RW endexine wall was fluorescent in all four wavelengths of light used for imaging. This finding points to a presence of sporopollenin-like material in the RW endexine that survives phosphoric acid treatment and retains autofluorescence.

FTIR analyses were used to determine chemical changes in pollens after chemical treatment. It is important to note that we performed FTIR for both intact and manually fractured pollens. Since the pollen wall is few micrometers in thickness and infrared beam in ATR-FTIR analysis method has a shallow depth of penetration,³¹ FTIR may not capture the vibrations of molecules from pollen core. Therefore, to perform a rigorous analysis, we fractured the pollens. The FTIR of intact pollens is presented as supplementary data. No significant differences were seen between the FTIR spectra of intact and fractured pollens.

FTIR analyses showed considerable differences in chemical composition of pollens before and after chemical treatment. Treatment with acetone and phosphoric acid removed

lipidic and proteinaceous components, respectively, from natural pollens. Unique fingerprint signatures were seen for pollens at wavenumbers less than about 1700 cm^{-1} . These fingerprint signatures could form the basis of assays for establishing batch-to-batch consistency. They could also be used to study pollen stability over time and to identify appropriate storage conditions by comparing the fingerprint spectra between different conditions.

FTIR data further showed that pollen shells of all the four species contained aromatic, carboxylic acid, and hydroxyl functional groups. Presence of these functional groups could be exploited for surface modification. Indeed, sporopollenin from lycopodium spore with similar functional groups has previously been shown to act as a solid support for catalytic reactions and enzyme immobilization.^{2,54} For example, the carboxylic acid group on the sporopollenin was converted into primary amine for ion-exchange applications,⁵⁵ and derivatized hydroxyl functional groups on sporopollenin surface were used to sequester oil from oil-water emulsions.⁵⁶ Thus, treated pollen shells of different species can similarly provide an excellent solid support to functionalize them with drugs and to develop multifunctional platforms through chemical modification of the functional groups. The FTIR spectra that we have obtained can guide investigators regarding the presence of specific functional groups on the different pollens after treatment, and can thus help in designing the appropriate chemical modification reactions.

TGA analysis showed that pollens have moisture adsorbed on their surfaces, which was also confirmed from FTIR spectra. Species-specific thermal stability was observed, with the treated RW pollen showing most thermal stability in comparison to BA, LQ, and SF pollens. Pollens after treatment appear to be stable up to at least $200\text{ }^{\circ}\text{C}$, suggesting that they could be suitable for use in applications that require exposure of pollens to moderately high temperatures. It is important to note that TGA was done in the absence of oxygen. It is possible that in the presence of oxygen, pollens might possess lower stability due to the possibility of combustion.

Pollens are morphologically diverse. Their physical and chemical characteristics can have an impact on their biological functions, especially in their use as an oral drug and vaccine delivery system.^{5,6} Recently, we proposed a possible mechanism to elucidate how protein-free RW pollen can mediate an immune response after oral vaccination.²⁷ It is possible that knowledge of chemical groups on the pollen surface could add to our understanding of this mechanism. However, the effects of physical and chemical properties of the pollen shells on biological activity still remain unexplored and need additional studies.

Pollen shells from new pollen species are continuously being developed for oral delivery applications. Shell wall structural integrity and residual protein content are the two major measures predominantly used to standardize pollen treatment methods. While intact pollen shell with minimum protein content is critical for oral delivery application, the analytical methods to evaluate these two criteria should be carefully chosen. Particularly, elemental analysis should not be used as a standalone method to set minimum nitrogen content in the treated pollen, since residual nitrogen can vary from species to species. More rigorous characterization methods are therefore necessary to benchmark pollen treatment. In this study, we have shown that FTIR analysis and gel electrophoresis can be used as complementary methods to evaluate pollen treatment efficiency. FTIR measurement can be readily adapted as an essential tool for pollen characterization during bulk manufacturing, long-term storage stability analysis, and functional modification to develop drug-pollen conjugates. In future, other sensitive methods may also be required to identify trace biomolecules in the treated pollen shells.

Conclusion

Pollens of four different species were treated with our newly developed chemical treatment process and characterized using different techniques to obtain a deeper understanding of the effects of the treatment. Intact and protein-free pollen shells were readily produced from natural pollens after chemical treatment. The pollen surface microarchitectures were preserved after chemical treatment. As a result of the treatment, microscopic apertures, which are naturally closed, got opened up. Using these apertures, the pollen core was filled with fluorescently labeled OVA as a model therapeutic molecule. Confocal microscopy showed that pollens of all species could be filled with OVA. The shell wall of treated pollens continued to show fluorescence under different excitation wavelengths similar to that exhibited by the untreated pollens. FTIR analysis showed that treatment with acetone and phosphoric acid removed lipidic and proteinaceous components, respectively. The FTIR spectra showed unique signatures for the pollens in the region less than around 1700 cm^{-1} . Different pollen shells had different thermal stability, however, typically they were stable up to about $200\text{ }^{\circ}\text{C}$ before they started showing weight loss. The data confirms that protein could not be detected even with gel-electrophoresis. Overall, these data provide a deeper insight into the chemical treatment process. Some of these assays could form the basis of developing a panel of quality control assays.

Acknowledgments and conflict of interest disclosure

We thank Rajeev Rajbhandari and Erandi Rajakaruna in the Department of Plant and Soil Science at Texas Tech University for their help with TGA and FTIR. This research was supported by the National Institutes of Health (NIH) [grant number DP2HD075691] and the Defense Advanced Research Projects Agency (DARPA) [grant number N66001-12-1-4251]. HSG is a co-inventor on a patent related to the development of pollen grains for oral vaccines. This potential conflict of interest has been disclosed and is managed by Texas Tech University.

Supporting Information.

The material includes additional confocal micrographs; FTIR spectra of intact pollen grains, chemically treated pollen shells, ragweed pollen extracts, and proteins (PDF)

References

1. Edlund AF, Swanson R, Preuss D 2004. Pollen and stigma structure and function: the role of diversity in pollination. *Plant Cell* 16(suppl 1):S84-S97.
2. Mackenzie G, Boa AN, Diego-Taboada A, Atkin SL, Sathyapalan T 2015. Sporopollenin, the least known yet toughest natural biopolymer. *Front Mater* 2:66.
3. Akyuz L, Sargin I, Kaya M, Ceter T, Akata I 2017. A new pollen-derived microcarrier for pantoprazole delivery. *Mater Sci Eng C* 71:937-942.
4. Sudareva N, Suvorova O, Saprykina N, Vilesov A, Bel'tiukov P, Petunov S, Radilov A 2017. Two-level delivery systems for oral administration of peptides and proteins based on spore capsules of *Lycopodium clavatum*. *J Mater Chem B* 5(37):7711-7720.
5. Atwe SU, Ma Y, Gill HS 2014. Pollen grains for oral vaccination. *J Control Release* 194:45-52.
6. Uddin MJ, Gill HS 2018. From allergen to oral vaccine carrier: a new face of ragweed pollen. *Int J Pharm* 545(1-2):286-294.
7. Hamad SA, Dyab AFK, Stoyanov SD, Paunov VN 2011. Encapsulation of living cells into sporopollenin microcapsules. *J Mater Chem* 21(44):18018-18023.
8. Barrier S, Rigby AS, Diego-Taboada A, Thomasson MJ, Mackenzie G, Atkin SL 2010. Sporopollenin exines: a novel natural taste masking material. *LWT - Food Sci Technol* 43(1):73-76.
9. Lorch M, Thomasson MJ, Diego-Taboada A, Barrier S, Atkin SL, Mackenzie G, Archibald SJ 2009. MRI contrast agent delivery using spore capsules: controlled release in blood plasma. *Chem Commun* (42):6442-6444.
10. Hesse M, Halbritter H, Weber M, Buchner R, Frosch-Radivo A, Ulrich S, Zetter R. 2009. Pollen terminology: an illustrated handbook. ed., New York: Springer-Verlag Wien.
11. Brooks J, Shaw G 1968. Chemical structure of the exine of pollen walls and a new function for carotenoids in nature. *Nature* 219(5153):532-533.
12. Brooks J, Shaw G 1978. Sporopollenin: A review of its chemistry, palaeochemistry and geochemistry. *Grana* 17(2):91-97.
13. Pacini E, Hesse M 2005. Pollenkitt – its composition, forms and functions. *Flora* 200(5):399-415.
14. Gadermaier G, Hauser M, Ferreira F 2014. Allergens of weed pollen: an overview on recombinant and natural molecules. *Methods* 66(1):55-66.
15. Atkin SL, Barrier S, Cui Z, Fletcher PDI, Mackenzie G, Panel V, Sol V, Zhang X 2011. UV and visible light screening by individual sporopollenin exines derived from *Lycopodium clavatum* (club moss) and *Ambrosia trifida* (giant ragweed). *J Photochem Photobiol B: Biol* 102(3):209-217.
16. Mundargi RC, Potroz MG, Park JH, Seo J, Lee JH, Cho N-J 2016. Extraction of sporopollenin exine capsules from sunflower pollen grains. *RSC Adv* 6(20):16533-16539.
17. Fadiran O. 2015. Characterization and use of pollen as a biorenewable filler for polymer composites, Ph.D. Thesis School of Chemical and Biomolecular Engineering, ed., Atlanta, GA: Georgia Institute of Technology.

