Natural Sunflower Pollen as a Drug Delivery Vehicle

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The fabrication of uniform micron-scale capsules with complex architectures is a longstanding goal for successful materials encapsulation strategies.[1,2] While various fabrication approaches have been devised in the past few decades, the biosynthetic capabilities of nature have evolved over much longer time scales to produce large quantities of exquisitely complex microcapsules with high fidelity. One such example includes micron-scale pollen grains, which effectively encapsulate sensitive biological materials for long durations under harsh conditions.[3,4] In nature, pollen grains ensure the reproductive capabilities of plants by protecting nucleic acids and other genetic materials from unfavorable environmental conditions such as prolonged desiccation, high temperatures, ultraviolet light, and microbial damage.[5] The genetic material is stored within the cytoplasmic core of the pollen grain and surrounded by a double layer shell consisting of an intine and an exine layer.[6,7] The outermost exine layer contains the sporopollenin biopolymer, which is considered to be one of nature's most resilient materials.[8,9] With growing recognition of pollen's unique material properties along with its organic production and simple collection, there has been renewed interest in exploring pollen and its components as promising biomaterials.

In recent years, the robust exine capsule, isolated from pollen grains and plant spores, has demonstrated strong potential as a delivery vehicle for the encapsulation of various materials including pharmaceutical drugs, vaccines, contrast agents, and oils.[10–21] In the materials encapsulation strategies explored thus far, high-purity exine capsules are required and obtained through vigorous and prolonged processing of pollen grains or spores under harsh conditions using organic solvents, acids and bases at elevated temperatures, or systematic exposure to numerous enzymes.[22] Indeed, it is commonly assumed that "It is possible to attach drugs to the outside of complete [pollen] particles, but the loading is restricted and the drug receives little protection and its release is relatively uncontrolled."[12] At the same time, as part of the natural pollen cycle, both the sporopollenin exine and cellulosic intine layers are permeable and undergo dehydration and hydration, which facilitates materials loading as the surrounding fluid is drawn into the internal pollen cavity.[23–26] This suggests that materials encapsulation inside unprocessed pollen grains should be possible provided a suitable encapsulation route is identified. The potential advantages of natural sunflower pollen grains as a drug delivery vehicle are enormous: (1) Proven track record as safe for human oral consumption due to use as a biosupplement and in herbal medicine; (2) Common constituent of "*bee pollen*" for human consumption for nutritional and therapeutic benefits; (3) Economical raw material for materials encapsulation without the need for the use of organic solvents and harsh ultrasonication or homogenisation conditions; and (4) Unique microstructure with uniform size distribution and inner cavity. The primary disadvantage of natural sunflower pollen grains is the presence of pores which result in rapid drug release; however, this can be addressed with suitable polymer coating techniques to modulate the release depending on the need to target different regions of the gastrointestinal track.

Motivated by the biological function of pollen grains, we hypothesize that encapsulation strategies can be devised to load biomacromolecules into natural pollen grains. This approach would drastically reduce processing requirements for materials encapsulation in exine-reinforced microcapsules while also taking advantage of the innate therapeutic benefits of natural pollen, which is often used as a dietary supplement and in traditional herbal medicine.[27,28] In particular, multifloral bee collected pollen, of which *Helianthus annuus* (sunflower) is a common constituent,[29,30] has been associated with a wide range of nutritional and therapeutic effects.[27,28] In relation to the allergenicity of pollen proteins, in all encapsulation experiments, commercial defatted pollen grains were used to avoid pollen surface proteins and lipids so as to provide pollen free from any possible surface coating allergenic pollen proteins. Also, due to the pollen being largely unregulated globally and considered by the USFDA to be generally regarded as safe, pollen products in general do not have to comply with special standards for human consumption.

