Pine pollen for molecular encapsulation and oral delivery applications

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2018

Prabhakar, A. K. (2018). Pine pollen for molecular encapsulation and oral delivery applications. Doctoral thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/75839>

<https://doi.org/10.32657/10356/75839>

NANYANG
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**Pine pollen for molecular encapsulation and oral delivery
applications**

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Interdisciplinary Graduate School
NTU Institute for Health Technologies

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NTU Institute for Health Technologies**

A thesis submitted to the Nanyang Technological University in partial
fulfillment of the requirement for the degree of
Doctor of Philosophy

2018

Statement of Originality

I hereby certify that the data and findings in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

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Date

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Arun Kumar Prabhakar

Abstract

Molecular delivery using carriers for biological applications has been a subject of interest for the past many years, as some molecules suffer from solubility (reduced bioavailability) and stability (pH & enzyme-sensitive molecules like proteins) issues, resulting in denaturation, inactivation & loss of function. This necessitates a robust delivery vehicle, capable encapsulating such molecules, protecting them till the site of release, followed by controlled tunable release as to avoid low or excess levels, keeping the molecular concentration in the effective therapeutic window, especially in case of drug delivery. Various nano and microparticles of different sizes and surface chemistry have been used for such delivery through different modes of administration with oral being most favoured, due to ease of dosage, along with high patient compatibility. Most of these particles are synthetic in nature, involving multiple processing steps and suffer from issues like large-scale synthesis, non-uniformity, particle stability (under gastric conditions etc), biocompatibility & biodegradability to name some pressing issues. Few natural and bio-inspired carriers, which have relatively much lesser safety concerns, have also been explored for this purpose successfully, which pushes us to look at other such naturally available resources. Plant pollen and spores, a natural product with uniform size, physico-chemical characteristics proves to be invaluable in this aspect, offering molecular loading and protection for oral delivery applications, with promising research done till date.

Pollen and spores have been shown to be promising prospects for encapsulating molecules due to their double layered wall, comprising of a cellulosic intine and a exine, composed of a supreme polymer called sporopollenin. Processed pollen namely, sporopollenin exine capsules (SECs), where pollen/spores are subject chemical treatment to remove sporoplasmic contents and the intine layer, were favoured due to lower allergenicity and more loading space, with most of the work focused on single-compartmental non-saccate pollen (*Lycopodium*, *Sunflower* etc.). Here we show that multi-compartmental pine pollen is also an effective vehicle to encapsulate and deliver molecules of interest. It has been consumed as a super- food with health-benefits such as enhanced immune and endocrine function (excellent source of testosterone), lowering of cholesterol levels along with anti-inflammatory, anti-arthritis, and anti-tumor activity and has not been explored for molecular encapsulation till date. Pine SECs however have been

produced by sequential organic solvent, acid/base and enzymatic processing, with no process optimization nor morphological characterisation done, followed by loading of a few molecules.

Here we looked at pine SECs got through acid-processing of defatted pine pollen initially, with various processing conditions like acids, concentrations and duration were explored, with the structural integrity of the capsules looked into for every processing condition with defatted pine as the source material. Given the rapid rise in protein therapeutics, SEC production was followed by protein (BSA) loading, where it was found to load thrice as much as defatted pollen of the same mass, with fluorescently tagged proteins (FITC-BSA) used to analyse the spatial loading. As the SECs suffered from structural issues, natural and defatted pollen(which involves relatively less processing) was explored more in detail in the next study, where natural pine pollen was ether-defatted. Natural pine pollen was process optimized for BSA encapsulation regarding the loading method (passive or vacuum-assisted loading), loading duration and protein concentration and then comparative protein (BSA) loading of natural with defatted pine pollen was done to quantify protein loading with the degree of defatting. Defatted pine loaded better here due to increased pore size as measured by nitrogen adsorption/desorption isotherms. Controlled release was shown using BSA-loaded defatted pollen as the carrier, with natural polymers as binders (Xanthan Gum) or using coatings (Sodium Alginate) with tableted formulations, with simulated gastric (pH 1.2) and intestinal fluids (pH 7), where the protein was protected in the gastric phase and released gradually in the intestinal phase. FITC-BSA loading showed that the protein loaded mainly into the air-sacs with minimal central cavity loading with vacuum loading for both natural and defatted pollen, which is different from SECs spatial loading pattern, which loaded all over the particle. Finally, natural pine pollen was explored for loading of other potential molecules like dyes and drugs apart from proteins (BSA & IgG) also, through simple passive loading and vacuum-assisted loading, where the large proteins were found to load exclusively in the air-sacs with vacuum loading and minimally into the central cavity with passive loading. The dyes and drugs however loaded into the central cavity alone irrespective of the loading method. This was followed by dual molecular loading, where the dyes and drug were loaded into the central cavity through passive loading, followed by vacuum loading of BSA, which resulted in

compartmentalization (distinct molecular loading pattern into air-sacs and central cavity) of molecules. The protein structure of BSA was checked into pre-loading and post-release and was found to be conserved.

All this show that pine pollen is capable of encapsulating, preserving and controlled-release of proteins and other molecules, opening up myriad applications in fields like drug delivery, molecular preservation, nutraceutical delivery etc.

Acknowledgements

I would first like to thank my supervisor, Assoc Prof Cho Nam Joon, Material Science Engg (MSE), NTU for guiding me all the way through. He has been a pillar of strength and believed in me, for which I am truly thankful and grateful. I would like to extend my most sincere gratitude to him, without whom it would not have been possible to complete my thesis. He gave me the freedom to explore my field of interest and was an excellent critique of my progress. He has morally and technically supported me right from the onset, which kept me going. I would also like to thank my co-supervisor, Prof Chen Peng, School of Chemical and Biomedical Engineering (SCBE), NTU for his invaluable guidance and expert advice. I am also highly thankful to my mentor, Prof Jeffrey Glenn (Stanford) for being there when I needed him and I appreciate all of their inputs in shaping my research successfully. I would also like to thank Dr. Eijiro Miyako for his invaluable support with my work.

I have been extremely lucky to have been a part of Interdisciplinary Graduate School (IGS) here in NTU. The IGS journey has been a delightful experience and gave me an opportunity to meet and interact with people from different thoughts and research interest, which widened my horizon and thinking level. I am extremely thankful to my research centre, HealthTech, for giving me this wonderful opportunity to conduct research in my field of interest.

My lab members have been helpful and kind throughout in creating a congenial atmosphere for me to work in and making me feel at home. A special thanks to Michael Potroz and Josh Jackman for their invaluable inputs and guidance at anytime asked for. I am highly indebted to all my friends in NTU, who have played their role in my life and made it memorable over the past 4 years.

Last but not the least, I would like to thank my beloved parents, family and friends for their constant encouragement and support throughout this challenging phase of my life.

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Abbreviations

2D	Two Dimensional
3D	Three Dimensional
a.u.	Arbitrary Unit
BSA	Bovine Serum Albumin
BJH	Barrett-Joyner-Halenda
BET	Brunauer, Emmet, and Teller
°C	Degree celsius
CD	Circular Dichroism
CD44	Cluster of Differentiation
CLSM	Confocal Laser Scanning Microscopy
CHN	Carbon, Hydrogen, Nitrogen
CNT	Carbon Nanotube
Da	Dalton
DI	Deionized
DIPA	Dynamic Imaging Particle Analysis
DDPP	Double Defatted Pine Pollen
EPA	Eicosapentaenoic Acid
FDA	Food and Drug Administration
FDDS	Floating Drug Delivery Systems
HPMC	Hydroxypropoyl Methylcellulose
g	Gram
GRAS	Generally Recognized as Safe
GI	Gastro-Intestinal
h/hrs	Hours
HA	Hyaluronic Acid
H ₂ SO ₄	Sulphuric Acid
H ₃ PO ₄	Phosphoric Acid
HCl	Hydrochloric Acid
IgG	Immunoglobulin G
kV	kilovolt
FITC	Fluorescein Isothiocyanate
FTIR	Fourier Transform Infrared

M	Molar
mbar	millibar
mins	minutes
mg	milligram
MQ	MilliQ
ml/mL	millilitre
MRI	Magnetic resonance imaging
mV	millivolt
MW	Molecular Weight
mm	millimeter
MWCNT	Multi-Walled Carbon Nanotube
N ₂	Nitrogen
NPP	Natural Pine Pollen
NIR	Near InfraRed
nm	nanometer
PBS	Phosphate Buffered Saline
PLGA	Poly-Lactic-co-Glycolic Acid
PEG	Polyethylene Glycol
RBC	Red Blood Cell
RPM	Revolutions Per Minute
secs	Seconds
SDS	Sodium dodecyl sulphate
SEC	Sporopollenin Exine Capsule
SEM	Scanning Electron Microscopy
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
SiRNA	Small Interfering Ribonucleic Acid
uM	Micromolar
µm	micrometer
µl	microlitre
UV	Ultraviolet
UV/Vis	Ultraviolet–visible
XG	Xanthan Gum
% v/v	Volume / Volume Percent

% w/v Weight / Volume Percent

% w/w Weight /Weight Percent

Chapter 1 Introduction

Molecular encapsulation and delivery has been a core issue concerning many industries like pharmaceutical, nutraceutical and other healthcare related units. Synthetic molecules, which have been largely explored for this suffer from various issues like bio-incompatibility, difficulty of scale-up, non-uniform physico-chemical characteristics etc. to name a few. Natural sources offer solution to most of these issues faced and one of the most widely available option is plant pollen. This chapter provides an overview for the need of molecular encapsulation and delivery, cons of synthetic carriers, natural-origin and purely natural molecules as apt alternatives and how pollen perfectly fits in to this end, with a brief introduction of pine pollen as a compelling and valuable tool for microencapsulation applications.

1.1 Need for Molecular Encapsulation

Encapsulation of molecules for preserving them in their native or functional state until the site or time of release using microparticles is the basis for the field of microencapsulation¹ and is necessary for molecules which suffer from issues like stability, solubility, degradation etc., which render them dysfunctional or result in their low availability. Biological molecular delivery in specific, deals with encapsulating and transporting molecules vulnerable to the natural physiological conditions^{2,3} present, to the desired tissue microenvironment for enhancing their bioavailability. Proteins, peptides⁴ and nucleic acids⁵ are some such molecules that are vulnerable to low pH and high enzyme environment⁶, degrading in their free state under such conditions, before reaching their target tissue and thus need cover. Apart from degradation, foreign molecules are also subject to opsonisation⁷, where proteins attach to the molecule (complement activation) and are subsequently cleared away by the defense system. With drug delivery, the need for a guided system is greater, as the delivery must be specific, with minimal side effects, displaying localized drug concentrations within the therapeutic window limits. The process of drug discovery/development as such is time-consuming and costly with most of the potential molecules being eliminated in the process⁸⁻¹¹ and so the ones that make it through have to be delivered near perfectly for the desired therapeutic effect. Molecular delivery can also be used for diagnostic needs to detect and track abnormalities using MRI¹², fluorescence¹³, colorimetric¹⁴, PET¹⁵ etc where the diagnostic agent is loaded or attached into a carrier molecule, which targets and labels an environment based on certain stimuli like excess cell receptors etc. Apart from molecules for medical purposes, nutraceuticals comprise a majority of other substances delivered to humans. These are basically products which provide additional health benefits apart from the basic nutritional value and thus are also known functional or super foods too^{16,17} and include a wide variety of molecules like ginseng, *Echinacea*, green tea, glucosamine, omega-3, lutein, folic acid, and cod liver oil. Some nutraceuticals also have some therapeutic value¹⁸ too. These also have to be delivered in a stable form for their effective action. So for all these above stated sensitive molecules, carriers or delivery vehicles play a significant role, where they encapsulate the molecule of interest and deliver it to the target organ/tissue¹⁹⁻²⁶ in a specific manner.

1.2 Molecular Encapsulation and Delivery

1.2.1 Carriers used- Shape, size, functionalization & loading

Carrier molecules are of varied sizes, shapes and surface properties as to avoid the defence system and reach the biological environment of interest with the molecular payload intact. According to their size, they are classified majorly as microparticles (0.1-100 μm) and nanoparticles (1-100nm)²⁷. Nanoparticles comprise of liposomes²⁸, micelles²⁹, quantum dots³⁰, hydrogels³¹, inorganic particles³², nanocapsules³³, nanotubes³⁴ etc. , while microparticles involve microspheres, microcapsules etc. and are mostly made of polymers, ceramic and glass³⁵. Their size dictates their method of delivery mostly with nanoparticles favoured for systemic delivery³⁶ where they can penetrate into the tissues and cells, as compared to bigger microparticles, which may cause blood vessel blockage. Smaller microparticles have a relatively greater circulation time as compared to larger ones and lesser tissue penetration with respect to nanoparticles. Thus bigger microparticles are more suited for non-systemic delivery methods such as oral^{37,38}, with nanoparticles also being used³⁹. Most of the particles produced are spherical in shape as to minimize the contact surface area with other biological molecules during transit, with other shapes also being synthesized and their effects on cellular uptake and biodistribution studied⁴⁰⁻⁴². The surface properties of these particles also play a major role in deciding inter-molecular interactions and cell uptake^{43,44} and circumventing the above mentioned issues associated with free molecular delivery. Possible surface-associated effects would be ionic interactions arising from charge effects, physisorption and chemisorption, non-covalent and covalent (rare) bonding, apart from complement activation and non-specific cell uptake. Cationic particles are found to have a greater cell uptake, due to the negative cell membrane charge. Attachment with PEG⁴⁵ has been a common way to avoid opsonisation and ensure long-term system circulation, while targeting⁴⁶ is necessary to make the delivery site-specific.

The molecule to be delivered is encapsulated into the carrier and delivered through a preferred route of administration depending on the target tissue. Loading methods include various techniques like emulsion-based methods, solvent -exchange, using ionic and pH gradients, simple passive loading etc. Multiple theranostic and diagnostic molecules have been loaded into nano and microparticles successfully with varied degrees of loading⁴⁷⁻⁵¹. This loading could be a single or a multi-step process and

has to be optimized to achieve greater therapeutic efficiency per dose to reduce the need for recurrent doses.

1.2.2 Route of administration

Post-molecular loading the formulation has to be administered suitably, as the route of administration plays a vital role in the deciding the bioavailability of the delivered molecules and thus the frequency of administration. The route of administration is decided on the target site considering factors like minimal interaction with non-target cells, possibility of clearance (low circulation times), time-varying concentrations in target tissue etc. Various routes include intranasal⁵², intravenous⁵³, transdermal^{54,55}, intramuscular⁵⁶, oral^{19,21,57}, subcutaneous⁵⁸, implantable systems⁵⁹ etc. with each one of them having pros and cons⁶⁰⁻⁶². Each of these demand a distinct formulation with nasal delivery favouring sprays, while intravenous uses liquid formulations and oral delivery involves tablets to name a few. Of all routes possible, oral still remains the most favoured one due to its ease of ingestion, painless nature, versatility (various types of drugs can be encapsulated), and, most importantly, patient compliance^{19,63,64}, even though it does suffer from variable absorption rates, bioavailability issues, acid degradation, first-pass effect^{4,5,65}.

1.2.3 Micro and nanoparticles for oral drug delivery

Synthetic Polymeric delivery vehicles

Various nano^{39,66,67} and microparticles^{27,37,68} have been used for oral delivery and most of these have been of polymeric nature^{43,66,69-72} since they offer the advantage of versatility apart from improved drug stability. These are synthesized from starting polymers and other reagents mostly through solvent processing techniques using top-bottom or bottom-up approaches⁴⁰ and are mostly rigorous and demanding requiring the use of multiple chemicals, organic solvents and stabilisers⁷³⁻⁷⁵. As concerning synthetic molecular carriers, biocompatibility and biodegradability have been pressing issues⁷⁶⁻⁷⁹ and the so FDA approved polymers like polylactic acid (PLA), polyglycolic acid (PGA), polylactic glycolic acid (PLGA), and polycaprolactone (PCL) are primarily used^{69,80-84}. Some polymers such as polyvinyl alcohol (PVA), ethylene vinyl acetate are biocompatible, but not biodegradable and thus aren't favored even though they can give long-term near-zero-order

drug release kinetics. Gastro-intestinal protection against low pH, enzymes (proteases, lipases etc.)⁴ and crossing the mucus barrier⁸⁵ are the foremost challenges as concerning oral delivery applications and this is achieved using coatings (eudragit, sodium alginate), binders (release retarding polymers like gums, HPMC etc.) for controlled release^{20,64,86-88} and by using mucoadhesive polymers like chitosan, PLGA for increasing intestinal retention time through mucal attachment⁸⁹⁻⁹² followed by tissue targeting⁹³. Thus the carrier particle, apart from being biocompatible and biodegradable, has to be suitably functionalized too in order to meet the above needs, which further complicates the process. As more and more processing steps are involved, the yield is lowered along with the level of control and homogeneity, with the final product exhibiting significant batch to batch variability in its physical, chemical and mechanical properties.

Figure 1.1 A schematic of drug delivery using nanoparticles⁹⁴.

Natural-polymer based Carriers

Apart from synthetic polymers, natural polymers like starch, alginate, xanthan gum, gelatin, guar gum, collagen, chitosan, and albumin, silk and biomolecules like proteins, lipids etc.⁹⁵⁻⁹⁹ have also been used for drug delivery¹⁰⁰. These are cheaper, abundant and bio-friendly, which make them preferable over synthetic polymer-based carriers. Lipid-based carriers have been well characterized over a long period of time and involve

liposomes mainly, which are synthetic in nature and are currently in the market for drug delivery applications¹⁰¹, whereas protein based carriers are upcoming^{99,102}. Lipid based vesicles have stability issues and thus have to be mixed with structurally rigid materials like synthetic polymers, which result in hybrid materials combining the properties of both constituents¹⁰³. Some natural polymers, which also suffer from mechanical stability issues have been blended with synthetic polymers¹⁰⁴ to form functional delivery agents known as biosynthetic polymeric materials. Hybrid biomaterials^{105,106} have been explored for encapsulation and delivery applications with promise. Polymers of animal and bacterial origin suffer from antigenic issues for human applications as compared to plant-based natural polymers, that are more favoured. All these polymers have to be extracted from their sources, which itself is quite laborious and they have to be modified or processed to produce microcapsules, which is an additional step. For example, chitin extraction from natural sources is a multi-step process by itself¹⁰⁷, after which it is deacetylated to get chitosan. The same can be said for gelatin¹⁰⁸ and alginate¹⁰⁹ extraction too, which are highly-used for drug delivery research. Post-extraction, the polymers are to be suitably processed or modified to make them suitable for drug delivery. Processing involves polymer modification and creating microspheres, capsules or other micro-sized particle out of the polymer by synthetic methods^{110,111}. The plant-based polysaccharides¹¹² and other natural polymers¹¹³ which are available in abundance like cellulose, pectin, starch and other gums have to be modified in ways like silanisation, esterification, cross-linking etc. in order to create functional microparticles suitable for molecular encapsulation and delivery. Post-modification, the polymers undergo synthetic processes like emulsification, spray drying etc. to generate varying microparticles according to the synthesis protocol adopted. Microcapsules offer more volume for molecules as compared to microspheres, which are more solid¹¹⁴ and hence microcapsules face the problem of fragility, governed by membrane thickness. This could lead to leaky capsules with rapid release profiles if the membrane is destabilized or ruptured¹¹⁵. So microcapsule synthesis would involve more of polymer modification or synthesis of hybrid materials to impart the necessary mechanical strength.

The lipid and other biopolymer based microparticles may have controlled physico-chemical properties and a non-toxic nature, but have poor circulation times and

targeting abilities and have to be suitably surface functionalized similar to synthetic polymers mentioned above apart from the initial modification steps to make them molecular- delivery fit.. All these factors limit their value, but these happen to be best one can get out of synthetic or modified particles. So even with natural polymers, apart from their arduous extraction process, synthetic steps are also involved, which would make the final carrier clinically unsuitable.

Natural Encapsulants

Natural products with minimal or no process requirements needed for molecular loading prove invaluable by avoiding the drawbacks of the fore-mentioned delivery systems. Such natural particles have been used for microencapsulation and to good effect because of their innate targeting and penetration abilities for their own needs^{116,117}. These properties can be tapped into thus simplifying the overall process. These include virus^{118,119}, bacteria^{120,121}, human cells (RBCs¹²², macrophages¹²³, lymphocytes¹²⁴ and stem cells¹²⁵) and their mimetics¹²⁶⁻¹²⁸ used primarily for delivering therapeutics in humans. Bacteria and virus evade physiological defence system and enter cells, which is ideally desirable for any drug carrier and thus were used in making pathogen-mimicking particles. These molecules were modified to an extent as to render them non-toxic for such delivery purposes (i.e) the virulent genes of the virus removed, using bacterial ghosts with inner contents removed etc. Even though these are considered non-infective, safety concerns still linger over their use. Even yeast^{129,130} and algal^{131,132} cells have also been used for microencapsulation applications with microorganisms looked at potential microencapsulants right from 1970s. Again the probable microorganism-related safety issues makes this option undesirable, even though the cells have shown good loading. Human cell-based carriers represent a far more bio-friendly option, apart from untracked circulation within the body due to ideal surface properties. Red blood cells offer an attractive option, given their abundance, size and shape and have been used for delivering various kind of drugs^{133,134}. PLGA and PEG¹³⁵, which are bio-friendly polymers were used to form particles, that were able to mimic the RBC structure(the CD47 marker was missing) and were loaded with molecules successfully. Immune cells like macrophages and lymphocytes have also been used where the actual carrier nanoparticle is bound to their surface (as is done with stem cells too) or

the nanoparticle is engulfed and targeted to hypoxic areas (cancer-specific) respectively. Thus human cell-based carriers seems to be most promising of all options and considering all relevant factors with RBCs being the most effective cell-type. RBC isolation from whole blood isn't too tedious a process but does involve use of anti-coagulants and has storage issues. An effective alternative would be a plant-based natural products, which are available in plenty with a simple isolation method.

Figure 1.2 A comparison of the synthetic and natural delivery systems and how the gap between them could be bridged¹³⁶.

Plants produce a lot of useful metabolites and products like waxes, resins, anti-microbial compounds, herbal extracts etc. that are useful for mankind, apart from being a wholesome source of food. Most plant-based products are deemed safe for human consumption after proper processing to removal natural sources of contamination. Plant cells have been used for oral delivery of proteins^{137,138}, but these proteins were produced by transgenic plants and not loaded into them externally. Following ingestion, the gut microbes digest the plant cell wall followed by release of the protein. Transgenic plants were also used to express virus –like particles which were also used for drug delivery¹³⁹. Plant cells thus seem a viable option for microencapsulation, but no extraneous molecular loading has been tried with plant cells till date. The small pore sizes of plant cells could be a limiting factor¹⁴⁰. Another plant material, whose application potential is massive¹⁴¹ as concerning molecular encapsulation are pollen¹⁴² and spores¹⁴³, which have been explored to an extent, indicative of huge prospects in store.

Pollen as plant-based natural microencapsulant

Pollen carries the male gametes of plants and protects its sporoplastic (living material) constituents from extreme conditions of light, temperature, dehydration etc.^{144,145}. It is thus naturally designed to hold sensitive material making it an ideal choice for encapsulation of molecules like proteins, nucleic acids etc. Pollen has been researched upon for sometime now with structures of multiple pollen species being well-known and documented¹⁴⁶⁻¹⁴⁸. There are various types of pollen and spores with different architecture, overall structure (saccate and non saccate, etc.), sizes and this depends on their mode of pollination, environmental conditions etc. Species-specific pollen are unique in their morphology, size etc. and this varies across species thus providing numerous options to choose from^{149,150}. Most of the pollinating species have double-layered protective structure around the pollen grain consisting of cellulosic intine followed by the robust exine¹⁵¹⁻¹⁵³, which makes it a potential sturdy delivery vehicle. In most biotically pollinated plants, the pollen exine is covered by a thick lipid coat necessary for sticking to the vectors (insects etc.) body¹⁵⁴ whereas pollen pollinated by other means (wind, water) it is much thinner. Few natural pollen species and spores¹⁵⁵⁻¹⁵⁷ have been looked at for molecular encapsulation and have shown promise. Most of the applications till date with pollen and spores involve a processed form known as Sporopollenine Exine Capsules (SECs), which is similar to the bacterial and viral ghost shells and will be discussed in the next chapter. These SECs have been developed for multiple pollen species with distinct morphologies and permeabilities. This pushes us to explore other pollen species too given the vast number of options available.

Pine is one such species and belongs to family of gymnosperms. It is structurally different from the most common ones previously explored, as it is bi-saccate in nature. It has various types like *Pinus longaeva*, *Pinus ponderosa*, *Pinus sylvestris*, *Pinus Taeda* etc. Pine has been described as super-food due to its nutritional value¹⁵⁸ and has been found to have intrinsic therapeutic value¹⁵⁸ as well. There have been no reports of natural pine pollen used for molecular encapsulation and a few reports regarding pine SEC production and proof-of concept encapsulation of a few molecules into SECs, but none were rigorous and truly exploratory as concerning practical applications. Thus pine seems a naturally exciting

option to be explored for molecular encapsulation and delivery given its unique structure and its fitness for human consumption.

1.3 Objective and aims

The goal of this thesis is to explore pine pollen in its natural and processed forms as a suitable microencapsulant for nutraceutical and therapeutic purposes. As to that end, the below specific aims have been proposed:

Aim 1: To produce pine pollen sporopollenin exine capsules (SECs) from defatted pine and analyze them physically and chemically with various processing conditions and test relative protein loading with defatted pine pollen.

Aim 2: To defat natural pine pollen, and compare natural and and defatted pine pollen for protein loading and achieve spatial and temporally controlled release of loaded molecule (BSA) using tableting, binders and coatings.

Aim 3: To look at natural pine pollen for loading and release of hydrophilic and hydrophobic small molecules (dyes and drug) and large proteins.

1.4 Dissertation overview

This thesis has been divided into six chapters. Chapter One gives the background information on the need for encapsulants for molecular delivery, current trend of particles used for oral delivery: both natural and synthetic and why purely natural ones are more favourable and finally how pollen fits in as a suitable choice. Chapter Two deals with literature regarding pollen formation and structure, pollen processing and applications explored till date with various pollen and spores; both in their natural & processed forms and a comparison of pollen with commonly used microencapsulants. Chapter Three deals with production of pine pollen sporopollenin exine capsules through acidic processing, with a discussion of the processed capsule structure, followed by comparative protein loading of processed capsules with defatted pollen. Chapter Four deals BSA loading process optimization with natural pine pollen and defatting of natural pine pollen and its effects on BSA loading. This was followed by tuning the BSA release through tableting with coating and binders. In Chapter Five, natural pine pollen as a viable microparticle for molecular loading and release of large proteins and small molecules such as dyes and drugs

individually and dually is explored, with the loaded protein (BSA) structure probed for its conservation. Lastly, Chapter Six concludes this dissertation by summarizing the findings, followed by proposed future work and listing potential applications, based on the infinite potential of this natural carrier, pine pollen.

