Sporoderm microcapsule extraction & application

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**NANYANG
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UNIVERSITY**

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**SPORODERM MICROCAPSULE EXTRACTION &
APPLICATION**

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING
2018

SPORODERM MICROCAPSULE EXTRACTION & APPLICATION

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

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in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2018

Statement of Originality

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

4 August 2017

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Date

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Michael Graeme Potroz

Abstract

The sporoderms of pollen and plant spores provide an ideal source of robust monodisperse capsules for microencapsulation applications. Plant-based sporoderms are nature's solution to protecting sensitive genetic material from harsh environmental conditions and offer a promising natural solution to the large-scale manufacture of microcapsules. The outer sporoderm layer (exine) of pollen and spores typically comprises a biopolymer, sporopollenin, with remarkable physicochemical stability, and the inner sporoderm layer (intine) typically comprises a combination of common cellulosic materials. It is important to note that the sporopollenin and cellulosic layers comprising the sporoderm are free from allergenic compounds commonly associated with pollen. Through standard wet chemistry processing methods, allergenic proteins and other contaminants may be completely removed. There currently exist a wide range of chemical processing protocols for extracting sporopollenin sporoderm microcapsules (S-SMCs), as well as various techniques for the loading of compounds within S-SMCs. However, there is still a need for a systematic analysis of the efficacy of S-SMC extraction steps, and key factors influencing the loading and release of compounds within S-SMCs. Therefore, in this thesis, detailed studies were undertaken of the most commonly utilized S-SMC extraction protocol, the applicability of this protocol to genetically distant sources of S-SMCs was explored, and the dynamics of compound loading and release from S-SMCs were elucidated. Standard S-SMC extraction steps involve, defatting of surface lipidic compounds, base-hydrolysis of proteinaceous cytoplasmic contents, acid-hydrolysis of cellulosic materials, and washing for removal of processing residues, under varying experimental conditions. Through these studies, it was shown that base-hydrolysis is redundant and may actually damage the integrity of the sporoderm from some plant species. It is now clear that defatting and acid-hydrolysis alone are adequate to isolate S-SMCs. Acid-hydrolysis temperatures can be reduced from 170°C to 70°C, and processing times can be reduced from 7 days to 5 - 30 hours. Additionally, it was observed that excessive duration acidolysis, based on existing standard protocols,

destabilizes the S-SMC microstructure and causes capsule fragmentation. With regards to mechanisms of S-SMC loading and release, it was identified that air bubble collapse drives S-SMC loading, and that methods of assisted loading are important to ensure the full loading of all S-SMC cavities. Through optimization of macromolecule loading it was possible to elucidate practical loading efficiencies and distributions, as well as to estimate theoretical maximum loading efficiencies. *In vitro* release studies resulted in both qualitative and quantitative analysis of release dynamics. Further studies with enteric coencapsulation allowed for the development of an S-SMC system for targeted intestinal delivery with tunable controlled release. Overall, these studies provide important insights into the ongoing exploration of these promising natural microcapsules and their potential for utilization in a wide range of industrial applications.

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From here I intend to go on and utilize my education to further humankind in the pursuit of a beautiful world for all. Some of us are blessed to have such gifts and opportunities in life. These gifts bring the capacity to help others and to create a kind and joyful world where all people are nurtured and can pursue their dreams. Many thanks to all.

Table of Contents

Abstract.....	i
Acknowledgements.....	iii
Table of Contents	v
Table Captions.....	xi
Figure Captions	xiii
Abbreviations.....	xxv
Chapter 1 Introduction	1
1.1. Hypothesis.....	2
1.2. Objectives and Scope	2
1.3. Dissertation Overview.....	3
1.4. Findings and Outcomes/Originality	5
Chapter 2 Background & Literature Review	9
2.1. Overview	10
2.1.1. Pollen Structure	10
2.1.2. Sporopollenin Overview	11
2.1.3. S-SMC Extraction Methods.....	16
2.1.4. S-SMC Compound Loading	19
2.1.5. Microcapsule Compound Release	21
2.2. Relevance of Thesis Work	22
2.2.1. S-SMC Extraction – <i>L. clavatum</i>	22
2.2.1. S-SMC Extraction – <i>H. annuus</i>	23
2.2.3. S-SMC Loading & Controlled Release	24
2.3. PhD in context of literature	26
References	27
Chapter 3 Experimental Methodology	35
3.1. Rationale for selection of methods and materials	36
3.1.1. S-SMC extraction	36