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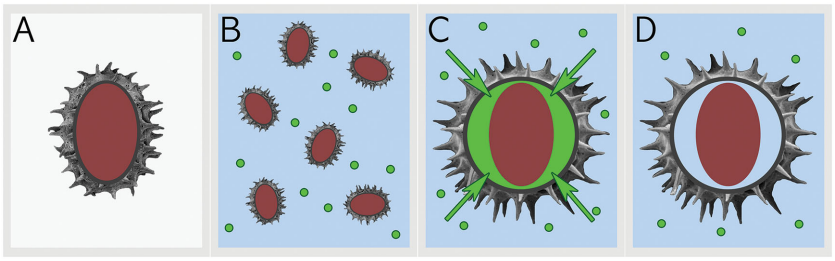

Scheme 1. Schematic of natural sunflower pollen grains processing to encapsulate macromolecules by different techniques. A) Dried natural pollen grains exhibiting a characteristic oval shape with uniform spikes on the surface. B) Pollen grains suspended in an aqueous solution of macromolecules for encapsulation by passive, compression, and vacuum techniques. C) A fully hydrated natural pollen grain loaded with macromolecules, indicated by green, along with the original pollen contents. D) A fully hydrated natural pollen grain after the release of macromolecules from the natural pores within the pollen grain walls.

As proof-of-concept to test our hypothesis, we investigated natural, unprocessed sunflower pollen grains as microscale materials for the efficient encapsulation of biomacromolecules. Through the comparison of three different encapsulation strategies (passive hydration, hydraulic compression, and vacuum-assisted), we demonstrate multiple routes to achieve high-efficiency protein loading with bovine serum albumin (BSA) as a model biomacromolecule. Importantly, the methods used are environmentally friendly and preserve the complex architecture of the natural pollen grains, including size, uniformity, and surface features. Furthermore, we demonstrate that a controlled release profile is achievable by encapsulating pollen grains inside alginate hydrogel beads. Taken together, our findings offer compelling evidence that natural pollen grains are excellent drug delivery vehicles.

Scheme 1 outlines the basic hydration and encapsulation process, then subsequent release. Hydration begins with taking dried sunflower pollen grains with the cytoplasmic material intact (Scheme 1A) and combining with a BSA solution (Scheme 1B). The BSA solution is absorbed into the pollen grain as the pollen grain swells and the BSA solution fills the additional volume created by the swelling process (Scheme 1C). This process may be natural, as in the passive loading method, or assisted, as in the compression or vacuum loading methods. After the BSA is released in the simulated intestinal or gastric fluid, the pollen grains remain swollen although all BSA is released (Scheme 1D).

To demonstrate a platform technology to utilize natural pollen grains as a drug delivery vehicle, the grains have been characterized methodically to assess uniformity in size, morphology, and physical changes arising during the course of the encapsulation process. In order to characterize natural pollen grains we employed dynamic imaging particle analysis (DIPA)[31,32] (see the Supporting Information). **Figure 1**A shows representative data by curve fitting histograms of equivalent spherical diameter (ESD) versus frequency with an average ESD of 37.93 ± 1.41 μm for natural pollen grains and an ESD of 36.54 ± 1.45 μm, 36.95 ± 1.35 μm, and 36.17 ± 1.36 μm, respectively, for passive, compression, and vacuum-loaded pollen grains. The natural pollen grains uniformity with reference to size was evident from ESD data before and after macromolecule loading, which is an important prerequisite of particulate drug delivery systems.[33] In addition to pollen size uniformity, the pollen circularity was measured before and after BSA loading and the data are represented by curve fitting to histograms of circularity versus frequency as shown in Figure 1B. The shape of pollen capsules before and after BSA loading are considered noncircular due to the characteristic distribution of spikes on the pollen surface with the resulting circularity value <1 (ideal circle = 1). The quality of the image focus of the images used for data analysis is evident from Figure 1C, and the edge gradient versus frequency data which is represented indicates that highly focused pollen grains were used during DIPA analysis.