1.5 Research Findings

This dissertation had the following novel outcomes:

- Acid processing of pine pollen to get pine SECs followed by thorough physico-chemical characterization and showing enhanced protein encapsulation into pine SECs as compared to unprocessed pollen on a mass basis
- Increasing protein loading as compared to natural pine pollen through defatting, which preserves the pine pollen morphology and achieving controlled release under intestinal conditions
- Using natural pine pollen as a microencapsulant for compartmentalized uni and dual molecular loading and release of proteins and other small molecules like dyes and drug

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Chapter 2 Literature Review

Pollen in general and specifically pine pollen has been a hugely unexplored asset for microencapsulation applications till date, which makes this research truly necessary. This chapter provides an overview of pollen formation, which leads to its unique structure and in turn opens up its value as microencapsulant with suitable physico-chemical and mechanical properties. The exine structure with emphasis on sporopollenin structure and properties is discussed. Work done till date using pollen and spores both in natural and processed forms has been documented, followed by a comparison with commonly used microencapsulants. Finally the rationale for this study based on literature is elaborated upon to show how pine pollen fits as an ideal tool for microencapsulation needs with its myriad potential applications.

2.1 Pollen and spores

Spores are produced by plants¹ and microbes like bacteria², algae³, fungi⁴ etc. for their proliferation, where they fuse (sexual) to form a zygote or give rise to another of their own kind (asexual). Pollen is the mature form of the secreted microspores and is naturally capable of protecting the plant's genetic material and cytoplasmic contents against unfavourable and harsh environmental conditions due to its mechanically rigid and physico-chemically stable double-layered structure made up of an inner intine followed by the exine^{5,6}. Intine layer consists of polysaccharides like cellulose, hemicellulose and pectin and are the immediate protectants of the sporoplasm. Intine can be divided into endintine and exintine, which can be isolated through differential staining. The intine is followed by the more robust exine composed mostly of a super-resilient polymer called sporopollenin⁷. The layers of the pollen are quite porous and allow for flow of water and nutrients across to serve the nutritive needs of the developing grain^{8,9}. This is facilitated by the nanochannels on the exine and the hydrophilicity of the intine.

2.1.1 Formation and development of pollen and spores

Pollen formation is a multi-stage process highly regulated by the tapetum. It consists of three stages: (i) microsporogenesis; formation and differentiation of the sporogenous cells and their meiosis; (ii) Post-meiotic development of the microspores and finally (iii) mitosis of the microspores^{10,11}. In plants, spores are produced in the sporangia (sexual organ) by the tapetum, which lines the sporangia and provides essential precursors for pollen development¹². Spores are usually secreted as tetrads (group of four) and are surrounded by a callose wall (from the tapetum) initially, which dissolves to release the tetrad as individual spores¹³. Spores may be tetrads or multiples forming polyads of 8, 16, 32, or 64 grains, or more complex arrangements too. Amongst tetrads, 0, 1, 2, 3, or all 4 (nullads, monads, dyads, triads, or tetrads respectively) grains may be fertile, with the sterile grains remaining attached. Every spore has its own wall and is segregated from the rest. This wall then begins to develop thoroughly, which dictates the physico-chemical and mechanical properties of the pollen species. Ubisch bodies are secreted by the tapetum, which have sporopollenin precursors and are involved in the exine formation^{5,14}. Thus the tapetum is responsible for structural wall for the development of the microspore during

meiosis and further maturation. Pollen wall deposition is regulated partly by tapetum, and also by the developing microspores too¹⁵. Lipid bodies accumulate in the tapetum and are exported by exocytosis and polymerize to form sculptured exine wall precursors¹⁶. Primexine, a polysaccharide matrix is initially formed as a base¹³ onto which sporopollenin deposits to form the final exine structure. Secreted bodies fuse and the tapetal cells accumulate large cytoplasmic lipid bodies, and become more vacuolated. Post-mitotic division, the tapetum breaks down (programmed cell death) enabling release of its contents for deposition onto the developing pollen grains¹⁷. This material is called the “pollenkitt” fills the the pores to form the pollen coat. This whole process is highly regulated by a genetic network as verified by gene-specific mutants¹⁸. Pollen terminology is mostly associated with seed-producing plants (spermatophytes), whereas spores correspond to clubs, mosses, ferns etc, where they form the reproductive unit sexually or asexually. The study of spores and pollen is called palynology.

Figure 2.1 Pollen development and structure. Microspore undergoes meiosis followed by mitosis to generate tetrads, which split up and mature. The final pollen structure comprises of an outer shell protecting the inner cytoplasmic contents. The outer shell is comprised. of intine followed by exine. The exine surface is coated with a layer of lipids known as pollenkitt, which is missing in gymnosperms.

2.1.2 Pollen: Contents, Variability and Pollination

Pollen grains protect and carry the male genetic material until the time of pollination, when they are transferred to the stigma of a plant through biotic or abiotic transfer¹⁹. They contain the necessary genetic information to give rise to an entire haploid plant organism or they unite with the female gamete to form a diploid zygote, which develops into a new sporophyte. The pollen grain could be bi, tri or pluricellular²⁰ and consists of the vegetative cell, enclosed sperm cells and tube cell, which gives rise to the pollen tube for pollen transfer during pollination. The male gametes are contained within the cytoplasm of the vegetative cell, which is filled with storage reserves. Pollen kitt is found to coat the exine and is composed of hydrophobic lipidic compounds and is found in angiosperms almost exclusively^{21,22}. Its primary function is to make the pollen sticky so that it can be transported by vectors across long distances. Its other functions include: preventing water loss from pollen, antifungal and anti-bacterial action, pollen protection from predators etc.²³. In gymnosperms, the pollen have a waxy coating as opposed to the pollen kitt or are covered with natural plant secretory substances like resin etc., which are also lipidic in nature.

Pollen size is quite variable and varies from 1-250 μm across species²⁴. The morphology too is quite variable with the existence of simple uni-compartment pollen like sunflower to saccate pollen, which have atleast two compartments (central cavity + air sac) for their pollination. They are also diverse in terms of structural elements like structural arrangements, apertures, sexine patterning, sporopollenin content, pollen wall thickness etc.^{25,26}. Out of all these apertures are probably the most diverse and unique amongst pollen grains and help in species identification²⁷. Apertures are soft spots on the pollen grain, where the exine is thinner and facilitates pollen tube growth. Pores and colpi are the most prominently observed apertures and depending on the number of pores and colpi present they can be classified as mono, di, tri porate or colpate etc. If both colpi and pores are present then the pollen is termed colpate. Pollen without apertures are rarely found, while some pollen/spores do fall into the above mentioned categories: fenestrate, which has holes in its tectum; syncoplate with fused colpi.

The main modes of pollination are either biotic (vector-mediated) or abiotic (environment-mediated). Pollen which are transferred biotically have an outer coat of lipids

known as the pollenkitt, which sticks to the vector (insects, birds, mammals etc.) to make the transfer possible. This kind of transfer is referred to as entomophily (insects) or zoophily (animals) depending on the vector²³. The pollen quantity produced here is lesser and is characteristic of angiosperms (flower-producing) like oak, maple, lilies etc. As concerning abiotic pollen transfer this occurs through wind (anemophily) or water (hydrophily) and doesn't require the pollenkitt and is characteristic of gymnosperm (non-flower producing) plants like pine, some grasses and palm species, sedges, although this has also been observed with angiosperms too²⁸. Abiotically transferred pollen is produced in large quantities²⁹, as the plant expends all its energy in producing only pollen with no nectar, flower production etc to attract vectors. Also, as compared to biotic pollination, abiotic is less effective due to environmental factors (not targeted) and thus needs more material to ensure success. Thus the mode of pollination decides the structure and quantity of the pollen produced, which in turn decides their price (the more the cheaper) and degree of processing needed for encapsulation and other applications. The physico-chemical and mechanical properties are however decided by the outer layer of the sporoderm (pollen wall) namely the exine, whose formation and substructure are discussed below.

2.2 Exine

This peripheral pollen membrane provides most of the protection to the inner content of the pollen and is crucial for microencapsulation applications, where the molecular loading is dictated by the exine pores, formed by the nanochannels of the sporopollenin subunits. The exine formation and its substructure are discussed below.

2.2.1 Formation and Substructure of exine

Exine Formation

Exine is the robust, major protective outer layer of the pollen and its primary component is sporopollenin, a polymer whose formation and deposition is ordered and happens in a multi-step regulated process. As mentioned previously, the tapetal layer is responsible for providing the precursors for the exine synthesis. Glycocalyx³⁰, a polysaccharide-protein fibrillary template serves as the base for the exine features. A cellulose layer (intine) is formed beneath the callose, followed by lipoprotein complexes called probacula forming

rod-like structures over this cellulose layer. These rod-like structures merge to form the foot-layers and the tectum after which sporopollenin deposition starts on this base structure known as primexine³¹. As the tetrads separate, the primexine stretches with sporopollenin deposition still happening. Endexine starts to develop prior to tetrad breakup with the probacula arranged tangentially. Primexine and endexine develop inside the callose layer with orbicules³² from the tatpetum providing the precursors. After the callose breaks down, large-scale sporopollenin precursors deposit onto the spores. These travel directionally through strands (30-40 nm), which break down post-sporopollenin deposition and give rise to the channels found in the sporopollenin network. It is these channels that enable molecular diffusion and loading. These strands are endexine tufts, which run into the ectexine radially aiding material transfer. Tectum forms during the mid to late microspore developmental stage followed by tectal elements known as columellae, which are then formed on the tectum and this network is called the sexine and is species dependent. The endexine and the foot layer form the nexine. Structurally the exine similar to the intine is divided into the endexine, a thin layer (made up of sporopollenin precursors) and ectexine, which is sporopollenin-rich and thus provides much of the resistance. This pattern of sporopollenin formation has been observed mainly in angiosperms and somewhat similar in gymnosperms³³⁻³⁶ too although some notable differences exist. In gymnosperms, a microspore surface coat is formed instead of a primexine onto which the sexine formation happens followed by nexine deposition below the coat³⁷. The microspore surface coat is formed from the plasma membrane and has sporopollenin reception sites on which the tectum and infratectum builds on. The endexine begins to form first followed by the foot layer and the ectexine simultaneously. The ectexine forms from outside to the inside, with delayed foot layer deposition onto the endexine. Sculptured exine decoration are formed during the late microspore stages and are myriad in number. such as reticulate, striate, baculate etc. The sporopollenin makes the exine a highly desirable membrane, giving it supreme properties and its arrangement makes up the exine substructure.

Figure 2.2 Schematic representation of pollen shell substructure. The intine composed of cellulose, hemicellulose and pectin is followed by endexine layer of the exine. The foot layer lies above this and these two make up the nexine. The ectexine is composed of foot layer, and structural elements like columella and tectum. On the very top there are sculptured elements that are species-characteristic. Sexine is composed of columella, tectum and the outer sculptured elements (Adapted from Punt et al³⁸).

Substructure of exine

The exine substructure consists of sporopollenin arrangement, which follows a pattern with definite inter and intra-subunit spacing varying from species to species. With *Fagus sylvatica*³⁹, it was seen that the substructure was made up of granules that were either spheroidal or ellipsoidal, where the exine was extracted using acetolysis and further dissolved in amino-ethanol, with the structure becoming more loose with time. The granules are arranged irregularly and higher order structures involve densely packed granule clumps or ladder-like arrangements, which was observed with other species too^{40,41}. Atomic force microscopy and scanning tunneling microscopy revealed similar exine substructures for *Lycopodium* spores and pollen of *Alnus*, *Betula*, *Fagus* and *Rhododendron*, consisting of helical or spheroid structures (with diameters ranging from 10-120 nm) arranged in an overall helical fashion^{42,43}. Microchannels were shown in the tectum and the helical subunits were also spaced out by about 45 nm, thus providing potential pathways for molecular diffusion. The overall subunit structure is cylindrical with variable pitches and arrangements. With gymnosperms, the nexine is mostly lamellar, with the sexine being granular or alveolate. Granular-homogenous sporopollenin arrangement has been observed with angiosperms⁴⁴ while the arrangement is characteristic of gymnosperms⁴⁵ with multiple lamellae constituting the endexine. The lamellae itself are shown to be made up of granular structures which are about 58 Angstrom in diameter and

about 5 granule layers per lamella with the orbicules are rooted in the outer part of the wall⁴⁶. This micro-scale arrangements gives rise to the nanopores in the sporopollenin network, which facilitates molecular diffusion.

2.3 Pine Pollen

2.3.1 Structure

Pine falls under the conifers, which are gymnosperms, under the genus *Pinus* belonging to the family Pinaceae. Gymnosperms are broadly divided into Cycadales, Ginkgoales, Coniferales and Gnetales, of which conifers are the most diverse. The genus *Pinus* is divided into three subgenera: *Pinus*, *Ducampopinus* and *Strobus*; which are distinguished by cone, seed, and leaf characters. There is a wide variety of species present⁴⁷, mostly monoecious; (have male and female cones on the same tree) with some dioecious species also present.

Pine trees produce lot of pollen²⁹, which are light-weight as they have to be wind-dispersed for pollination. The pollen are saccate in nature, which is widely found with conifers⁴⁸. Wings, saccus or bladders are found only in *Phyllocladus*, *Pinus*, *Picea*, *Abies*, *Cedrus*, *Pseudolarix* and *Podocarpus* under conifers, which are usually bisaccate with trisaccate pollen also present. The size of pine pollen is about 45-75 μm in size with a tripartite structure⁴⁹, comprising of two empty air sacs (for maintaining buoyancy during wind pollination⁵⁰) and a central compartment (corpus) holding the genetic material. The size and orientation of the body and sacs vary across pine species. The air sacs are fragile in nature comprising of a thin sexine membrane, which is made up of tectum and supporting columellae. The central cavity (corpus) is more thick-walled and consists of the sexine plus the nexine (endexine +foot layer)⁴⁹ and is thus more selective. The sporoplasm consisting of the genetic material and other cytoplasmic contents fall below the intine layer.

Figure 2.3 Schematic representation of pine pollen structure. The central cavity holds the genetic material and other biomolecules, which constitute the sporoplasm. The two air-sacs have a sexine membrane, while the central cavity comprises of sexine and nexine. The intine lies below the exine.

Pine species exhibit some variability in their size⁵¹, morphology⁵², structure (air sac central cavity orientation), with some of them being widely-spaced with others closely packed. Environmental conditions impact their structure too (i.e) the air sacs shrink and come closer to the central cavity during dehydration. The spore walls in pine are formed during the tetrad, which helps in distal sacchi formation as the proximal poles are contained within the tetrad⁵³. These sacchi are wettable, but don't sink and this helps in the proper pollen orientation during pollination⁵⁴.

2.3.2 Current Uses and Future Potential

Pine pollen is a nutrient rich source (vitamins, hormone booster etc.)^{55,56} and has been considered as a super-food for a long time now, along with anti-inflammatory (contains methylsulfonylmethane) & anti-oxidant properties too⁵⁷⁻⁵⁹. It has been used in Asia, especially China and Korea for thousands of years now for its nutritional value and as a supreme source of testosterone.

Pine pollen is also cell-wall broken sometimes to enhance digestibility for greater nutrient uptake. Pine pollen nullifies xenoestrogens, which block normal hormonal functioning, leading to breast and prostate cancers and balances the androgen/estrogen ratio within the body and endocrine system. Thus pine pollen is a natural nutraceutical molecule with immense potential of microencapsulation given its size and empty air sacs. Its size⁶⁰ makes it too big for pulmonary uptake, which could be an issue with other air-borne pollen,

although nasal uptake is possible. Allergenicity is an issue for some natural pollen, but with pine it has been found to be minimal^{61,62}. Currently there are no regulations limiting the consumption of pollen^{63,64}, which makes it fit for human consumption with no safety concerns. Its pricing is also quite similar to other natural substances like gelatin, chitosan⁶⁵ being used, which makes it affordable on a large scale for industrial applications. Due to its fragile nature, rigorous processing may not be productive, but mild defatting (removing lipid content) could make it safer to work with in terms of immunogenicity^{66,67}. The empty air-sacs could be seen as naturally available microcapsules with a thin membrane protecting it, capable of rich molecular loading apart from the central cavity. The air-sacs naturally don't allow water-penetration, but water-filling has been observed with sacci treated with an organic solvent⁶⁸, indicating molecular loading possibilities. The membrane thickness of the corpus and the air-sacs along with their volumes have been estimated using models^{50,54,69}. This shows that even if the central cavity is assumed to be full of cellular contents, the air-sacs which contribute over 1/3 rd of the total pollen volume are still available for loading. Thus the potential of pine pollen as a microencapsulant is immense and remains to be tapped into.

2.4 Natural vs Processed Pollen

2.4.1 Natural Pollen

Natural pollen is pure and thus ideal to work with in terms of applications as minimal processing is needed with the particles being uniform and sturdy, but pollen does have a history of being allergic, especially those that float around in the atmosphere and are more prone for uptake. This allergy is termed as pollinosis and symptoms observed include ocular pruritus with conjunctival hyperemia, coryza, sneezing, nasal or pharyngeal-palatal pruritus etc⁷⁰. Bronchial hyper-reactivity with associated asthma may be seen in some cases, while conjunctival hyperemia and ocular pruritus are almost always present in pollinosis. Inflammation is found in the airway, with the nasal mucosa showing an increased eosinophil count (above 10%). Food hypersensitivity has also been observed in some cases⁷¹. Pollen allergy induces immunological changes in the body⁷², with increased resistance observed post-exposure. Most of these allergenic pollen comes from grasses and weeds⁷³⁻⁷⁸ like ragweed, mugwort etc., which grow in large quantities and thus produce

large amounts of pollen, increasing the likelihood of allergenicity. Trees also produce allergenic pollen which include birch, hazel and alder. These allergenic pollen have to be processed to remove the cytotoxic proteins, lipids⁶⁶ etc in order to make it fit for human consumption and related applications. Ideally the pollen kit would have to be processed away to avoid any kind of immunogenicity in case of therapeutic applications and also because of the fact that pollen removal would open up more pores creating multiple pathways for molecular penetration and loading for microencapsulation purposes. From a nutritive outlook however, natural pollen is desirable with some pollen being highly nutrient-rich composed of simple sugars⁷⁹, essential vitamins, proteins, hormones etc. An example would be bee-pollen which has the following composition: 40-60% simple sugars (fructose and glucose), 20-60% proteins, 3% minerals and vitamins, 1-32% fatty acids, and 5% diverse other components and thus is known to be a super-food^{80,81}. Pine pollen as stated above is also another nutrient-rich pollen species. Thus, it can be seen that natural pollen has its pros and cons, with the natural form being allergenic in some cases, but also highly nutritive in nature. So from a microencapsulation perspective, if the desired application for nutritive purpose, natural pollen would be the best choice.

2.4.2 Processing of Pollen

So as to circumvent the above mentioned issues with natural pollen and to make it more encapsulation-friendly, pollen is processed and this involves use of acids, bases and organic solvents mainly to remove the outer lipid layer, and the inner cytoplasmic contents. The inner intine layer is also removed so that robust microcapsules composed of exine sporopollenin, favouring loading even under extreme conditions are got. Most of the applications till date have revolved around such processed pollen namely sporopollenin exine capsules (SECs) and this has been mainly due to the superior properties of the polymer, sporopollenin which forms the major part of the pollen exine, with its structure and properties is discussed below.

Sporopollenin Structure

Sporopollenin is highly cross-linked polymer composed of carbon, hydrogen, and oxygen⁸² and has been found to be super-stable organic material⁸³, as evidenced by its intactness in

sedimentary rocks over a million years old⁸⁴. Sporopollenin has been found to be inert both in pollen^{85,86} and spores^{87,88} displaying similar characteristics, indicating its conserved properties across species. This inertness contributes to its stability where it is found to dissolve in very few solvents (2-aminoethanol, 3-aminopropanol, 2,2'2''-nitriltriethanol, and 4-methylmorpholine-N-oxide) and its insensitivity towards enzyme and most chemical reactions, excluding oxidation⁸⁹. In fact it was once described as the insoluble residue post-acetolysis due to its stable chemical nature⁹⁰. The structure of sporopollenin, which imbibes these supreme properties has been actively probed for a long time now across species, with the first explorations done in 1930s. Initially it was thought to be composed of terpenoids⁸⁷, which are known for their aromatic property and then later comprised of fatty acids-and lignin-like components^{91,92}, which is also another stable natural biopolymer. A carotenoidic⁹³ structure was also speculated with similarities observed with poly-B-carotene, which was contradicted by a following study⁹⁴. In 1980s, sporopollenin structure was proposed to contain phenols^{95,96} like coumaric and ferulic acids, which gave rise to its antioxidant properties, while solid-state NMR studies showed the presence of a core consisting of aliphatic compounds^{97,98} thus supporting the lipidic nature of the polymer. Oxygenated aromatic units were observed in sporopollenin^{95,96,99} and thus the existence of a two unique sporopollenin structures having either an aliphatic or an aromatic structure was deemed possible.^{100,101}. Soon a consensus structure with an aliphatic core crosslinked by aromatic components^{102,103} was arrived at experimentally using tracers in the 1990s, where phenylalanine was seen to be an important precursor in sporopollenin synthesis¹⁰⁴. Tracers in fact were highly dominant in probing sporopollenin building structure and included molecules like glucose, mevalonate, stearic acid, B-carotene, tyrosine etc. The presence of phenolics was disputed and the sporopollenin was considered a aliphatic lipidic polymer too around this time by Dominguez et al¹⁰⁵. In 2004, van Bergen et al. proposed a aliphatic backbone cross-linked by aromatic side chains structure (Figure 2.3)¹⁰⁶, compiling all of the research done.

Unsaturated fatty acids like oleic acid¹⁰⁷, linoleic and linolenic acids were also experimentally proved to be a part of the backbone structure, linked by ether bonds¹⁰⁵. A relatively recent study showed the presence of both saturated and unsaturated linear acids as well as phenolic compounds using FTIR¹⁰⁸ in sporopollenin. This further strengthens

the structure proposed by Bergen et al in comprising of an aliphatic backbone cross-linked with aromatic side chains.

Sporopollenin composition has also been probed from its degradation studies using fused potassium hydroxide, and in oxidizing mixtures such as hypochlorite/hydrochloric acid, potassium dichromate/sulphuric acid, hydrogen peroxide/sulphuric acid, and ozonolysis. Early studies explored potash fusion ^{91-93,109}, where hydroxybenzoic and alkanolic acids as degradation products were observed.

Figure 2.4 Proposed structure of a sporopollenin building block, where linear aliphatic carbon chains are cross-linked by aromatic oxygenated molecules linked by ether bonds (adapted from van Bergen et al. ¹⁰⁶).

Saponification and nitrobenzene oxidation revealed the presence of coumaric acid and benzaldehyde. Ozonolysis from *Lycopodium* gave out simple mono and dicarboxylic acids, while pine gave paraffins and fatty acids. Short alkyl chains were also shown to be present in sporopollenin according to recent studies ^{110,111}. All these further corroborate the results obtained from anabolic methods mentioned above as to the sporopollenin composition, but a final binding structure is still pending as such with the general consensus that sporopollenin composition is not the same amongst all organisms, but rather a related family of polymers varying to a degree.

Sporopollenin properties

Sporopollenin apart from its physical, chemical (apart from oxidation) and biological inertness^{112,113} and stability^{87,114,115}, has also been found to possess UV absorption properties¹¹⁶⁻¹¹⁹, thus making it useful for skin applications. It does have an amphiphilic nature due to the presence of aromatic as well as carboxyl and hydroxyl groups, which makes it useful for encapsulating various compounds in different solvents⁶⁵. The fact that exines have been found in sedimentary rocks millions of years old¹¹² reaffirms to their mechanical strength under high pressure, an essential property to withstand harsh processing conditions. Fragile species also do exist which have much thinner exine and intine layers and thus collapse under pressure^{95,120,121}. As for its thermal stability, sporopollenin has been found to withstand temperatures upto 250°C, but after that, it does undergo changes¹²². Biologically, sporopollenin has been found to degrade under enzyme action⁸² and its degradation in blood was also studied¹²³, which addresses the biodegradability issue. Most significantly, sporopollenin has been found to have nanochannels¹²⁴ or apertures³⁸ in its structure thus facilitating molecular diffusion and encapsulation into pollen and spores. With processing these channels are expected to open up making molecular entry easier with increased loading. Sporopollenin can also be functionalized or surface modified through various reactions^{112,125}, which increases its potential applications such as targeting and immobilization to surfaces. Due to such rich properties of sporopollenin along with the presence of permeable channels, the making of sporopollenin exine capsules for molecular encapsulation was actively pursued for a long time with various species being investigated.

Sporopollenin Exine Capsule production

Initial SEC production involved the use of chemical methods like successive treatments with hot acetone, potassium hydroxide, and phosphoric acid^{87,88}. Organic solvents like acetone, ether etc. dissolve the lipids, pollenkitt and thus open up more pores for further treatment, apart from reducing the immunogenicity of the pollen. Bases like sodium and potassium hydroxide clear out the protein and other biomolecules present in the cytoplasm, while the acid (hydrochloric, sulphuric or phosphoric) dissolves the polysaccharic intine layer leaving behind a hollow microcapsule with an intact exine. Thus sporopollenin exine

capsules resembling the natural pollen shape and a unique external architecture, but lacking the sporoplasm, lipidic coat and intine with a far more porous structure is produced. Intine processing had to be optimized by varying acids and their concentration as to maximize removal and avoid contamination^{91,92,112,126}, where some species were found to collapse with the above sequential treatment^{91,92}. One-pot methods were looked into to make the processing easier and less time-consuming with methods like acetolysis^{121,127} treatment with hydrochloric acid^{128,129}, hydrofluoric acid¹³⁰, and combinations of aqueous 4-methylmorpholine-*N*-oxide and sucrose^{98,107} explored. Acidic processing was able to cleanse and make the capsules nitrogen-free but not always intine-free and also induced some structural changes as did acetolysis. Physical methods like ultrasonication, grinding, autoclaving¹³¹ were also tried out and resulted in fragmented exines and an intact protoplast and were then mostly used as pre-step for further SEC production.^{95,99} As mentioned earlier some pollen and spore species (*Fraxinus excelsior* L. (ash), *Populus nigra* L. etc.) were unable to withstand the chemical processing. Saccate pollen too fall under this category because of their fragile nature. For such pollen, milder SEC extraction techniques were designed using enzymes, where a mixture of enzyme consisting of cellulose, pectinase, lipases etc. was chosen as to degrade/digest individual components of the pollen grain^{95,99,132}. The most explored species for SEC production has been *Lyopodium Clavatum*, which has been used widely¹³³⁻¹³⁵, due to its low cost and superior robustness²⁴. Most of the lycopodium SEC production techniques associated were long and rigorous running upto days¹³⁶⁻¹³⁸. That was optimized with the processing time brought down to 30 hours using phosphoric acid¹³⁹. A similar optimization was done to produce sunflower SECs too¹⁴⁰. Most recent species explored for SEC production using acid or/and base processing are *Zea Mays*¹⁴¹, *Phoenix Dactylifera*¹⁴², *Corylus Avellana*¹⁴³, *Platanus Orientalis*¹⁴⁴ and *Betula Pendula*¹⁴⁵. More recently a green approach to produce SECs was achieved using ionic liquids¹⁴⁶. *Populus deltoides* pollen grains were treated with ionic liquids like tetra n-butylphosphonium hydroxide, 1-butyl-3-methylimidazolium chloride, 1,8-diazabicycloundec-7-eneninium hydrogen sulphate for a short time at high temperatures.