3.1.2. S-SMC compound loading	39
3.1.3. S-SMC tableting & coating	40
3.2. Sample Preparation	41
3.2.1. Materials	41
<i>Chapter 4. L. clavatum S-SMC extraction</i>	41
<i>Chapter 5. H. annuus S-SMC extraction</i>	41
<i>Chapter 6. H. annuus S-SMC loading & controlled release</i>	42
3.2.2. S-SMC extraction	42
<i>Chapter 4. L. clavatum S-SMC extraction</i>	42
<i>Chapter 5. H. annuus S-SMC extraction</i>	43
3.2.3. S-SMC compound loading	44
<i>Chapter 4. L. clavatum S-SMC extraction</i>	44
<i>Chapter 6. H. annuus S-SMC loading & controlled release</i>	45
3.2.4. S-SMC tableting and coating.....	46
<i>Chapter 6. H. annuus S-SMC loading & controlled release</i>	46
3.2.5. S-SMC compound release	47
<i>Chapter 6. H. annuus S-SMC loading & controlled release</i>	47
3.3. Characterization – Principles and Analysis.....	48
3.3.1. Elemental Analysis (Carbon, Hydrogen, Nitrogen - CHN).....	48
3.3.2. Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry	50
3.3.3. Dynamic Imaging Particle Analysis	52
3.3.4. Scanning Electron Microscopy.....	55
3.3.5. Confocal Laser Scanning Microscopy (CLSM)	57
3.3.6. UV/Vis Spectrophotometry	59
3.3.7. Statistical Analysis	61
References	61
Chapter 4 <i>L. clavatum</i> S-SMC extraction	63
4.1. Introduction	64
4.2. Experimental Methods	65
<i>Materials</i>	65
<i>Extraction of Sporoderm microcapsules (S-SMCs)</i>	66

<i>Surface Morphology Evaluation by Scanning Electron Microscopy (SEM)</i>	66
<i>Elemental CHN Analysis</i>	66
<i>Dynamic Image Particle Analysis (DIPA)</i>	66
<i>Confocal Laser Scanning Microscopy (CLSM) Analysis</i>	66
<i>Biomacromolecule Encapsulation by Vacuum Loading Technique</i>	66
<i>Encapsulation Efficiency</i>	66
<i>Statistical analysis</i>	66
4.3. Principle Outcomes	67
4.3.1. Analysis of S-SMC acid/base hydrolysis extraction	67
<i>Morphology of S-SMCs during extraction</i>	67
<i>Protein content of S-SMCs during extraction</i>	70
<i>Micromeritic properties of S-SMCs during extraction</i>	72
<i>Fragmentation of S-SMCs during extraction</i>	76
4.3.2. Acid hydrolysis only extraction	77
4.3.3. Macromolecular loading of S-SMCs	79
<i>Loading efficiencies & observation</i>	79
<i>Morphology & micromeritic properties of compound loaded S-SMCs</i>	82
4.4. Conclusions	83
References	84
Chapter 5 <i>H. annuus</i> S-SMC extraction	89
5.1. Introduction	90
5.2. Experimental Methods	92
<i>Materials</i>	92
<i>Extraction of sunflower sporoderm microcapsules (S-SMCs)</i>	93
<i>Elemental analysis</i>	93
<i>Dynamic image particle analysis (DIPA)</i>	93
<i>Confocal laser scanning microscopy analysis</i>	93
<i>Surface morphology evaluation by scanning electron microscopy (SEM)</i>	93
<i>Matrix-assisted laser desorption/ionization - time of flight (MALDI-TOF) Mass Spectrometry (MS)</i>	93

<i>Statistical analysis</i>	93
5.3. Principle Outcomes	94
5.3.1. Acid/Base hydrolysis extraction exploration.....	94
<i>Morphology of S-SMCs during HCl hydrolysis extraction optimization</i>	94
<i>Morphology & micromeritic properties of S-SMCs from H₃PO₄ extraction</i>	95
<i>Protein content of S-SMCs from various extraction protocols</i>	99
5.3.2. H ₃ PO ₄ hydrolysis S-SMC extraction optimization and analysis .	100
<i>Morphology & protein content of S-SMCs during H₃PO₄ extraction</i>	100
5.4. Conclusions	109
References	110
Chapter 6 <i>H. annuus</i> S-SMC loading & controlled release ...	113
6.1. Introduction	114
6.2. Experimental Methods	116
<i>Materials</i>	116
<i>Dynamic image particle analysis (DIPA)</i>	116
<i>Confocal laser scanning microscopy (CLSM) analysis</i>	116
<i>Surface morphology evaluation by scanning electron microscopy (SEM)</i>	117
<i>BSA loading into sunflower S-SMCs - mechanistic model</i>	117
<i>Bovine serum albumin (BSA) encapsulation into sunflower S-SMCs</i>	119
<i>Encapsulation efficiency</i>	119
<i>Tableting and enteric coating</i>	119
<i>In vitro drug release in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)</i>	119
<i>Statistical analysis</i>	119
6.3. Principle Outcomes	120
6.3.1. Mechanistic evaluation of protein loading	120
<i>Real-time compound loading observations</i>	120
<i>Mechanistic loading model</i>	121
6.3.2. Loading optimization and analysis	124

<i>Morphology & micromeritic properties of compound loaded S-SMCs</i>	<i>124</i>
<i>Loading efficiencies & distributions</i>	<i>125</i>
<i>Theoretical maximum loading efficiencies.....</i>	<i>129</i>
6.3.3. S-SMC Protein Release Analysis	130
6.3.4. Tableted S-SMC formulation for intestinal protein release.....	133
<i>Morphological analysis of tableted S-SMCs.....</i>	<i>134</i>
<i>Analysis and optimization of controlled release</i>	<i>136</i>
6.4. Conclusions	138
References	139
Chapter 7 Future Outlook.....	143
7.1. Introduction - S-SMCs from Pollen, Spores, & Algae.....	144
7.2. S-SMCs for Microencapsulation.....	146
7.2.1. S-SMCs for Microencapsulation in Functional Foods	150
7.2.2. S-SMCs for Microencapsulation in Herbal Therapeutics.....	153
7.3. S-SMCs as Novel Polymeric Biomaterials	156
7.3.1. S-SMCs for Cosmetics & Personal Care	160
7.3.2. S-SMCs for Novel Composite Biomaterials	163
7.4. Conclusions	165
References	167
Appendix	173
Research Publications	173
Patents	175