In addition to quantitative data and images, Figures 1D–G indicate the structural similarity of pollen before loading as well as after passive, compression, and vacuum loading techniques, respectively. In addition to DIPA, we characterized each representative batch of pollen grains using scanning electron microscopy (SEM) to examine any morphological changes[13] and these images are displayed as Figures 1H–K, respectively, for pollen before loading and after loading by passive, compression, vacuum loading techniques. These structural and morphological observations indicate that our pollen grain formulations have maintained their structural integrity without any denaturation, and exhibit size uniformity after macromolecule encapsulation using different techniques. This is important, as any proposed drug delivery vehicle for controlled and targeted delivery demands uniformity in size and shape for efficient quality control and performance, and it can be a major challenge in encapsulation processess to control the product quality.[34] It is clearly evident from the morphological observations that pollen apetures exist and that each spike on these pollen grain surfaces is surrounded by pores which both allow for the possible uptake of macromolecules and facilitate the pollen hydration process.[23–26] The results from quantitative determination after macromolecule encapsulation in terms of loading and encapsulation efficiencies in pollen grains are depicted in **Table 1**. The encapsulation efficiency (EE) of passive and compression loading is similar 37.2% ± 4.4% and 37.8% ± 3.2%, whereas with the vacuum loading process a statistically significant ($p < 0.05$) higher EE of 65.7% ± 1.8% was achieved. The possible reasons for higher macromolecule encapsulation are evident from applying a vacuum as an external force in addition to macromolecule uptake during the natural pollen rehydration process.[23–26] Although some existing reports discuss the encapsulation of drugs into extracted exine capsules,[15] we have focused on encapsulation processes involving natural pollen grains through the encapsulation of a model macromolecule.

In order to directly visualize qualitatively and understand the intrapollen presence of loaded macromolecules in the presence of pollen cytoplasmic constituents, we encapsulated FITC-conjugated BSA into natural pollen grains by the three