18. Sargin I, Akyuz L, Kaya M, Tan G, Ceter T, Yildirim K, Ertosun S, Aydin GH, Topal M 2017. Controlled release and anti-proliferative effect of imatinib mesylate loaded sporopollenin microcapsules extracted from pollens of *Betula pendula*. *Int J Biol Macromol* 105:749-756.
19. Chiappe C, Demontis GC, Di Bussolo V, Rodriguez Douton MJ, Rossella F, Pomelli CS, Sartini S, Caporali S 2017. From pollen grains to functionalized microcapsules: a facile chemical route using ionic liquids. *Green Chem* 19(4):1028-1033.
20. Fadiran OO, Meredith JC 2014. Surface treated pollen performance as a renewable reinforcing filler for poly(vinyl acetate). *J Mater Chem A* 2(40):17031-17040.
21. Fan T, Park JH, Pham QA, Tan E-L, Mundargi RC, Potroz MG, Jung H, Cho N-J 2018. Extraction of cage-like sporopollenin exine capsules from dandelion pollen grains. *Sci Rep* 8(1):6565.
22. Gonzalez Cruz P, Uddin MJ, Atwe SU, Abidi N, Gill HS 2018. Chemical treatment method for obtaining clean and intact pollen shells of different species. *ACS Biomater Sci Eng* 4(7):2319-2329.
23. Zimmerman B, Tafintseva V, Bağcıoğlu M, Høegh Berdahl M, Kohler A 2016. Analysis of allergenic pollen by FTIR microspectroscopy. *Anal Chem* 88(1):803-811.
24. Bağcıoğlu M, Zimmermann B, Kohler A 2015. A multiscale vibrational spectroscopic approach for identification and biochemical characterization of pollen. *PLoS ONE* 10(9):e0137899.
25. Pappas CS, Tarantilis PA, Harizanis PC, Polissiou MG 2003. New method for pollen identification by FT-IR spectroscopy. *Appl Spectrosc* 57(1):23-27.
26. Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B, Orecchia P, Zardi L, Righetti PG 2004. Blue silver: A very sensitive colloidal Coomassie G-250 staining for proteome analysis. *Electrophoresis* 25(9):1327-1333.
27. Uddin MJ, Gill HS 2017. Ragweed pollen as an oral vaccine delivery system: mechanistic insights. *J Control Release* 268:416-426.
28. Gottardini E, Rossi S, Cristofolini F, Benedetti L 2007. Use of Fourier transform infrared (FT-IR) spectroscopy as a tool for pollen identification. *Aerobiologia* 23(3):211-219.
29. Fraser WT, Scott AC, Forbes AES, Glasspool IJ, Plotnick RE, Kenig F, Lomax BH 2012. Evolutionary stasis of sporopollenin biochemistry revealed by unaltered Pennsylvanian spores. *New Phytol* 196(2):397-401.
30. Bağcıoğlu M, Kohler A, Seifert S, Kneipp J, Zimmermann B 2017. Monitoring of plant–environment interactions by high-throughput FTIR spectroscopy of pollen. *Methods Ecol Evol* 8(7):870-880.
31. Zimmermann B, Kohler A 2014. Infrared spectroscopy of pollen identifies plant species and genus as well as environmental conditions. *PLoS ONE* 9(4):e95417.
32. Zimmermann B 2010. Characterization of pollen by vibrational spectroscopy. *Appl Spectrosc* 64(12):1364-1373.
33. Dell'Anna R, Lazzeri P, Frisanco M, Monti F, Malvezzi Campeggi F, Gottardini E, Bersani M 2009. Pollen discrimination and classification by Fourier transform infrared (FT-IR) microspectroscopy and machine learning. *Anal Bioanal Chem* 394(5):1443-1452.
34. Kanter U, Heller W, Durner J, Winkler JB, Engel M, Behrendt H, Holzinger A, Braun P, Hauser M, Ferreira F, Mayer K, Pfeifer M, Ernst D 2013. Molecular and immunological characterization of ragweed (*Ambrosia artemisiifolia* L.) pollen after exposure of the plants to elevated ozone over a whole growing season. *PLoS ONE* 8(4):e61518.

35. Zimmermann B, Bağcıoğlu M, Sandt C, Kohler A 2015. Vibrational microspectroscopy enables chemical characterization of single pollen grains as well as comparative analysis of plant species based on pollen ultrastructure. *Planta* 242(5):1237-1250.
36. Jardine PE, Abernethy FAJ, Lomax BH, Gosling WD, Fraser WT 2017. Shedding light on sporopollenin chemistry, with reference to UV reconstructions. *Rev Palaeobot Palynol* 238:1-6.
37. Blokker P, Boelen P, Broekman R, Rozema J 2006. The occurrence of p-coumaric acid and ferulic acid in fossil plant materials and their use as UV-proxy. *Plant Ecol* 182(1):197-207.
38. Wehling K, Niester C, Boon JJ, Willemse MTM, Wiermann R 1989. p-Coumaric acid — a monomer in the sporopollenin skeleton. *Planta* 179(3):376-380.
39. Dreischmeier K, Budke C, Wiehemeier L, Kottke T, Koop T 2017. Boreal pollen contain ice-nucleating as well as ice-binding 'antifreeze' polysaccharides. *Sci Rep* 7:41890.
40. Fang K, Wang Y, Yu T, Zhang L, Baluška F, Šamaj J, Lin J 2008. Isolation of de-exined pollen and cytological studies of the pollen intines of *Pinus bungeana* Zucc. Ex Endl. and *Picea wilsonii* Mast. *Flora* 203(4):332-340.
41. Lahlali R, Jiang Y, Kumar S, Karunakaran C, Liu X, Borondics F, Hallin E, Bueckert R 2014. ATR-FTIR spectroscopy reveals involvement of lipids and proteins of intact pea pollen grains to heat stress tolerance. *Front Plant Sci* 5:747.
42. Jardine PE, Fraser WT, Lomax BH, Gosling WD 2015. The impact of oxidation on spore and pollen chemistry. *J Micropalaeontol*.
43. Max J-J, Chapados C 2004. Infrared spectroscopy of aqueous carboxylic acids: comparison between different acids and their salts. *J Phys Chem A* 108(16):3324-3337.
44. Monsoor MA, Kalapathy U, Proctor A 2001. Determination of polygalacturonic acid content in pectin extracts by diffuse reflectance Fourier transform infrared spectroscopy. *Food Chem* 74(2):233-238.
45. Yang H, Yan R, Chen H, Lee DH, Zheng C 2007. Characteristics of hemicellulose, cellulose and lignin pyrolysis. *Fuel* 86(12):1781-1788.
46. Ghaffari A, Navaee K, Oskoui M, Bayati K, Rafiee-Tehrani M 2007. Preparation and characterization of free mixed-film of pectin/chitosan/Eudragit® RS intended for sigmoidal drug delivery. *Eur J Pharm Biopharm* 67(1):175-186.
47. Reitsma T 1969. Size modification of recent pollen grains under different treatments. *Rev Palaeobot Palynol* 9(3):175-202.
48. Mariotti F, Tome D, Mirand PP 2008. Converting nitrogen into protein—beyond 6.25 and Jones' factors. *Crit Rev Food Sci Nutr* 48(2):177-184.
49. Roulston TH, Cane JH 2000. Pollen nutritional content and digestibility for animals. *Plant Syst Evol* 222(1):187-209.
50. Vanderplanck M, Leroy B, Wathelet B, Wattiez R, Michez D 2014. Standardized protocol to evaluate pollen polypeptides as bee food source. *Apidologie* 45(2):192-204.
51. Pablos I, Wildner S, Asam C, Wallner M, Gadermaier G 2016. Pollen allergens for molecular diagnosis. *Curr Allergy Asthma Rep* 16(4):31.
52. O'Connor DJ, Iacopino D, Healy DA, O'Sullivan D, Sodeau JR 2011. The intrinsic fluorescence spectra of selected pollen and fungal spores. *Atmospheric Environ* 45(35):6451-6458.
53. Mundargi RC, Potroz MG, Park S, Shirahama H, Lee JH, Seo J, Cho N-J 2016. Natural sunflower pollen as a drug delivery vehicle. *Small* 12(9):1167-1173.

54. de Souza SP, Bassut J, Marquez HV, Junior II, Miranda LSM, Huang Y, Mackenzie G, Boa AN, de Souza ROMA 2015. Sporopollenin as an efficient green support for covalent immobilization of a lipase. *Catal Sci Technol* 5(6):3130-3136.
55. Barrier S, Lobbert A, Boasman AJ, Boa AN, Lorch M, Atkin SL, Mackenzie G 2010. Access to a primary aminosporopollenin solid support from plant spores. *Green Chem* 12(2):234-240.
56. Diego-Taboada A, Cousson P, Raynaud E, Huang Y, Lorch M, Binks BP, Queneau Y, Boa AN, Atkin SL, Beckett ST, Mackenzie G 2012. Sequestration of edible oil from emulsions using new single and double layered microcapsules from plant spores. *J Mater Chem* 22(19):9767-9773.

Figure legends

Figure 1. Scanning electron micrographs of black alder (BA) and lamb's quarters (LQ) pollen showing effects of chemical treatment. (A) BA pollens before treatment, (B) equatorial view of a raw BA (arrow points to aperture), (C) exine surface of a raw BA showing closed aperture (indicated by arrow), (D) manually broken raw BA showing cellular material inside the core, (E) BA after treatment showing intact pollens, (F) equatorial view of a treated BA (arrow points to an aperture), (G) enlarged view of a treated BA showing an open aperture (indicated by arrow), (H) manually broken treated BA showing clean core, (I) raw LQ pollens before chemical treatment, (J) high magnification view of a raw LQ showing surface morphology (arrow points to aperture), (K) spinules (indicated by dotted circle), puncta (indicated by dotted square), and a closed aperture (indicated by arrow) in a raw LQ, (L) manually broken raw LQ showing cellular material inside the core, (M) LQ after treatment showing intact pollens, (N) high magnification view of a LQ after treatment (arrow points to open aperture), (O) an open aperture after treatment (indicated by arrow), spinules (indicated by dotted circle), puncta (indicated by dotted square), and (P) manually broken treated LQ showing a clean core.