Table Captions

Table 4.1 Sporoderm microcapsules (S-SMCs): CHN composition

Table 4.2 Encapsulation of Bovine serum albumin into S-SMCs

Table 5.1 DIPA data of sunflower sporoderm microcapsules

Table 5.2 Sunflower sporoderm microcapsules: CHN composition

Table 5.3 Sunflower sporoderm microcapsules (S-SMCs): CHN composition

Table 5.4 Equivalent spherical diameter of sunflower S-SMCs

Table 6.1 Efficiency of Bovine Serum Albumin (BSA) encapsulation into S-SMCs

Table 6.2 Estimation of S-SMC theoretical maximum BSA-loading efficiency

Table 6.3 Details of tableted BSA-loaded S-SMCs

Table 6.4 Details of control study tablets

Figure Captions

Figure 2.1 Schematic depicting general basic structure of pollen. A sporoderm shell surrounds a cytoplasmic interior, with the sporoderm comprising an outer exine and inner intine. The exine typically comprises a robust biopolymer, sporopollenin. The intine typically comprises a combination of cellulosic compounds, such as, cellulose, hemicellulose, and pectin. The surface of many pollen species is coated with a thin layer of primarily lipidic compounds, collectively referred to as pollenkitt.

Figure 2.2 Hypothetical simplified structure of a sporopollenin building block. The substituents R can either be linked by ether bonds or, in the case of the two cinnamic acids, also through ester bridges (adapted from van Bergen et al. [24] and Barrier et al. [16]) NOTE: Some important confirmed characteristics and observations have not been taken into account: 1) methyl and hydroxyl groups have been neglected; and 2) the proportion of oxygen has largely been under evaluated.

Figure 2.3 Example of conventional acid/base-hydrolysis extraction method as utilized in literature [77] and comparison with an optimized extraction protocol resulting from studies outlined in Chapter 4.

Figure 2.4 Schematic of common S-SMC loading techniques and loading mechanisms applied to natural *L. clavatum* spores. A) Spore microstructure depicting uniform ridges distributed on the surface with natural sporoplasm constituents contained inside. B) Natural spores suspended in a macromolecule-containing solution for the uptake of macromolecules, the enlarged inset depicts macromolecule entry via nanochannels located within the *L. clavatum* microstructure. C) Spores encapsulating macromolecules indicated as green along with the natural sporoplasm constituents. D) Passive macromolecule loading technique involving the incubation of natural spores in the aqueous macromolecule solution. E) Compression technique involving the compression of a dry spore powder and incubating the resulting spore tablet in

the macromolecule solution for the uptake of macromolecules by the spores. F) Vacuum loading technique involving the application of a vacuum to a suspension containing natural spores and macromolecules. Adapted with permission from [82].

Figure 2.5 Trends in publications relating to S-SMCs for 2007 to 2017, comparing total publication numbers with the keywords “sporopollenin” and “microcapsule”, with total publication numbers with the keyword “sporopollenin” only.

Figure 3.1 Visual depiction of primary steps used in optimized acid/base-hydrolysis extraction protocol from first S-SMC study in Chapter 4. (A) Untreated spores. (B) Spore defatting using a refluxing set-up at 50 °C for 6 hr. (C) Solvent filtration and collection of defatted spores. (D) Drying of defatted spores at room temperature. (E) Spore acidolysis in polytetrafluoroethylene (PTFE) flask using 85% (v/v) phosphoric acid at 70 °C for 30 hr. (F) Acid filtration using vacuum filtration set- up. (G) S-SMC washing using a series of water, acid, base, and solvents washing steps. (H) S-SMC drying using vacuum oven at 60 °C and 1 mbar vacuum for 10 hr. (I) Intact, clean, and dried S-SMCs as a final product.

Figure 3.2 Visual depiction of primary steps used in vacuum-loading protocol from first S-SMC study in Chapter 4. (A) Intact, clean, and dried S-SMCs as a final product. (B) Mixing of S-SMCs and BSA solution. (C) Homogenization of S-SMCs and BSA solution. (D) Vacuum loading of BSA into S-SMCs. (E) BSA-loaded S-SMCs.

Figure 3.3 Schematic of elemental analysis system structure depicting basic operational principle. Sample loading, sample combustion, gas separation, detection, and analysis.

Figure 3.4 Schematic of matrix assisted laser desorption/ionization-time of flight mass spectrometer structure depicting basic operational principle.

Figure 3.5 Schematic of dynamic imaging particle analysis structure depicting basic operational principle. Images are collected of samples passing through the flow cell.

Figure 3.6 Dynamic imaging particle analysis (DIPA): Polystyrene microspheres as a standard ($50 \pm 1 \mu\text{m}$) (Thermoscientific, USA). Plots are representative graphs of (A) diameter, (B) circularity, (C) aspect ratio, and (D) edge gradient, obtained by the spline curve fitting of histogram data from 1000 well-focused particle images after triplicate measurements ($n=3$). (E) Representative microsphere images from DIPA.

Figure 3.7 Schematic of scanning electron microscope structure depicting basic operational principle. Images are built up line by line as electron beam scans over the specimen.