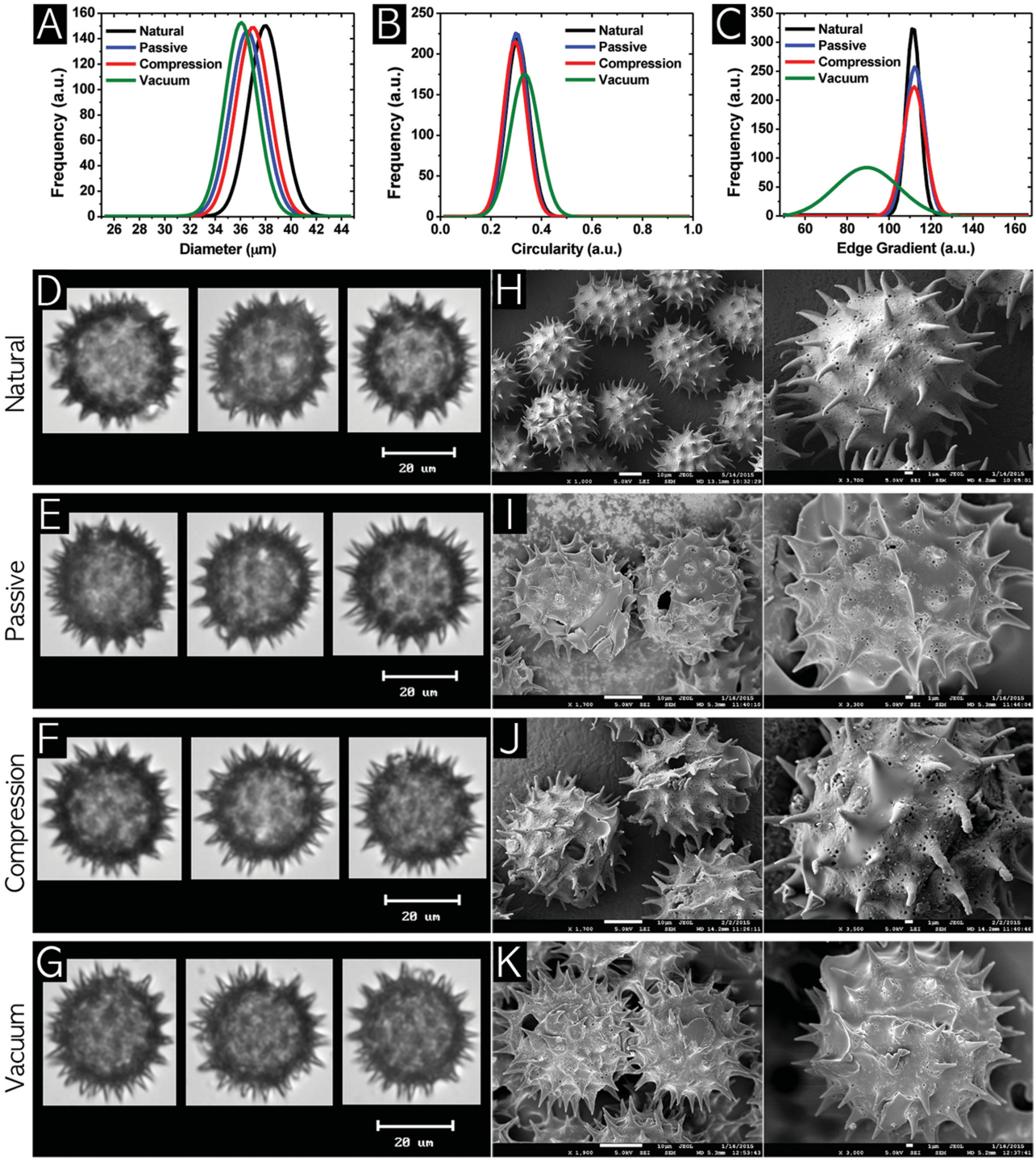

Figure 1. Characterization of natural sunflower pollen before and after BSA loading by FlowCam and SEM: Size and circularity by dynamic imaging particle analysis (DIPA, FlowCam) with a particle count of 10,000 pollen grains before and after BSA loading. Representative graphs from curve fitting to histograms of A) equivalent spherical diameter versus frequency, B) circularity versus frequency, and C) edge gradient versus frequency. Images (D)–(G) represent natural pollen grains before loading as well as, after passive, compression, and vacuum loading techniques captured in FlowCam at 20× magnification, respectively. SEM image H) represents natural pollen grains before loading and images (I)–(K), respectively, indicate macromolecule-loaded pollen grains by passive, compression, and vacuum loading techniques captured by FESEM (JEOL, Japan).

aforementioned techniques and performed analysis using confocal laser scanning microscopy (CLSM). All images were captured focusing on the middle section of pollen grains. Confocal microscopy images of natural sunflower pollen grains before macromolecule loading are presented in **Figure 2**A. It is evident from row (A) that no green color contributing to FITC-BSA was observed with pollen grains before loading, where as blue and red channels show strong autoflorescence from pollen constituents which is even more evident from their overlay image.

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Table 1. BSA-loaded Sunflower pollen: formulation parameters.

Natural sunflower pollen[a)]	Theoretical BSA loading [%][b)]	BSA loading [%]	BSA encapsulation efficiency [%][c)]
Passive loading	50	18.6 ± 2.2	37.2 ± 4.4
Compression loading	50	18.8 ± 1.5	37.8 ± 3.2
Vacuum loading	50	32.8 ± 0.9	65.7 ± 1.8

[a)] Results are means of three batches ($n = 3$) with standard deviation; [b)] Theoretical loading is based on 50% weight of natural pollen; [c)] BSA encapsulation efficiency is determined using 5 mg BSA-loaded natural pollen grains.

As a pollen grain, a male gametophyte exhibits autofluorescence based on the presence of compounds like carotenoids, phenolics, and terpenoids, and this supports the observed autofluorescence in natural sunflower pollen.[35] In the case of FITC-BSA loading by the passive technique (Figure 2B), a strong green fluorescence was observed confirming macromolecular loading into the natural pollen grains and is clearly evident from the overlay of all channels indicating FITC-BSA along with the natural pollen constituents. In the case of the compression loading of macromolecules (Figure 2C), we also observed a bright green fluorescence. However, in addition, the pollen grains appeared as compressed although still retaining their structural integrity. Relatively higher overall fluorescence is observed in the case of vacuum loading (Figure 2D) and this supports our assertion that higher encapsulation efficiency may be obtained with the application of an external vacuum force in addition to the natural pollen rehydration process.[23–26] CLSM Z-stack imaging was performed for FITC-BSA-loaded pollen grains loaded by vacuum loading techniques (Figure S2 in the Supporting Information) to provide further confirmation of the encapsulation of FITC-BSA into natural pollen grains.