Pine SECs have been produced, initially by a pure enzymatic treatment⁹⁵ given their fragile structure and then by the use of organic solvents, acids followed by

enzymes^{49,147}. All the SEC production techniques have quantified the nitrogen content but almost none of them have ever quantified the SEC morphology post-processing. This is significant as the chemicals used for processing have been reported to change the sporopollenin polymer and thus this could induce breakage, collapse etc. Corn pollen (thin-walled) SECs¹⁴¹ were morphology characterized where collapse was observed with short-term acid treatment. Even lycopodium SECs, which are relatively thick-walled, were seen to be fragmented during acid-hydrolysis¹³⁹, thus necessitating the need for structural inspection. This morphological assessment becomes even more critical when it comes to saccate or wind-pollinated pollen, which have much thinner walls and no such study has been reported till date for pine pollen.

Figure 2.5 Mild and harsh SEC extraction methods used on pollen and spores (Adapted from Barrier²⁴)

2.5 Microencapsulation using natural pollen, spores and SECs

Natural pollen, spores and their associated SECs produced from various plant species using the above methods are potent microcapsules in terms of size, size uniformity and spatial requirements (hollow) and molecules have been loaded into these SECs. Their varied sizes and morphology open up multiple options tailored for specific usage. Pollen shape and size uniformity was so sought after that inorganic capsules were produced using pollen as

templates^{148,149} but these were not defect free and so not promising enough. The irregular rough surfaces of these SECs also imbibe the property of mucoadhesiveness, where they bind to tissue, which increases the retention time and aids in specific cell-targeted release. The outer sculptured surface of the exine also avoid helps to avoid particle aggregation, especially if the surface is spiky whereby the interactive surface area is minimal. The sporopollenin has natural nanochannels and micro-sized apertures as mentioned earlier and these are expected to become more porous with chemical treatment, thus facilitating loading, which is discussed below.

2.5.1 Loading methods

Many molecules such as proteins, nucleic acids, dyes, drugs and bioactives have been loaded into natural pollen, spores and SECs²⁴ and the loading methods used for encapsulation mainly include simple passive, vacuum-assisted, centrifugation and compression loading.

Passive Loading

Simple passive loading involves mixing of the pollen/spore along with the encapsulate in solution for a fixed period of time, where the molecule loads through the available pores mostly through solvent diffusion, followed by mild centrifugation, dialysis or solvent evaporation to remove the unloaded molecule⁶⁵. Pine SECs were passively loaded with dyes, sugars and it was found that the air-sacs could load molecules upto 200 nm while the central cavity had a limit of 4 nm⁴⁹. This is mainly used for small molecules and solutions of low viscosity like waxes^{65,150} and has been shown to display lower loading as compared to other methods like vacuum and compression loading.

Vacuum Loading

Vacuum-assisted loading^{65,128}, involves use of low vacuum to force molecules into the hollow space through suction-induced low pressure, which as opposed to passive loading is a more active process. Since an external driving force is present here, the loading is expected to be relatively higher and this has been observed with some natural pollen species in case of protein^{151,152} and drug loading¹⁵³ as compared to passive, where

encapsulation is restricted to size, orientation and charge effects. Lycopodium SECs have shown good loading through vacuum-assisted loading for various molecules like dyes, proteins¹³⁹, oils, enzymes and resin⁶⁵. Vacuum loading was visually seen to load better than passive loading using FITC-BSA with sunflower SECs¹⁵⁴ and the mechanism of vacuum loading was probed here, where it was seen that air bubbles in the SECs shrink with time forcing solution inside to aid loading. Vacuum loading is also used in case of viscous solutions where an external force helps in better solution flow and has been shown with oil loading^{136,155,156}.

Compression Loading

Compression loading takes advantage of the elasticity of the exine¹⁵⁷, which is one more factor why the inorganic capsules, made from pollen templates were not quite as successful. These SECs can be compressed into tablets and dropped into the encapsulate solution whereupon they swell up with the pores reopening allowing loading. Pollen tablets have been made with pine and bee pollen and consumed for health purposes and thus compression loading seems practical. Compression loading was tried with natural pollen and was found to load slightly better as compared to passive loading but lower than vacuum loading technique. The pollen were found to be structurally stable with no damage observed due to compression^{151,152}. Later, sunflower SECs were tableted by compression and imaged through SEM, which showed close packing of the SECS with no visible damage¹⁵⁴. One of the most important applications achieved through compression loading was encapsulation of individual cells into SECs, where yeast cells entered lycopodium SECs through pores opened up during compression¹⁵⁸. Encapsulation of multiple molecules was also probed using compression loading method, where the SEC was used a reactor in which the molecules were loaded sequentially and reacted to form a product¹⁵⁹. This affirms the use of SECs as an interaction medium.

Centrifugal Loading

Centrifugation based filling is employed for highly viscous compounds where high speeds result in better flow of the solvent⁶⁵ and could be an alternative for vacuum loading in the

absence of vacuum pumps. The centrifugal speeds have to be optimized so that the pollen doesn't aggregate and settle down, but is able to load the encapsulate.

Figure 2.6 Schematic of loading techniques used for pollen and spore loading shown here with natural *L. clavatum* spores. (A) An extracted lycopodium spore (B) Lycopodium spore suspended in a solution of encapsulate, which loads through the nanochannels (C) Natural spores encapsulating the molecules (green) (D) Passive loading technique with natural spores and the encapsulate both in solution and mixed (E) Compression loading with the spores pressurised to a tablet form, which uptakes the encapsulate once in solution (increased pore size) (F) Vacuum loading technique involving the use of vacuum to a mixed suspension containing natural spores and macromolecules (Adapted from Mundargi et al ¹⁶⁰)

2.5.2 Loading, Release and Activity retention of encapsulated molecules using pollen and spores

Molecular Loading & other applications

Encapsulation using natural pollen and spores has been explored rarely as compared to SECs and involved protein^{52,140,160} and drug loading¹⁴⁰, where various loading methods as stated above were used. SECs have been used widely for microencapsulation, with *Lycopodium* being the most widely used species as stated earlier and some of them are

listed below. Oil loading was explored with *Lycopodium* SECs, where the encapsulated cod liver oil was tested for its freshness using peroxide value and was found to be protected from oxidation⁶⁵. Enhanced fish oil eicosapentanoic acid (EPA) bioavailability was observed when *Lycopodium* SECs were used to encapsulate EPA, with the taste also being masked here¹⁵⁵. Another taste masking study involved use of *lycopodium* SEC encapsulation of cod liver and sunflower oil with human volunteers¹⁵⁶. High oil loading was observed in all the above cases, where the SECs still maintained their powdered form. Various functional dyes like Evans blue, Nile red, Rhodamine etc have been encapsulated into SECs^{49,65,161} and these could be used for purposes like diagnosis, E-Ink¹⁶¹ etc. Magnetic SECs have also been prepared with iron oxide nanoparticles loaded into SECs¹⁶¹. A closely related application would be use that of Gadolinium loaded SECs, which were used as MRI contrast agents¹⁶². Ovalbumin was loaded into *Lycopodium* SECs and this was used for oral vaccination, where enhanced immune response was observed as compared to free antigen¹³⁸. Most interestingly, the SECs were shown to cross the intestinal barrier here as seen using fluorescence. A similar study was also done using ragweed¹⁶³, where pollen interaction with intestinal epithelial cells and uptake by macrophages was observed, with “persorption” indicated as the mechanism of pollen penetration into the intestine. Pollen have been shown to be capable of absorption through the GI mucosal barrier and have been found in bloodstreams^{164,165}, where they were shown to degrade^{162,165}. The various sizes of available pollen would be really useful here with larger ones (>100 μm) used for topical or oral delivery applications or as drug delivery system to the GI tract, while the smaller-sized species (*Lycopodium Clavatum* 25 μm) can be used for systemic delivery. *Lycopodium* SECs were used to encapsulate whole yeast cells as mentioned previously and have also been used for drug delivery applications, where ibuprofen was encapsulated¹³⁷. Rape pollen shell was shown to adsorb BSA¹⁶⁶ and date palm SECs were also shown to encapsulate paracetamol, ibuprofen^{142,167}. Other species used for drug delivery applications include *Corylus Avellana*¹⁴³, *Platanus Orientalis*¹⁴⁴ and *Betula Pendula*¹⁴⁵. Sequential multiple molecular loading has also been done with SECs with *Lycopodium* SECs being used as microreactors for preparation of organic and inorganic nanomaterials¹⁵⁹. SECs have been shown to bind to heavy metals and thus can play a role in metal remediation^{168,169}. Sporopollenin has also been derivatised (i.e.)

thiolation, amination etc. and been used as a solid-support for peptide synthesis, enzyme immobilisation¹⁷⁰ as an ion-exchange resin, catalyst^{112,171,172} too, apart from microencapsulation.

Figure 2.7 Confocal Laser Scanning Microscopy images of Lycodium SECs encapsulated with (A) Fish oil containing lycopene (B) Malachite Green (C) Evans Blue (D) Nile Red (E) Evans Blue Evans Blue stained alpha amylase (F) LR white resin encapsulated TEM section (Adapted from Barrier et al¹¹⁹)

Tunable release profiles

Post-loading, the compounds have to be released in a specific environment at a desirable rate and release from these microcapsules happens in a suitable solvent characteristic of the loaded molecule; hydrophilic or lipophilic. Specific spatial release is necessary to avoid side-effects, and to protect acid-sensitive molecules in case of oral delivery, while temporally tunable release is necessary to keep molecular concentrations in the therapeutic window range, which is more crucial as concerning drug delivery. For SEC drug delivery applications, ibuprofen was shown to be encapsulated into Lycodium SECs, where drug remained encapsulated in the simulated gastric fluid and released in the simulated intestinal

fluid¹³⁷. The rape pollen shell used for BSA adsorption released it in a pH dependent manner¹⁶⁶. Date palm SECs encapsulated paracetamol, ibuprofen and released them in a controlled manner using coatings^{142,167}. With drug delivery applications using *Corylus Avellana*¹⁴³, *Platanus Orientalis*¹⁴⁴ and *Betula Pendula*¹⁴⁵, the release was seen to be relatively controlled as compared to the free drug, but still significant release was observed in HCl(gastric phase). Protein (BSA) release was found to be rapid with natural sunflower and lycopodium while coating with alginate salt was seen to modulate the release more gradually with increase in alginate concentration^{151,152}. In case of sunflower SECs, tableting was shown to retard BSA release as compared to a powder form, while coating the tablet with Eudragit gave a near perfect release profile¹⁵⁴. Use of coencapsulants like waxes, cocoa butter has also been used to control release and has been successful, where the encapsulate is loaded first followed by the co-encapsulant¹⁵⁰, which blocks the diffusive route of the encapsulates and retards its release.

Figure 2.8 Controlled Released from tableted sunflower SECs using Eudragit coating, with uncoated tablets and BSA tablets without SECs (Eudragit as the matrix of release) as controls(Adapted from Michael et al¹⁵⁴).

Activity of encapsulates post-release

For active molecule loading, it is vital to show activity retention post-loading, lacking which the loaded molecules would be ineffective, since the process of loading could have induced structural changes (in case of proteins) or chemical modifications. This is problematic especially with proteins, where exposure to organic solvent or hydrophobic interactions with the encapsulating polymer, may denature it. Enzymes namely horseradish peroxidase and alkaline phosphatase that were encapsulated in lycopodium SECs were tested for their post-release activity using 3,3',5,5' tetramethylbenzidine and 9H-(1,3-dichloro-9,9-dimethylacridin-2-one)-phosphate as substrates and were shown to retain their activity with insignificant loss⁶⁵. Yeast cells that were encapsulated were shown to be viable post-encapsulation¹⁵⁸ and this opens up potential applications of probiotics, studying cell-cell interactions in a controlled environment etc. Thus it can be seen that the encapsulates retain their function post-release, which is crucial and this expands the potential list of molecules that could be encapsulated using pollen/spores. The table below summarises the different pollen and spore species used for microencapsulation purposes till date.

Table 2.1. Pollen/Spore Species used for microencapsulation

Pollen/Spore Species	Natural/Processed	Application	Reference
Lycopodium	Natural	Protein encapsulation and controlled delivery	151
		Controlled Drug Delivery (5 fluorouracil)	153
Lycopodium	Sporopollenin Exine Capsule	Oil Sequestration	136
		Ibuprofen Delivery and taste Masking	137
		Oral Vaccination	138
		MRI Contrast Agent Delivery	162
		Oil Loading & Taste Masking	156
		Sporopollenin Microreactors	159
		Microencapsulant and Antioxidant for oil, Enzyme Encapsulation	65
		Enhanced Bioavailability of Fish Oil	155
		Protein Encapsulation	139
		Live Cell Encapsulation	158
Sunflower	Natural	Protein encapsulation and controlled delivery	152

Sunflower	Sporopollenin Exine Capsule	Controlled Protein Release	154
Lycopodium clavatum and Ambrosia trifida	Sporopollenin Exine Capsule	UV & Visible Light Screening	119
Platanus orientalis	Sporopollenin Exine Capsule	Controlled Drug Delivery (5 fluorouracil)	144
Corylus avellana	Sporopollenin Exine Capsule	Drug Delivery (Pantoprazole)	143
Betula pendula	Sporopollenin Exine Capsule	Drug Delivery (imatinibmesylate)	145
Phoenix dactylifera L.)	Sporopollenin Exine Capsule	Drug Delivery (ibuprofen)	167
Rape	Sporopollenin Exine Capsule	Protein Adsorption and pH-dependent release	166
Date palm (Phoenix dactylifera L.)	Sporopollenin Exine Capsule	Controlled Drug Delivery (Paracetamol)	142
Pine	Sporopollenin Exine Capsule	Encapsulation of dyes, polymers	49

2.6 Biocompatibility of processed spores

Biological applications entail the encapsulant to be biocompatible and biodegradable as to avoid toxicity issues. Pollen generally as such has been classified as safe for consumption, but its processed forms have to be tested individually since the processing involves the use of multiple chemicals and organic solvents. Lycopodium SECs, which have been commonly used were tested for their cell compatibility with endothelial cell line and were found to be biocompatible within the working concentration range¹³⁷.

Thus pollen, spores and their associated SECs have been explored for their potential as a microencapsulant and have been promising. We will have a comparative look at how pollen matches up with conventionally used microencapsulants with the focus on PLGA microparticles mainly, which have been largely used for molecular encapsulation and delivery, due to their biocompatible and biodegradable nature.

2.7 Comparison of pollen/spores with other commonly used micro/nanoparticles

Microencapsulants like PLGA, PLA, PGA microparticles, liposomes and chitosan have been widely explored given their bio-friendly nature¹⁷³⁻¹⁷⁶ and have shown good loading and release profiles. As mentioned in the Introduction, making microparticles from the polymers is a arduous process itself apart from polymer synthesis, which is the initial step. However, let us compare the properties, loading and release of some of these particles, which can be looked at as standards in the field of microencapsulation.

2.7.1 Material Preparation/Isolation and Scale-Up

Pollen isolation is a simple and straightforward process, which is collected from trees or from hives (bee pollen) followed by purification^{177,178}, whilst the polymer synthesis and formation of microspheres/capsules is a multi-step process involving numerous chemicals, that can be complex and demanding¹⁷⁹⁻¹⁸¹. As for material availability and synthesis scale-up, synthetic microspheres can be made anytime, subject to availability of starting material and reagents, but scale-up could result in non-uniform particles with varied morphologies and properties. In case of pollen, production and viability depends on the species and environmental factors involved, with pollen collection being seasonal¹⁸²⁻¹⁸⁴ 185. However since the pollen amount produced is massive (with wind-pollinated species mostly (i.e) pine

pollen) and with proper storage, this pollen can be stored and used throughout the year with no scale-up procedures involved. Pollen too exhibit variability in size to an extent, but morphology and other physico-chemical properties are more or less uniform amongst particles.

2.7.2 Loading

Compounds have been loaded into synthetic microparticles using various loading techniques like solvent based single and double emulsions (oil in water etc), spray-drying, in-situ formation, melting and salting out technique etc., where the drug¹⁸⁶⁻¹⁸⁸ or encapsulate is mostly encapsulated during the particle formation process, while loading with pollen has been limited to fewer techniques (as mentioned in section 2.5). Hydrophobic drug loading has been easier as compared to hydrophilic/amphiphilic molecular loading due to solubility issues, with strategies developed to improve this loading and thus opening up more options and related applications using PLGA microparticles. Thus molecular loading using synthetic particles exhibit high loading efficiencies¹⁸⁶. In fact even dual molecular loading was accomplished with PLGA nanoparticle¹⁸⁹, with both hydrophilic and hydrophobic molecules loaded in a single-step process which opens up dual drug delivery applications, while multiple sequential molecular loading has been shown with SECs¹⁵⁹. Microencapsulation using pollen is relatively more recent and has shown good loading¹⁵², which could further be improved with more optimization. PLGA microparticles have been used for loading for variety of molecules like therapeutic peptides^{186,190}, bacterial membrane proteins¹⁹¹ for vaccination, curcumin¹⁹², hormones¹⁹³ apart from synthetic drugs like vancomycin, phenobarbital¹⁸⁷, cyclosporin, indomethacin etc., with pollen and spores have also been looked at for encapsulation of multiple molecules like drugs¹⁵³, dyes⁶⁵, oils¹⁵⁵ and proteins¹⁵².

2.7.3 Release

The significance of controlled release has been mentioned in **Section 2.5.2** and PLGA could be looked at as the gold standard for this, with sustained release profiles produced due to cleavage of the polyester backbone over time, keeping the molecular concentrations stable. Compounds controllably released include hormones¹⁹⁴, small molecule drugs¹⁹⁵,

peptides, and proteins, cytokines¹⁹⁶, chemotherapeutics¹⁹⁷, antibiotics, insulin etc where release occurred over a number of days. Natural and processed pollen on the other hand are much leakier and require coatings or co-encapsulants to control their release¹⁵², given their relatively larger pore size, which has also been observed with porous PLGA microparticles too¹⁹⁸. As long as the excipient (coating, binder etc.) used for release is a natural substance that is biocompatible and biodegradable, this shouldn't limit the use of pollen for controlled release systems.

2.7.4 Molecular activity post-release

Moving onto another important aspect of molecular microencapsulation, which is activity retention of the encapsulate which can be measured quantitatively or observed biologically like cell death or tumour volume reduction in case of cancer therapy. In-vivo testing of loaded-PLGA microparticles was observed to induce the desired effects such as lowering of sugar levels with insulin, weight increase with growth hormone¹⁹⁰, which shows that the functionality of the peptide is retained post-encapsulation and release, as was shown with pollen/ spore SECs too⁶⁵ with enzymes.

2.7.5 Storage

Shelf-life is another factor that determines the choice of encapsulant. Ideally the carrier and the formulation (carrier+ drug) should be storable at room temperature conditions with no special storage requirements. For synthetic particles, this is a problem, where storage affects the physical and chemical state with particles undergoing aggregation, oxidation, dissolution, degradation etc depending on size, shape, charge and chemical composition^{199,200}. With silver nanoparticles, the above mentioned changes were seen to increase the toxicity of the nanoparticles with aged particles being more toxic than freshly synthesized ones²⁰⁰. Temperature is another crucial storage parameter, where most polymeric particles are stored at 4⁰ C. Higher temperatures were found to degrade the polymers rapidly²⁰¹. pH and type of polymer²⁰² too are vital for particle stability and have to be optimized too. Lyophilisation has been used to improve the long-term stability of the particles given their solution-storage issues²⁰³, which result in dry powders. Cryoprotectants (eg: Trehalose. Sucrose etc.) have to be used during the process for

minimizing structural damage with freezing and drying conditions optimized. Drug loaded formulations with excess free drug result in aggregated and unstable formulations²⁰⁴ and so formulations have to be cleansed well to avoid this. Pollen, being natural is much less stringent as concerning storage with dry pine pollen having almost infinite shelf-life if stored in a cool, dry place. Even if opened, it would be good for a year for consumption when stored under dry conditions. Morphologically pine pollen retains its overall shape with slight variations in its air-sac orientation depending on the atmospheric moisture content.

2.7.6 Shell Structure

Capsules, while presenting more loading space are also more prone to rupture as compared to solid microspheres due to the thin shell present and thus more care has to be taken in case of synthetic micro/nanocapsules²⁰⁵, but with pine pollen due to the presence of sporopollenin, the SECs prepared are relatively more rigid, with the air-sac membrane thickness being the limiting factor. As compared to plant cells (which have lignin at a certain percentage), pollen wall is much more stable due to the presence of sporopollenin (major component), which makes it a more favourable plant-based microencapsulant.

So on the whole, pollen matches conventionally used microencapsulants for molecular loading and release, with room for loading optimization. This is really promising for future exploration of pollen as a diverse natural microencapsulant with immense potential. Given the wide range of available options, the bio-friendly nature of both natural and processed pollen, this pushes us to explore more species for their applicability in this wide and diverse field.

2.8 Rationale of the proposed work based on literature

It can be seen that pollen and spores can be used for microencapsulation applications both in their natural and processed forms, with the latter being favoured more for encapsulation applications due to lower immunogenicity, a more hollow cavity and porous exine structure enabling rapid and greater loading as compared to natural species. This also depends on the application per se with nutraceutical-based delivery systems more likely to use natural pollen because of its rich nutrient values^{81,206,207}, and for medical purposes, the

processed forms, to reduce undesirable pollen-leucocyte interaction and the following allergenic reactions involved.^{70,208}

Here we propose working with pine pollen, which as stated above is highly nutritional as to be considered a superfood and has been shown to have minimal allergenicity. It has been shown to have therapeutic properties such as anti-oxidative, anti-inflammatory too making it all the more-valuable. Pine pollen being bisaccate falls under the category of thin-walled pollen, which are more fragile and thus are not processing-friendly as explained earlier. We start off by looking at SECs first, with pine SECs production through acidolysis using defatted pollen as source, where the SEC structure was analysed for damage with every condition, followed by comparative protein loading amongst the SECs and defatted pine pollen. Saccate pollen has never been morphologically characterised and quantified for its processed structure, with only basic loading performed on pine SECs till date and thus this study covers that research gap, which is important for thin-walled pollen. Most if not all of the encapsulation work done using pollen and spores have focused on single-compartmental pollen with *Lycopodium* most explored. As mentioned previously, pine pollen has empty air-sacs, which constitute a significant volume of the pine pollen particle. So, this naturally available space can be used for molecular loading, which makes processing of pine pollen unnecessary, thereby skipping multiple steps, which makes it a better option as even compared to plant cells and mono-compartmental pollen/spores which are relatively full of biomolecules. We can thus use the inherent nutritive properties of pine pollen in addition to the molecules we can potentially load into the air-sacs. Thus we then move onto microencapsulation using natural pine pollen, where we optimize the BSA loading conditions with natural pine pollen and extend this to defatted pine pollen to compare the relative protein loading. Controlled release was also shown from BSA-loaded defatted pine pollen using coating and binders of natural origin on tablets. Finally as to expand the loading potential of natural pine, we looked at standard passive and vacuum loading of therapeutic proteins (BSA & IgG, which were FITC tagged), hydrophilic and hydrophobic dyes (Nile Red, Calcein) and an anti-cancer drug doxorubicin, which are all fluorescent into natural pine pollen as to look at potential molecules that could be encapsulated. This unimolecular loading was then extended to dual molecular loading where Nile Red/Doxorubicin and the protein (BSA) were loaded into natural pine pollen

sequentially. Thus pine pollen was subject to decreased processing with every study with its morphological, chemical and loading properties carefully assessed and optimized.

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Chapter 3 Pine pollen sporopollenin exine capsules (SECs) *

Sporopollenin exine capsules are favoured for microencapsulation applications, due to their low immunogenicity and higher spatial volume available. The SEC production process has been streamlined over time with mostly uni-compartmental pollen explored for process optimization, with pine SECs produced mostly through enzymatic and multi-step processing, with no structural analysis done post-processing. This chapter deals with the bisaccate pine SEC production through acidolysis with morphological characterisation for structural damage assessment along with sporoplasmic removal for every processing condition involved. The SECs from the optimized processing condition were also shown to be capable of protein encapsulation, which points to their practical usability.

* This chapter was published substantially as: (1) Arun Kumar Prabhakar, Hui Ying Lai, Michael G. Potroz, Michael K. Corliss, Jae Hyeon Park, Raghavendra C. Mundargi, Daeho Cho, Sa-Ik Bang, Nam-Joon Cho, “Chemical processing strategies to obtain sporopollenin exine capsules from multi-compartmental pine pollen” J. Ind. Eng. Chem. (2017), <http://dx.doi.org/10.1016/j.jiec.2017.05.009>

3.1 Introduction

There is broad interest in utilizing natural resources to develop functional materials for a wide range of applications in chemistry and materials science¹⁻⁴. Given their diverse range of highly controlled sizes and structures that vary according to the plant species, pollen grains as well as related spores are a promising example of a natural resource that offers many robust options for microencapsulation strategies⁵⁻⁷. They possess a large cavity surrounded by an inner structural support layer (the intine) that is composed of cellulose, hemicellulose, and pectin, which is itself surrounded by a rigid outer coating (the exine) that is composed mainly of a biopolymer, sporopollenin⁸⁻¹⁰. As a key structural component that supports pollen grains' natural function to protect genetic material, sporopollenin possesses high physical and chemical resistance, ultraviolet shielding, and antioxidant capability¹⁰⁻¹⁴. Owing to these features, one of the most promising directions to utilize pollen grains for microencapsulation involves the chemical processing of pollen grains to yield sporopollenin exine capsules (SECs), which faithfully preserve the architectural features of the original grains while lacking sporoplasmic and intine contents¹⁵⁻²⁵. The resulting SECs are largely devoid of protein, making them less allergenic and offering more volume for molecular encapsulation²⁶. Combined with high natural abundance and renewable production, these advantageous properties make SECs an ideal delivery vehicle for encapsulating therapeutic drugs, nutrients, and microorganisms as well as for other material science applications²⁶⁻³⁶.

Pollen processing has mainly focused on single-compartment pollen species, typically ones which function *via* biotic pollination and hence possess thicker exine walls^{37,38}. Early attempts to prepare SECs involved sequential treatments of organic solvents, alkali, and acid in order to remove lipids, proteins, and intine layers³⁹⁻⁴². One-pot methods aimed at streamlining the process were later introduced and included individual treatments with hydrochloric acid^{15,16}, acetic acid^{18,19}, hydrofluoric acid¹⁷, and combinations of aqueous 4-methylmorpholine-*N*-oxide and sucrose^{24,25}. Multi-compartmental pollens, however, including saccate pollens that travel by wind dispersal, are generally more fragile due to thinner exine walls and often rupture during harsh chemical treatments. Indeed, these challenges have proven a common theme among thin-walled pollen, and have motivated the search for improved processing strategies⁴³. As a result, milder processing methods

such as purely enzymatic treatments have been developed^{41,44}. However, enzymatic processing is not practically feasible at larger scales, because the necessary enzymes are costly and required in relatively high concentrations. Developing robust chemical methods to prepare SECs from multi-compartmental pollen grains would increase the general utility of pollen SECs, especially since these grains are among the most abundant in nature³⁸.