Figure 2. SEM images of ragweed (RW) and sunflower (SF) pollen showing effects of chemical treatment. (A) raw RW pollens before chemical treatment, (B) high magnification view of a raw RW showing surface morphology (arrow points to aperture), (C) closed aperture (indicated by arrow) in a raw RW pollen, (D) manually broken raw RW showing cellular material inside the pollen core and two distinct pollen walls, (E) RW after chemical treatment showing intact pollens, (F) high magnification view of a treated pollen showing no visible damage (arrow points to aperture), (G) open aperture after treatment (indicated by arrow), (H) manually broken RW showing a clean core, (I) raw SF pollens before chemical treatment, (J) high magnification view of a raw SF showing surface morphology (arrow points to aperture), (K) zoom in view of a SF surface (arrow points to closed aperture), (L) manually broken SF showing cellular material inside the pollen core and two distinct pollen walls, (M) SF after chemical treatment showing intact pollens, (N) high magnification view of a treated SF showing no visible damage (arrow points to aperture), (O) open aperture (indicated by arrow) on SF surface after treatment, and (P) manually broken SF showing a clean core. *ekt.* = *ektexine*, *end.* = *endexine*.

Figure 3. Elemental analysis and SDS-PAGE analysis of pollen grains. (A) carbon, (B) hydrogen, and (C) protein content in pollens before and after chemical treatment. Values shown are means $\pm$ SD for three independent experiments. $p \leq 0.05$ [*], $p \leq 0.01$ [**], $p \leq 0.0001$ [***], ns = not significant. (D) Crude extracts were obtained from raw or chemically treated pollens. Lane 1: molecular weight marker; lane 2: raw BA; lane 3: treated BA; lane 4: raw LQ; lane 5: treated LQ; lane 6: raw RW; lane 7: treated RW; lane 8: raw SF; lane 9: treated SF. The gel image is a representative of three independent experiments with similar results.

Figure 4. Confocal micrographs of chemically treated pollen grains loaded with FITC-OVA. Natural pollens show autofluorescence in their core and the wall. Treated pollens retain autofluorescence of the pollen wall. FITC-OVA loaded pollen shells showed a fluorescent core.

Figure 5. Fourier transform infrared (FTIR) spectra of manually broken raw black alder (BA), lamb's quarters (LQ), ragweed (RW), and sunflower (SF) pollen grains.

Figure 6. Fourier transform infrared (FTIR) spectra of manually broken ragweed pollen (RW) after different chemical treatments. Raw RW pollens were successively treated with acetone, phosphoric acid, and KOH to obtain treated RW.

Figure 7. Fourier transform infrared (FTIR) spectra of manually broken black alder (BA), lamb's quarters (LQ), and sunflower (SF) pollen grains after full chemical treatment (i.e., after KOH step).

Figure 8: Thermogram and first derivative thermogravimetry (DTG) of pollen grains before and after chemical treatment. (A-B) Black alder (BA) pollen, (C-D) lamb's quarters (LQ) pollen, (E-F) ragweed (RW) pollen, (G-H) sunflower (SF) pollen.

Table 1: Assignment of functional groups to the FTIR spectrum bands of pollens

Absorbance (cm ⁻¹)	Assignment	References
~3360	O–H stretching in alcohol/phenolic group of pectin, cellulose, lignin, water (broad peak)	25,28,29
~3012	=C–H stretching in lipids or alkyl-esters	28,29
~2925	out-of-phase CH ₂ stretching in lipids, carbohydrates or proteins (sharp peak)	25,29
~2854	in-phase CH ₂ stretching in lipids, carbohydrates or proteins (sharp peak)	25,29
~1743	C=O stretching in lipids, vibration of alkyl-esters	25,28-30,42
~1700	C=O stretching in carboxylic acids	30,42
~1660	phenolic ring in sporopollenin, adsorbed water	34,39
~1640	C=O stretching in amide I (protein)	30,33,39
~1630	C=C bond	25
~1605	phenyl ring vibration in sporopollenin	24,30
~1600	–COO ⁻ bending, C=C in p-coumaric acid and ferulic acid	25,28,45
~1579	asymmetric stretching in carboxylate anions (COO ⁻)	43
~1540	N–H bending and C–N stretching in amide II (protein)	30,31,33,40
~1515	C=C stretching in phenolic ring of sporopollenin	29,31,36
~1440	sporopollenin, CH ₂ stretching	36
~1410	symmetric stretching in carboxylate anions (COO ⁻)	33,39
~1375	CH ₃ bending	29,40
~1322	C–C and C–O skeletal vibrations in cellulose	25
~1281	vibration of C–O–C in pectin	34,45
~1236	PO ₂ ⁻ asymmetric stretching in nucleotides (DNA, RNA) or phospholipids, amide III (protein)	25,32
~1170	phenolic ring vibration in sporopollenin, C–O–C stretching in cellulose	24,30
~1070	C–O stretching in arabinogalactan, amylose (carbohydrates)	24,28,40
~1031	C–O–C stretching in cellulose	24,28
~995	C–O–C stretching in cellulose, amylose, nucleotides	24
~980	C–O in carbohydrates	42
~852	phenylpropanoids in sporopollenin	35
~833	p-coumaric acid in sporopollenin	31,35
~720	CH ₂ rocking	29,31