The encapsulation of macromolecules into natural pollen grains was evident and we further studied in vitro release profiles of BSA from these natural pollen grains separately in simulated intestinal (PBS, pH 7.4) and gastric conditions (pH 1.2). The in vitro release of BSA in PBS (**Figure 3**A) indicates 80% release in the first 5 min and complete release was observed in 30–60 min. There was no significant difference among the release from BSA-loaded pollen grains prepared using different techniques ($p \geq 0.05$). The high percent of release is expected to be due to the high number of pores and apertures in pollen grains resulting in rapid release which was also similar to the rate of drug release reported earlier with *Lycopodium clavatum* exine capsules.[15] In simulated gastric conditions (Figure 3B) a similar burst release was observed with all three different loading techniques, suggesting no significant release differences in simulated gastric and intestinal conditions.

Further, in order to control the macromolecule release, we selected vacuum-loaded BSA pollen grains to be incorporated into natural biopolymer alginate beads by ionic crosslinking using calcium chloride. Interestingly, after macromolecule loading, the pores of the pollen grains are

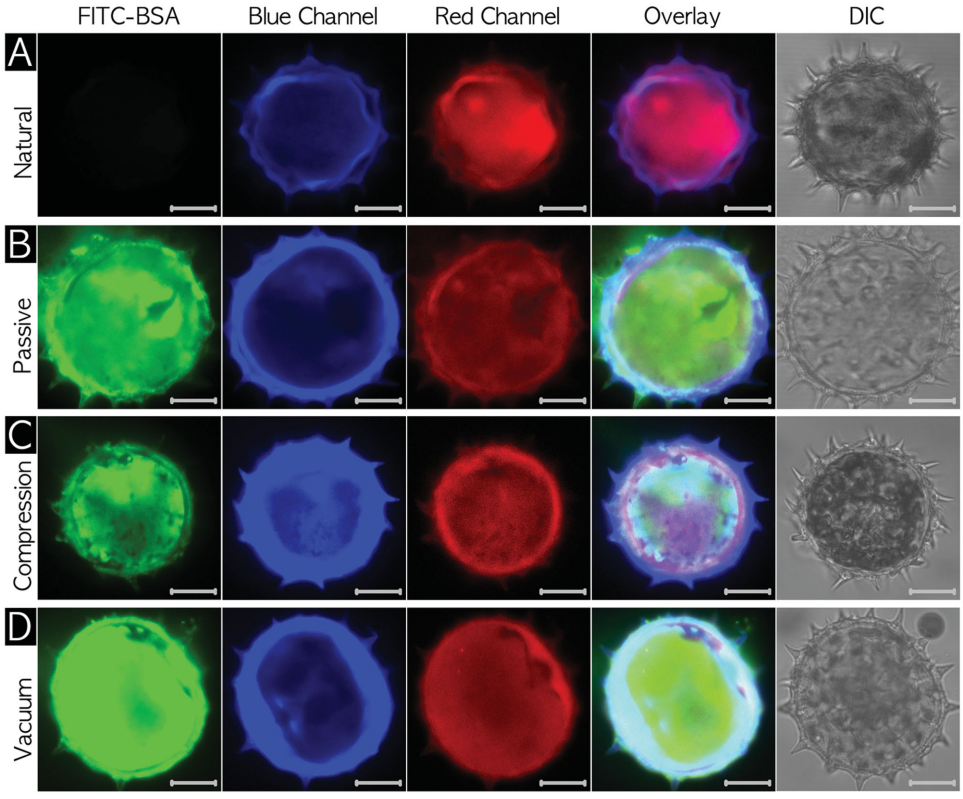

Figure 2. Confocal microscopy analysis of natural sunflower pollen grains before and after macromolecule loading. CLSM images in row (A) are natural sunflower pollen grains before BSA loading. Row (B) are BSA-loaded pollen grains using the passive loading technique. Row (C) are BSA-loaded pollen grains using the compression loading technique. Row (D) are BSA-loaded pollen grains using the vacuum loading technique (scale bars are 10 μm).