In this regard, pine (*Pine taeda*) pollen is noteworthy because it has been widely used in traditional Chinese medicine for thousands of years⁴⁵ and its structure has been rigorously studied in the biological sciences⁴⁶⁻⁴⁹. Pine pollen has two lightweight, empty air sacs attached to its sporoplastic central cavity that aid in wind pollination and the compartments collectively form a micron-scale size (45-75 μm) tripartite structure^{50,51}. These relatively fragile sacchi possess higher permeability on account of porous features that are nearly two orders of magnitude larger than those of the central cavity (200 nm vs 4 nm)⁵², and understanding how these different permeability barriers might be useful for microencapsulation remains to be explored^{5,53,54}. Indeed, while the membrane permeability properties of isolated pine pollen exines have been investigated in the context of fundamental pollen biology^{44,51,52}, there have been no attempts to develop focused strategies for preparing pine pollen SECs. Addressing this gap would significantly advance efforts to achieve scalable chemical processing methods for obtaining high-quality pine pollen SECs as well as to explore the feasibility of loading the SECs with macromolecules for microencapsulation applications. Indeed, while pine pollen has been shown to have relatively low allergenicity (see, *e.g.*, its low skin sensitivity^{55,56} and steric limitations for pulmonary uptake⁵⁷, SEC development could further improve this species' immunogenic profile⁵⁸ as well as increase the scope of applications based on enhanced loading of selected compounds into hollow cavities.

This chapter explores Pine SEC with relatively more rigorous processing involved. Herein, the first systematic evaluation of one-pot acidolysis protocols for pine pollen SEC extraction was done with comprehensive characterization of pine pollen SEC morphology and encapsulation efficiency. Processing times, storage methods, temperatures, and acid type were varied in order to achieve maximum removal of potentially allergenic protein components while preserving microcapsule architecture. In a selected case, additional enzymatic treatment with trypsin was used to further demonstrate the potential of fully

optimizing sporoplasmic removal. Finally the SEC from the most optimized conditions was tested for Bovine Serum Albumin (protein) loading against defatted pollen.

3.2 Experimental Section

3.2.1 Materials

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

3.2.2 Extraction of pine sporopollenin exine capsules (SECs)

Acidolysis Processing

Defatted pine pollen (2 g) was placed into a round-bottomed perfluoroalkoxy (PFA) flask fitted with a glass condenser and refluxed (70 °C) in aqueous 85 % phosphoric acid solution (15 mL) at a stirring rate of 200 rpm for one hour. Samples were collected by vacuum filtration and washed sequentially with warm distilled water (150 mL x 5), acetone (100 mL x 1), 2 M HCl (100 mL x 1), distilled water (100 mL x 5), acetone (100 mL x 1), and ethanol (100 mL x 2). Washed microcapsules were dried in an oven at 60 °C for 6 h, followed by storage at room temperature as a dry powder. This protocol was repeated for different batches of the same pollen, using different reflux durations (1 h, 2.5 h, 5 h, 10 h, and 20 h) and samples were collected for analytical characterization. The reflux duration that demonstrated the highest yield of intact pollen microcapsules was re-tested using one or more of the following adjustments: wet storage, as a suspension of SECs in distilled water (kept at room temperature) without oven-drying; lowered reflux temperatures (50 °C and 25 °C); and different acid reagents (hydrochloric and sulphuric acids).

Enzymatic Treatment with Trypsin

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

3.2.3 Pine sporopollenin exine capsules characterization*Micromeritic Evaluation by Dynamic Imaging Particle Analysis (DIPA)*

DIPA is an analytical technique where physical particle parameters like diameter, roughness, circularity, aspect ratio etc. and particle numbers are calculated from a particle population as they pass through a flow cell and images are captured by a camera. The flow cell and the lens have to be matched to get good images. Single particle images are stored and the data is plotted to give histograms, which can be compared across particle sets. Particle morphology can also be determined visually using DIPA where broken or fragmented particles can be distinguished from intact ones. Parameters that can be optimized include particle concentration, flow rates and camera settings as to avoid aggregates, highly rapid flow; which makes imaging ineffective and low quality images resulting from poor lighting. DIPA was performed using a FlowCam® benchtop system (FlowCamVS, Fluid Imaging Technologies, Maine, USA) equipped with a 200 µm width flow cell (FC-200) and 20× magnification lens (Olympus®, Japan). The flow cell was visually inspected and cleansed with ethanol prior to each sample run. Polystyrene microspheres (50 ± 1 µm diameter) served to calibrate the microscope focus, and a representative histogram was plotted to verify the proper operation under the defined measurement settings. Defatted pine pollen and Pine SECs (at 2 mg/mL concentration) were sonicated in a water bath for 10 minutes and filtered through 100 µm diameter filter meshes prior to experiment to remove aggregates. The samples were then manually added into the flow cell *via* a pump-controlled syringe and analysed at a flow rate of 0.1 mL/min.

10,000 particles were scanned out of which 300 well-focussed particles were chosen from every run (n=3) and their morphology was assessed.

Surface Morphology Evaluation by Scanning Electron Microscopy (SEM)

SEM works on the principle of an electron beam interacting with the sample and producing secondary electrons, that are basically signals detected by the electron detector, which converts it into images based on the laser beam. Samples are coated with a layer of conducting material like gold before imaging. It is a high resolution technique, used to see the surface details of the sample, which has to be preferably in a dry state to avoid charging effects, although techniques like environmental SEM do exist, which image samples in their native state. Imaging was performed using a JSM 5410 (JEOL, Tokyo, Japan). Samples were sputter-coated with a 10 nm-thick gold film using a JFC-1600 instrument (JEOL, Tokyo, Japan) at 20 mA for 60s. Images were captured at an accelerating voltage of 5 kV at different magnifications and both interior (cross-sectional) and exterior morphological changes of defatted pine pollen and SECs were observed.

Elemental CHN Analysis

Elemental Analysis is used to analyse the elemental composition of a sample and so can be used to assess its purity. CHN looks at specific elements namely: Carbon, hydrogen and nitrogen. It is done by combusting samples at very high temperatures in the presence of oxygen resulting in the formation of gases like carbon dioxide, water, and nitric oxide, which are passed through a thermal conductivity detector. Here thermal conductivity changes with a reference gas are detected and signals corresponding to weight % are produced. Since sporopollenin as proposed is nitrogen free, the nitrogen present is entirely due to the sporoplasm composed of nucleic acids, proteins and other biomolecules, while carbon and hydrogen% correspond to the whole pollen particle. A VarioEL III elemental analyser (Elementar, Hanau, Germany) provided CHN analysis to determine the amount of residual protein. All samples were dried at 60 °C for 1 h before being combusted in excess oxygen at high temperature to release compositional carbon, hydrogen, and nitrogen.

3.2.4 Encapsulation of Bovine Serum Albumin (BSA) and Loading Efficiency estimation

Vacuum Loading

BSA protein loading was achieved by using vacuum loading protocols^{59,60}. 50 mg of unprocessed pollen and select batches of dry-stored processed SECs (85% phosphoric acid for 5 h at 70 °C, and 18% hydrochloric acid for 5 h at 70 °C, with additional 24 h trypsin treatment) were suspended in 0.5 mL of 50 mg/mL aqueous BSA solution within polypropylene tubes and mixed *via* vortexing (IKA, Staufen, Germany) for 1 min. The mixture was then subjected to vacuum treatment at 0.006 mbar for 2 h). The tubes were collected and the loaded particles were washed by centrifugation with 1 mL of water at 12,000 rpm for 3 min and then freeze-dried overnight. Blank batches of untreated pollen and SECs were prepared similarly without BSA loading. In addition, FITC-BSA was encapsulated in the same way and imaged *via* CLSM in order to observe the molecular localization of loaded components within SEC particles.

UV/Vis Spectrophotometry

Ultraviolet-visible spectrophotometry is used to analyse and quantify samples based on their absorbance pattern and peak absorbance. Molecules absorb photons from the spectra and move to their excited state from the ground state by energy transfer. The technique works on the principle of the Beer-Lambert law, where the absorbance of a solution is directly proportional to the concentration of the absorbing sample in the solution at a fixed path length. Typically a quartz cuvette of 1 cm length is used for such measurements

The spectrophotometer measures and compares the intensity of light after and before it passes through a sample and this ratio is called transmittance (%T). Absorbance (A) is calculated in the machine using the equation: $A = -\log (\%T / 100\%)$.

BSA Standard Curve

The BSA calibration or standard curve is made by preparing known concentrations of BSA and measuring its absorbance at 280 nm (characteristic of protein) using UV/vis spectrophotometry after which a linear plot is made by plotting the concentration against

the absorbance value, which is used to find out unknown sample concentrations from the measured absorbance. BSA concentrations of 93.75, 187.5, 375, 750 and 1500 ug/ml were made in PBS and their absorbances were measured at 280 nm (all absorbance values should be below 1) and this was used to plot the below calibration curve:

$$y = 0.0006x + 0.0038$$

where 'y' is the absorbance at 280 nm and 'x' is the unknown concentration.

The mass of BSA in the sample is got multiplying 'x' with the volume of the solution.

Loading Efficiency (LE) Estimation

10 mg of BSA-loaded pollen were crushed for 5 mins using a mortar & pestle, mixed with 2 ml PBS, vortexed for 5 min and centrifuged at 15000 rpm for 5 mins as to expel the pollen contents. The supernatant was filtered using a 0.45 µm PES syringe filter (Agilent, CA, USA) to separate out BSA from pollen constituents. The absorbance values were measured at 280 nm (Boeco-S220, Germany) using a unloaded pollen as blank and the amount of BSA in the pollen was calculated using the BSA standard curve with the below formula:

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

It is the amount of BSA loaded per unit weight of the formulation.

3.2.5 Encapsulation of FITC-Bovine Serum Albumin (BSA)

Confocal Laser Scanning Microscopy Analysis (CLSM)

CLSM is a microscopic technique used to visualise fluorescent molecules, where you can penetrate the sample slice by slice stepwise using the laser, which runs from 400 to 800 nm. A pin-hole is used to focus the light, whose aperture size can be modified to vary the slice thickness. The light intensity focused can be modified by varying the gain, which can be used to set the baseline fluorescence for the sample. 3D images can be got from reconstruction of the slices. Pine pollen samples that were loaded as stated above were mounted on sticky slides with Vectashield® and scanned *via* confocal laser scanning microscopy (Carl Zeiss LSM700, Germany). Laser excitation lines were set to 405 nm, 488 nm, and 561 nm at a scan speed of 67 s per phase. Images were collected with differential

interference contrast at 405 nm (6.5 %), 488 nm (6 %) and 561 nm (6 %) using enhanced-contrast (EC) Plan-Neofluar 20X, 40X and 63X oil objective M27 lenses. The fluorescence emission was collected in photomultiplier tubes equipped with different filters (416-477 nm, 498-560 nm, and 572-620 nm) and analyzed by using the ZEN software.

Fluorescein isothiocyanate-bovine serum albumin (FITC-BSA)-loaded defatted pine pollen and pine SECs were mounted on sticky slides with Vectashield® and imaged using CLSM as mentioned above.

3.3 Results and Discussion

3.3.1 Process Development

Phosphoric acid (H_3PO_4) is widely used in consumer products, generally-recognized-as-safe, and affordable, and has consequently proven an attractive solvent for SEC processing^{11,20,61}. Pollen SEC extraction using phosphoric acidolysis has been conventionally performed at temperatures up to 180 °C for durations as long as one week (168 h)^{26,32-35,53,62}. While such protocols may suit SEC extraction from single-compartment, thick-walled pollen grains and spores such as *L. clavatum*, they can cause significant damage to the tripartite microstructure of *Pine taeda* pollen grains, including the fragile air sacs⁵². In recent work, our group has successfully established streamlined acidolysis protocols to extract SECs from *Helianthus annuus* (sunflower)⁴³, *Lycopodium clavatum* (moss)⁵⁹ and *Zea mays* (corn)⁴³ using highly efficient acidolysis protocols that require much shorter time intervals. Building on these efforts with single-compartment pollen grains and in light of the challenges associated with multi-compartment pine pollen, the following experimental and analytical characterization strategies were aimed at identifying suitable processing strategies to extract pine pollen SECs.

Figure 3.1 Chemical processing strategy to extract pine pollen sporopollenin exine capsules (SECs). Defatted *Pine taeda* pollen grains, dispersed by wind, are collected in the natural state and prepared by incubation in diethyl ether to remove lipid components. Then, the grains are subjected to acidolysis in a specified acidic solvent (strong or weak acid) and incubation conditions to remove proteinaceous content. Sequential washings are performed to remove residual solvent from processed capsules, and the capsules are subjected to analytical characterization for quality control, including morphological assessment and protein removal efficiency.

Chemical processing strategy to extract pine pollen sporopollenin exine capsules (SECs). Defatted *Pine taeda* pollen grains, dispersed by wind, are collected in the natural state and prepared by incubation in diethyl ether to remove lipid components. Then, the grains are subjected to acidolysis in a specified acidic solvent (strong or weak acid) and incubation conditions to remove proteinaceous content. Sequential washings are performed to remove residual solvent from processed capsules, and the capsules are subjected to analytical characterization for quality control, including morphological assessment and protein removal efficiency.

Processing Scheme

As presented in **Figure 3.1**, one-pot phosphoric acid processing was systematically tested across a variety of durations, temperatures, and storage methods based on the following SEC extraction process: Natural pine pollen was first defatted with diethyl ether, refluxed with acid under the appropriate conditions, and then washed and dried. Extracted pine SECs were stored in either dry (conventional) or aqueous wet conditions (to mitigate

potential collapse of the thin-walled pollen), and characterized using various analytical techniques in order to evaluate the degree of structural preservation as well as the removal of sporoplasmic and protein contents. From these experiments, optimal conditions were identified and then extended to strong acids and, in some cases, the treatment protocols included an additional enzymatic processing step ^{15,16}. A detailed description of all processing conditions is provided in **Table 3.1**.

Table 3.1. Processing parameters used for extraction of *Pine taeda* sporopollenin exine capsules.

Conditions		Processing Parameters			
Reagent	Study	Time (h)	Conc. (w/v)	Storage	Temp. (°C)
Unprocessed	-	-	-	-	-
H ₃ PO ₄	Time	1	85%	Dry	70
		2.5	85%	Dry	70
		5	85%	Dry	70
		10	85%	Dry	70
		20	85%	Dry	70
	Storage	5	85%	Wet	70
		5	42%	Wet	70
	Temperature	5	85%	Wet	50
		5	85%	Wet	25
HCl	Strong Acid	5	18%	Wet	70
		5	27%	Wet	70
H ₂ SO ₄		5	25%	Wet	70

3.3.2 Physical Characterization

SEC structure classification by DIPA

Depending on the extraction process, the structural integrity of the resulting SECs will vary and the morphological structure of SEC particles can be analysed at the single-particle level in high-throughput DIPA measurements ⁵⁹. We divided the pine pollen SECs into three groups for classification: “intact,” “fractured,” and “collapsed”. Representative examples of particles that were assigned to each group are presented in **Figure 3.2**. Preserved particles closely resemble unprocessed pine pollen, with both air sacs attached to the central cavity in a tripartite microstructure and show no significant breaks or cracks. However, they may have minor deviations in shape, average diameter, or increases in surface roughness, as compared to the untreated case. Fractured SECs have clearly visible holes, cracks, or missing sections in one or more compartment, but at least one uncompromised, fully enclosed compartment remaining based on the visual inspection. Examples of fractured SECs may include a complete central cavity that has damaged or missing air sacs, or an intact air sac that is attached to other damaged/missing compartments. Collapsed SECs appear shrivelled and flat and have very little inner volume for loading. These classifications provide a starting point to aid the development of optimized processing strategies. Following this characterization strategy, we initially examined the effects of processing duration, storage method, and reflux temperature on SEC particle morphology, and the results are summarized in **Table 3.2**. Before processing, the natural pollen grains were 99% intact with only trace amounts of collapsed and fractured particles (**Figure 3.3A**).

Figure 3.2 Representative optical micrographs of processed SECs with different morphological states. Based on visual inspection of the optical micrographs, individual particles were classified as **(A)** intact (preserved tripartite microstructure with no ostensible breaks or cracks), **(B)** fractured (cracked or missing portions, with at least one fully preserved compartment, or **(C)** collapsed (significantly shrivelled with low encapsulation volume).

Effect of processing time on SEC morphology

The first parameter that was then tested was the time scale of pollen grain processing in 85% phosphoric acid at 70 °C, followed by conventional dry storage. After 1 h of processing, 45% of particles were intact while 32% were collapsed. With increasing processing time, the percentage of intact particles increased to ~60% while the percentage of collapsed particles decreased to approximately 10% and 2% after 5 h and 20 h, respectively. This decrease in the fraction of collapsed particles at longer processing times supports past accounts of pine pollen grains undergoing rapid collapse due to acid-shock resulting from phosphate ion exchange between membranes³² and removal of the intine

layer and was most prominent after 1 hr of treatment after which the % of collapsed particles go down and for longer times, the % of broken particles go up resulting in similar number of intact capsules. This acid-shock induced buckling or collapse was also evident with another thin walled pollen namely corn⁶³, which was also subject to acid for microcapsule extraction. In fact, pollen has been observed to buckle or collapse when subject to dessication^{7,64}.

At the same time, processing times of 10 h or longer led to a large number of fractured particles reaching around 40% (Figure 3.3).. Optimal results in this test series were obtained with processing in 85% phosphoric acid at 70 °C for 5 h, with ~60% intact particles as well as ~30% fractured and ~10% collapsed.

Figure 3.3 DIPA representative images of (A) unprocessed and (B-F) processed pine SECs using 85% phosphoric acid at 70°C for different durations: Particles processed for short timeframes suffer dramatic collapse that recovers and maintains shape after 5 hours of processing after which greater breakage is observed.

Effect of storage & phosphoric acid concentrations on SEC morphology

As the sporopollenin exine walls of pine pollen are relatively thin and hence fragile, we next explored whether a wet storage environment that provides structural stability⁴³ could

reduce the percentage of collapsed particles and improve the overall yield. To explore this option, the particles were processed in 85% phosphoric acid at 70 °C, followed by wet storage. In this case, the percentage of intact particles increased to 78% while the percentage of collapsed particles decreased to 0.5%. These findings support that wet storage facilitates particle intactness by avoiding the structural collapse/buckling that occurs with dehydrated pollen microcapsules⁶⁵⁻⁶⁸, although dry storage would likely increase durability and shelf-life of the SEC particles for industrial applications. Similar results were also obtained with processing in 42% phosphoric acid (**Figure 3.4**), where the intact numbers were even lower supporting that the high acid concentration is suitable for SEC production.

Figure 3.4 DIPA representative images of processed pine SECs using different phosphoric acid concentrations (A) 85% (B) 62% (C) 42 % at 70°C for 5 hours: Lower acid concentrations led to a surprising rise of collapsed particles.

Effect of temperature on SEC morphology

The optimized condition (5 h phosphoric acid, wet storage) was next investigated for the effect of temperature and the number of intact particles increased at lower temperature, reaching 93% intact particles when processed at 25 °C (**Figure 3.5**). Hence, good control

over the processing steps could be achieved as indicated by retention of pine pollen exine morphology in the SECs

Figure 3.5 DIPA representative images of processed pine SECs using different temperatures (A) 70 °C (B) 50 °C (C) 25 °C with 85% phosphoric acid for 5 hours: Lower temperatures resulted in similar structure, albeit less effective sporoplasmic removal.

Effect of strong acids on SEC morphology

For comparison, we used a similar standard protocol (5 h at 70 °C) to test two strong acids, including hydrochloric (HCl) and sulphuric (H₂SO₄) acids. Processing in 18% hydrochloric acid successfully yielded 92% intact and 7% fractured particles while processing in 27% hydrochloric acid was less optimal, resulting in 83% intact and 17% fractured particles. On the other hand, processing in sulphuric acid yielded 77% intact and 23% fractured particles (**Figure 3.6**). As these two strong acids are known to dissolve cellulosic intine materials but not proteinaceous materials⁶⁹, we also explored the feasibility of adding an additional processing step with trypsin protease (as described in Ref. ⁵²) to the 18% hydrochloric acid protocol and identified that the number of intact particles decreased to ~75%.

Overall, the weak phosphoric acid was used at a similar concentration(85%) as was used in the corn pollen study given the thin-wall air-sac structure of the sacs of bisaccate pine

pollen, where even at this high concentration, 80% particles were found to be intact for the optimized processing condition of 70 C for 5 hrs followed by wet storage. The same conditions explored with stronger corrosive acids namely hydrochloric and sulphuric acids where even at a much lower concentration (27 & 25% respectively), resulted in similar number of intact particles (77 & 83% respectively), which is indicative of their acid strengths. A lower concentration of the HCl (18%) did result in a higher structurally preserved number (92%), where the acids caused broken structures rather than collapsed ones. This shows that the collapse phenomena is caused due to the acid-shock effect, which is time-dependent rather than acid-dependent, after which pollen particles start breaking up.

Collectively, the findings demonstrate that both weak and strong acids that are widely used in SEC extraction protocols successfully work with pine pollen grains, and that the fragile nature of this thin-walled pollen species is an important factor for optimizing the processing conditions.

Figure 3.6 DIPA representative images of pine SECs processed with strong acids (A) 18% HCl (B) 27% HCl (C) 25% H₂SO₄ at 70°C for 5 hours: Intactness suffers as acid concentration increases with more broken particles observed.

Table 3.2 Effect of different processing conditions on the morphological properties of processed SEC samples. The number of intact, fractured and collapsed particles are expressed as percentages from data collected for >300 particles.

Morphological Characterization			
Processing Condition	Intact (%)	Fractured (%)	Collapsed (%)
Unprocessed	99.0 ± 0.3	0.8 ± 0.2	0.1 ± 0.2
85% H ₃ PO ₄ at 70 °C for 1 h (dry)	44.8 ± 6.6	23.2 ± 6.0	31.8 ± 4.2
85% H ₃ PO ₄ at 70 °C for 2.5 h (dry)	56.6 ± 5.8	24.1 ± 2.4	18.6 ± 2.6
85% H ₃ PO ₄ at 70 °C for 5 h (dry)	60.6 ± 0.8	29.8 ± 1.9	9.4 ± 2.3
85% H ₃ PO ₄ at 70 °C for 10 h (dry)	55.2 ± 8.7	41.2 ± 9.2	3.7 ± 0.50
85% H ₃ PO ₄ at 70 °C for 20 h (dry)	55.2 ± 3.8	43.2 ± 4.1	1.6 ± 0.6
85% H ₃ PO ₄ at 70 °C for 5 h (wet)	80.6 ± 4.2	18.7 ± 4.3	0.5 ± 0.00
42% H ₃ PO ₄ at 70 °C for 5 h (wet)	76.1 ± 8.7	23.3 ± 8.7	0.5 ± 0.5
85% H ₃ PO ₄ at 50 °C for 5 h (wet)	82.0 ± 3.6	18.0 ± 3.6	0.0 ± 0.0
85% H ₃ PO ₄ at 25 °C for 5 h (wet)	93.3 ± 3.2	6.7 ± 3.2	0.0 ± 0.0
18% HCl at 70 °C for 5 h (wet)	92.2 ± 2.0	7.2 ± 2.1	0.1 ± 0.2
27% HCl at 70 °C for 5 h (wet)	77.1 ± 6.4	22.8 ± 6.4	0.0 ± 0.0
25% H ₂ SO ₄ at 70 °C for 5 h (wet)	83.0 ± 3.7	17.0 ± 3.7	0.0 ± 0.0

Based on the data collected, the initial evidence suggests that a large percentage of intact particles can be obtained with single-pot phosphoric acid processing or a combination of strong acid (*e.g.*, hydrochloric acid) process followed by enzymatic treatment with a protease enzyme.

Micromeritic Properties

In addition to assessing the structural integrity of chemically processed samples, DIPA analysis was conducted in order to determine the number-weighted average particle diameter of each sample, as presented in spline curve fit histograms in Figure 3.7. While unprocessed pollen grains had an average diameter of 62 μm , the average diameter shrank to around 56 μm for samples treated with 85% (70 °C) phosphoric acidolysis for 2.5 h or shorter pollen grains had an average diameter of 62 μm , the average diameter shrank to around 56 μm for samples treated with 85% (70 °C) phosphoric acidolysis for 2.5 h or shorter durations. With longer processing times, the average particle diameters once again approached the values for unprocessed pollen grains (Fig 3.7A), and the average diameter of particles processed for 5 h duration or longer was around 59 μm . Initially the reduction in diameter for short-term treatments (upto 2.5 hrs) was due to collapsing of the pine pollen particles due to the acid-shock effect. The overall % of structurally intact particles is more or less the same for treatment times (≥ 2.5 hrs from the table, with the % of collapsed particles going down and % of broken particles going up). If the collapsed particles broke apart, then the diameter of the particles treated for 5 hrs and above should be similar or smaller than that of a 2.5 hr treatment batch. But it is not so with the average diameter actually being 3 μm more in size. So it is possible that the pine pollen recovers its morphology for longer processing times(between 2.5 & 5 hrs) after which it fractures (cracks mostly and doesn't break into pieces) and so the average diameter once again rises.

Figure 3.7 Size Histograms of Processed SEC Particles. (A) Effect of processing time in 85% phosphoric acid at 70 °C. (B) Effect of phosphoric acid concentration. The time and temperature were fixed at 5 h and at 70 °C, respectively. (C) Effect of processing temperature for 5 h processing in 85% phosphoric acid. (D) Alternative processing strategies with strong acids (5 h at 70 °C). Data is collected from >300 individual particles per sample.

Furthermore, the average diameters of particles treated with 85% or 42% phosphoric acid were similar, as were the samples processed at different temperatures (wet-stored), once again demonstrating that the high acid concentration and high temperature are suitable for the processing step (Figs 3.7B, C). The strong acid treatments also did not affect the particle size (Fig 3.7D). Taken together, the data reinforce that an optimal processing time (in the range of 5-10 h, and defined to be 5 h for our experiments) overcomes initial particle collapse due to acid shock while avoiding excessive particle damage due to fracturing.

Morphological Investigation using SEM

Representative SEM micrographs of pine pollen grains after phosphoric acid acidolysis for different processing times are presented in **Figure 3.8**. The cross-sectional image of the unprocessed pine pollen grains reveals the presence of pollen constituents inside the large central inner cavity (**Fig 3.8A**). As discussed above, the majority of SEC particles appeared to collapse after acidolysis for a period of 1 h and some were still present after 2.5 h (**Figs 3.8B,C**). However, at longer processing times, the fraction of collapsed particles decreased and the 5 h time point again showed an optimal balance of intact particles (**Figs 3.8D-F**).

Figure 3.8 SEM micrographs of (A) unprocessed pine pollen and (B-F) SEC capsules processed with 85% phosphoric acid at 70 °C for varying durations of processing time.

In addition, the SEM micrographs for pine pollen SECs prepared using strong acids are presented in **Figure 3.9**. In all three tested cases, the SECs appear largely intact as expected. While most particles were observed to be intact, the fraction of damaged particles were generally more fractured than collapsed with the number increasing with higher acid concentration. The cross-sectional images obtained from highly damaged particles showed that inner components are mostly removed with a smooth lining seen in the central cavity. These aspects are further discussed below in the context of elemental analysis and highlight that phosphoric acid is advantageous for removing sporoplasmic contents in general, while strong acids require an additional processing step with proteolytic enzyme to aid SEC production, which help to preserve the capsule morphology (**Figure 3.10**)

Figure 3.9 SEM micrographs of SEC capsules processed with different strong acids (A) 18% HCl (B) 27% HCl (C) 25% H₂SO₄ for 5 h at 70 °C.