Figure 1

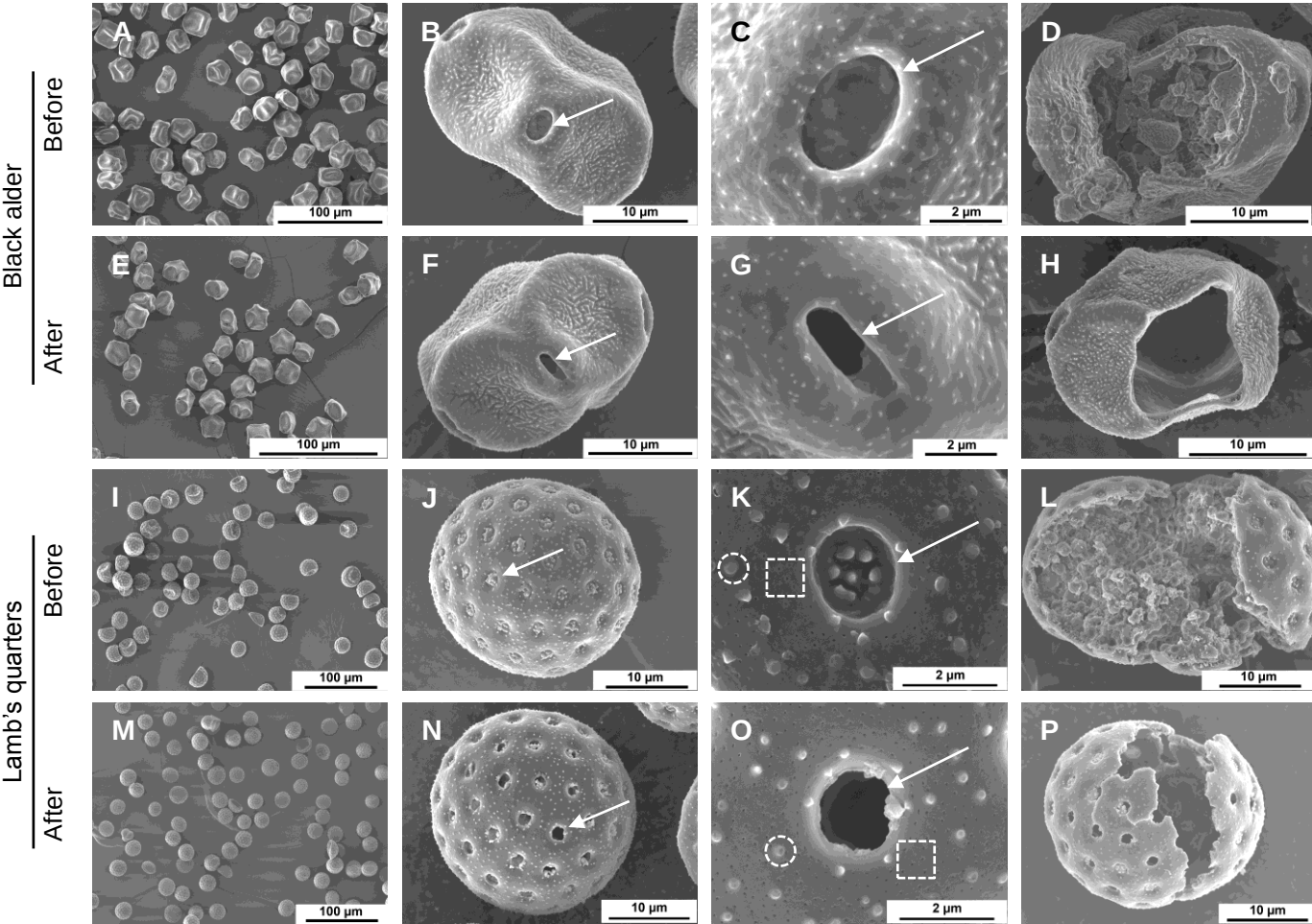


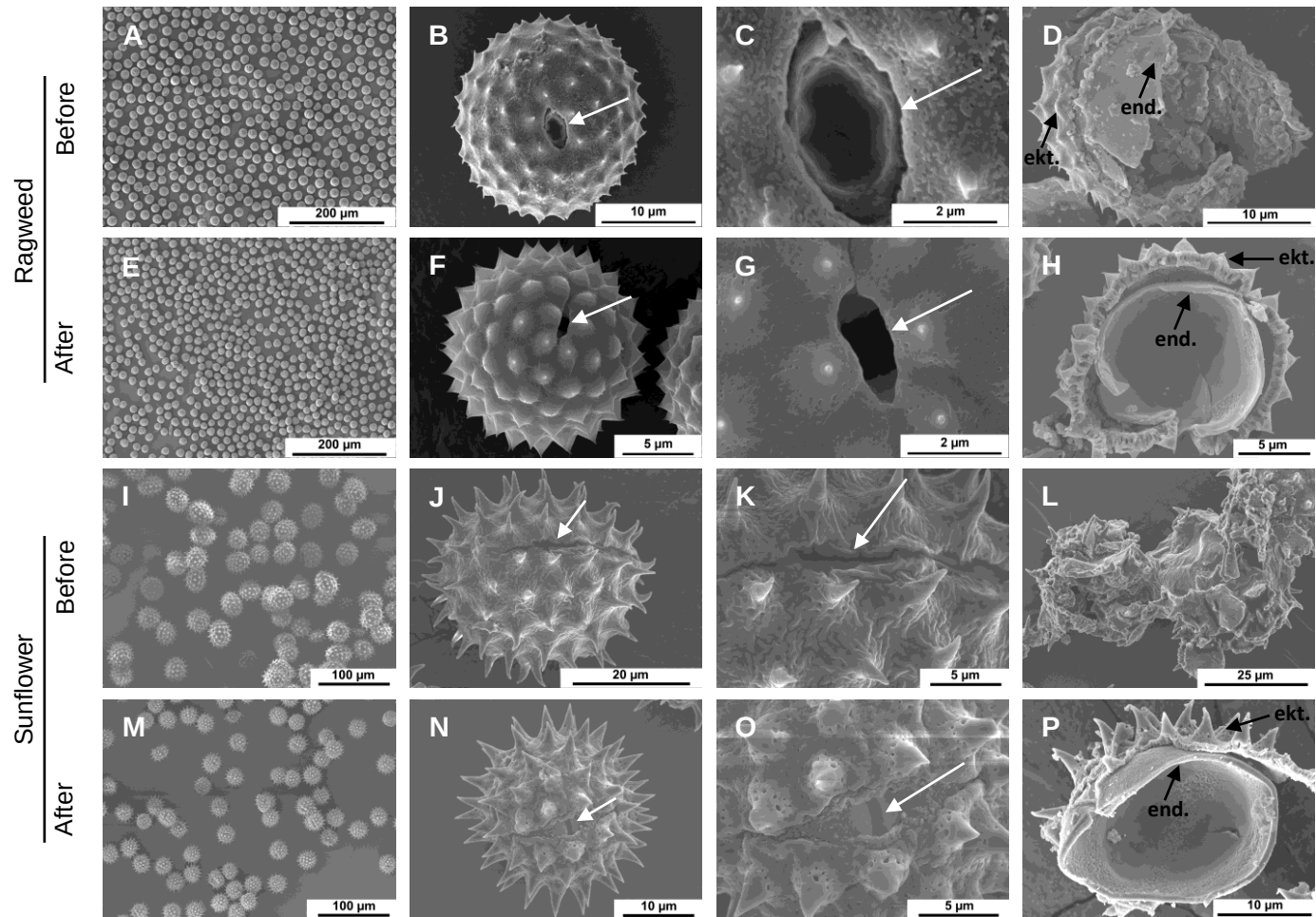
Figure 2

Figure 3

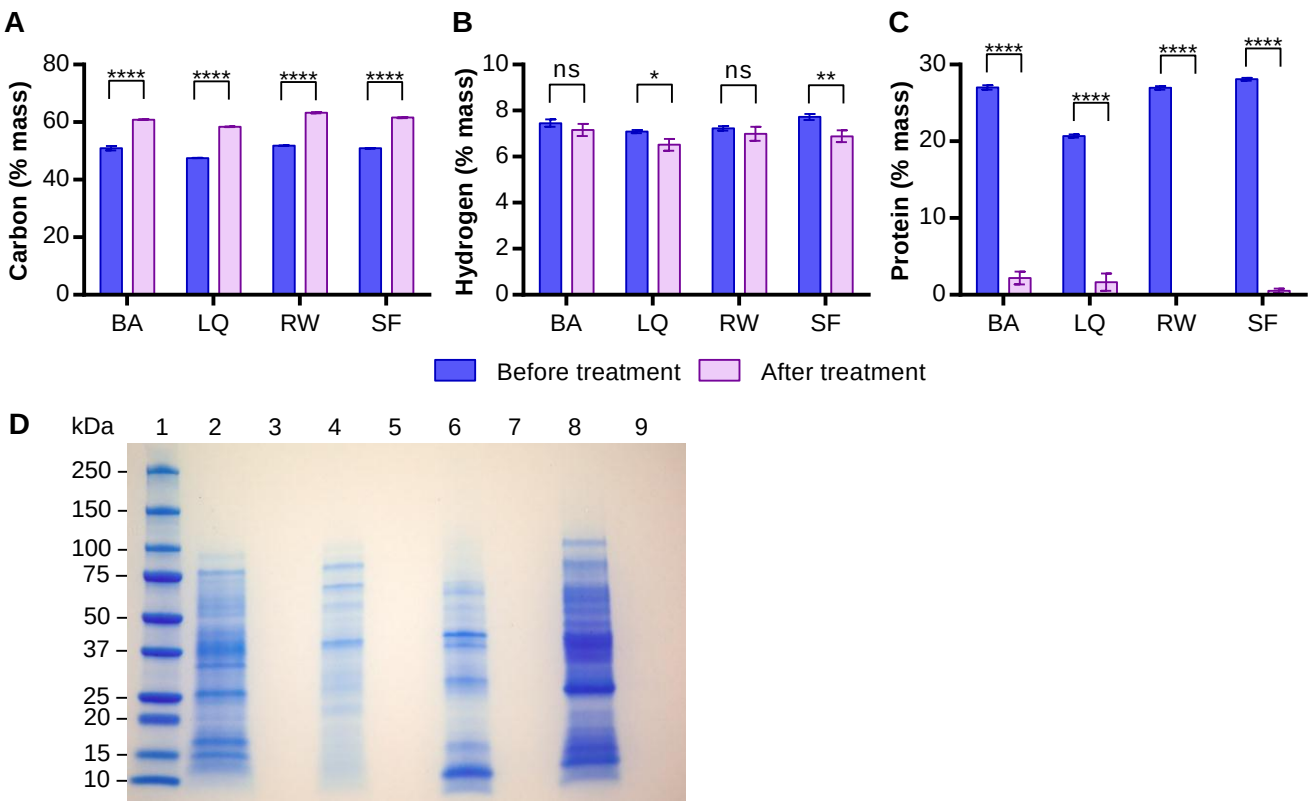


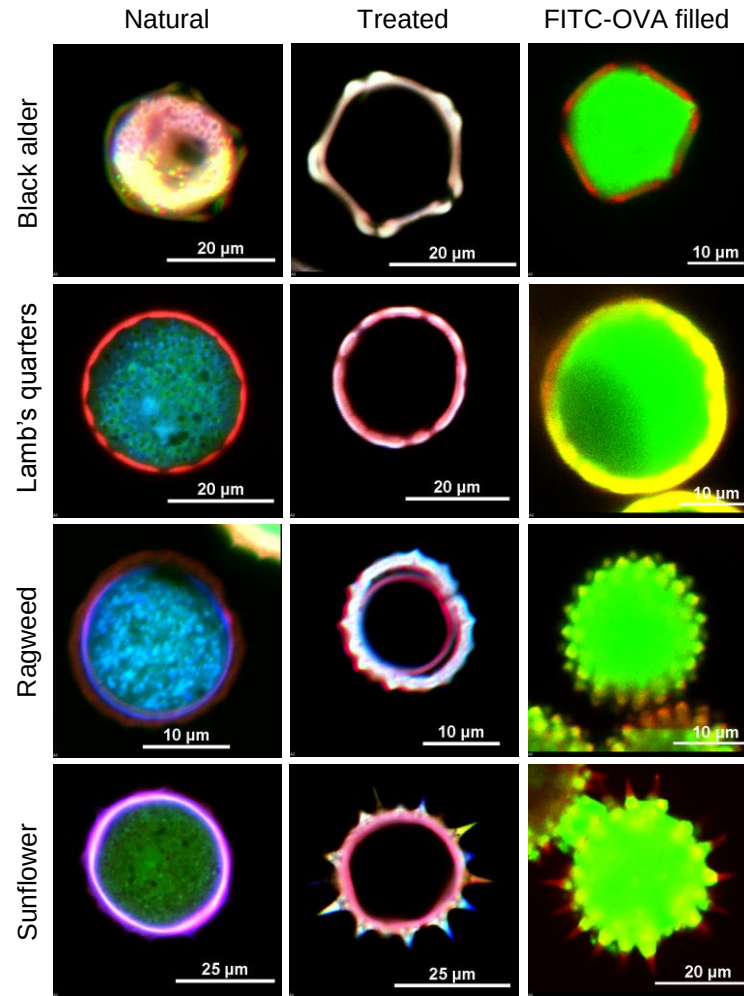
Figure 4

Figure 5

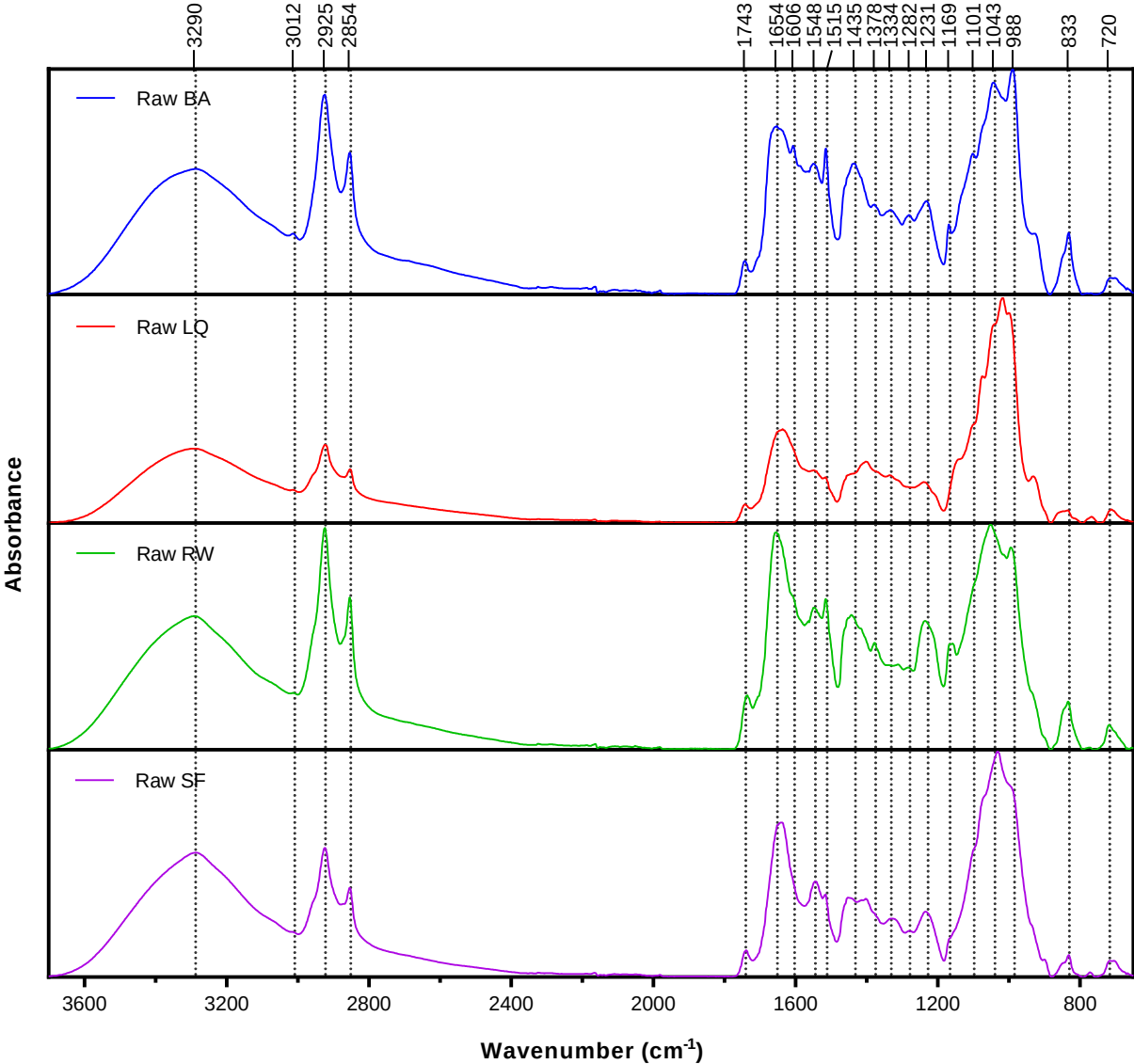


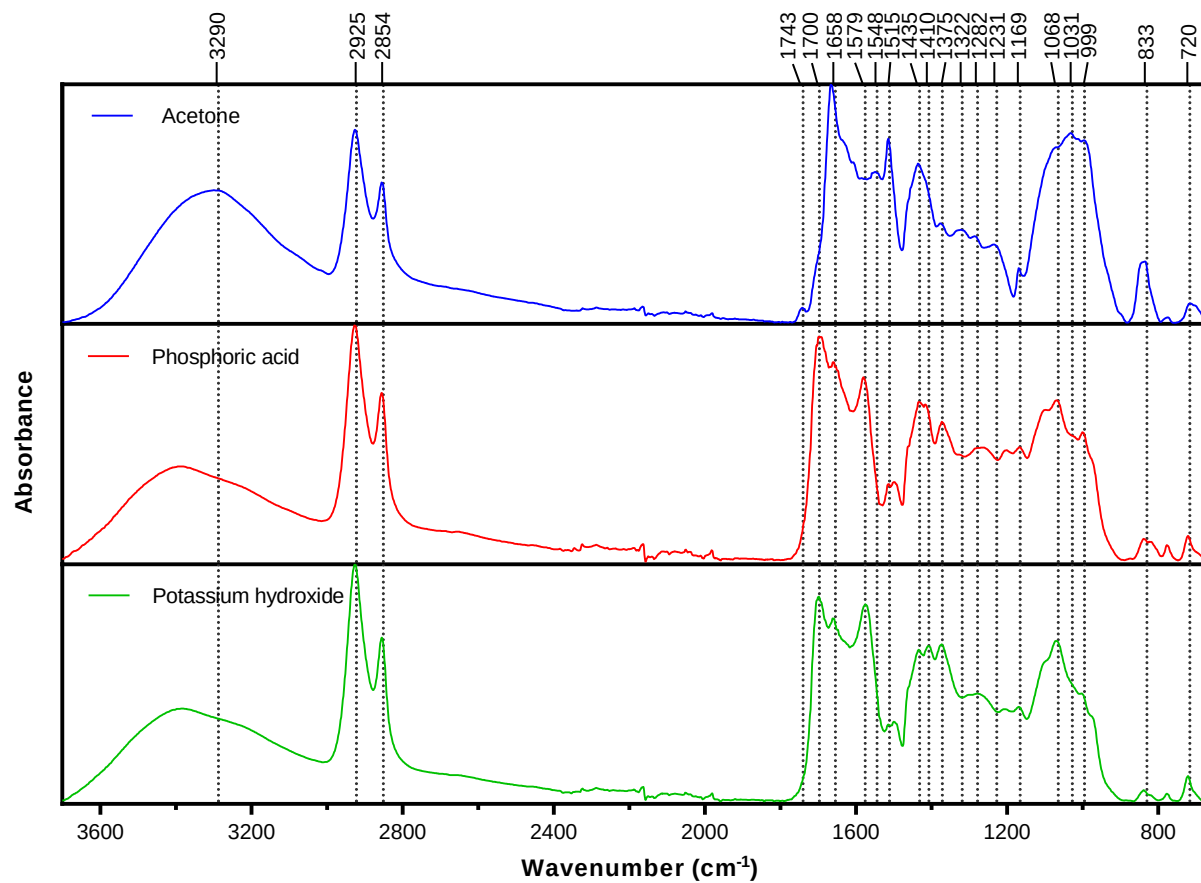
Figure 6

Figure 7

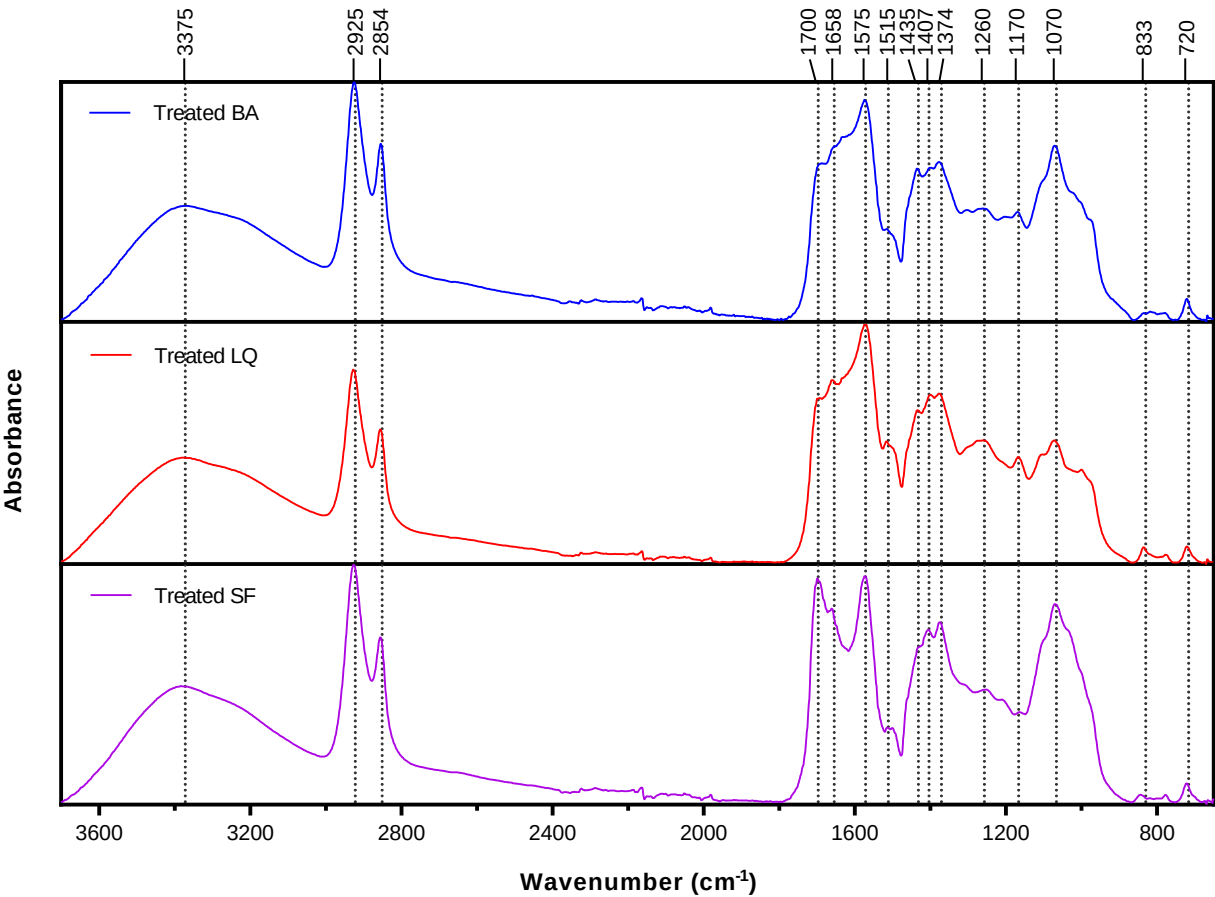
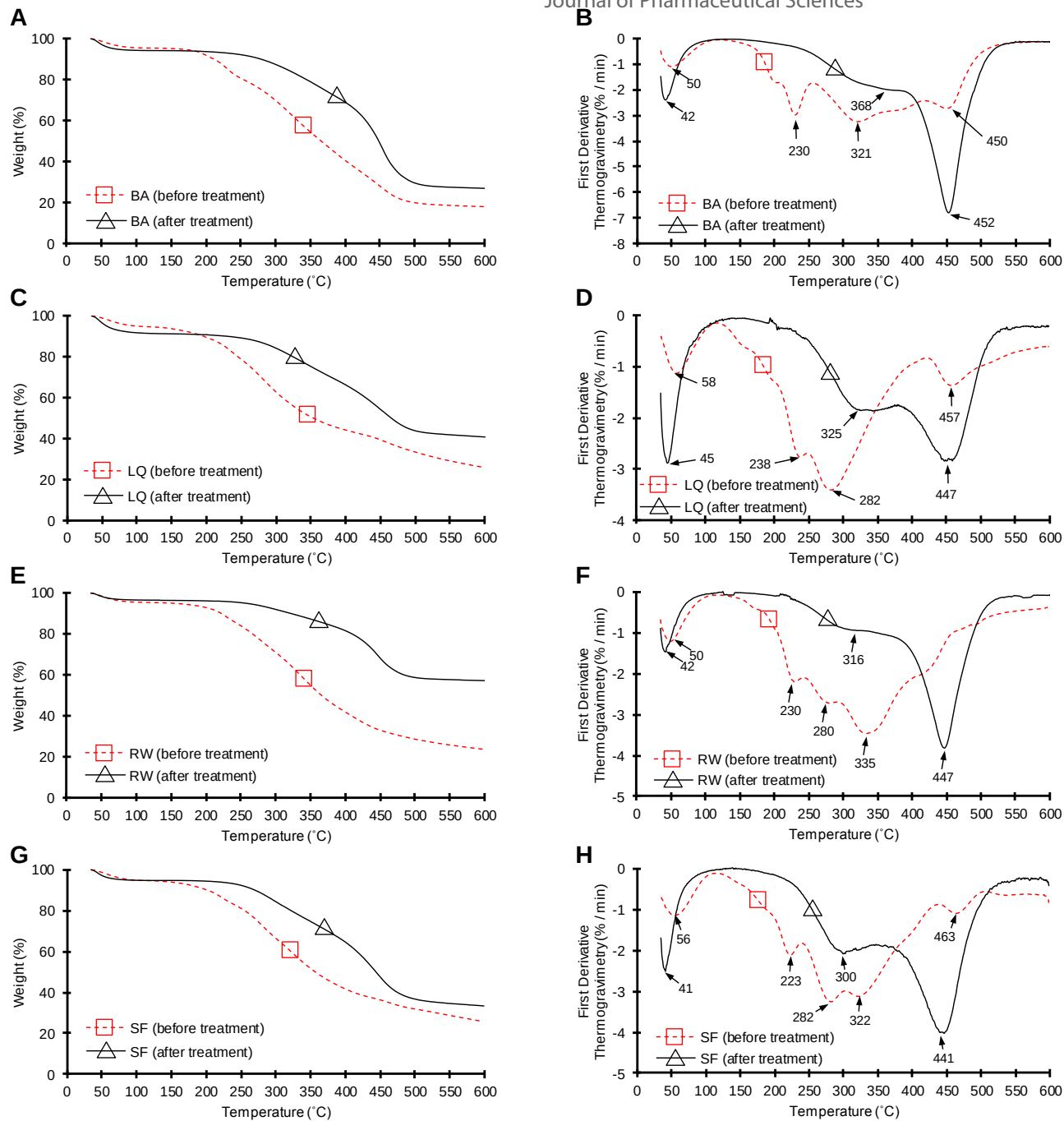


Figure 8



Supporting information

Physical and biochemical characterization of chemically-treated pollen shells for oral therapeutic delivery