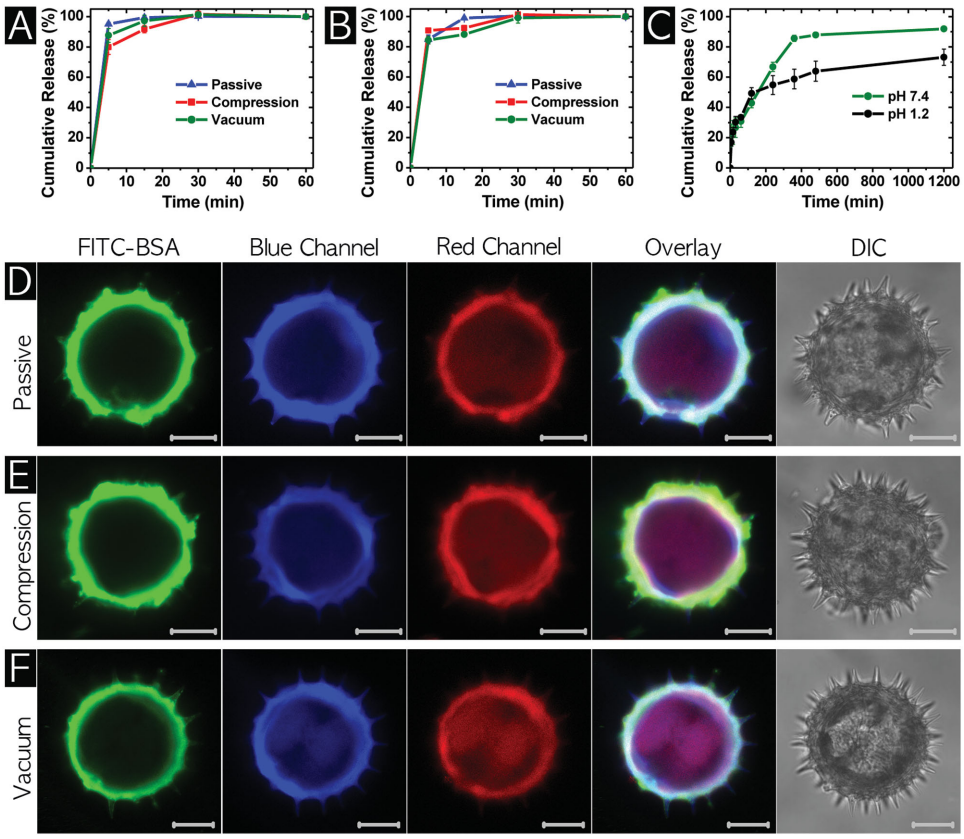

Figure 3. In vitro release profiles: A) simulated intestinal fluid, pH 7.4 media; B) simulated gastric fluid, pH 1.2 media; and C) release profile of BSA-loaded pollen grains coated with alginate. CLSM images after FITC-BSA release from pollen grains prepared by different techniques in pH 7.4 media: D) passive technique; E) compression technique; and F) vacuum technique (scale bars are 10 μm).

predominantly not covered, however, after alginate coating substantial closure of pollen pores is clearly indicated, thus acting as a barrier for macromolecule release. Our initial pollen coating optimization using 0.1% and 0.5% alginate provided a thin alginate coating but was not suitable for modulating the release of macromolecules. Further optimization using a 2% alginate solution (see Figures S3 and S4 in the Supporting Information) was found to provide an adequate barrier for delayed macromolecule release and was also necessary to form a viscous enough hydrogel so as to prevent BSA leaching during the coating process. By using 2% alginate as the hydrogel medium, the release was retarded and extended up to 20 h and there was a significant difference ($p \leq 0.05$) with release after coating compared to release before coating in both simulated conditions. Among the BSA release from coated pollen grains in simulated gastric and intestinal conditions, a more retarded release is observed in gastric conditions. A similar release profile was reported in a previous study also using alginate gel microspheres[36] and alternatively, controlled release of drugs was also reported by constructing a self-assembly of human serum albumin and l-α-dimyristoylphosphatidic acid (DMPA) to form layer-by-layer assembly on drug crystals.[37–39] The DMPA based multilayer approach controlled the drug release based on capsule wall thickness[37] and this provides further evidence that greater control over protein release may be obtained by modulating the permeability of the coating layer.