Figure 3.8 Schematic of confocal laser scanning microscope structure depicting operational principle. Images are built up line by line as electron beam scans over the specimen in x-axis and y-axis directions, and it is possible to create 3D images by scanning of multiple focal planes in a z-axis direction.

Figure 3.9 Schematic of ultraviolet-visible spectrophotometer depicting operational principle. Varying wavelengths of monochromatic light are passed through a sample and transmittance is measured.

Figure 4.1 Process of extracting sporoderm microcapsules (S-SMCs) from spores. (A) Schematic of plant spores and S-SMCs, (i) *L. clavatum* in its natural form, (ii) Spores containing sporoplasmic organelles, and (iii) S-SMC after removal of sporoplasmic organelles and biomolecules. (B) Flowchart of more eco-friendly processes to translate plant spores into S-SMCs.

Figure 4.2 Characterization of *L. clavatum* spores during different stages of pre-acid treatment by scanning electron microscopy. (A) Untreated *L. clavatum* spores are intact with uniform size, well defined microstructure, and cross section indicating sporoplasmic cellular organelles and biomolecules. (B) Defatted spores at different magnifications are intact and the cross section

reveals sporoplasmic biomolecules. (C) and (D) respectively show S-SMCs after 6 h and 12 h alkaline lysis indicating intact capsules with the cross section revealing residual proteinaceous debris and the cellulosic intine layer.

Figure 4.3 Scanning electron microscopic images of sporoderm microcapsules (S-SMCs) during different stages of acidolysis using 85 % (v/v) phosphoric acid. (A) S-SMCs after 5 h acidolysis indicating an intact, well defined microstructure with a clean empty inner cavity. (B), (C), and (D) respectively indicate empty, intact S-SMCs after acidolysis for 10 h, 20 h, and 30 h durations at different magnifications.

Figure 4.4 Characterization of sporoderm microcapsules (S-SMCs) after prolonged acidolysis by scanning electron microscopy. (A) S-SMCs after 60 h acidolysis indicating an intact, well defined microstructure with a clean empty inner cavity. (B) and (C) respectively indicate S-SMCs after acidolysis for 90 h and 120 h. (D) Commercial L-type *Lycopodium* S-SMCs at different magnifications.

Figure 4.5 Protein content in sporoderm microcapsules (S-SMCs) at different stages of treatment. The protein content is obtained from CHN elemental analysis data and is a measure of sporoplasm constituents in untreated spores and treated S-SMCs. Data represented is an average of triplicate measurements with standard deviation (n = 3).

Figure 4.6 Characterization of sporoderm microcapsules (S-SMCs) at various stages of treatment using dynamic imaging particle analysis (DIPA). Micromeritic properties of S-SMCs are displayed as column (A) Pre-acid treatment, column (B) Acid treatment using 85 % (v/v) phosphoric acid at 70°C, and column (C) Intact S-SMCs from 22 to 38 µm in diameter after acidolysis. Plots are representative graphs of diameter, circularity, aspect ratio, and edge gradient, obtained by the spline curve fitting of histogram data from 1000 well-focused particle images after triplicate measurements (n=3).

Figure 4.7 Representative DIPA images of intact sporoderm microcapsules (S-SMCs) at various stages of treatment.

Figure 4.8 Dynamic imaging particle analysis (DIPA): Commercial sporoderm microcapsules (S-SMCs). Plots are representative graphs of (A) diameter, (B) circularity, (C) aspect ratio, and (D) edge gradient, obtained by the spline curve fitting of histogram data from 1000 well-focused particle images after triplicate measurements (n=3). (E) Representative S-SMC images from DIPA.

Figure 4.9 Sporoderm microcapsule (S-SMC) fragmentation analysis based on dynamic imaging particle analysis (DIPA) at various stages of processing. (A) Fragment count from 1000 well-focused particle images based on particles in the size range of 7 to 22 μm . (B) Representative DIPA images of particles in the size range of 7 to 22 μm from natural untreated spores. (C) Representative DIPA images of particles in the size range of 7 to 22 μm after 120 h acidolysis treatment. Data represented is an average of triplicate measurements with standard deviation (n = 3). (scale bar is 20 μm).

Figure 4.10 Characterization of 30 h acidolysis-only sporoderm microcapsules (S-SMCs) using scanning electron microscopy (SEM), dynamic imaging particle analysis (DIPA), and confocal laser scanning microscopy (CLSM). (A) SEM images indicating an intact, well defined microstructure with a clean empty inner cavity. (B) Micromeritic properties of BSA-loaded S-SMCs. Plots are representative graphs of diameter, circularity, and aspect ratio, obtained by the spline curve fitting of histogram data from 1000 well-focused particle images after triplicate measurements (n=3).

Figure 4.11 Schematic of microencapsulation of bovine serum albumin (BSA) into intact sporoderm microcapsules (S-SMCs). (A) BSA loading by incubating S-SMCs in aqueous BSA solution under vacuum. (B) Uptake of BSA through nanochannels in S-SMC walls. (C) and (D) respectively represent a S-SMC during the loading process and a finally prepared BSA-loaded S-SMC.