Md Jasim Uddin^a, Sumedha Liyanage^b, Nouredine Abidi^b, and Harvinder Singh Gill^{a,*}

^a *Department of Chemical Engineering, Texas Tech University, Lubbock, TX 79409, USA*

^b *Department of Plant and Soil Science, Texas Tech University, Lubbock, TX 79409, USA*

*Corresponding author:

Dr. Harvinder Singh Gill

Texas Tech University

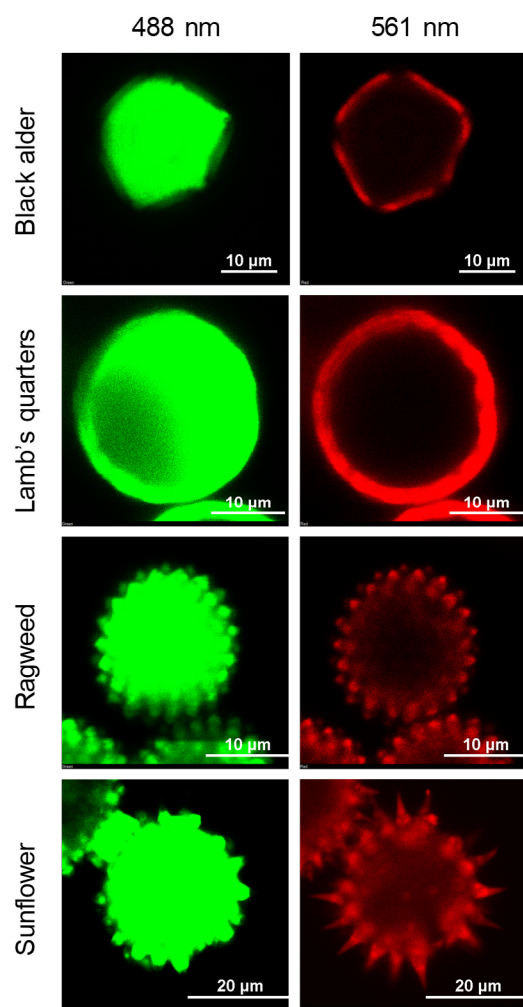
Department of Chemical Engineering

8th Street and Canton Ave, Mail Stop 3121, Lubbock, TX 79409-3121, USA.

E-mail address: harvinder.gill@ttu.edu

Table of Contents	Page
Confocal micrographs of OVA loading into chemically treated pollen shells	S-2
Confocal micrographs of natural pollen grains	S-3
Confocal micrographs of chemically treated pollen shells	S-4
FTIR spectra of intact pollen grains	S-5
FTIR spectra of extract from ragweed pollen grain	S-6
FTIR spectra of proteins from ragweed pollen grain	S-6
FTIR spectra of intact chemically treated pollen shells	S-7
Confocal micrographs of defatted sunflower pollen grain	S-8

S-2

Confocal micrographs of OVA loading into chemically treated pollen shells**Figure S-1.** Confocal micrographs of chemically treated pollen shells loaded with FITC-OVA.