To observe the condition of the pollen grains after FITC-BSA release, we used pollen grains after in vitro release in simulated intestinal media and performed confocal microscopy analysis. Figures 3 D–F clearly indicate the release of FITC-BSA from pollen grains prepared using three different techniques and the pollen structure was found to remain intact. It is also evident from the CLSM images that a small amount of BSA binding to the exine has occurred and is clearly visible by the resulting "*green ring*."

Taken together, for the first time, we have demonstrated the use of natural pollen grains as a drug delivery vehicle by encapsulating BSA as a model macromolecule using encapsulation techniques based on passive, compression, and vacuum loading. Encapsulation of up to 65% was achieved using the vacuum loading technique, suggesting a simple means of encapsulating therapeutic compounds into natural pollen grains. Using these methods, different natural pollen grains can be explored for encapsulation of small molecules, proteins, peptides, growth factors, and biosimilars in a simple process without the need for the use of harsh encapsulation conditions where the stability of therapeutic molecules may be compromised. In addition, we have demonstrated a way to retard the release of macromolecules with pollen grains by the use of a natural biopolymer, through crosslinking alginate with calcium ions to achieve a controlled release up to 20 h. This is of particular interest in the field of the controlled delivery of therapeutic molecules, where different drug

release profiles are needed to improve the therapeutic benefit of active ingredients. Our group is currently pursuing ongoing studies into the use of natural pollen grains and their exine capsules as efficient drug delivery carriers of natural origin.

Experimental Section

Materials and Chemicals: Defatted natural sunflower pollen grains were procured from Greer Labs (NC, USA) and pollen defatting was achieved by washing of pollen grains using ACS grade ethyl ether. Bovine serum albumin (BSA), FITC-conjugated BSA, and other reagents were purchased from Sigma (Singapore). Vectashield (H-1000) medium was procured from Vector labs (CA, USA) and Sticky-slides, D 263 M Schott glass, No.1.5H (170 µm, 25 mm × 75 mm) unsterile were procured from Ibidi GmbH (Munich, Germany).

Encapsulation of Macromolecules into Natural Pollen Grains: 75 mg BSA (50 wt% based on pollen grains weight)[1] was dissolved into 0.5 mL purified water in a 1.5 mL polypropylene tube and 150 mg of natural pollen grains was suspended in the BSA solution. The suspension was mixed by vortexing (VWR, Singapore) for 5 min and the tube was transferred to a thermoshaker (Hangzhou Allsheng Inst. Singapore) at 4°C and 500 rpm for passive loading. In the case of compression loading, a compressed tablet was prepared by using a hydraulic press at 5 ton pressure for 20 s, and the tablet was soaked in BSA solution to allow for BSA uptake by the pollen grains (Dimensions of compressed tablets are provided in the Supporting Information). The 5 ton compression pressure (diameter 13 mm; 132.75 mm^2) employed is able to retain the intact sunflower pollen structure with some portion of pollen cytoplasmic constituents, as indicated by red and blue channel CLSM autofluorescence. For the vacuum loading technique, the BSA and pollen grains suspension was used, and a 2 mbar vacuum was slowly applied in a freeze dryer (Labconco, MO, USA). The quantity of BSA, pollen grains, and incubation time (2 h) were maintained constant for all batches, and after incubation the BSA-loaded pollen grains were collected by centrifugation at 12 000 rpm for 4 min, washed using 0.5 mL water, and then centrifuged to remove surface adhered BSA. The pollen grains were frozen in a freezer at –70 °C for 30 min and freeze dried for 24 h, and then the final BSA-loaded pollen grains were stored at –20 °C until further characterization. The placebo pollen grains were prepared with the same procedure without BSA and preserved at –20 °C.