Figure 4.12 Confocal laser scanning microscopy (CLSM) analysis of sporoderm microcapsules (S-SMCs) before and after treatment and BSA encapsulation. CLSM images in the first row are unprocessed *L. clavatum* spores indicating autofluorescence due to sporoplasmic cellular organelles and biomolecules. The second row indicates intact S-SMCs after 30 h acidolysis treatment with a clean and intact large inner cavity, and the third row indicates FITC-BSA loaded into 30 h acidolysis treated S-SMCs. (Scale bars are 10 μ m).

Figure 4.13 Confocal laser scanning microscopy (CLSM) Z-stack analysis of 30 h acidolysis-only S-SMCs loaded with FITC-BSA.

Figure 4.14 Characterization of BSA-loaded sporoderm microcapsules (S-SMCs) using scanning electron microscopy (SEM) and dynamic imaging particle analysis (DIPA). (A) SEM images of BSA-loaded S-SMCs. (B) Micromeritic properties of BSA-loaded S-SMCs. Plots are representative graphs of diameter, circularity, aspect ratio, and edge gradient, obtained by the spline curve fitting of histogram data from 1000 well-focused particle images after triplicate measurements ($n = 3$).

Figure 5.1 One-pot process to extract intact, clean, and conserved distinct morphological sunflower sporoderm microcapsules (S-SMCs); Schematic shows (A) and (B) Harvesting of natural sunflower pollen grains and processing to remove plant-based debris. (C). Scanning electron microscopic images of natural pollen with different magnifications. (D) S-SMC extraction processes involving alkaline lysis with KOH, acidolysis with HCl or phosphoric acid to remove all pollen constituents. (E) Washing steps using organic solvents and dilute acids to remove residual pollen materials to yield clean, intact S-SMCs. (F) Scanning electron microscopic images of S-SMCs after complete processing at various magnifications. (G) Different one-pot process approaches used to extract intact sunflower S-SMCs.

Figure 5.2 Scanning electron microscopic images of sunflower pollen grains and sporoderm microcapsules (S-SMCs) obtained from different extraction processes: (A) Natural sunflower pollen grains intact with uniform size and

well-defined spikes, pores and cross-section reveal pollen constituents. (B) S-SMCs after alkali (6 % potassium hydroxide) treatment (12 h). (C) S-SMCs after acidolysis using hydrochloric acid (6 M) process for 48 h.

Figure 5.3 Scanning electron microscopic images of sunflower sporoderm microcapsules (S-SMCs) after acidolysis using hydrochloric acid and extensive washing for different time points: (A) 10 h. (B) 20 h. (C) 30 h. (D) Trypsin washed S-SMCs, (E) Tween-20 washed S-SMCs.

Figure 5.4 Sunflower sporoderm microcapsules (S-SMCs) produced after one-pot process using phosphoric acid: (A) Scanning electron microscopic images of S-SMCs after acidolysis at different magnifications and a cross-section image. (B) Confocal microscopy analysis of natural sunflower pollen grains before and after one-pot processing: CLSM images in first row are natural pollen grains before processing and indicate autofluorescence due to the presence of terpenoid, phenolic, and carotenoid molecules. Second row indicates clean S-SMCs with complete removal of pollen constituents. (Scale bars are 10 μm).

Figure 5.5 Characterization of sunflower sporoderm microcapsules (S-SMCs) after one-pot extraction process. (A) Frequency histogram of particle diameter (μm) for 1000 untreated pollen and S-SMCs. Optical micrographs of (B) untreated pollen grains and (C) S-SMCs after one-pot acidolysis using phosphoric acid.

Figure 5.6 Protein content in sunflower sporoderm microcapsules (S-SMCs) produced by different chemical processes from CHN elemental analysis. The protein content is a measure of pollen protein constituents in unprocessed and processed S-SMCs and determined by using the percent nitrogen from elemental analysis with a multiplication factor of 6.25 to convert percent nitrogen into percent protein. Data represented is average of triplicate measurements with standard deviation ($n = 3$).

Figure 5.7 Optimized sunflower pollen sporoderm microcapsule (S-SMC) extraction: (A) Comparative analysis of protein removal efficacy following H_3PO_4 acidolysis, based on % nitrogen content; data is presented as the average of triplicate measurements with standard deviation ($n = 3$); (B) Scanning electron microscope cross-section images of a defatted sunflower pollen grain and S-SMC following 5 h H_3PO_4 acidolysis; and (C) Confocal microscopy analysis of a defatted sunflower pollen grain and S-SMC following 5 h H_3PO_4 acidolysis.

Figure 5.8 Scanning electron microscope images of sunflower sporoderm microcapsules (S-SMCs) following different durations of H_3PO_4 acidolysis: (A) without acidolysis; (B) 1 h; (C) 5 h; (D) 10 h; (E) 20 h.

Figure 5.9 Confocal microscopy analysis of sunflower sporoderm microcapsules (S-SMCs) following varying durations of H_3PO_4 acidolysis. (Scale bars 10 μm).

Figure 5.10 Residual protein content in sunflower sporoderm microcapsules (S-SMCs) following different periods of H_3PO_4 acidolysis, as measured with CHN elemental analysis. Data is presented as the average of triplicate measurements with standard deviation ($n = 3$).