Confocal micrographs of natural pollen grains

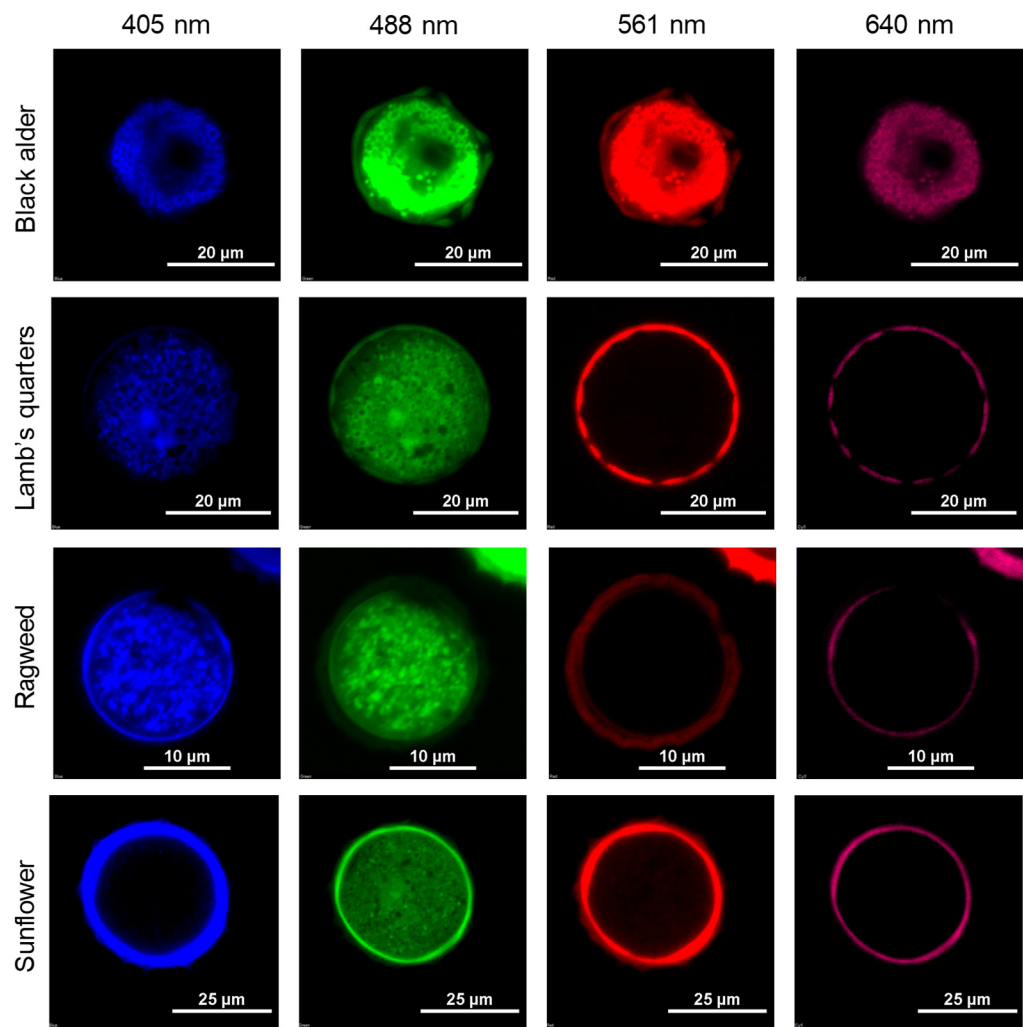


Figure S-2. Confocal micrographs of natural pollen grains showing autofluorescence of cellular material in their core and of the wall.

S-4

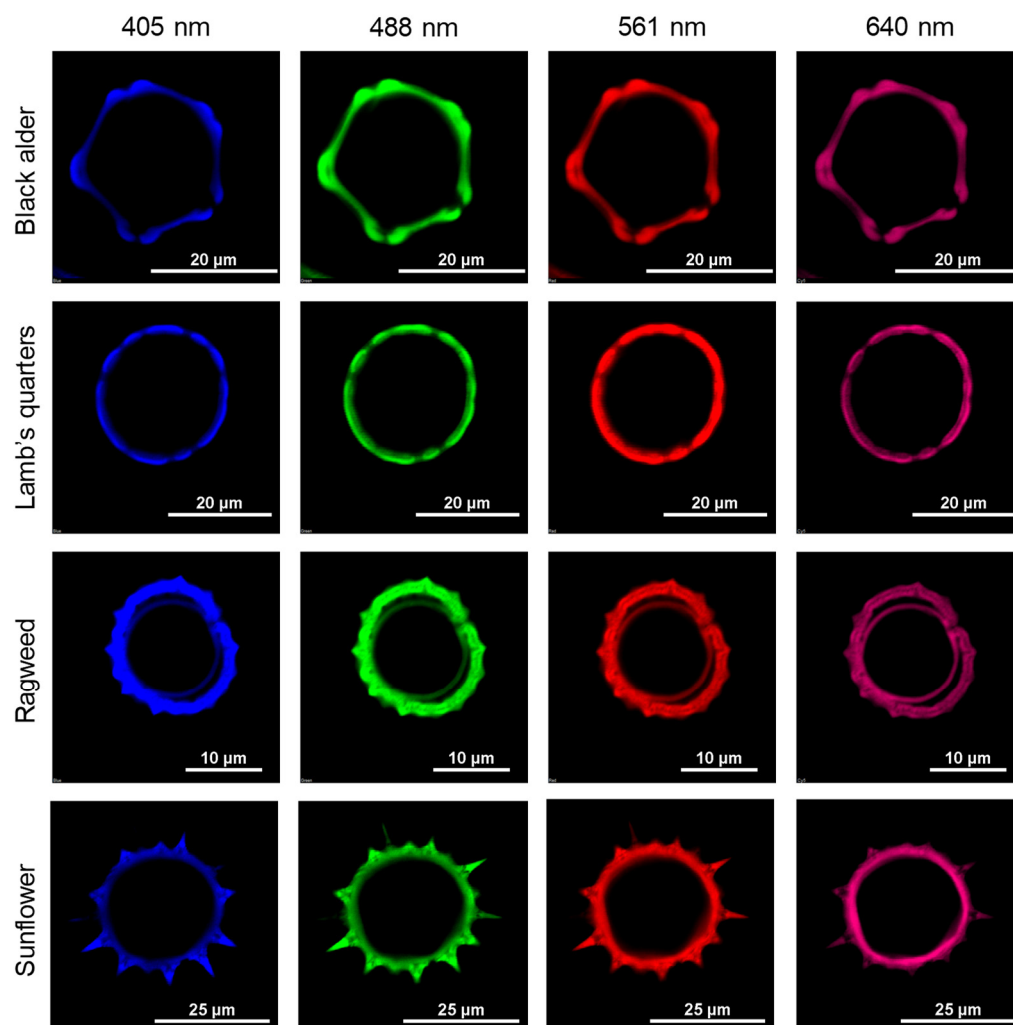
Confocal micrographs of chemically treated pollen shells

Figure S-3. Confocal micrographs of chemically treated pollen shells showing autofluorescence of the pollen wall.

FTIR spectra of intact pollen grains

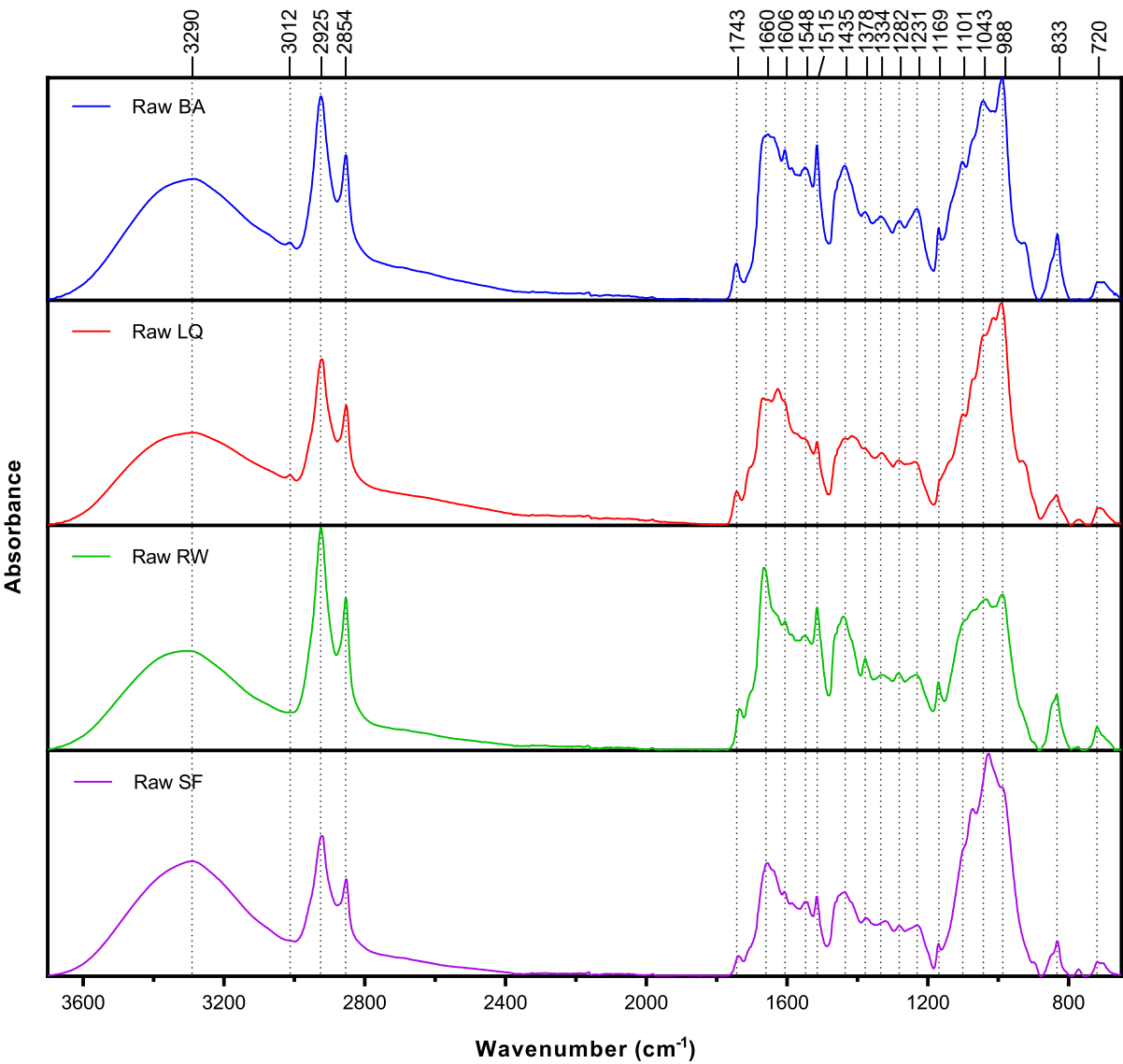


Figure S-4. Fourier transform infrared (FTIR) spectra of intact raw black alder (BA), lamb's quarters (LQ), ragweed (RW), and sunflower (SF) pollen grains.