Dynamic image particle analysis (DIPA) by FlowCam: A FlowCam benchtop system (FlowCam vs Fluid Imaging Technologies, Maine, USA). 0.5 mL (2 mg mL^{-1}) was used. Natural sunflower pollen grains and macromolecule-loaded grains were analyzed using a prerun volume of 0.5 mL (primed manually into the flow cell) with a flow rate of 0.1 mL min^{-1} and a camera rate of 10 frames s^{-1} leading to a sampling efficiency of about 9%, and 1000 highly focused pollen grain images were segregated by edge gradient ordering. Representative data is plotted as a histogram and fitted with a Gaussian curve and values are reported with standard deviations. (For a detailed description refer to supplementary section.)

Surface Morphology Evaluation by Scanning Electron Microscopy (SEM): SEM imaging was performed using an FESEM 7600F (JEOL, Japan). Samples were coated with platinum at a thickness of 10 nm by using a JFC-1600 (JEOL, Japan) (20 mA, 60 s) and images were recorded by employing FESEM with an acceleration voltage of 5.00 kV at different magnifications to provide morphological information.

Confocal Laser Scanning Microscopy (CLSM) Analysis: CLSM analysis was performed using a Carl Zeiss LSM700 (Germany) confocal microscope. Laser excitation lines 405 nm (6.5%), 488 nm (6%), and 633 nm (6%) with DIC in an EC Plan-Neofluar 100 × 1.3 oil objective M27 lens were used. Fluorescence from natural and macromolecule-loaded pollen grains were collected in photomultiplier tubes equipped with the following emission filters: 416-477, 498-550, and 572-620. The laser scan speed was set at 67 s per each phase (1024 × 1024:84.94 μm^2 sizes) and plane mode scanning with a 3.15 µs pixel dwell was used and at least three images were captured for each sample and all images were processed and converted under the same conditions using software ZESS 2008 (ZEISS, Germany).

Encapsulation Efficiency: 5 mg BSA-loaded pollen grains were suspended in 1.4 mL PBS, then vortexed for 5 min and probe sonicated for 10 s (three cycles, 40% amplitude). The solution was filtered to collect the extracted BSA using 0.45 µm PES syringe filters (Agilent, USA). The absorbance was measure at 280 nm (Boeco-S220, Germany) using a placebo extract as a blank to compute the amount of BSA in the pollen grains.

In Vitro Drug Release Studies: 5 mg of BSA-loaded pollen grains and placebo were suspended in pH 1.2 (0.1 m HCl), and phosphate buffer saline (PBS), pH 7.4, solutions. The suspensions were incubated at 37 °C at 50 rpm in an orbital shaker, the release samples were collected at specified time intervals by centrifugation at 14 000 rpm for 30 s, and replenished with fresh release media. The release sample was filtered using PES filters and absorbance was measured at 280 nm using a placebo release sample as a blank. The amount of BSA released was calculated using a BSA standard curve.

Formulation of Macromolecule-Loaded Natural Pollen Grains into Alginate Beads: 150 mg of BSA-loaded (vacuum loading) pollen grains were mixed homogeneously with 1.5 mL of alginate (2%) in water. The resulting suspension was slowly added to 10 mL calcium chloride (8%) using a blunt 18 G needle under magnetic stirring, the stirring was continued for 5 min, and the calcium chloride was removed by centrifugation (1500 rpm, 2 min). The beads were washed twice using 1.5 mL water and freeze dried for 24 h. All formulations were stored at –20 °C until further studies and 10 mg of alginate coated pollen grains were used for in vitro studies.

Statistical Analysis: Statistical analysis was performed using two-tailed t -tests and $p < 0.05$ was considered as statistically significant. Encapsulations with natural pollen and release experiments were repeated at least three times and all data are expressed as mean ± SD.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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