Figure 5.11 Large molecule content analysis by MALDI-TOF for sunflower pollen and sporoderm microcapsules (S-SMCs) based on sample grinding and ethanol extracts: (A) Mass-to-charge ratio spectrum for pollen, S-SMCs, and MALDI matrix (CHCA) from 1000 m/z to 20000 m/z ; (B) Major mass-to-charge ratio peaks for S-SMCs; (C) Major mass-to-charge ratio peaks for pollen; and (D) Relative peaks heights of major pollen peaks, CHCA peaks, and major S-SMC peaks. Data is presented as representative of triplicate measurements.

Figure 5.12 Characterization of sunflower sporoderm microcapsules (S-SMCs) before and after extraction using phosphoric acid at varying time points: (A) Phosphoric acid treated S-SMCs; (B) Only intact S-SMCs.

Figure 5.13 Optical micrographs of sunflower pollen grains and S-SMCs produced by varying periods of H_3PO_4 acidolysis.

Figure 6.1 Schematic diagram showing the development of tableted sporoderm microcapsules (S-SMCs) for intestinal protein delivery.

Figure 6.2 Confocal laser scanning microscopy of BSA loaded sunflower S-SMCs based on passive and assisted loading. The second row depicts an enlarged view of a representative S-SMC indicating the evacuation of air from the S-SMC inner cavity. (Scale bars 10 μm).

Figure 6.3 Liquid loading into S-SMCs, measured by tracking encapsulated air bubbles in S-SMCs using confocal microscopy: (A) a representative time evolution of a bubble radius; (B) a comparative lag time (t_0) distribution for the 45 S-SMCs that showed loading; (C) a comparative loading time (τ) distribution for the 45 S-SMCs that showed loading; and (D) a comparison of Equation 7 and experimental data for 10 S-SMCs.

Figure 6.4 Scanning electron microscopic images of BSA-loaded sunflower sporoderm microcapsules (S-SMCs) with varying BSA-loading proportions: (A) 23 wt.%; (B) 37.5 wt.%; and (C) 54.5 wt.%. (Scale bars 10 μm).

Figure 6.5 Micromeritic properties of BSA-loaded sunflower S-SMCs: (A) diameter (μm); (B) circularity; (C) aspect ratio; for 1000 sunflower S-SMCs prior and following BSA-loading. The data obtained by the spline curve fitting of histogram data from triplicate batches ($n = 3$).

Figure 6.6 Confocal laser scanning microscopy (CLSM) analysis of FITC-BSA-loaded sunflower S-SMCs: (A) unloaded and FITC-BSA loaded S-SMCs; (B) 3D images constructed using z-stacks illustrate the inner cavity of the S-SMCs; (C) diagram depicting the average BSA loading based on fluorescence intensity per S-SMC, vol.%; and (D) comparative analysis of BSA-loading fraction, BSA : S-SMC, and S-SMC average loading, vol.%, with estimation of theoretical maximum loading. (Scale bars 10 μm).

Figure 6.7 Confocal laser scanning microscopy (CLSM) Z-stack analysis of sunflower S-SMCs before and after FITC-BSA loading at three different BSA-loading proportions.

Figure 6.8 Confocal laser scanning microscopy (CLSM) of sunflower S-SMCs before and after FITC-BSA loading at three different BSA-loading proportions: (A) Unloaded S-SMCs, and FITC-BSA loading at three different loadings; (B) Quantification of fluorescence intensity from FITC-BSA loaded S-SMCs at three different BSA-loading proportions.

Figure 6.9 *In vitro* release of BSA from S-SMCs, comparing BSA release study data and fluorescence intensity analysis data: (A) BSA-loaded S-SMCs in simulated gastric fluid, pH 1.2; (B) BSA-loaded S-SMCs in simulated intestinal fluid, pH 7.4. BSA release profile data is presented as the average of triplicate measurements with standard deviation ($n = 3$). Fluorescence intensity analysis data is presented as analysis of 10 regions ($n = 10$).

Figure 6.10 Scanning electron microscopic images of tableted BSA-loaded S-SMCs with and without 15 % (w/v) Eudragit L100: (A) surface prior to enteric coating; (B) cross-section prior to enteric coating, arrow indicates the cross-sectional direction; (C) surface following enteric coating; and (D) cross-section following enteric coating, arrows indicate the enteric coating layer.

Figure 6.11 *In vitro* release of BSA from enteric coated S-SMC tablets and controls. Tablet release profiles are based on incubation of 2 h in simulated gastric fluid, pH 1.2, and 8 h in simulated intestinal fluid, pH 7.4. Data is presented as the average of triplicate measurements with standard deviation ($n = 3$).

Figure 6.12 Scanning electron microscopic images of sunflower S-SMCs following their *in vitro* release SGF and SIF. (Scale bars 10 μm).

Figure 7.1 Overview of the potential for exploring pollen, spores, and algae as solutions to microencapsulation, novel composite biomaterials, and the implications for other and related areas of research and industry.

Figure 7.2 Overview of microencapsulation as a key research project. There exists potential for use in diverse industrial fields, and clear research goals and objectives which may be pursued.

Figure 7.3 Overview of microencapsulation for functional foods as a key application example. As a natural food-based microencapsulant, taste masking, improved product stability, ease of loading, and unique product branding are some advantages which may be obtained. A uniform dry-powder product line is a good example of a specific potential application of microencapsulation for functional foods.

Figure 7.4 Overview of microencapsulation for herbal therapeutics as a key application example. As a natural food-based microencapsulant, taste masking, improved product stability, ease of loading, and unique product branding are some advantages which may be obtained. A range of herbal therapeutics encapsulated within synergistic pollen species is a good example of a specific potential application of microencapsulation for herbal therapeutics.