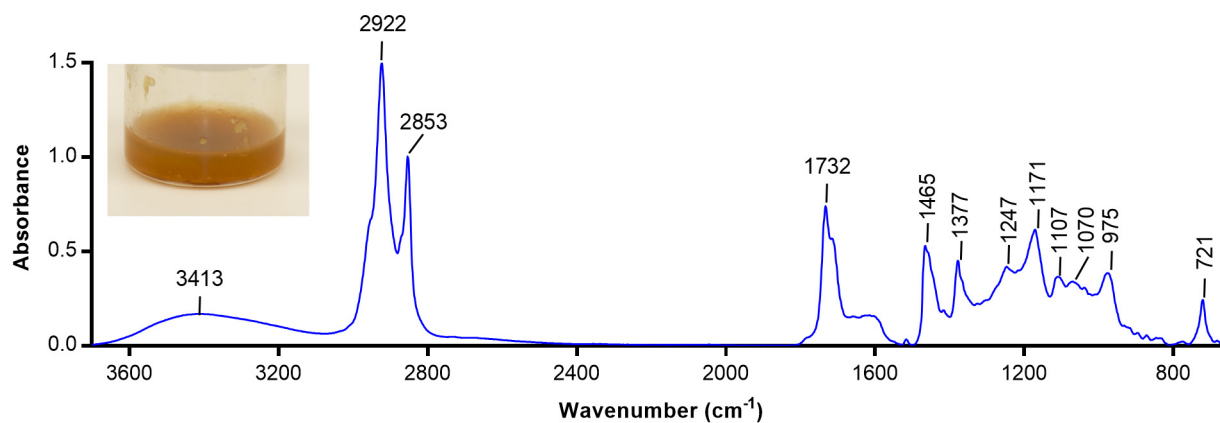
FTIR spectra of extract from ragweed pollen grain

Figure S-5. Fourier transform infrared (FTIR) spectra of waxy material (photograph) extracted from ragweed pollen using acetone.

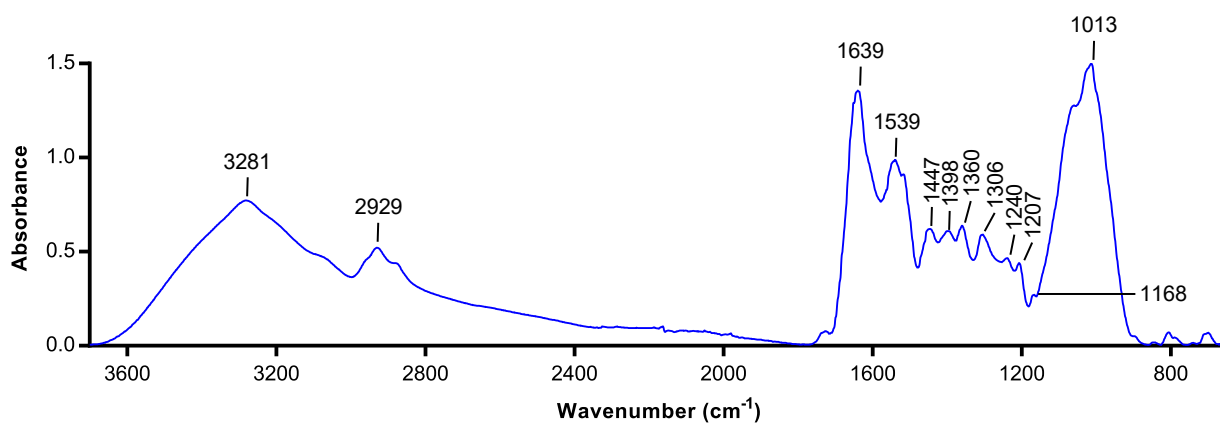
FTIR spectra of proteins from ragweed pollen grain

Figure S-6. Fourier transform infrared (FTIR) spectra of commercially available lyophilized proteins extracted from ragweed pollen.

FTIR spectra of intact chemically treated pollen shells

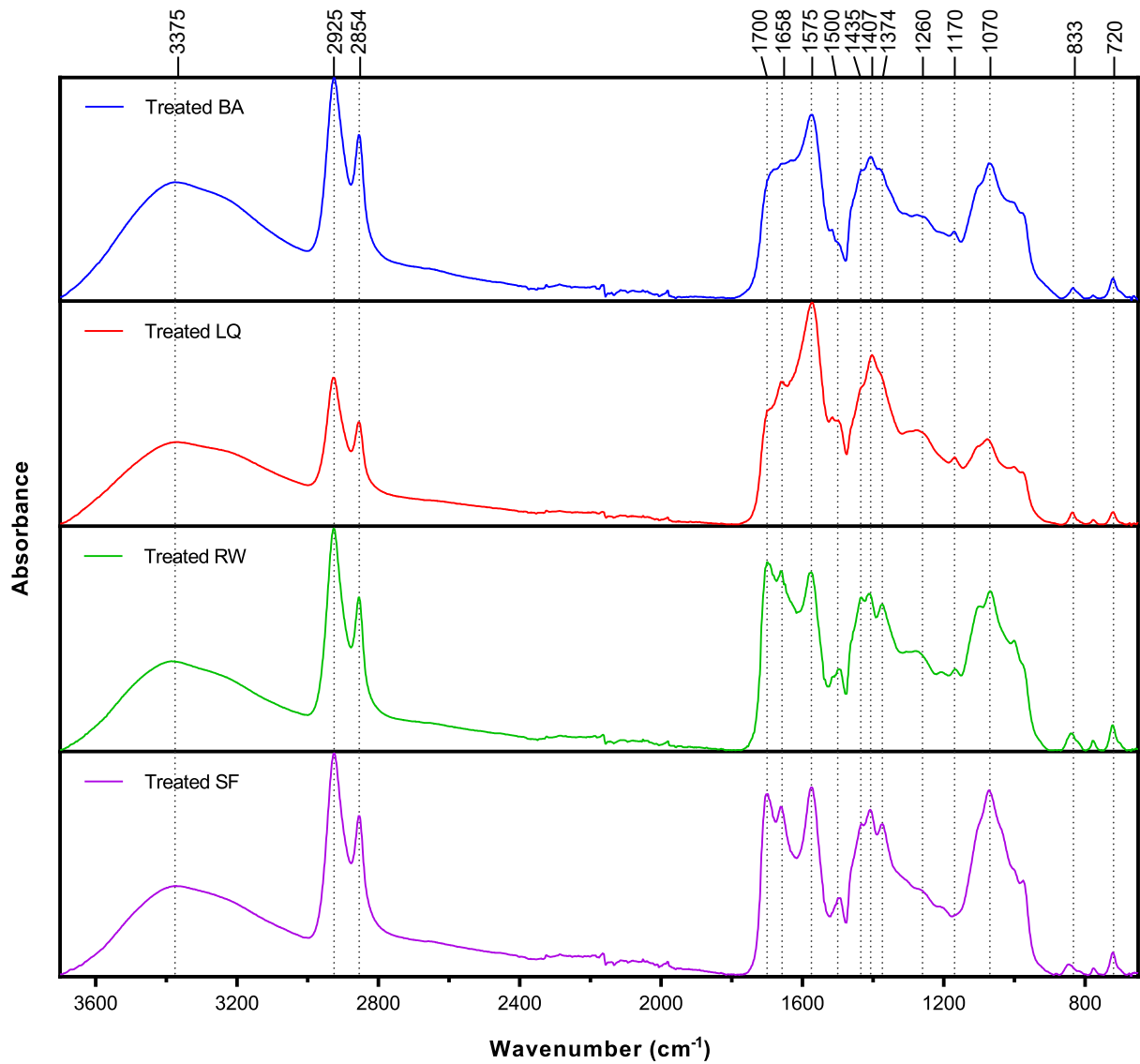


Figure S-7. Fourier transform infrared (FTIR) spectra of intact black alder (BA), lamb's quarters (LQ), ragweed (RW), and sunflower (SF) pollen grains after full chemical treatment (i.e., after KOH step).

S-8

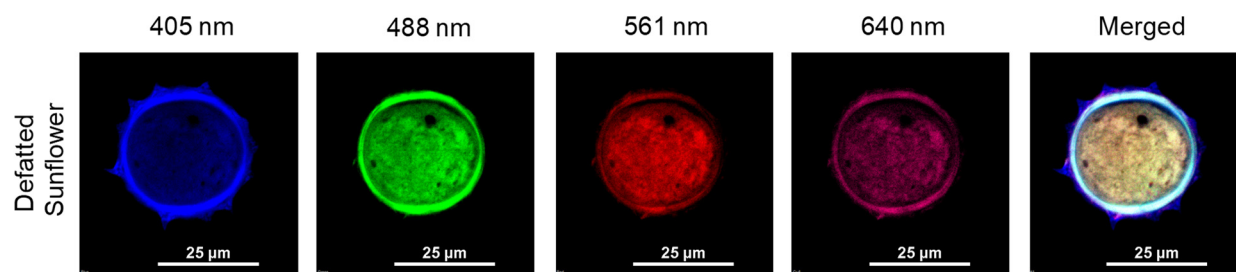
Confocal micrographs of defatted sunflower pollen grain

Figure S-8. Confocal micrographs of defatted sunflower pollen showing strong autofluorescence from pollen exine and core contents.