Figure 3.10 SEM images SECs processed with 6M Hydrochloric Acid (5 h at 70°C) + 24 h Trypsin

3.3.3 Chemical Characterization

Assessment of Protein Removal

In addition to retaining morphological structure, a key requirement of SEC production is the removal of potentially allergenic proteins from the pollen microcapsules. To verify protein removal, CHN elemental analysis based on high-temperature combustion was conducted on untreated and treated pine pollen samples, and the percentage of protein removal was determined based on the amount of nitrogen remaining in the samples. The key measurement principle behind this approach is that it is known that proteins are the only major component of pollen grains which contain nitrogen, and hence measuring the nitrogen content of SEC particles provides an indication of how much protein was removed as a result of chemical processing⁵³. The percentage of cytoplasmic removal (complementary to protein removal) was also determined by the following equation:

$$\% \text{ Cytoplasmic Removal} = \frac{\%N_{\text{Unprocessed}} - \%N_{\text{Processed}}}{\%N_{\text{Unprocessed}}} \times 100\%$$

As presented in **Figure 3.11**, unprocessed pine pollen was determined to have a protein content of around 11%, which agrees well with literature values for other pollen

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

Figure 3.11 Protein content and protein removal efficiency for processed SEC samples as determined by CHN analysis. The removal efficiency is determined based on the protein content of an unprocessed sample relative to the processed samples.

Pure trypsin treatment alone, even though is relatively milder was less effective in removing the sporoplasm (**Fig 3.12**) as compared to an acid pre-treatment.

Figure 3.12 Sporoplasmic removal after pure trypsin action alone for 24 hrs and Acidolysis (6M HCl-5 h-70 °C) followed by 24 hr trypsin treatment showing that acidolysis facilitates enhanced sporoplasmic removal by trypsin.

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

On the other hand, treatments with strong acids were less effective at removing protein. In all cases, one-pot treatment of pine pollen grains with strong acids (5 h treatment at 70 °C) led to protein removal efficiencies around 57-65%. These values are consistent with the previous observations that indicate that strong acids are typically less effective at cleaning SECs. To improve protein removal, trypsin was added to the hydrochloric acid-treated pollen sample and this two-step protocol had a protein removal efficiency around 93%. As a control, it was also identified that trypsin alone was ineffective for removing protein. Hence, sequential treatments with hydrochloric acid and trypsin were selected as the two-step method of choice for preparing SECs for further exploration for microencapsulation.

3.3.4 Evaluation of Loading Efficiency

Washing

Post-loading the samples had to be washed to remove any free BSA and BSA bound to the surface. This was done using a one-step with water after which SEM imaging was performed to look at the cleanliness of the washed samples and it can be seen that the matrix and the surface are clean (**Fig 3.13**).

Figure 3.13 SEM images of BSA-loaded Pine Pollen SECs (A) Unprocessed pine pollen (B) 6M Hydrochloric Acid for 5 h at 70°C + 24 h Trypsin treatment (C) 85% Phosphoric Acid for 5 h at 70 °C. All SECs were washed thoroughly prior to loading measurements to remove adsorbed BSA. All scale bars are 10 μ m.

Loading efficiency using UV absorbance

To verify protein loading, absorbance measurements were also conducted in order to quantitatively determine the loading efficiency of the different samples. The vacuum loading method was utilized, and the loaded samples were extensively washed before measurement. The natural pine pollen had a loading efficiency of $7.9 \pm 1.5\%$, which is comparable to other pollen species. This low efficiency is likely attributed to the presence of sporoplasmonic contents in the central cavity. By contrast, the loading efficiency of the SECs was around three-times greater, with values in the range of 23-26 % that demonstrate excellent loading capacity. The phosphoric acid-treated SECs had a loading efficiency of $23.0 \pm 2.6\%$, whereas the hydrochloric acid-treated SECs (with additional trypsin

treatment) had a loading efficiency of 26.5 ± 3.7 %. These findings strongly support the CLSM results, and demonstrate that pine pollen SECs can be prepared, which are morphologically intact, devoid of protein contents, and capable of efficient loading. The increased loading of the SECs can be explained by the removal of sporoplasmic contents (greater available loading volume per particle and hence a higher number of particles per unit mass) as well as dissolution of the intine layer, which increases access to the nanoscale channels facilitating protein encapsulation. Of note, while loading appeared to be greater in the air sacs for the natural pine pollen grains, the loading appeared to be greater in the central cavity for the SECs. This difference in loading properties indicates that chemical processing affects the molecular permeability of one or both cavity types. In particular, these findings support that the permeability of the central cavity in natural pine pollen grains is largely controlled by the molecular properties of the intine layer and that chemical processing removes this intine layer ⁷⁰. As demonstrated in this work, systematic investigation of chemical processing strategies identified that 85% phosphoric acid for 5 h at 70 °C is optimal while other strategies are also possible.

Spatial localization using CLSM

Figure 3.14 presents confocal laser scanning microscopy (CLSM) images characterizing the loading properties of unprocessed and processed pollen grains. As discussed above, two SEC samples were selected for this evaluation based on one-pot treatment (85% phosphoric acid for 5 h at 70 °C) and two-step treatment (18% hydrochloric acid for 5 h at 70 °C, followed by trypsin incubation), respectively. The CLSM approach is useful because the pollen exine wall as well as its sporoplasmic content is known to autofluoresce across a wide range of excitation wavelength, hence providing a visual means to assess structural integrity as well as qualitatively verify protein removal ^{35,59}.

Cross-sectional images of the unloaded natural pollen and two SEC samples confirm that the processing steps effectively removed sporoplasmic contents from inside the exine capsules, whereas an extensive quantity of autofluorescent contents was visible inside the natural pollen sample. All the contributing slices to the 3D reconstructed image are shown in **Fig 3.15**. FITC-labeled BSA protein was next loaded by vacuum methods into the samples as previously described, and the loading of the fluorescently labelled

protein could be visualized. Under equivalent image settings, cross-sectional slices were collected and reconstructed to form a three-dimensional representation of the individual particles. It was observed that the fluorescence intensity of loaded protein was typically greater for SEC samples than for natural pine pollen grains, suggesting that the SECs have higher loading efficiencies than natural pollen. In particular, the loading inside the central cavity appeared to be greater for SECs versus the natural pine pollen. Co-localization of the fluorescence signals from the pollen exine wall (blue channel) and the loaded protein (green channel) further supports that the protein is encapsulated within the capsules in all three cases.

Figure 3.14 CLSM images of unprocessed pine pollen and processed SEC samples without and with loaded BSA. The processing conditions were either 85% phosphoric acid for 5 h at 70 °C or 18% hydrochloric acid for 5 h at 70 °C followed by trypsin treatment. Left column: Cross-section of capsules before protein loading. The blue autofluorescence corresponds to the pollen contents. The other columns present 3D reconstructions of BSA-loaded capsule samples, for which the dual-channel CLSM images show pollen contents (blue) and loaded FITC-labelled BSA (green). All scale bars are 20 μ m.

Figure 3.15 Z-stack CLSM array for unloaded and FITC-BSA-loaded (A) Pollen and (B,C) SECs. All scale bars are 20 μm .

3.4 Conclusion

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

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Chapter 4 Defatting of natural pine pollen with protein loading and controlled release

Defatting is a mild process involving organic solvents like ether, acetone etc., which opens up pores by dissolving the lipids. It reduces the allergenic content and aids molecular loading by creating new pathways for molecular penetration. Pine pollen being fragile in nature, was not able to withstand harsh SEC processing techniques and thus defatting was chosen as a milder alternative. This chapter shows how simple defatting could improve loading, with no morphological damage caused and attaining controlled release of the loaded defatted pine pollen using natural options.

* This chapter was submitted substantially as: Arun K. Prabhakar, Michael G. Potroz, Ee-Lin Tan, Haram Jung, Jae Hyeon Park, and Prof. Nam-Joon Cho*;"Microencapsulation with Plant-Based Multi-Cavity Microparticles: Nanoporous Microstructures, Loading Optimization, and Controlled Release" to ACS Applied Materials & Interfaces

4.1 Introduction

Identifying novel natural materials for use in microencapsulation is of significant interest to a wide range of industries.^{1 2 3 4 5 6}. Pollen is a key example of nature's own evolutionary microencapsulation solution for the protection of sensitive genetic material crucial for reproductive success.^{7 8}. In general, pollen presents a wide range of properties desired of an ideal microencapsulant, such as, monodispersity, morphological stability, physicochemical resilience, and biocompatibility^{9 10 11}. Most existing attempts to utilize pollen as a microencapsulant have involved the extraction of pollen sporoderm hollow shells through the use of various chemical extraction processes.^{12 13 14 15 16 17}. Sporoderm microcapsules (SDMCs) have been shown to provide a range of appealing properties, such as, taste-masking,¹⁸ anti-oxidant protection,¹⁹ UV protection,²⁰ immunomodulatory properties,²¹ and ease of compound loading.²². However, a primary limitation of SDMCs is that they require additional regulatory approval before being utilized for oral delivery applications in food and medicine. Whereas, natural pollens have a long history of use as food and medicine,^{23 24 25} with natural pollen considered to be a regulation-free food ingredient in most parts of the world.^{26 27}.

Natural pine pollen possesses a range of ideal characteristics for developing natural pollen microencapsulation technology. Firstly, pine pollen possess a triple cavity structure well suited to being used for microencapsulation, with two hollow air-sac (bisaccate)^{28 29} cavities providing ample space for compound loading³⁰. Secondly, pine pollen is available in large industrial-scale quantities and is priced similar to numerous other commonly used microencapsulant base materials, such as, alginate, food gums, chitosan, gelatin, etc.^{31 32}. Thirdly, pine pollen is one of the most widely utilized natural pollens, with a prestigious history in Chinese, Korean, Japanese, Indian, native American, and other cultures as a food and potent medicine.³³. Overall, the utilization of pine pollen as a natural microencapsulant, offers the potential for developing a range of novel food, cosmetics, and therapeutic products with minimal processing or regulatory hurdles^{34 35 36}. In particular, pine pollen microencapsulation presents a compelling technology for helping to modernize the vast fields of traditional and modern herbal therapeutics^{37 38 39} without the need for introducing synthetic or highly processed materials.

Understanding and tuning the properties of the dual air-sac pine pollen is crucial for successful compound loading within the air-sac hollow cavities. Pine pollen is an anemophilous (wind-loving) pollen, wherein the hollow semi-porous pollen air-sacs represent a naturally optimized hierarchical micro/nano-structure (**Fig 4.1a**) for reducing pollen density, facilitating improved wind-dispersion properties,⁴⁰ and ensuring the pollen grain remains afloat and correctly oriented during pollination.^{41 42} Studies have shown that the outer sporopollenin sexine shell of pine pollen air-sacs is porous and permeable, however, these studies utilized air-sacs extracted with extensive harsh chemical processing to isolate the shell material (sporopollenin) only.^{43 44} Although the air-sac structure is porous, studies with natural pine pollens have shown that a waterproofing layer of lipidic compounds reduces permeability and inhibits water uptake (**Fig 4.1b**)^{45 46}. Gymnosperms, such as pine, are not known to produce pollen coated with pollenkitt, a sticky composition aiding in biotic pollination and typically found in angiosperms.⁴⁷ The specific waterproofing lipidic compounds found on pine pollen have not been identified, however, it has been proposed that pollens may become coated with resinous residues from resin producing parent plants,⁴⁸ such as pine trees. The removal of the lipidic compound waterproofing layer from pine pollens with various organic solvents has been shown to facilitate water uptake⁴⁹ and may further facilitate compound loading within pine pollen sacci.

Figure 4.1 Schematic diagram showing the development of natural pine pollen multiparticulate tablets for intestinal protein delivery: **(a)** Scanning electron micrograph of pine pollen capsule cross-section, with emphasis on a single porous air-sac (saccus) structure; **(b)** Schematic representation of natural pine pollen structure, emphasizing resilience to water absorption; **(c)** Defatting of pine pollen removes the external lipidic layer and enhances pollen water uptake; **(d)** BSA becomes trapped in the defatted pine pollen porous sexine structure; **(e)** Alginate-coated multiparticulate pine pollen tablet provides controlled release suitable for intestinal delivery of proteins.

Herein we explored the utilization of pine pollen (*Pinus sylvestris*) for compound loading and developed a multiparticulate tableted oral delivery formulation, exhibiting controlled release suitable for intestinal delivery. Washing and defatting of raw pollen was conducted to obtain monodisperse natural pine pollen (NPP), single-defatted pine pollen (SDPP), and double-defatted pine pollen (DDPP). Fundamental morphological and compositional particle properties were analyzed to determine the influence of defatting. Changes in surface porosity and wetting due to defatting were examined and related to

particle water absorption dynamics (**Fig 4.1c**). Compound loading optimization was conducted with bovine serum albumin (BSA), as a representative protein, to determine the vacuum-assisted loading and washing parameters required for optimal compound loading. Compound loading distribution studies were performed to gain insight into compound loading dynamics and further elucidated compound loading potentials (**Fig 4.1d**). Compound release studies were conducted with tableted BSA-loaded DDPP formulations, comprising either xanthan gum as a binder, or ionotropically crosslinked sodium alginate as an enteric coating. Finally, tablets exhibiting ideal compound release profiles were examined to elucidate the multiparticulate tablet morphological properties (**Fig 4.1e**).

4.2 Experimental Section

4.2.1 Materials

BSA, FITC-conjugated BSA, xanthan gum, sodium alginate, calcium chloride, and diethyl ether were purchased from Sigma-Aldrich (Singapore). Raw pine pollen (*Pinus sylvestris*) was purchased from Xi'an Yuensun Biological Technology Company Limited (China). Vectashield (H-1000) was purchased from Vector labs (CA, USA). Sticky-slides, D 263 M Schott glass, No.1.5H (170 μm , 25 mm $\times$ 75 mm) unsterile were purchased from Ibidi GmbH (Munich, Germany). Milli-Q water was used in all experiments. Perfluoroalkoxy polymer flasks were purchased from Vitlab (Grossostheim, Germany). A stainless steel pellet press die (13 mm) was purchased from Specac (Kent, UK).

4.2.2 Pollen Volumetric Calculations

Based on models presented from previous studies,^{30 40} the central cavity volume was calculated based on an ellipsoid, and the air-sac volumes were calculated as half of an ellipsoid each. Central cavity ellipsoid radii: $r_1 = a / 2$, $r_2 = b / 2$, $r_3 = b / 2$. Air-sac ellipsoid radii: $r_1 = d$, $r_2 = c / 2$, $r_3 = c / 2$. Dimensions a, b, c, d, and e, defined in Fig 4.3b.

4.2.3 Washing and defatting of Natural Pine Pollen

Washing of Natural Pine Pollen

Natural pine pollen (30 g) was suspended in deionized (DI) water (1 liter) and vacuum-filtered a total of four times to remove dust and other smaller plant debris, followed by an

additional filtration step with a nylon mesh (100 μm) to separate out any large debris, while allowing the smaller pollen particles to pass through. The pollen was then freeze-dried to obtain clean dry natural pine pollen powder.

Defatting of Natural Pine Pollen

Natural pine pollen (30 g) was treated with diethyl ether (300 ml) for 3.5 h with stirring (200 rpm), after which the solution was vacuum-filtered and vacuum-oven dried until stable weight (100 mbar, 40 ° C, 30 min) to remove any traces of ether. Then the dried pollen was cleansed similarly to the natural pine pollen as mentioned above. A single defatting step produced single-defatted pollen. Single-defatted pine pollen (10 g) was treated with diethyl ether (100 ml) with stirring (200 rpm) for 3.5 h, after which the pollen was vacuum-filtered and dried (100 mbar, 40 ° C, 30 min) to obtain dry double-defatted pollen.

4.2.4 Physical and chemical characterisation of natural and defatted pollen

Dynamic Imaging Particle Analysis (DIPA)

DIPA imaging and analysis was performed as explained in section 3.2.3 on natural and defatted pine pollen. A minimum of 10,000 particles was scanned and three separate measurements were performed. Data analysis was carried out using 3000 well-focused particles.

Contact Angle

This technique is used to measure the angle a liquid (polar or non-polar) makes when dropped onto a thin layer of the particle of interest (solid). The lesser the angle made (< 90 degrees) with the corresponding solvent, the more hydrophilic or lipophilic the particle is and it denotes the wettability of the solid by the liquid. This contact angle is unique to the temperature and pressure, which dictates the liquid-vapour-solid system's equilibrium condition.

A single layer of carbon tape was stuck onto glass slides after which the dry pollen powder (natural, single & double defatted) was dropped onto the surface as to make a thin layer.

Water (2 ul) was dropped onto the layer and the contact angle was measured over the next 10 secs.

Pore Size estimation (N₂ adsorption-desorption)

Since defatting is expected to make the pollen more porous, it is essential to measure the pore size distribution of natural and defatted pollen for comparison. This is done by using nitrogen adsorption-desorption isotherms graphs. It is a plot of relative pressure vs. volume adsorbed obtained by measuring the amount of nitrogen gas that adsorbs onto the surface of interest and the subsequent amount that desorbs at a constant temperature. Nitrogen is chosen so that it does not chemically react with the sample. BET theory is used to calculate the specific surface area of the particle assuming multilayer adsorption, while BJH method is used to determine the pore size distribution. Barrett-Joyner-Halenda (BJH) theory is originally designed for relatively wide-pore adsorbents with a wide pore size distribution, but was later shown to be applicable to all types of porous materials. The model assumes that pores have a cylindrical shape and the desorption branch of isotherm in the a specific pressure range (0.4-0.967) is generally used as initial data for BJH calculations.

300 mg of natural and defatted pollen were added into glass tubes and degassed for 2 hrs at 130° C to remove any bound molecules. Liquid nitrogen was filled into the Dewar flasks and then the tubes were fixed onto the apparatus (ASAP tristar II 3020) and the setup was left overnight for the complete adsorption-desorption cycle.

Elemental CHN Analysis

Elemental CHN analysis was performed as explained in section 3.2.3. on natural and defatted pine pollen.

4.2.5 Aqueous Permeability

Passive Loading

Natural, single & double defatted pine pollen (10 mg each) was added to 1 ml of water (for complete wetting) and the samples were mixed at 500 rpm and then observed under the optical microscope after fixed time points (5,15,30 mins, 1, 2 & 24 hrs).

Vacuum Loading

Natural, single & double defatted pine pollen (10 mg each) was added to 1 ml of water and the samples were subject to vacuum loading at 0.01 mbar for 5 mins and then observed under the optical microscope.

Particle fraction of completely water-filled pollen was quantified (%) at each time point (for both passive and vacuum-aided methods) by looking at three frames (with a minimum of 30 pollen particles per frame).

4.2.6 BSA loading into natural and defatted pine pollen*Passive Loading*

50 mg of natural pine pollen was taken and 50 mg/ml (1 ml) BSA solution was added and the mixture was put on a shaker at 500 rpm for 1 hr.

Vacuum Loading

50 mg of natural pine pollen was taken and 50 mg/ml (1 ml) BSA solution was added and the mixture was vortexed for 30 seconds to ensure uniform mixing, after which it was subject to vacuum loading (0.01 mbar) for varying time points (5, 15, 30 mins, 1 & 24 hrs). The formulations were washed with water and freeze-dried. This was as to optimize the time point for loading (indicated by saturation). The vacuum pressure was then varied: 1 & 100 mbar to probe suction effects on BSA loading. Then at the optimized conditions, two other different BSA concentrations were tried out keeping the BSA mass fixed at 50 mg (2 ml of 25 mg/ml and 4 ml of 12.5 mg/ml) as to see which concentration loads best (pollen mass fixed at 50 mg). After optimizing the time point, vacuum pressure and concentration, both single and double defatted loaded with BSA to arrive at the best pollen (based on degree of defatting) for loading as indicated by the loading efficiency. Finally, different loading pollen: BSA ratios were tried out (1:2, 1:3 & 1:4, 2:1, 3:1 & 4:1) to maximize BSA encapsulation with a fixed mass of the pollen (25 mg) that loaded best.

Washing of BSA-loaded pollen

Natural and defatted pollen were water-washed, followed by scraping of bound-BSA to give formulations, which were SEM-imaged to confirm surface cleanliness.

Surface Morphology Evaluation by Scanning Electron Microscopy (SEM)

SEM was performed as explained in section 3.2.3 on natural and defatted pine pollen.

Loading Efficiency Estimation

BSA calibration curve was made in HCl similar to that of PBS (section 3.2.4) and the resulting calibration curve is:

$$y=0.0005x - 0.009$$

where 'y' is the absorbance at 280 nm and 'x' is the unknown concentration.

Loading Efficiency estimation was performed as explained in section 3.2.4

4.2.7 FITC-BSA loading into natural and defatted pine pollen*Confocal Laser Scanning Microscopy Analysis (CLSM)*

Fluorescein isothiocyanate-bovine serum albumin (FITC-BSA)-loaded natural and defatted pine pollen were mounted on sticky slides with Vectashield® and imaged through CLSM as explained in section 3.2.5 and the fluorescence intensities of samples were compared through Image J.

4.2.8 Release Profile Testing*Powdered Formulation*

To test the BSA release profiles of the loaded samples double defatted pollen powder (with 2mg BSA equivalent) was dissolved in 4 ml of simulated gastric fluid (SGF) & simulated gastric fluid (SIF) separately and 1 ml of the solution was taken out after fixed time points (replaced with 1 ml fresh solution) and the absorbance was read at 280 nm. A blank formulation (unloaded pollen) was also tested in the same way as to negate the intrinsic protein content. The difference in the absorbance values of the sample and the blank gave

the BSA mass released, which was plotted against the time points to give the release pattern.

Tableted Formulation

Double defatted pollen formulations (150 mg blank and equivalent BSA loaded formulations) were tableted using a hydraulic press at 5 tonnes (for 20 secs)⁵⁰. The tableted formulations were dissolved in 20 ml of SGF for initial 3 hrs after which the release medium was replaced with SIF for 24 hrs. 1ml of the solution was taken out after fixed time points (replaced with 1 ml fresh solution) and the absorbance was read with a reference blank similar to the above method.

Tableted Formulation with Xanthan Gum (Binder)

Double defatted pollen formulations (150 mg blank and equivalent BSA loaded formulations) were tableted using a hydraulic press at 5 tonne (for 20 secs) using xanthan gum as binder (1, 2.5, 5, 10, 20 & 30 % w/w). The tableted formulations tested for release in a similar way to the tablets without the binder. A tablet control using only xanthan gum (150 mg) and BSA(13 mg) was also done to test the pollen effect on release.

Tableted Formulation with Calcium Alginate Coating

Tablets(without binders) were made as described above and then dipped in sodium alginate (2 % w/v in water) for 5 sec followed by dipping in calcium chloride (4 % w/v in water) for 10 secs (1, 2 & 3 cycles) after which it was air-dried overnight, followed by vacuum drying at 200 mbar for 24 hrs. The coated tablets formulations were tested for release in a similar way to the non-coated ones. A tablet control using only sodium alginate (150 mg) and BSA(13 mg) followed by a double- coat cycle was also done to test the pollen effect on release.

Where there is no other indication, all the data was collected and is presented in triplicate (n=3). Significance testing was performed using two-tailed t-tests and $P < 0.05$ was considered as statistically significant.

4.3 Results & Discussions

4.3.1 Pine Pollen -Central cavity and Air-Sac Structure and Volumetric Calculations

To determine the overall potential of compound loading within pine pollen, it is important assess the structure and volume of the hollow regions within the pollen. Morphological observations of pine pollen cross-sections indicate that the air-sacs may be categorised into two distinct regions, the outer porous sexine structure and the inner hollow cavity region (**Fig 4.2, 4.3a**). It should be noted that the sexine region around the central cavity does comprise a thin porous layer, however, volumetric estimations of only the air-sacs may be adequate for determining overall loading potential as the central cavity is packed with vegetative and tube cells along with organelles and energy reserves like starch grains etc.. The outer porous sexine structure comprises micron-sized cavities separated by smooth cavity walls (**Fig 4.3a**). The presence of nanopores on the sexine walls can be clearly seen along with stratified pollen wall consisting of sexine, nexine and the intine. Pollen morphology analysis resulted in the determination that pine pollen sacchi constitute over a third of the total volume of a pollen particle, with the sacchi porous sexine structure constituting approximately a fifth of the total pollen volume (**Fig 4.3b**). A potential volumetric cargo capacity of 37.5 ± 7.1 % strongly supports the potential use of pine pollen for microencapsulation applications.

Figure 4.2 Scanning electron micrographs of (a) pine pollen cross-section, with close-up images of the sexine structure, and (b) sexine, nexine, and intine structure around the central cavity.

Figure 4.3 Pine pollen micro/nano-structure and cargo capacity: **(a)** Scanning electron micrograph of (i) a pine pollen cross-section, with close-up images of (ii) sexine, nexine, and intine structure around the central cavity, (iii) an underside of the sexine structure with the nexine removed, (iv) cross-section of the sexine structure around the saccus, (v) cross-section of a single sexine cavity with external wall pore, (vi) internal sexine structure, and (vii) sexine wall; **(b)** Schematic diagram and tables defining key pine pollen dimensions used for calculating volumes and volume proportions of particle, central cavity, sacci, and sacci sexine. Scale bars: (i) = 10 μm ; (ii) (iii) (iv) (vi) = 1 μm ; (v) (vii) = 100 nm.

4.3.2 Washing and Defatting of Natural Pine Pollen

Pre-processing and defatting of raw pine pollen was undertaken for the removal of contaminants and surface adhered lipidic compounds, and resulted in substantial reductions in final pollen yields. Initial pre-processing steps of water washing, filtration, and freeze-drying removed contaminants and produced a more homogenous dry pollen sample with a yield of 77.6 ± 1.1 %. Pollen defatting further decreased pollen yields, with a single defatting step and double defatting step producing final yields of 74.3 ± 3.1 % and 62.9 ± 5.5 %, respectively. The reduction of pollen yields from defatting highlights that NPP comprises a large portion of surface adhered lipidic compounds.

4.3.3 Physical characterisation of natural and defatted pine pollen

Dynamic Imaging Particle Analysis (DIPA)

Dynamic imaging particle analysis (DIPA) indicated pollen defatting had negligible effect on pollen morphology (**Fig 4.4a,b,c**). The average particle diameter and particle size distribution of NPP, SDPP, and DDPP was similar, at 58.57 ± 1.16 μm , 58.21 ± 0.62 μm , and 62.19 ± 1.93 μm , respectively (**Fig 4.4d**). With regards to shape, all of NPP, SDPP, and DDPP exhibited similar morphological features such as aspect ratio and circularity (**Fig 4.4e** and **4.4f**). These parameters show that pine pollen is physically intact after defatting with no significant collapsing or breaking occurring, thus confirming the mild nature of the defatting process.