Figure 7.5 Overview of novel polymeric biomaterials as a key research project. There exists potential for use in diverse industrial fields, and clear research goals and objectives which may be pursued.

Figure 7.6 Overview of cosmetics and personal care as a key application example. As a multi-functional natural material, UV protection, detoxification, improved product stability, and unique product branding are some advantages which may be obtained. A range of potential products, such as, novel sunscreens, novel skin detoxification creams, and functional microbeads, are good examples of specific potential cosmetics and personal care applications.

Figure 7.7 Overview of novel composite biomaterials as a key application example. As a multi-functional natural material, chemical resistance, UV

protection, biocompatibility, and mechanical robustness are some advantages which may be obtained. A range of potential products, such as, natural chemically resistant biopolymers, composite biopolymer sheets, and soft and flexible electronics, are good examples of specific potential novel composite biomaterials applications.

Abbreviations

2D	Two Dimensional
3D	Three Dimensional
ACS	American Chemical Society
BSA	Bovine Serum Albumin
CHCA	Cyano-4-Hydroxycinnamic Acid
CLSM	Confocal Laser Scanning Microscopy
CHN	Carbon, Hydrogen, Nitrogen
DHB	2,5-dihydroxybenzoic acid
DI	Deionized
DIPA	Dynamic Imaging Particle Analysis
EA	Elemental Analysis
FC	Flow Cell
FITC	Fluorescein Isothiocyanate
FTIR	Fourier Transform Infrared
GC-MS	Gas Chromatography–Mass Spectrometry
GRAS	Generally Recognized as Safe
H ₂ SO ₄	Sulphuric Acid
H ₃ PO ₄	Phosphoric Acid
HCl	Hydrochloric Acid
HF	Hydrofluoric Acid
HPLC	High-Pressure Liquid Chromatography
KOH	Potassium Hydroxide
m/z	mass-to-charge
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization – Time-of-Flight
MMNO	4-methylmorpholine N-oxide monohydrate
MQ	MilliQ
MS	Mass Spectrometry
NaOH	Sodium Hydroxide
NMR	Nuclear Magnetic Resonance
PBS	Phosphate Buffered Saline

PES	Polyethersulfone
PFA	Perfluoroalkoxy
PTFE	Polytetrafluoroethylene
RCF	Relative Centrifugal Force
RPM	Revolutions Per Minute
SEC	Sporopollenin Exine Capsule
SEM	Scanning Electron Microscopy
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
S-SMC	Sporopollenin Sporoderm Microcapsule
TCD	Thermal Conductivity Detector
TLC	Thin-Layer Chromatography
TOF	Time-of-Flight
UV	Ultraviolet
UV/Vis	Ultraviolet–visible
U.S. FDA	United States Food and Drug Administration
v/v	Volume / Volume
vol%	Volume Percent
w/v	Weight / Volume
wt%	Weight Percent

Chapter 1

Introduction

Microcapsules derived from various sporoderm sources represent huge potential as a biocompatible microencapsulation material. Pollen, spores, and algae are nature's solution to the microencapsulation of sensitive genetic material for protection against environmental extremes so as to ensure the evolutionary success of a multitude of species on earth. Microencapsulation has become a core technology at the heart of many industries and consumer products, yet nature's own solution has been largely overlooked as a source of potential encapsulants. Through further exploration and improved understanding of sporoderm microcapsule extraction, compound loading, and compound release, it may be possible to begin to find better, more ecofriendly, and more health promoting solutions to the key industrial technology of microencapsulation. This chapter provides an overview of the motivations driving this research, the objectives and scope of this work, the flow of the work that was pursued, and the primary findings of these endeavors.

1.1. Hypothesis

The overall hypothesis of this dissertation is that microcapsules derived from various sporoderm sources represent huge potential as a biocompatible microencapsulation material. Pollen, spores, and algae are nature's solution to the microencapsulation of sensitive genetic material for protection against environmental extremes so as to ensure the evolutionary success of a multitude of species on earth. Microencapsulation has become a core technology at the heart of many industries and consumer products, yet nature's own solution has been largely overlooked as a source of potential encapsulants. Through further exploration and improved understanding of sporoderm microcapsule extraction, compound loading, and compound release, it may be possible to begin to find better, more ecofriendly, and more health promoting solutions to the key industrial technology of microencapsulation. Towards these goals, the following testable hypotheses serve as the overall motivation for this work:

- Greater understanding of commonly used sporoderm microcapsule (S-SMC) extraction methods may allow for increased extraction protocol optimization.
- Commonly used S-SMC extraction methods may need to be modified for application to a diverse range of sporoderm sources.
- Greater understanding of commonly used S-SMC loading methods may allow for increased optimization of loading protocols and loading efficiency potentials.
- Utilization of SMDCs with standard controlled release methodologies may allow for development novel multiparticulate targeted delivery systems.

1.2. Objectives and Scope

The primary objective is to provide a more detailed analysis of techniques and methods commonly used in the extraction, loading, and application of S-SMCs. The goal is to contribute a series of systematic studies to the field of S-SMC research to further develop a solid foundation from

which others can continue to explore with greater clarity and standardization. This goal was pursued based on the following objectives.