Figure 4.4 Dynamic imaging particle analysis (DIPA) of natural and defatted pine pollen: Optical images of (a) NPP, (b) SDPP, (c) DDPP; and micromeritic characterization of NPP, SDPP, and DDPP using parameters including, (d) Diameter, (e) Aspect Ratio, and (f) Circularity. NPP: Natural Pine Pollen, SDPP: Single-Defatted Pine Pollen, DDPP: Double-Defatted Pine Pollen. Scale bars: 20 μm .

Contact Angle

Contact angle measurements indicated that pine pollen becomes more hydrophilic with increased defatting. Microparticle coatings of NPP exhibit a contact angle of $83.3 \pm 0.4^\circ$, with SDPP and DDPP exhibiting contact angles of 50.7 ± 1.9 and $17.3 \pm 9.6^\circ$, respectively (**Table 4.1**). The increase in hydrophilicity observed with defatting may be attributed to the removal of surface adhered lipidic compounds which are inherently hydrophobic.

Surface and Porosity Analysis

Nitrogen adsorption-desorption isotherm analysis showed that pollen defatting increased specific surface area, surface area of pores, volume of pores, and decreased average pore width. The N_2 sorption isotherms are characteristic of type IV isotherms typically associated with mesoporous materials.⁵¹ The NPP exhibited a Brunauer–Emmett–Teller (BET) theory surface area of $0.53 \pm 0.08 \text{ m}^2 / \text{g}$, and SDPP and DDPP with $1.36 \pm 0.15 \text{ m}^2 / \text{g}$ and $1.37 \pm 0.22 \text{ m}^2 / \text{g}$, respectively (**Table 4.1**). The increase in specific surface area with defatting may be attributed to exposing additional nanopores during the defatting process. The cumulative surface area of pores increased with the degree of defatting, with

NPP exhibiting $0.23 \pm 0.05 \text{ m}^2 / \text{g}$, and SDPP and DDPP with $0.46 \pm 0.05 \text{ m}^2 / \text{g}$ and $0.51 \pm 0.08 \text{ m}^2 / \text{g}$, respectively. Correspondingly, the cumulative volume of pores also increased with the degree of defatting, with NPP exhibiting $3.91 \pm 0.21 \times 10^9 \text{ m}^3 / \text{g}$, and SDPP and DDPP with $5.16 \pm 0.51 \times 10^9 \text{ m}^3 / \text{g}$ and $5.44 \pm 0.70 \times 10^9 \text{ m}^3 / \text{g}$, respectively. The increase in cumulative surface area and cumulative volume of pores indicates that defatting increases pine pollen porosity. The BET average pore size of defatted pollen is smaller than natural pollen, with NPP exhibiting an average pore width of $29.7 \pm 4.4 \text{ nm}$, and SDPP and DDPP with $16.0 \pm 0.8 \text{ nm}$ and $16.5 \pm 0.8 \text{ nm}$, respectively. This is due to the creation of multiple nano-sized new pores in defatted pollen that make the average pore size smaller as compared to natural pollen, which has a smaller pore number but a relatively greater pore size. The Barrett-Joyner-Halenda (BJH) desorption pore diameter distribution indicates that all of NPP, SDPP, and DDPP, exhibit a peak pore diameter of $\sim 45 \text{ nm}$ (**Fig 4.5a**), with SDPP and DDPP exhibiting a distribution of pores below 4.6 nm . The presence of nano-pores below 4.6 nm may be attributed to the removal of surface adhered lipidic compounds from the multilevel helical sporopollenin subunits of the exine as defined in previously published studies.⁵²

Morphological observations of the pollen surface, by SEM, before and after defatting, indicated that defatting effectively removes surface adhered lipidic material and exposes a higher density of nano-pores in the outer exine shell surrounding both the central cavity and the air-sacs (**Fig 4.5b**). Exposed visible pore sizes are in the range of 30 to 100 nm , with the majority of nano-pores being $\sim 50 \text{ nm}$. The exposure of numerous small pores may be expected to aid in enhanced and more rapid compound loading.

Table 4.1 Contact angle, cumulative pore surface area and volume and average pore width of Natural Pine Pollen, Single & Double Defatted Pine Pollen.

	NPP	SDPP	DDPP
Contact angle (degrees)	83.3 ± 0.4	50.7 ± 1.9	17.3 ± 9.6
BET surface area (m^2/g)	0.53 ± 0.08	1.36 ± 0.15	1.37 ± 0.22
Cumulative surface area of pores (m^2/g)	0.23 ± 0.05	0.46 ± 0.05	0.51 ± 0.08

Cumulative volume of pores (* 10⁹ m³/g)	3.91 ± 0.21	5.16 ± 0.51	5.44 ± 0.70
Average pore width (nm)	29.7 ± 4.4	16.0 ± 0.8	16.5 ± 0.8

NPP - Natural Pine Pollen, SDPP - Single-Defatted Pine Pollen, DDPP - Double-Defatted Pine Pollen

4.3.4 Chemical characterisation of natural and defatted pine pollen

Elemental analysis indicated that pollen defatting caused no removal of the pine pollen proteinaceous cytoplasmic contents. Percent nitrogen content of plant-based materials can be used to estimate percent protein content by application of a multiplication factor of 6.25.⁵³ The percent nitrogen content for NPP, SDPP, and DDPP was found to be 1.6 ± 0.0 %, 1.6 ± 0.1 %, and 1.6 ± 0.1 %, respectively (**Table 4.2**). The stability of percent nitrogen suggests that the defatting process has no significant effect on cytoplasmic constituents of the pollen.

Table 4.2 Elemental analysis of natural and defatted pine pollen

	NPP	SDPP	DDPP
C (%)	53.8 ± 0.3	53.4 ± 0.4	52.7 ± 0.1
H (%)	8.20 ± 0.21	7.90 ± 0.01	7.86 ± 0.03
N (%)	1.58 ± 0.01	1.62 ± 0.05	1.62 ± 0.06

NPP – Natural Pine Pollen, SDPP – Single-Defatted Pine Pollen, DDPP – Double-Defatted Pine Pollen

4.3.5 Aqueous permeability of natural and defatted pine pollen

Particle water uptake was explored by exposing natural and defatted pine pollen to both passive and vacuum-assisted loading methods. Passive and vacuum-assisted loading of water into natural and defatted pine pollen indicated that water filling rates increase with increased defatting, with DDPP exhibiting the fastest overall water uptake and greatest portion of particles loaded.

Passive filling

Passive loading showed that NPP is poorly penetrated by water with only 7.2 ± 0.8 % of the particles completely filled (becoming transparent) even after 24 h (**Fig 4.5c, d & 4.6**). Whereas, for passive loading, the proportion of full SDPP increases to 61.1 ± 12.0 % within 60 min and remains stable to 24 h, and the proportion of full DDPP increases to 73.7 ± 2.8 % within 30 min and remains stable to 24 h (**Fig 4.5d & 4.6**). Overall, these observations suggest that defatted pollen may facilitate the loading of hydrophilic molecules faster and more efficiently than natural pollen due to enhanced water flux.

Figure 4.5 Surface properties and water uptake of natural and defatted pine pollen: (a) Nitrogen adsorption-desorption isotherms with pore diameter estimations of NPP, SDPP, and DDPP; (b) Scanning electron micrographs of central cavity and air-sac surfaces of NPP, SDPP, and DDPP; (c) Optical microscope images of unfilled and water-filled NPP; (d) Proportion of pollen particles with water-filled air-sacs with passive and vacuum-assisted loading. NPP: Natural Pine Pollen, SDPP: Single-Defatted Pine Pollen, DDPP: Double-Defatted Pine Pollen. Scale bars: (b) = 500 nm, (c) = 10 μ m.

Vacuum filling

Vacuum loading showed that air-sac water filling could be enhanced for defatted pine pollen with the application of a vacuum for only 5 min, while providing no enhancement with NPP. Short duration vacuum loading achieved 82.9 ± 14.3 % and 94.3 ± 4.1 % of full particles for SDPP and DDPP, respectively, with NPP exhibiting only 0.7 ± 0.9 % of full particles (**Fig 4.5d & 4.6**). The increased proportion of filled air-sacs and overall filling, observed with vacuum loading of defatted pollen, may be attributed to water being drawn into the air-sac cavity due air being forcefully extracted by a negative pressure differential resulting from the creation of an external low pressure region by the application of a vacuum.⁵⁰

Figure 4.6 Optical microscope images of water-filling of natural and defatted pollen by passive and vacuum-assisted loading methods at various time points. NPP: Natural Pine Pollen, SDPP: Single-Defatted Pine Pollen, DDPP: Double-Defatted Pine Pollen. Scale bars: 100 μ m.

4.3.6 BSA Encapsulation Optimization with natural pine pollen

Optimization of BSA loading in natural pollen was explored with vacuum loading to gain insight into the ideal parameters and limits of compound loading in NPP. After significant parameter variation, it was determined that the application of a vacuum with a pressure of 1 mbar, for 5 min, with a BSA loading concentration of 50 mg / ml, could achieve a maximum BSA loading efficiency in NPP of 5.8 ± 0.7 % (**Fig 4.7a,b,c**).

Loading Method and Parameter Optimization

Vacuum loading was shown to produce greater loading efficiencies than passive loading. During the first step of vacuum-assisted BSA loading optimization, it was determined that vacuum pressures of 1 mbar and 0.01 mbar produced maximum loading efficiencies which were statistically equivalent ($p = 0.25$). Overall, loading pressures of 1000, 100, 1, and 0.01 mbar produced loading efficiencies of 2.3 ± 0.4 %, 2.5 ± 0.5 %, 5.8 ± 0.7 %, and 6.5 ± 1.7 %, respectively (**Fig 4.7a**). Vacuum loading at 0.01 mbar achieved ~ 3 times greater

loading than passively loaded samples (1000 mbar), which is in line with existing research with other pollen species and particles^{54,55}.

During the second step of vacuum-assisted BSA loading optimization, it was determined that a vacuum application duration of 5 min was sufficient to achieve the maximum loading efficiency. Overall, vacuum application durations of 5, 15, 30, and 60 min produced loading efficiencies of 6.5 ± 1.7 %, 7.0 ± 0.8 %, 6.4 ± 0.9 %, and 6.5 ± 1.7 %, respectively (**Fig 4.7b**). Statistical analysis of this data indicates that loading efficiencies for varying vacuum application durations are statistically equivalent ($p = 0.95$ for the time points 5 min and 15 min). From observations during sample preparation, it appears that short vacuum application durations are adequate due to rapid evaporation of aqueous loading solutions under vacuum conditions.

Figure 4.7 Loading parameter and washing optimization for BSA compound loading in natural and defatted pollen: **(a)** Effect of vacuum pressure on BSA loading potential; **(b)** Effect of vacuum duration on BSA loading potential; **(c)** Effect of BSA loading solution concentration on BSA loading potential; **(d)** Scanning electron micrographs of BSA-loaded NPP at each washing step; **(e)** Loading efficiency of BSA-loaded NPP at each washing step; **(f)** Loading efficiency of BSA-loaded NPP, SDPP, and DDPP with application of optimized NPP washing protocol; **(g)** Loading efficiency of BSA-loaded NPP, SDPP, and DDPP with optimized washing protocols; **(h)** Scanning electron micrographs of BSA-loaded NPP, SDPP, and DDPP after application of optimized

washing centrifugation duration. NPP: Natural Pine Pollen, SDPP: Single-Defatted Pine Pollen, DDPP: Double-Defatted Pine Pollen. Scale bars: 10 μ m.

During the third and final step of vacuum-assisted BSA loading optimization, it was determined that a BSA loading solution concentration of 50 mg / ml achieved the maximum loading efficiency. Overall, BSA loading solution concentrations of 12.5 mg / ml, 25 mg / ml, and 50 mg / ml produced loading efficiencies of 3.0 ± 0.3 %, 4.5 ± 1.1 %, and 6.5 ± 1.7 %, respectively (**Fig 4.7c**). The data indicates that the amount of BSA in the BSA loaded pine pollen is directly proportional to the amount of BSA in the BSA loading solution.

Comprehensive optimization of BSA loading parameters for vacuum assisted loading of NPP highlighted the importance of high loading solution concentration and the application of an adequately low vacuum pressure, and indicated that maximum loading may be achieved within a short duration of vacuum application. To allow for the potential of further system variation, the loading parameters used for the remainder of this study were determined to be, the application of a vacuum with a pressure of 0.01 mbar, for 5 min, with a BSA loading concentration of 50 mg / ml.

4.3.7 Washing & loading optimization using defatted pollen

The influence of the washing of BSA loaded natural pollen was explored to optimize the washing conditions so as to achieve maximum loading efficiencies while ensuring adequate removal of surface adhered BSA. Based on SEM analysis of BSA-loaded NPP particle cleanliness for zero, one, and two water washes, it was determined that two water washes (0.5 ml each) resulted in adequate removal of surface adhered BSA (**Fig 4.7d & Fig 4.8a**). Overall, zero, one, and two water washes of BSA-loaded NPP produced loading efficiencies of 28.4 ± 2.4 %, 13.8 ± 1.0 %, and 6.5 ± 1.7 %, respectively (**Fig 4.7e**). However, application of the NPP two-wash protocol to BSA-loaded SDPP and DDPP resulted in lesser loading, with SDPP and DDPP producing loading efficiencies of 4.2 ± 2.2 % and 2.9 ± 2.0 %, respectively (**Fig 4.7f**). Due to defatted pollen exhibiting a more rapid uptake of water, further optimization of the washing protocol was undertaken with a reduction of washing cycles and centrifugation time. Centrifugation durations of 3 min (2 washes of 0.5 ml each), 2 min (1 wash of 1 ml), and 1 min (1 wash of 1 ml), for NPP,

SDPP, and DDPP, respectively, were found to produce loading efficiencies of $6.5 \pm 1.7 \%$, $8.7 \pm 1.2 \%$, and $10.6 \pm 0.9 \%$ (**Fig 4.7g**), and resulted in adequate removal of surface adhered BSA (**Fig 4.7h & Fig 4.8b**). Variations in loading and washing dynamics of natural and defatted pine pollen may be attributed to increases in porosity and water permeability with increased defatting.

Figure 4.8 Scanning electron micrographs of natural and defatted BSA-loaded pine pollen during washing protocol optimization: (a) washing optimization of NPP; and (b) optimized washing for NPP, SDPP, and DDPP. NPP: Natural Pine Pollen, SDPP: Single-Defatted Pine Pollen, DDPP: Double-Defatted Pine Pollen.

Based on DDPP facilitating maximum loading, further variation of loading solution volumes indicated that the initial loading solution volume of 1 ml per 50 mg pollen (20 μ l / mg) achieved an optimal loading efficiency of 10.6 ± 0.9 %. By retaining a loading solution concentration of 50 mg / ml and reducing the amount of loading solution used, it was determined that volumes of 0.25, 0.33, and 0.5 ml, produced maximum loading efficiencies of 4.5 ± 0.6 %, 5.3 ± 1.3 %, and 4.5 ± 0.5 %, respectively (**Fig 4.9**). The reduction in loading efficiencies associated with reduced loading volumes may be attributed to an inadequate volume of loading solution to completely wet the 50 mg of pine pollen used for loading. By increasing the volume of loading solution, it was determined that volumes of 1, 2, 3, and 4 ml, produced maximum loading efficiencies which were statistically equivalent to ($p = 0.41$ for 1 ml and 4 ml), with loading efficiencies of 10.6 ± 0.9 %, 10.1 ± 1.4 %, 8.5 ± 1.3 %, and 9.6 ± 1.6 %, respectively (**Fig 4.9**).

Figure 4.9 Loading efficiency quantification for double-defatted pine pollen (DDPP) with varying loading solution volume.

4.3.8 Loading Distribution Analysis using CLSM

Loading distribution analysis based upon CLSM imaging of FITC-BSA-loaded NPP, SDPP, and DDPP, indicated that under optimum loading conditions, the majority of pollen particles exhibit some degree of loading, and that loading occurs predominately in air-sacs (**Figs 4.10a and 4.10b, 4.11 & 4.12**). However, air-sac loading is typically restricted to the outer portion of the air-sac cavity and the central portion of the air-sacs remains empty. Based on pollen volumetric measurements above, the outer porous sexine structure comprises ~ 58 % of total air-sac volume. Calculations for estimating 100 vol.% loading of DDPP, indicate that the theoretical maximum potential BSA loading of pine pollen air-sacs equates to a BSA loading efficiency of ~ 18.0 %. Therefore, the BSA loading efficiency of 10.6 ± 0.9 % obtained with DDPP, highlights that the optimized BSA loading protocols which have been utilized are highly effective.

Based on the FITC-BSA loading being restricted to the outer porous sexine structure, as well as the previous observations of water loading of air-sacs, it appears that the intricate porous structure of the air-sac wall (sexine) acts as a filter, trapping large molecules, such as BSA (~ 65 kDa), within the porous shell structure (**Figs 4.10b and 4.10c**). To support this assertion, the 3D reconstructions of CLSM z-stacks (**Fig 4.12**) depicted in **Fig 4.10b**, show non-uniform loading of the NPP porous sexine structure, indicating that BSA loading solution may not pass freely between all sexine porous structure cavities. The reason for this effect being highlighted in NPP may be attributed to the presence of lipidic compounds blocking surface nano-pores present in the outer exine layer, which may inhibit initial loading of the some sexine cavities. Whereas, when the lipidic compounds are uniformly removed in the SDPP and DDPP, the loading of the porous sexine structure may be more uniform due to the passage of BSA loading solution through sexine surface nano-pores, rather than from internal flow between sexine cavities. Additionally, 2D z-stack slices of FITC-BSA-loaded DDPP show regions where FITC-BSA is restricted to the micron-sized sexine cavities (**Fig 4.10c**).

Loading proportion data from CLSM image analysis, and loading efficiency data from loading quantification studies, were normalized and compared, with both data sets exhibiting similar trends (**Fig 4.10d**). The similarity in loading proportion and loading efficiency trends provides support for the robustness of the loading analysis, and suggests

that the previous loading optimization studies have been effective. Overall, accurately elucidating compound distribution via CLSM helps to explain the apparently low loading efficiencies observed with natural and defatted pine pollen, and provides valuable insight into the potential for further loading optimization.

Figure 4.10 Confocal laser scanning microscopy (CLSM) analysis of vacuum-assisted FITC-BSA-loaded natural and defatted pine pollen: **(a)** Multi-particle images of DDPP without FITC-BSA loading, and NPP, SDPP, and DDPP with FITC-BSA loading; **(b)** Single-particle 3D z-stack reconstructions of DDPP without FITC-BSA loading, and NPP, SDPP, and DDPP with FITC-BSA loading; **(c)** 2D and 3D images of FITC-BSA loaded NPP and DDPP highlighting FITC-BSA entrapped within the pine pollen porous sexine structure; **(d)** Comparison of trends between

normalized CLSM loading proportion data and conventional loading efficiency data, indicating a high degree of similarity. NPP: Natural Pine Pollen, SDPP: Single-Defatted Pine Pollen, DDPP: Double-Defatted Pine Pollen. Scale bars: (a) (b) = 10 μm ; (c) = 2 μm .

Figure 4.11 Confocal laser scanning microscopy (CLSM) images of FITC-BSA-loaded pine pollen for natural and defatted pollen. NPP: Natural Pine Pollen, SDPP: Single-Defatted Pine Pollen, DDPP: Double-Defatted Pine Pollen. Scale bars: 10 μm .

Figure 4.12 Confocal laser scanning microscopy (CLSM) z-stack images of unloaded natural pollen and FITC-BSA-loaded pine pollen for natural and defatted pollen. NPP: Natural Pine Pollen, SDPP: Single-Defatted Pine Pollen, DDPP: Double-Defatted Pine Pollen.

4.3.9 Tableted Formulations and Release profiles

Achieving targeted delivery of sensitive compounds, such as proteins, to the intestinal tract typically requires the use of a co-encapsulant to provide adequate gastric protection and allow compound release in a particular environment at a fixed rate.^{50,54 56} In this study, only natural co-encapsulants were explored for facilitating the delivery of sensitive proteins to the intestinal tract. BSA-loaded DDPP tablets were prepared with either xanthan gum or alginate. Xanthan gum based multiparticulate tablets utilized dry xanthan gum powder as a binder in varying proportions, whereas, alginate based multiparticulate tablets utilized ionotropically cross-linked alginate as a coating layer, with varying numbers of coatings.

Release from pollen-BSA powder & tablet

Initial release studies were conducted with BSA-loaded DDPP powder before and after tableting. Before tableting, BSA-loaded DDPP powder exhibited a burst release profile, wherein 100 % release was observed within 5 min in both simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) solutions (**Fig 4.13a**). The same release pattern was observed with natural pine pollen as well, indicating the natural pore size of pine pollen is large enough to facilitate instant release. After the tableting of BSA-loaded DDPP powder only, some delayed release of BSA was observed. Tablets were exposed to SGF for three hours, followed by exposure to SIF so as to simulate gastrointestinal tract transit resulting from oral delivery. Three hours in SGF resulted in a release of 80.1 ± 3.0 % with the remaining BSA releasing within another 15 min in SIF (**Fig 4.13b**). The delayed release observed from the tableting alone may be attributed to the physical robustness of the tablet, with internal BSA-loaded DDPP requiring greater time to become hydrated and release BSA.

Tableting using binder & Coating of BSA -loaded pollen

The use of xanthan gum as a binder in the tableting of BSA-loaded DDPP powder produced varying degrees of sub-optimal controlled release for targeted intestinal delivery depending on the proportion of xanthan gum used. Overall, the addition of xanthan gum in weight fractions of 1, 2.5, 5, 10, 20, and 30 % w/w, resulted in the release of 70.6 ± 2.0 %, 38.5 ± 14.5 %, 28.3 ± 4.8 %, 14.1 ± 9.9 %, 8.1 ± 9.4 %, and 14.7 ± 2.45 % of BSA, respectively,

within an initial three hours in SGF, with an additional release of 27.4 ± 0.6 %, 46.7 ± 4.7 %, 46.7 ± 8.7 %, 40.2 ± 2.8 %, 44.9 ± 7.7 %, and 18.1 ± 3.4 % of BSA, respectively, with another 24 hours in SIF (**Fig 4.13c**). A formulation using only Xanthan Gum-BSA mixture as tablets (Cont.) was also explored to see if the pollen had any significant effect on the release profiles. There was minimal release observed both in SGF (16.5 % in 3hrs) & SIF (7% for 24 hrs) with a total of 23.6 % release at the end of the study. The data indicates that increasing the xanthan gum % slows the release of BSA in SGF, but also results in incomplete drug release especially with higher xanthan gum fractions. Delayed and incomplete overall BSA release may be attributed to increasing proportions of xanthan gum increasing tablet stability, leading to limited compound diffusion from intact stable tablets. However, the addition of xanthan gum during tableting, in a proportion of 20 % w/w, provides the best sub-optimal controlled release profile with only 8.1 ± 9.4 % release in SGF for three hours, and an additional 44.9 ± 7.7 % release in SIF for another 24 hours.

Figure 4.13 *In vitro* release profiles of BSA from powdered and tableted BSA-loaded DDPP: (a) BSA release from BSA-loaded DDPP in SGF (pH 1.2) and SIF (pH 7); (b) BSA release from BSA-loaded DDPP powder and tablets with 3 h SGF incubation followed by SIF incubation; (c) Xanthan gum weight % effect on BSA release from BSA-loaded DDPP tablets with xanthan gum as a binder, with 3 h SGF incubation followed by SIF incubation; (d) Alginate coating number effect on BSA release from BSA-loaded DDPP tablets coated with ionotropically crosslinked sodium alginate, with 3 h SGF incubation followed by SIF incubation. DDPP: Double-Defatted Pine Pollen, SGF: Simulated Gastric Fluid, SIF: Simulated Intestinal Fluid.

The use of alginate for coating BSA-loaded DDPP powder tablets provided the most ideal release profile for targeted delivery to the intestinal tract. A single coating cycle was achieved by dipping tablets in a 2 % aqueous sodium alginate solution, followed by ionotropic cross-linking in a 4 % calcium chloride solution. Overall, the addition of ionotropically cross-linked alginate coatings with 1, 2, and 3 coating cycles, resulted in the release of 88.1 ± 4.9 %, 2.6 ± 0.3 %, and 2.0 ± 0.0 % of BSA, respectively, within an initial three hours in SGF, with an additional release of 15.8 ± 8.3 %, 96.0 ± 2.9 %, and 25.0 ± 10.8 % of BSA, respectively, with another 24 hours in SIF (**Fig 4.13d**). The data indicates that a single coating cycle is inadequate to provide desired release dynamics, and that three coating cycles excessively inhibits BSA release in simulated intestinal conditions. However, the application of two coating cycles provides optimal controlled release profile with minimal release in SGF for three hours (2.6 ± 0.3 %), and near complete release in SIF over an additional 24 hours (96.0 ± 2.9 %). Another control tried out was BSA (13 mg) + 150 mg of sodium alginate double coated with sodium alginate cross-linked by Calcium Chloride (marked as Cont. in **Fig 4.13d**). This is a control that is close to our working formulation except that the defatted pollen has been replaced with sodium alginate to make a tablet. There was minimal release observed both in SGF & SIF with a total of 5.39% release at the end of the whole study. This shows that DDPP does play a role in modulating the BSA release favorably. Based on the these observations, tableting and dual coating of BSA-loaded DDPP may be used to provide an effective all natural formulation for targeted delivery to the intestinal tract.

Tablet Mass and Dimensions

Tableting of BSA-loaded DDPP powder tablets with varying portions of xanthan gum or coatings of sodium alginate resulted in some variation in tablet morphology. Basic DDPP

tablets were prepared with 163.1 ± 0.6 mg of BSA-loaded DDPP, with a diameter of 13.04 ± 0.01 mm and thickness of 1.21 ± 0.01 mm (**Table 4.3**). Xanthan gum based tablets were prepared by incorporating an additional 1 to 30 wt% of dry xanthan gum, with final tablet weights ranging from 164.2 ± 0.2 mg to 234.3 ± 0.3 mg, diameters ranging from 13.04 ± 0.01 mm to 13.10 ± 0.05 mm, and thicknesses ranging from 1.21 ± 0.01 mm to 1.76 ± 0.01 mm. Alginate based tablets were prepared by providing 1 to 3 coats of ionotropically cross-linked alginate, with final tablet weights ranging from 168.8 ± 1.9 mg to 219.0 ± 4.8 mg, diameters ranging from 12.97 ± 0.05 mm to 12.92 ± 0.03 mm, and thicknesses ranging from 2.27 ± 0.08 mm to 3.05 ± 0.12 mm. Overall, the weight and thickness of the tablets increased with the % of xanthan gum and number of alginate coatings, while the diameter remained nearly constant due to the die press used.

Table 4.3 Details of tableted BSA-loaded DDPP, with and without binder (xanthan gum) or coating (sodium alginate).

	Mass (mg)	Diameter (mm)	Thickness (mm)
DDPP Tablet (No coating or binder)	163.1 ± 0.6	13.04 ± 0.01	1.21 ± 0.01
DDPP + 1% XG tablet	164.2 ± 0.2	13.09 ± 0.05	1.21 ± 0.01
DDPP + 2.5% XG tablet	166.2 ± 0.6	13.11 ± 0.07	1.22 ± 0.03
DDPP + 5% XG tablet	169.0 ± 0.6	13.13 ± 0.02	1.29 ± 0.03
DDPP + 10% XG tablet	180.7 ± 0.9	13.12 ± 0.06	1.36 ± 0.01
DDPP + 20% XG tablet	204.0 ± 0.8	13.11 ± 0.03	1.42 ± 0.03
DDPP + 30% XG tablet	234.3 ± 1.3	13.10 ± 0.05	1.76 ± 0.01
DDPP Tablet + 1 coat cycle	168.8 ± 1.9	12.97 ± 0.05	2.27 ± 0.08
DDPP Tablet + 2 coat cycle	174.7 ± 1.1	12.68 ± 0.46	2.87 ± 0.24
DDPP Tablet + 3 coat cycle	219.0 ± 4.8	12.92 ± 0.03	3.05 ± 0.12

Figure 4.14 Scanning electron microscope (SEM) analysis of multiparticulate BSA-loaded defatted pine pollen tablets before and after optimized alginate coating: **(a)** SEM micrographs of an uncoated multiparticulate BSA-loaded DDPP tablet depicting the tablet surface and cross-section, with the white arrow indicating the edge of the tablet cross-section; **(b)** SEM micrographs of an alginate-coated multiparticulate BSA-loaded DDPP tablet depicting the tablet surface and cross-section, with the white arrow indicating the surface of the tablet, and the red and blue arrows indicating the layers of crosslinked alginate. White boxes indicate areas of magnification. DDPP: Double-Defatted Pine Pollen.

Tablet Morphology

Based on two-coat alginate-based tablets providing the most ideal targeted delivery formulation we proceeded with morphological analysis of tablet surfaces and cross-sections, by SEM, before and after alginate coating. Tablets comprising only BSA-loaded DDPP exhibit a rough surface and layered cross-sectional structure due to close packing of compressed discrete pine pollen particles (Fig 4.14a). Tablets with an additional double coating of ionotropically cross-linked alginate exhibit a smooth surface and a coating layer of $\sim 30\ \mu\text{m}$, comprising 2 distinct sub-layers resulting from the double coating process (Fig 4.14b).

4.4 Conclusion

Pine pollen may be utilized as a microencapsulant for compound loading, and completely natural formulations based upon pine pollen microencapsulation technology can be used for targeted oral delivery applications. The removal of the lipidic compounds adhered to the outer pollen surface improves pollen wetting and exposes nano-channels present in the outer sexine layer, leading to increased water absorption and improved compound loading. Ensuring appropriate vacuum strength, vacuum duration application, loading solution concentration, and washing conditions, is required to achieve optimal compound loading. The porous sexine structure is shown to trap large BSA molecules ($\sim 65\ \text{kDa}$), and BSA loading is typically limited to the outer region of the hollow air-sac cavity with the central cavity region remaining empty. However, uniform exposure of nano-channels, resulting from defatting, ensures uniform filling of the micron-sized pores of the sexine, allowing for greater overall compound loading.

The development of a multiparticulate tableted formulation, with the application of a natural binder or enteric coating, achieved controlled release properties suited to targeted intestinal delivery of compounds, such as therapeutic proteins. Xanthan gum as a binder provided great ease in tablet preparation, however, exhibited incomplete compound release over a 24 h period. Ionotropically crosslinked alginate coating of defatted pine pollen tablets is a simple multi-step process, resulting in ideal release dynamics for intestinal delivery.

Overall, pine pollen exhibits many highly attractive microencapsulant properties and has been shown to provide an effective vehicle for microencapsulation. The large cargo capacity, ease of compound loading, competitive cost, abundant availability, extensive historical usage, and regulation-free oral consumption nature of pine pollen makes it very appealing for a wide range of practical applications, such as, foods, natural cosmetics, traditional herbal therapeutics, or synergistic treatments incorporating modern pharmaceutical compounds, such as therapeutic proteins.

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Chapter 5 Dual molecular encapsulation with natural pine pollen

Natural pine pollen has been a rich source of nutrients over millennia now and has a unique bisaccate structure. It remains unexplored till now as to its potential for encapsulating molecules. Here we show uni-molecular loading of protein (FITC-BSA), dyes and drug using natural pine pollen, which displays distinctive compartmentalized air-sac loading(using vacuum) prominently with the large protein FITC-BSA , which was also verified later with FITC-IgG and pure central cavity loading with small dyes and drugs. Dual molecular encapsulation and delivery has been superior over single molecular equivalents in terms of therapeutics and offering multiple options for various medical purposes, like tracking and diagnosis. It has been challenging as well because of more involved variables and has been shown with synthetic particles mostly, which suffer from bio-toxicity issues at working concentration. Here we also show dual molecular loading of dye and FITC-BSA, drug and FITC-BSA into natural pine pollen through simple loading methods. This study builds toward the use of natural particles for dual-drug therapy and as theranostic models.

* This chapter was submitted substantially as: Arun K. Prabhakar, Michael G. Potroz, Soohyun Park, Eijiro Miyako* and Nam-Joon Cho* titled: "Microencapsulation with Plant-Based Multi-Cavity Microparticles: Nanoporous Microstructures, Loading Optimization, and Controlled Release" to Particles & Particle Systems Characterisation

5.1 Introduction

Pine SECs were found to suffer from structural issues and defatted pine pollen, even though retained the natural morphology, they are subject to regulations given the organic solvent treatment. Natural pine pollen on the other hand has been consumed by humans for health-benefits and is regulation-free. In the previous chapter, natural pine pollen has been shown to be capable of protein encapsulation and this necessitates the need for further exploration for its loading potential (i.e.) types of molecules that can be encapsulated, which is done here. Molecular encapsulation and delivery using carriers have been pressing issues as concerning molecules, which are unstable and are thus prone to degradation during transit in the biological system. This instability could be due to pH¹, aggregation², denaturation³ etc. and is crucial especially in the field of drug delivery, where drug design or discovery is super-expensive and highly time consuming⁴. The encapsulate molecules can be either therapeutic (drugs) or diagnostic (dyes) in nature, which are loaded individually or in multiple, where more than a single molecule is loaded into an encapsulant. In the field of drug delivery, enhanced targeted cytotoxicity and thus better therapy has been shown with dual-drug loaded delivery systems⁵⁻⁷ as compared to single drug molecules, where it overcomes drug resistance and reduces side-effects associated with increased single drug concentrations. Molecular carriers, apart from displaying good loading, should also be tracked within the body to ensure site-specific delivery (for minimal side effects) and to also assess their clearance post-delivery, which has been done using MRI⁸, fluorescence⁹ and other techniques¹⁰. Another application that deals with multiple molecular encapsulation is theranostics, which amalgamates both therapeutics and diagnostics^{11,12} and plays a significant role in personalised healthcare. There are few cases where a single molecule can serve both purposes (i.e) a NIR dye with photothermal effect for killing cancer cells¹³, but mostly involves using multiple molecules like quantum dots with drugs, micelles with encapsulated dyes and siRNA etc.¹⁴⁻¹⁷. The dual-molecular loading potential of the carrier augments its biological value, but also complicates the loading process as extra care has to be taken to optimize the loading and preserve both the loaded components. If both molecules involved are of similar size, charge, hydrophilicity etc., the loading process is simplified multi fold as compared to physico-chemically distinct molecules, which would require multiple loading steps. Another issue with loading

multiple molecules is that the more of the encapsulant would be needed to get significant concentrations of each component as the loading space is now divided between more components as compared to one. This amplifies the need for a greater amount of material and thus large-scale synthesis of a highly biocompatible material and here synthetic or highly processed carriers pose serious problems. Various nano¹⁸ and microparticles^{19,20}, mostly synthetic in nature and of polymeric origin²¹ have been used for molecular delivery and even though these have shown promise, they do suffer from biotoxicity issues with increasing concentrations, which limits their utility²²⁻²⁴. Natural particles^{25,26} or particles of natural origin^{27,28} with minimal processing are thus best suited for this and there have been some natural-origin particles used as theranostic models^{16,29-31}. Plant pollen is a natural encapsulant, which protects the male genes and other enclosed biomolecules with its double-layered wall structure composed of a cellulosic intine and a sporopollenic-exine and is produced in copious amounts, which makes it a perfect fit for such applications. Natural spores and pollen like lycopodium³² and sunflower³³ have been used for microencapsulation purposes displaying good loading. In terms of overall process simplification, it is preferable to use natural pollen, but most of pollen/spore applications has revolved around Sporopollenin Exine Capsules (SECs)³⁴⁻³⁷, one of its processed forms since they offer more loading space with the sporoplasm and intine being removed, apart from reduced allergenicity. These SECs have been used for multiple encapsulation applications with all of them dealing with single type of molecule like proteins, cells etc.³⁸⁻⁴¹. Dual or multiple molecular loading has been mostly experimented with synthetic materials as mentioned previously with natural particles mildly explored and none so involves pollen, one of nature's most abundant resources. As pollen naturally encapsulate multiple biomolecules necessary for plant life, they seem a very practical option for multiple molecular loading with space being the limiting factor. This is where multi-compartmental (i.e) saccate pollen come into play, where the empty air-sacs can be looked at natural SECs themselves. They offer vital space for molecular loading apart from their sporoplasm-holding central compartment. More the number of air-sacs the better it is for encapsulation purposes, facilitating multiple molecular loading with little or no processing involved in the process.

In this study, five molecular compounds with different sizes and charges were chosen as a therapeutic and diagnostic model to explore their encapsulation behavior into natural pine pollen followed by release. The encapsulated molecules were fluorescein-isothiocyanate-labeled bovine serum albumin (FITC-BSA) and FITC-modified immunoglobulin G (IgG) as model therapeutic proteins; doxorubicin HCl, an anti-cancer drug; and two organic dyes: Nile Red and Calcein. In addition, the structure of BSA molecules in natural pine pollen was also investigated by circular dichroism (CD) to determine whether pollen can stabilize and protect encapsulated molecules and thereby function as future effective drug delivery systems.

5.2 Experimental Section

5.2.1 Materials

Natural *Pinus Massoniana* pollen was purchased from China. Nile Red, Doxorubicin HCl, Calcein, BSA, and FITC-BSA were obtained from Sigma-Aldrich (Singapore).

5.2.2 Washing of natural pine pollen

Natural pine pollen (30 g) was washed with water and vacuum-filtered 4 times (1 liter each) to remove dust & other smaller plant debris, followed by filtration through a 100 μ m nylon mesh to separate out the larger fibers, while the smaller pollen particles passed through. The pollen was then freeze-dried to get the clean dry natural pine pollen powder.

5.2.3 Dimensions and Zeta Potential measurements

All dimensions were estimated using the Draw ProteinDimensions plugin in Pymol using PDB or chemical files.

Zeta Potential is the potential difference between the material its solution near its surface, where an double layer is formed due to ionic attractions. The first layer (stern layer) is formed by strongly attached counter-ions followed by a loosely attached ionic layer (diffuse layer) consisting of a mixture of ions. Slipping plane is the outermost boundary of the diffuse layer after which the ions in solution move independently of the material surface. The charge distribution of the layers is determined by the surface charge of the material. The potential difference between the material surface and the slipping plane is

the zeta potential and is an indicator of the material stability in solution. Zeta Potential is measured by measuring the electrophoretic mobility of the material in solution under a voltage.

Zeta potential of all the encapsulates: FITC-BSA (10 mg/ml), Calcein (0.1 mg/ml), Doxorubicin (0.5mg/ml), Nile Red (1mg /ml), FITC-IgG (10 mg/ml) was measured using a Zeta Cell (DTS-1070) with 1ml of solution used.

5.2.4 Molecular loading of natural pine pollen & loading efficiency calculation

Passive loading

Natural pine pollen (10 mg) was mixed with 200 ul of FITC-BSA (10,20 & 50 mg/ml), Calcein (0.1 mg/ml), Doxorubicin HCl (0.5mg/ml), Nile Red (1 mg /ml), FITC-IgG (10 & 20 mg/ml) all separately, followed by putting them on a shaker (at 500 rpm) for 24 hrs after which they were washed with suitable solvents (water or ethanol), freeze-dried and observed using CLSM.

Vacuum Loading

Natural pine pollen (10 mg) was mixed with 200 ul of FITC-BSA (10 mg/ml), Calcein (0.1 mg/ml), Doxorubicin HCl (0.5mg/ml), Nile Red (1 mg /ml), FITC-IgG (10 mg/ml) all separately and the samples were subject to a vacuum of 100 mbar for 2 hrs after which they were washed with suitable solvents (water or ethanol), freeze-dried and observed using CLSM.

Dual molecule loading

Natural pine pollen (10 mg) was added to 200 ul of the following encapsulates for passive loading first : Doxorubicin HCl (1 mg/ml) and Nile Red (0.5 mg/ml) both separately followed by washing. Then 1 ml of FITC-BSA(10 mg/ml) was added and subject to vacuum loading at 100 mbar for 2 hrs after which the formulation was washed, freeze-dried and observed using CLSM.

Loading Efficiency estimation

Natural pine pollen (50 mg) was mixed with 2 ml of FITC-BSA (10 mg/ml), Calcein (0.1 mg/ml), Doxorubicin HCl (2.5mg/ml), Nile Red (0.1 mg /ml) all separately and the samples were subject to a vacuum of 100 mbar for 2 hrs after which they were washed with suitable solvents (water or ethanol), freeze-dried and estimated for their loading efficiencies as explained in section 3.2.4 (5mg of formulation was used) using their individual calibration curves.

5.2.5 Molecular Release from natural pine pollen

10 mg of the above loaded formulations were immersed in 4ml of HCl (20% ethanol for Nile Red alone) and PBS (20% ethanol for Nile Red alone) separately with suitable blanks (unloaded natural pine pollen) with 1ml of solution taken out at suitable time points and replaced with fresh solution and release profiles calculated as in section 4.2.8.

5.2.6 Confocal Laser Scanning Microscopy Analysis (CLSM)

FITC-BSA, FITC-IgG, Nile Red, Calcein and Doxorubicin HCl-loaded natural pine pollen were mounted on sticky slides with Vectashield® and scanned via CLSM as explained in in section 3.2.5.

5.2.7 Circular Dichroism (CD)

The secondary structure of presented proteins was characterized by circular dichroism (CD), which uses circularly polarised light and measures the absorption difference between left and right handed circularly polarised light. A beam of polarized light oscillates sinusoidally in a single plane. The sinusoidal wave is the resultant of two vectors of equal length (tracing out circles), one which rotates clockwise (E_R) and the other which rotates counterclockwise (E_L) and the waves are 90 degrees out of phase with each other. Asymmetric molecules when they interact with light show differential absorption towards right and left handed circularly polarized light. CD can be used to study protein folding, study mutation effects on protein stability and also protein interactions. It requires very less sample concentration (in μM) and the results are got in few hours.

Chirascan spectropolarimeter (Model 420, AVIV Biomedical Inc.) with a 0.01 cm path length cuvette (Hellma) was used for this. The concentration of all prepared samples was measured by UV spectrometer (Boesco-S220, Germany) at 280 nm. Spectra data were collected in triplicate at 25 °C, wavelengths ranging from 190 to 260 nm with 1 nm bandwidth with a step size of 0.5 nm and time constant of 0.1s. Baseline scans were recorded using the same parameters as the protein solvent (PBS or natural pine pollen protein in PBS) and subtracted from the respective data scans with protein samples. The final corrected averaged spectra were expressed in mean residue molar ellipticity ($[\theta]$) and presented spectra were smoothed by the Savitzky-Golay method. Proteins have alpha helices, beta sheets and random coils as part of their secondary structure. The fractional helicity (fH) of the peptides was calculated as follows^{42,43} :

$$fH = ([\theta]_{222}^{obs} - 3000)/(-36000 - 3000)$$

where $[\theta]_{222}^{obs}$ is the molar ellipticity at 222 nm.

5.2.8 BSA-Loading, release and CD

Vacuum Loading

100 mg of pollen was mixed with 2 ml of 50 mg/ml of BSA and the mixture was vortexed for 5 secs followed by vacuum loading at 100 mbar for 2 hrs after which it was washed. This was done in triplicates.

Release

55 mg of the above formulation was dissolved in 1ml of PBS (pH 7) to give a final BSA concentration of 8.72 uM after 100 % release, which was in 15 mins. Pure BSA was made upto a concentration of 11 uM was used as pre-loading reference for CD.

CD of native and post-release BSA

CD was performed as explained in section 5.2.6. During release along with BSA, protein from natural pine pollen, is also released and so a solution containing purely protein from natural pine pollen is used as the blank for post-release secondary structure estimation.

5.3 Results and Discussion

5.3.1 FITC-BSA loading

Passive loading

Standard passive and vacuum loading techniques, which have been used with other natural pollen³³ were used to load the above molecules into natural pine pollen. The highest concentration (50 mg/ml) of FITC-BSA was used initially for passive loading (2 hrs) to ensure maximum loading, but even this resulted in low loading(**Fig 5.1A**). Extended passive loading showed that with more time, the loading increased (**Fig 5.1B**), where with greater FITC-BSA uptake into the pine pollen, the background becomes lighter. Both the time-point samples (2 & 24 hrs) were partially washed as to avoid high background fluorescence but not excessively as to preserve the loading pattern. Blue colour is from pollen autofluorescence and helps to identify the shape outline. Passive loading of lower FITC-BSA concentrations (10 & 20 mg/ml) after 24 hrs resulted in relatively much poorer uptake (**Fig 5.2**), with all the particles being unloaded. Even with 50 mg/ml, the overall loading pattern was still poor with only a few particles filling up and that too only in the central cavity (**Fig 5.3B, D**).

Figure 5.1 CLSM images of natural pine pollen particles encapsulated with FITC-BSA (50 mg/ml) by passive loading for (A) 2 hrs (B) 24 hrs. Scale bars are all 20 μ m.

Vacuum loading

In case of BSA encapsulation of pollen, vacuum loading has been shown to load better as compared to passive over shorter times previously^{32,33}. This was verified visually where partially washed (to avoid highly fluorescent background) passively loaded particles for 2 hrs were almost completely empty (**Fig 5.1A**) as compared to 2 hour vacuum loaded particles, which even with a 5-fold lower BSA concentration (10 mg/ml) showed good air-sac loading (**Fig 5.3A,C**) and minimal or no central cavity loading.

Figure 5.2 CLSM images of natural pine pollen particles passively loaded with FITC-BSA at a concentration of (A) 10 mg/ml (B) 20 mg/ml. Scale bars are all 20 μm.

Compartmentalized Loading

The poor passive loading efficiency could be due to the negative charge on FITC-BSA (**Table 5.1**), where a previous study indicated that a small negative charged molecule (Evans Blue of size 1.4 nm) was unable to load into the central cavity of processed pine SECS⁴⁴. BSA is a relatively bigger molecule and so both size and charge could play a role in its exclusion from the central cavity. The same study gives a molecular size cutoff of 4 nm for the central cavity and thus supports this strongly, as SECs are supposed to be more porous as compared to natural pollen due to processing effects. The absence of air-sac loading with passive loading can be explained by the fact that air sacs, even though are water-wettable, however don't fill up to maintain buoyancy⁴⁵, while the central cavity is much more water-permeable to avoid dehydration of the sporoplasm⁴⁶. Applying a vacuum

however, forces the BSA molecules into the air-sacs facilitating active loading through suction created out of low pressure⁴⁷, with such loading absent in the central cavity due to its packed nature (lack of air spaces) along with a relatively more elaborate membrane structure. As for intra-compartmental spatial localization, in case of passive loading, the FITC-BSA mostly loads into the exine of the central cavity (**Fig 5.3B**), while vacuum loading fills up the sexine of air sacs completely, with the exemption of the small inner space which is protected by a meshy network (**Fig 5.3A**). As mentioned above, BSA has already been looked into for encapsulation with pollen (Sunflower) and spores (Lycopodium) with its spatial localization looked into using FITC-BSA. All the above pollen species are mono-compartmental, with molecules loading into the corpus (pollen body) whereas pine pollen being bi-saccate (corpus + two saccus) is multi-compartmental and thus unique. This structural uniqueness gives it a distinct loading-method dependent spatial pattern, where vacuum loading was able to load the air sacs quite efficiently with no central cavity loading and this was reversed with passive loading, but with much a weaker pattern. For such molecules passive + vacuum loading would be best to load the pine pollen to its fullest capacity, where all the compartments would be loaded (**Fig 5.4**).

Table 5.1 Molecular Weight, Dimensions and Zeta Potential of all the molecules encapsulated into natural pine pollen

Encapsulates	MW (Da)	Dimensions (nm)	Zeta Potential (mV)
Calcein	622.5	$1.6 \times 0.9 \times 0.7$	1.33 ± 0.01
Doxorubicin HCl	543.5	$1.5 \times 1.0 \times 0.4$	-2.91 ± 0.06
Nile Red	318.3	$1.4 \times 0.7 \times 0.4$	-0.52 ± 0.17
FITC- BSA	>66500	$7.8 \times 7.3 \times 5.9$	$- 23.3 \pm 4.61$
FITC- IgG	>150000	$16.3 \times 12.6 \times 6.9$	-5.64 ± 1.46

[MW]:Molecular Weight

Figure 5.3 CLSM images of vacuum and passive loading of FITC-BSA into natural pine pollen: Single particle image of (A) Vacuum loaded sample (10 mg/ml) & (B) Passively loaded sample (50 mg/ml). Multiple particle image of (C) Vacuum loaded sample (10 mg/ml) & (D) Passively loaded sample (50 mg/ml). Scale bars are all 10 μm .

Figure 5.4 CLSM image of natural pine pollen particle fully loaded with FITC-BSA by passive loading (50 mg/ml) initially for 24 hrs followed by vacuum loading (10 mg/ml) at 100 mbar for 2 hrs. Scale bars is 20 μm .

5.3.2 Dyes and drug loading

After this interesting observation, the other 3 molecules were experimented with to see if they also exhibited similar compartmentalization. No such pattern was observed, with all

of them loading into the central cavity irrespective of the loading method. Nile red (a near charge neutral molecule) and Doxorubicin HCl (slightly negatively charged) (**Table 5.1**, loaded appreciably into central cavity within 2 hrs of passive loading (**Fig 5.5**) given their smaller size as compared to FITC-BSA. Passive loading was done for 24 hrs for all the molecules, while vacuum loading was done for 2 hrs, based on the FITC-BSA loading times. Calcein, Doxorubicin HCl and Nile Red exhibited strong central cavity loading with both passive and vacuum loading (**Fig 5.6 & 5.7**). This also shows that the central cavity isn't full, but does have empty space.

Figure 5.5 CLSM images of natural pine pollen particles passively loaded with (A) Doxorubicin HCl (0.5 mg/ml) (B) Nile Red (1 mg/ml) for 2 hrs. Scale bars are all 20 μm .

Figure 5.6 CLSM images of natural pine pollen encapsulated with Calcein (0.1 mg/ml), Doxorubicin HCl (0.5 mg/ml) & Nile Red (0.5 mg/ml) through: (A) Vacuum loading at 100 mbar for 2hrs and (B) Passive loading for 24 hrs. Scale bars are all 10 μ m.

Figure 5.7 CLSM images of natural pine pollen particles encapsulated with Calcein(0.1 mg/ml),

Doxorubicin HCl (0.5 mg/ml) & Nile Red(1 mg/ml) through (A) Vacuum (100 mbar- 2hrs) & (B) Passive loading (24 hrs). Scale bars are all 10 μm .

Washing and molecular retention

Calcein seems to load into the sexine of air sacs with partially washed particles, but it can be seen that after washing there isn't any left unlike FITC-BSA (**Fig 5.8**), which could be attributed to the size, where the bigger protein molecule is entrapped within the air sac mesh and is retained, while the smaller ones are washed out. The same could be said of Calcein and Doxorubicin too, while Nile Red being in the same size range, due to its strong lipophilicity remains attached to the exine even after the washing and stains it (**Fig 5.6 & 5.7**). Washing is a necessary step to obtain clean formulations and it does remove some of molecules from the central cavity also. Even with unwashed formulations, we can see that the relatively smaller molecules are also localised in the sexine, with no loading in the hollow space of the air-sacs, which highlights the impermeable nature of the sexine mesh. Thus from the above observations we can speculate that a molecule similar to FITC-BSA, in terms of size and charge would exhibit such compartmentalized loading as compared to smaller, neutral & positively charged molecules.

Figure 5.8 CLSM images of natural pine pollen particles vacuum loaded with Calcein (0.1 mg/ml) which are: (A) Partially washed (B) Fully washed. CLSM images of natural pine pollen particles vacuum loaded with FITC-BSA (10 mg/ml) which are: (C) Partially washed (D) Fully washed. Scale bars are all 20 μm .

5.3.3 Dual molecular loading

After single molecule loading was established using the above 5 molecules, dual molecular loading was explored using FITC-BSA, Nile Red & Doxorubicin HCl. Nile Red and Doxorubicin were loaded separately into the central cavity by passive loading (24 hrs) and the formulation was washed followed by vacuum loading of FITC-BSA, which loaded into the air-sacs as expected. This resulted in the unique compartmentalised dual molecular loading, where we have one molecule in the central cavity and another in the air sacs (**Fig 5.9A, B** respectively). Multiple particles showing similar trend shows that loading is consistent across the formulation (**Fig 5.9C, D**). So we have both a dual therapeutic (drug +protein in **Fig 5.9A**) and a natural theranostic (dye+ protein in **Fig 5.9B**) proof-of concept models here. This is highly interesting for applications as apart from the carrier being natural and safe, it offers the option of molecular segregation, where we can have different molecules in either compartment (central cavity or air sacs), which is useful for

preventing unfavourable interaction between the encapsulated molecules. Consider for example an enzyme and another molecule to be delivered to the GI tract where the molecule happens to be a substrate for the enzyme. If both these are packed into the same space then the molecule could be metabolized to its product state and would be delivered in a different state from the feed state and thus would prove ineffective. In such cases compartmentalized loading with no inter-molecular access would prove valuable. If both molecules involved are of similar nature, loading is simplified, but in this case the distinct nature of the involved molecules helps to achieve this unique loading pattern.

Figure 5.9 CLSM images of dual molecule loading into natural pine pollen: Single particle CLSM image of natural pine pollen encapsulated with (A) Doxorubicin passively followed by vacuum loading of FITC-BSA and (B) Nile Red passively followed by vacuum loading of FITC-BSA. Multiple particle CLSM images of natural pine pollen encapsulated with (C) Doxorubicin passively followed by vacuum loading of FITC-BSA and (D) Nile Red passively followed by vacuum loading of FITC-BSA. Scale bars are all 10 μm .

5.3.4 Immunoglobulin G loading

Extending this unique FITC-BSA loading trend to another large protein used in therapy, IgG⁴⁸⁻⁵¹ was chosen and subject to passive loading (10 & 20 mg/ml) for 24 hrs. There was no loading with both the concentrations as expected because of the bigger size of IgG as compared to FITC-BSA (**Fig 5.10 & 5.11B,D**). With vacuum loading (10 mg/ml) however, air sac loading similar to FITC-BSA was observed where the FITC-IgG was trapped inside the sexine of the air sacs (**Fig 5.11A,C**). The spatial localisation of FITC-IgG also seems similar to that of FITC-BSA with the sexine layer completely filled followed by the inner hollow space, which remains empty due to the impenetrable sexine.

This shows that size plays a major role in the air sac loading and that large proteins can be loaded into natural pine pollen air sacs, while small molecules take up the central cavity. These small molecules apart from synthetic drugs and dyes can also be small proteins with functional significance⁵²⁻⁵⁴ and thus potential dual protein delivery vehicles could also be developed. Apart from dyes and drugs, another group of medically significant small molecules are nutraceuticals⁵⁵ and have been found to have therapeutic action⁵⁶ too apart from nutritional effects. These include molecules like resveratrol, green tea extract, lycopene apart from proteins and peptides^{57,58} etc., some of which suffer from stability issues and need of an encapsulant⁵⁹ for their successful delivery. Thus from the above observation it can be seen that the potential of natural pine pollen as a delivery vehicle for bioactives comprising of pharma & nutraceuticals is immense indeed. It is of note that intact natural pine pollen is being used here and thus the permeability is limited, which gives rise to such compartmentalized loading. If defatted pine or pine SECs had been used, the small molecules⁴⁴ and large molecule (i.e) FITC-BSA³⁴ would have loaded into both the central cavity and the air sacs due to increased permeability as a result of processing. Dual molecule loading would still have been achieved, but would be non-compartmentalised.

Figure 5.10 CLSM images of natural pine pollen particles passively loaded with FITC-IgG (10 mg/ml) (A) Single unloaded particle and (B) Multiple particles showing poor loading. Scale bars are all 10 μm.

Figure 5.11 Single particle CLSM image of (A) Vacuum loaded FITC-IgG (10 mg/ml) & (B) Passively loaded FITC-IgG (20 mg/ml). Multiple particle CLSM image of (C) Vacuum loaded FITC-IgG (10 mg/ml) & (D) Passively loaded FITC-IgG (20 mg/ml). Scale bars are all 10 μ m.

5.3.5 Loaded molecules and their compartment of loading

Table 5.2 summarizes the spatial location of all the encapsulate molecules depending on the method of loading and it can be seen that all the small molecules (dyes and drugs) load in the central cavity irrespective of the loading method. The larger protein molecules load into the air sacs with vacuum loading and into the central cavity with passive. IgG being twice the size of BSA (**Table 5.1**) did not load into the central cavity at the tested concentrations.

Table 5.2 Spatial loading of all the molecules encapsulated into natural pine pollen

Encapsulate \ Loading Method	Passive	Vacuum
Calcein	C	C
Doxorubicin HCl	C	C
Nile Red	C	C
FITC- BSA	C	A
FITC- IgG	N	A

[C]: Central Cavity [A]: Air Sacs [N]: Neither Compartment

5.3.6 Molecular loading efficiency and Release Profiles

Molecular loading efficiency

Table 5.3 summarizes the loading efficiency (LE) of all the encapsulate molecules achieved though vacuum loading and it can be seen that the large protein molecule (BSA) has a low loading efficiency with 5% of the theoretical maximum loaded, which can be explained by its constrained loading space (only the air-sac sexine). Amongst the small molecules, calcein loads the best reaching 13.81% of the theoretical maximum followed

by Nile Red (7.53%) and Doxorubicin HCl (4.81%), all of which load into the central cavity.

Table 5.3 Loading efficiency of all molecules encapsulated into natural pine pollen

Encapsulates	Theoretical LE max(%)	LE (%)
BSA	50	2.49 ± 0.53
Calcein	0.398	0.055 ± 0.02
Doxorubicin HCl	9.09	0.438 ± 0.06
Nile Red	0.398	0.03 ± 0.007

Release Profiles

BSA, which was loaded into the air-sacs had a rapid release profile, where a 100% release was achieved in both HCl & PBS within 15 minutes (**Fig 5.12A**). Of all the small hydrophilic molecules loaded into the central cavity, Calcein released the quickest with all of it releasing in 5 minutes (**Fig 5.12C**), whilst Doxorubicin HCl had a relatively gradual release profile with a 100% release over a 1 hour period (**Fig 5.12B**). Nile Red had a much slower release profile, given its high lipophilicity, where it released upto 70 % over 1 hr (80% even after 24 hrs) in HCl and 100% in PBS during the course of an hour(**Fig 5.12D**), with a relatively flatter release pattern. This again shows the leaky nature of natural pollen, where co-encapsulants have to be used to control the release (as used in Chapter 4.). Extending this to dual molecular release, similar release patterns would be observed as the molecules would release from different compartments.

Figure 5.122 *In vitro* release profiles of molecules loaded into natural pine pollen through vacuum loading in HCl (gastric pH 1.2) & PBS (pH 7-Intestinal pH) separately (a) Bovine Serum Albumin (BSA) (b) Doxorubicin HCl (c) Calcein (d) Nile Red

5.3.7 Circular Dichroism of BSA pre-loading and post-release

Protein structure is highly significant for its effective function⁶⁰⁻⁶² and disruption of this structure results in a dysfunctional or non-functional protein. Structure decides the folding of the protein which exposes its active binding sites and thus makes it a functionally active (i.e) binding another protein, catalysing a reaction (enzyme) etc. As the BSA is forced into the air sacs, its structure has to be ascertained post-loading, which was probed using circular dichroism⁶³. The secondary structure was analysed pre-loading and post-release using % helicity (major secondary structural element of BSA), which was calculated using ellipticity and was found to be conserved (**Fig 5.13**). Pure BSA was found to be 71.03% helical and post release in the PBS, it was 72.29%. This shows that the loading process

doesn't affect the protein structure, which is very much conserved. All this points to the use of natural pine pollen as a promising carrier for protein therapeutics^{52,64-66}.

Figure 5.13 Circular Dichroism of native BSA before encapsulation and BSA post-release in PBS for 15 mins.

5.4 Conclusion

In summary, we have shown that large proteins can be loaded into the air-sacs of pine pollen through forced vacuum loading, demonstrated with bovine serum albumin initially and followed up with Immunoglobulin G, whilst passive loading was poor, with few particles showing pure central cavity loading. Small dye molecules like Calcein, Nile Red & FITC and a drug, Doxorubicin were also loaded through vacuum and passive loading techniques and were found to localize in the central cavity irrespective of the loading method. This is due to the size effects of the molecules, where the smaller ones are washed out of the air sacs, while the larger ones like BSA and IgG remain encapsulated. Of all the small molecules, FITC alone displayed poor loading, which could be because of its relative high positive charge. Dual molecular loading showed compartmentalized loading as expected from single molecule loading pattern where Doxorubicin or Nile Red loaded into the central cavity and FITC-BSA loaded into the air sacs with vacuum loading. Molecular loading was quantified and was found to be low due to compartmentalised loading, which was further restricted with BSA to only the sexine of air-sacs. The release was found to be

rapid as observed with other natural pollen like Lyocodium & Sunflower, with all the molecules releasing within 1 hr in HCl & PBS. Protein structure of BSA was conserved pre-loading and post-release from CD data, as evidenced from the helical content. This strongly supports the use of natural pine pollen for protein therapeutics and also as a natural theranostic model given its ability to encapsulate drugs and dyes too.

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Chapter 6 Conclusion and future work

The three previous chapters corroborate pine pollen as an effective microencapsulant, with its ability to encapsulate small and large molecules. The loading of proteins, dyes and drugs into natural pine pollen show that fragile pollen can be used in its unprocessed state, while defatting is a step-improvement to increase the loading. Pine SECs even though they suffer from structural concerns show that they are capable of protein loading, thus rendering all forms of pine pollen as a viable encapsulant and delivery vehicles, opening up its future applications which are discussed in this chapter.

6.1 Conclusion

Pollen and spores naturally protect the organism's genetic material and other sensitive biomolecules and keep it in a viable state for reproduction, thus proving to be highly effective natural encapsulants. This prompts the need to explore them for encapsulation of bioactives like therapeutic proteins, nucleic acids etc. apart from other functional molecules like synthetic dyes and drugs. This need is further instigated by the bio-toxicity issues faced by synthetic delivery systems like quantum dots, nanorods etc and the need for elaborate processing to convert natural and bio-friendly polymers into usable microcapsules. Many pollen and spore species have been explored for their processed forms lacking the allergenic protein, lipids, while few have actually gone onto demonstrate possible applications, with natural forms almost untouched upon. Pine pollen is one such mildly explored species, which is produced in large quantities, with its collection method being straightforward. It remains unexplored for its natural state loading potential and its rigorous processing to produce microcapsules, which has to be streamlined given its fragile nature to enable molecular loading in its interior.

Here initially pine sporopollenin exine capsules (SECs) were produced by single step acidolysis procedures using defatted pollen as the base with weak and strong acids. The processing conditions were optimized with weak acid (85% phosphoric acid), which has been used with SEC processing previously and then extended to strong acids: hydrochloric and sulphuric. The structure was inspected for its integrity through DIPA and SEM, with % intactness quantified for every processing condition used. Enzymatic action was used for one of the acidolysis procedure to demonstrate sporoplasmic removal with the structure being preserved. The two most optimized conditions, as determined by a balance between nitrogen removal and % of structurally intact particles, were then loaded with BSA through vacuum loading and then compared with unprocessed defatted pollen for its loading efficiency, where it was found to load thrice as effectively on a mass basis. Spatial loading patterns indicated that SECs loaded in both the central cavity and the air-sacs while the defatted pollen loaded heavily in the air-sacs with relatively lesser central cavity loading.

The field of protein therapeutics has been growing widely and so quantification of protein loading was done in the following study. Natural pine pollen was used, as SECs were found to suffer from structural issues. BSA was loaded into natural pine pollen using

passive and vacuum loading, where vacuum loading was found to be better using loading efficiency estimation. Vacuum loading was optimized for its loading conditions like duration, vacuum pressure and BSA concentration. These conditions were then extended to defatted pollen, where defatting is a much milder process with no accompanying morphological changes as was verified and was found to enhance protein loading. Tunable release profiles are necessary to customize the delivery according to the site of interest and this was achieved here using natural binders like xanthan gum and coatings such as cross-linked sodium alginate with tableted forms, which provided gastric protection to the encapsulated BSA, facilitating release under small intestinal conditions.

Finally natural pine pollen was investigated for the encapsulation of functional molecules, since this aspect has been not explored, with pine pollen being looked at as super-food primarily, displaying anti-oxidant and anti-inflammatory properties. Firstly relatively large therapeutic proteins (FITC-tagged): Bovine Serum Albumin (BSA) and Immunoglobulin G (IgG) were encapsulated using simple passive and vacuum-assisted loading. Passive loading was poor, but with vacuum loading, these were encapsulated into the air-sacs with minimal or no central cavity loading observed resulting in distinct compartmentalized loading, which was uniform across the sample. Hydrophilic (Calcein) and hydrophobic dyes (Nile Red, FITC) and a hydrophilic anti-cancer drug, Doxorubicin Hydrochloride, which are small molecules were also probed for their encapsulation into natural pine pollen and were observed to fill the central cavity with the air-sacs remaining empty with both vacuum and passive loading. Extending uni-molecular loading to dual molecular loading, an all-compartment loaded pine pollen particle was got as expected, with dyes and drug occupying the central cavity and the large proteins occupying the air-sacs. Thus the segregated encapsulates here show a unique loading pattern, with potential applications like theranostic models, dual drug or protein loading etc.

On the whole, the potential of pine pollen for microencapsulation has been demonstrated in both natural and processed forms, with structural issues involved with SEC production. Extraneous molecular encapsulation thus adds to the therapeutic value of pine pollen, which inherently is a nutritional source and with compounded therapeutic and nutraceutical effects, this could tilt the field of microencapsulation in favour of plant-based natural particles. Future work involving the optimisation of pine pollen processing, spatial

loading and fine-tuning the release, which along with some potential future applications of this exciting material are listed below.

6.2 Future Work

Drugs, dyes and proteins have been loaded into pine pollen using basic molecular loading methods namely passive and vacuum-assisted. Tunable release profiles have been shown with encapsulated BSA though use of binders and coatings in tablets. Both mild and harsh processing techniques have been used to increase loading, with their effects on morphology assessed. All these are fundamental studies regarding the use of pine pollen as a microencapsulant. There is however optimization required for the above-mentioned work to make pine pollen an excellent microencapsulant and improve its value in the field.

6.2.1 Retaining natural morphology and structural integrity with Pine SECs

Pine SEC processing showed that even for the most optimal processing condition, only 80% of the produced SECs were intact with the rest of the SECs mostly fractured and some collapsing (Chapter 3). This is a fundamental investigation into Pine SEC production, which could be improved through further experimentation with other weak acids, ionic liquids and enzymatic removal as to preserve the final morphology (close to 100%) with effective protein removal at the same time.

6.2.2 Fine Tuning release under gastro-intestinal conditions

The BSA release profiles from Chapter 4 show that the protein can be protected under gastric conditions, with release under intestinal conditions. If the intended molecule is for the small and the large intestine this release profile is good enough. If the release is intended only for the small intestine, this release should be improved to give 100% release in 3-4 hrs, which is the average small intestinal residence time. This could be attained by coating 1% sodium alginate as the second coat layer (instead of 2 %), after the first layer coating using 2%. However, if the encapsulate molecule is intended for systemic absorption, this release profile has to be more rapid (i.e) near 100% release in 30 mins under pH 7 as this is the average duodenal residence time, from where it goes onto the liver through the portal vein and then into the blood circulation. For this purpose, the sodium alginate could be

lowered to 0.5% or lesser for the second coat. Controlled release in the gastric phase would require a different coating material that dissolves under gastric conditions. Xanthan gum could also be used depending on how much of the encapsulate needs to be released as incomplete release profiles were observed with increasing Xanthan Gum %.

6.2.3 Improving air-sac loading

The loading of the drugs and dyes haven't been quantified, but it can be seen that they don't load into the air sacs (Chapter 5), which contribute over 1/3 rd of the total pine pollen volume. This loading has to be maximized to fill up the air-sacs too with alternative methods of loading like compression loading maybe, since pine pollen is found to be morphologically stable as observed with tablets (Chapter 4). Even large proteins like BSA don't load the air –sac completely, with only the sexine layer filling up and the central cavity almost empty (Chapter 4), which could also be improved upon. Compression loading could be tried since the pine pollen tablets seem structurally stable with the pollen structure intact (Chapter 4). Low loading would mean that more of the encapsulant has to be given for the encapsulate to reach the required concentration, which would not be an issue as the encapsulant; pine pollen is regarded fit for human consumption, but would still be good from an encapsulation perspective, if the loading could be enhanced. Pine SECs have been found to load quite well in the air-sacs, but since they suffer from structural disintegrity, alternative processing methods must be explored.

6.3 Potential Future Applications using Pine Pollen as a Microencapsulant and More

Pollen in general has the advantage of being classified as generally regarded as safe (GRAS) and has no regulations on being used for human consumption^{1,2}, thus alleviating any bio-concerns. Pollen has been part of ancient medicine because of its healing properties, apart from nutrient composition. Some notable species include pine and bee pollen as mentioned before. Other species include cattail pollen also used in traditional Chinese medicine for nosebleeds, wound healing and shrinking cancers and camellia, a vitamin, protein and nucleic acid-rich pollen, that aids detoxification, improves immune system and memory with enhanced brain functioning. Raw and processed pollen are viable options for encapsulation, with processed forms (i.e) SECs being favoured over natural

forms for therapeutic applications given the extra loading space available, but with relatively greater biocompatibility concerns too.

The work done in this thesis, opens up a lot of potential applications for pine pollen for biological applications in different areas. Microencapsulation still remains the most valuable application, with pollen serving as the delivery vehicle in therapeutics and nutraceuticals, whilst topical formulations could make use of inherent UV protection property of sporopollenin. The buoyancy of pine pollen makes it a candidate for aerosol involved applications, where it could be put to industrial uses apart from medical (i.e.) nasal sprays etc. Some potential biological and industrial applications using microencapsulation involving pine pollen are discussed below.

6.3.1 Biological Applications using Microencapsulation

Pine Pollen for Nutraceutical and other food-related molecular delivery

As mentioned in the Introduction, nutraceuticals comprise an important group of bioactive molecules³, which are the crux of functional foods, that provide high nutrition and help in controlling diet-related diseases like obesity, heart-diseases, diabetes, brain-disorders, osteoporosis etc. Some examples include curcumin, lycopene, *vitamins*, *minerals*, pro & pre-biotics⁴ etc. The therapeutic effects of nutraceuticals apart from providing basic nutrition include-anti-cancer, anti-oxidative and anti-inflammatory⁵. Some nutraceuticals, similar to pharmaceuticals also suffer from low bioavailability due to low solubility, pH instability and permeability issues and need carriers for their efficient delivery⁶. Molecules like B-carotene, which have solubility and oxidation issues and aspartame, which is hygroscopic and has thermal instability can be encapsulated into pine pollen for better absorption by the body. Nanoparticles⁷ and food-matrix based design⁸ have been used for this purpose with biodegradable and biocompatible polymers. Protein⁹, carbohydrate¹⁰ and lipid based systems are examples of such systems which include protein nanoparticles, liposomes etc. Natural pine pollen as such is considered a super-food due to its rich nutrient composition. The fact that we can load more molecules into natural pine pollen (Chapter 5) enhances its nutritive and therapeutic value. Since controlled release has been demonstrated too by the use of coating, we can deliver these nutraceuticals to the intestinal region for maximal systemic absorption. Sporopollenin can also be functionalized¹¹ and

this can make the delivery more effective by using targeting molecules, increasing mucoadhesion, adding permeation enhancers etc.⁴, which will be a natural delivery system with improved therapeutic and nutritive functionalities.

Figure 6.1 Carriers used for nutraceutical delivery (A) Polymeric vehicles like nanoparticles, micelles etc. (B) Lipid based vehicles including liposomes, solid lipid nanoparticles (SLN) etc. that are more biocompatible (C) Inorganic carriers like quantum dots, silica nanoparticles, carbon nanotubes etc that are relatively less biocompatible¹².

Pine Pollen as floating drug delivery systems

Floating drug delivery systems (FDDS) are buoyant enough to float on the gastric fluid and are known to increase drug efficacy¹³ though extended residence time in the stomach¹⁴, whereas non-floating units tend to be cleared away easily and have drug levels below the therapeutic window. Gastric emptying time is a variable, which results in fluctuating molecular concentrations making controlled drug release difficult. FDDS are less dense

relative to the gastric layer, which aids floating and this is similar to that of mucoadhesion in the intestinal tract¹⁵, where the residence time of the drug delivery system is increased. A large size (7.5 mm) would also help to increase gastro-retentivity by avoiding passage through the pyloric opening with swelling systems being a good example. These floating systems could be effervescent (gas bubble producing) or non-effervescent, single or multiple units^{14,16}. Natural pine pollen floats in acid due to the buoyancy of its air sacs but has lower loading as compared to defatted pollen and SECs, which sink as the pollen fills up, due to increased porosity, but have relatively greater loading. So natural pine pollen as such can be used with optional thin layer of coating, as the release is found to be rapid (Chapter 4), which would still help retain its buoyancy. For the uncoated defatted pine pollen, the release is too rapid as was seen in Chapter 4. So if a thin layer of coating is applied in such a way that it can keep block the pores, but still keep the pollen afloat and control the release, it could be a natural floating drug delivery system useful for treating gastric cancers, ulcers, stomach infections etc.

A schematic of natural pine pollen for treating gastric cancer using dual drug therapy in a targeted manner is shown. The pine pollen is first surface functionalized by conjugating hyaluronic acid (HA) onto the surface using PEG-diamine as a linker, since the sporopollenin doesn't contain any amine groups as such, but does have carboxyl groups present. This hyaluronic acid will act as a targeting agent and bind to overexpressed CD44 molecules on the surface of gastric cancer cells¹⁷. Then the anti-cancer molecules, Doxorubicin (hydrophilic) and Camptothecin (hydrophobic) are loaded into the surface-functionalised natural pine by simple passive loading method (which is possible extending from Chapter 5). This loaded pine pollen can then be coated with Eudragit (E-100)¹⁸, which dissolves in acidic pH. This Eudragit is positively charged and can be ionically-coupled with negatively charged hyaluronic acid on top of the coating, which helps in primary CD44 targeting. The hyaluronic acid coupled to the Eudragit helps in targeting the gastric cells initially and as the Eudragit coating dissolves, the hyaluronic acid on the pollen surface is exposed, which attaches to the CD44 molecules and helps in retaining the targeting. The dissolving of Eudragit controls the release of the loaded drugs thus preventing excess concentrations.

Figure 6.2 Schematic of coated, surface-functionalised natural pine pollen for gastric cancer therapy. PEG-Polyethylene Glycol, HA- Hyaluronic Acid.

Pine Pollen for multi stage drug delivery

Pine pollen is too big for entry across the intestinal epithelium and can thus only serve as vehicle to protect and deliver molecules prone to acidity and enzymatic action into the intestinal region through oral delivery, whereafter the molecules are absorbed into circulation, which may seem good enough for some bioactive molecules, while others may need another level of guidance, where they are delivered into the cells from the circulation in a specific manner¹⁹. Pollen itself has been found to be absorbed into the bloodstream²⁰ after ingestion and this points to the concept of pollen inside pollen (similar to vesosomes with lipids), where pine pollen can be processed to increase pore size and smaller pollen, spore species like Ragweed, Chlorella etc (which have been loaded with molecules of interest) can be encapsulated into the processed pine pollen. This could also be applicable to other smaller synthetic nanoparticle drug delivery vehicle like quantum dots, nanoclusters etc, which don't have a core shell-structure and are in the size range of > 10 nm and thus can be encapsulated using minimally processed pollen. The open structure of such nanoparticles causes the loaded drug to release prematurely. To increase drug-carrier interaction strength to prevent leakage, the drugs have to be chemically attached to the carrier, which could modify their action apart from leaving them vulnerable to action of

acid, enzymes. In such cases a natural microcarrier would be highly beneficial to deliver the nanoparticle-drug system to the intestine after which the nanoparticles can penetrate the enterocytes and M cells to enter the circulation and release their payload specifically. Below is an example of such a microparticle guided drug delivery system, where graphene quantum dots (GQDs), which are drug loaded are loaded into pine pollen through passive loading after which the formulation is coated to offer gastric protection. Thus the drug – loaded GQDs release in the small or large intestine (tunable according to the coating) after which they can pass through the intestinal cells or enter the systemic circulation post-first pass effect and then into the cells. The quantum dots can be functionalized to make the drug delivery more tissue-specific and this process can be optimized in to increase the efficacy.

Figure 6.3 Schematic of multiphase drug delivery with drug loaded-quantum dots encapsulated into pine pollen through oral delivery tuned for small intestinal release.

Pine pollen for nasal sprays

Pine pollen is bi-saccate travelling through air for its pollination and is thus a potential candidate for aerosol preparation, where it could be used with nasal sprays. In conditions like rhinitis, the pH of the nasal mucosa rises to 8 from 6²¹ and thus suitably coated pine pollen with encapsulated bioactive like antihistamines could be used as part of therapy. Pine pollen has been shown to possess anti-inflammatory effects²², which could be useful in nose-related allergies and non-allergenic (pathogenic) cases too, where it could work synergistically. It could also be functionalized with nasal drug absorption enhancers like fatty acids, starch for improved delivery²³, which has been done with chitosan and other polymeric delivery vehicles. The size of pine pollen makes it too big to be used for lung - associated disorder though.

Figure 6.4 Factors to be considered for nasal drug delivery systems²³.

Pine pollen for taste masking and delivery of bioactives

Adaptogens are compounds, which result in physiological stabilization like increased stress resistance etc. and natural pine pollen is an adaptogen by itself possessing all component molecules necessary for physiological homeostasis²⁴. More such compounds like gingseng, resihi mushrooms, show increased stamina and relieve fatigue. Ginsensoides have been proposed as the active ingredient of ginseng with many varieties available like Siberian Gingseng, American gingseng etc., which are all unique in their action. These are mostly given in herbal forms, where the individual herbs or plants are ground and mixed into a

concoction and consumed. These potions have a bad –taste, which makes them second-choice as compared to other available medication, even though these possess lesser side effects. Some other bioactives isolated from plants include alkaloids, terpenoids, flavonoids etc, which are produced as secondary metabolites by plants and have been found to have physiological benefits²⁵. Some examples include taxol (a diterpenoid), morphine (alkaloid), calycosin-7-glucoside (isoflavone), caffeine, polyphenols, choline, green tea extract etc. Some of these bioactive molecules have a bad taste (mostly bitter), as do some drugs²⁶ and since sporopollenin exine capsules have been shown to have taste masking effects²⁷, pine SECs could be used here to for the delivery of such bioactives and adaptogen molecular extracts after their isolation.

6.3.2 Industrial Applications using Microencapsulation

Pine pollen for cosmetics

The cosmetics and the personal care products represent a huge market and has been dominated by synthetic products mostly. Sporopollenin layer of the exine of pollen has been shown to have UV protection²⁸ and this makes it a potential natural sunscreen product. It could also be used for other cosmetics and personal care materials as natural materials are preferred for such usage as compared to synthetic ones²⁹⁻³², which can be toxic to the human body³³⁻³⁵ and also the environment³⁶. Pollen can also be used for aroma encapsulation, which could be used as scents etc. Pine pollen and its SEC form could be a natural solution to the above issues and could help expand the market by providing a novel option.

Figure 6.5 UV absorption profile of proposed sporopollenin constituents³⁷.

Overall pine pollen has a huge potential in the field of microencapsulation, where it is potentially an excellent natural vehicle for oral delivery applications. The pine SEC production could be optimized further to reduce structural damage, which would increase their loading efficiency on a mass basis and these could be readily used for SEC applications like taste masking, cell encapsulation, as a microreactor, vaccination, drug and oil loading etc. which have been explored with lycopodium (mostly) and a few other species till date. The tripartite structure of pine along with its inherent nature to be buoyant gives its distinct characteristics like membrane permeability, multi-compartmental nature, floating tendency etc. Few proteins, dyes and drugs have been shown to be encapsulated here with simple loading methods, indicative of the potential of this natural encapsulant and laying a solid base for further exploration. The fact that natural pine pollen has been long used as food & medicine makes it preferred for human applications with more processed forms relatively less favored and thus may have to be tested for their biocompatibility. Pine pollen seems an exciting prospect, with some basic applications shown here that would stretch its potential and help in advancement and bettering of natural materials for microencapsulation and much more.

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Appendix

List of publication

1. Arun Kumar Prabhakar, Hui Ying Lai, Michael G. Potroz, Michael K. Corliss, Jae Hyeon Park, Raghavendra C. Mundargi, Daeho Cho, Sa-Ik Bang, Nam-Joon Cho, "Chemical processing strategies to obtain sporopollenin exine capsules from multi-compartmental pine pollen" J. Ind. Eng. Chem. (2017), **53**: p. 375-385

List of manuscripts under review

1. Arun K. Prabhakar, Michael G. Potroz, Ee-Lin Tan, Haram Jung, Jae Hyeon Park, and Prof. Nam-Joon Cho*; "Microencapsulation with Plant-Based Multi-Cavity Microparticles: Nanoporous Microstructures, Loading Optimization, and Controlled Release" to ACS Applied Materials & Interfaces
2. Arun K. Prabhakar, Michael G. Potroz, Soohyun Park, Eijiro Miyako* and Nam-Joon Cho*: "Spatially controlled molecular encapsulation in natural pine pollen microcapsules" to Particles & Particle Systems Characterisation