1. Define and systematically explore the most commonly used S-SMC extraction protocol to determine the efficacy of each step, then, refine and optimize the explored S-SMC extraction protocol to improve protocol efficiency and to produce S-SMCs suitable for use in microencapsulation applications.
2. Explore the commonly used S-SMC extraction protocols with angiosperm-derived pollen to compare the efficacy of various established methods and determine the optimal S-SMC extraction method for application to angiosperm-derived pollen.
3. Analyze the application of standard S-SMC loading protocols to clearly elucidate loading mechanisms, requirements, results, and potential of compound loading within S-SMCs.
4. Apply standard controlled release methodologies to developing a multiparticulate targeted delivery system to achieve targeted delivery to the digestive tract.

1.3. Dissertation Overview

Studies began with a detailed analysis of the most commonly utilized S-SMC extraction protocol, applied to the most commonly extracted and studied S-SMC source material. The extraction method was based on acid/base-hydrolysis, and the raw source material was *Lycopodium clavatum* spores. The standard steps of defatting, base-hydrolysis, acid-hydrolysis, washing, and drying were applied to *L. clavatum* spores with experimental parameters based on the most commonly utilized protocols defined within S-SMC extraction literature. The primary goals of S-SMC extraction for capsules useful for microencapsulation are, the removal of cytoplasmic contents, removal of potentially allergenic proteins, and maintaining a stable hollow core-shell structure. Therefore, at each step, the particle morphology was carefully analyzed via scanning electron microscopy, confocal laser scanning microscopy, and dynamic imaging particle analysis, so as to visually confirm the degree of cytoplasmic content removal and the stability of the

hollow core-shell structure. The protein content was also determined at each step by elemental analysis so as to determine nitrogen content and the associated protein content. Finally, bovine serum albumin (BSA) was loaded, as a model macromolecule, so as to confirm the loading potential of the extracted *L. clavatum* S-SMCs.

To elucidate the generality of S-SMC extraction protocols, the application of commonly cited S-SMC extraction protocols were explored with *Helianthus annuus* pollen, as a representative angiosperm pollen with a distinctive tethered-spike surface morphology. Extraction processes were applied with various bases and acids commonly defined in the relevant literature, as well as additionally applying a process of enzymatic extraction. After determining the optimal hydrolysis solution, the hydrolysis extraction duration was further optimized. The particle morphology was carefully analyzed, at each step, via scanning electron microscopy, confocal laser scanning microscopy, and dynamic imaging particle analysis to confirm cytoplasmic removal and capsule stability. Protein content was analyzed by elemental analysis nitrogen content quantification and additionally supported by matrix assisted laser desorption/ionization time-of-flight mass spectrometry as a highly sensitive technique for protein detection.

With the successfully extracted *H. annuus* S-SMCs, loading mechanisms, washing requirements, loading distributions, and the theoretical maximum loading for BSA, as a representative macromolecule, were systematically explored. Commonly utilized S-SMC loading methods, passive and assisted loading, were implemented to elucidate aqueous solute loading mechanisms and requirements. This was followed up with determination of S-SMC washing requirements to effectively remove surface adhered loading compounds. Then loading efficiencies and distributions were determined for multiple loading solution concentrations, and from this it was possible to estimate theoretical maximum loading efficiencies. Confocal laser scanning microscopy (CLSM) was utilized to provide real-time loading observations and allow for mathematical modeling of loading dynamics. Scanning electron microscopy was implemented to determine loaded S-SMC washing requirements. Loading efficiencies were determined by ultraviolet-visible

(UV/Vis) spectrophotometry of aqueous extracts, and CLSM was utilized to observe loading distributions of fluorescently labeled BSA loaded into S-SMCs. Combining UV/Vis loading efficiency analysis data and CLSM loading distribution data allowed for robust estimations of theoretical maximum loading efficiencies.

To develop a multiparticulate targeted delivery system to achieve targeted delivery to the digestive tract, BSA-loaded S-SMCs extracted from *H. annuus* were utilized. *In vitro* compound release dynamics were explored from BSA-loaded *H. annuus* S-SMCs in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). Then standard pelleting and enteric coating methods were explored to develop a multiparticulate enteric-coated S-SMC pellet that may be used for targeted delivery of therapeutic compounds to the intestinal tract. BSA release profiles of non-pelleted BSA-loaded S-SMCs were obtained by UV/Vis spectrophotometry of aqueous extracts, and CLSM was implemented to provide real-time release observations of fluorescently-labeled BSA from multiple S-SMCs. Analysis of CLSM release data allowed for a direct comparison of UV/Vis release profile data. Pelleting of BSA-loaded S-SMCs was achieved by hydraulic pressing, and the morphology of non-coated S-SMC pellets and enteric-coated S-SMC pellets were observed by scanning electron microscopy (SEM). BSA release profiles of pelleted and enteric-coated BSA-loaded S-SMCs were obtained by UV/Vis of aqueous extracts at various time points during *in vitro* incubation in SGF followed by SIF. To highlight the stability of *H. annuus* S-SMCs, SEM was utilized to observe capsule morphology after all of BSA loading, washing, pelleting, coating, and release.

1.4. Findings and Outcomes/Originality

The systematic analysis of *L. clavatum* S-SMC extraction based on standard acid/base-hydrolysis was the first of its kind study looking at the efficacy of each extraction step. Until this time, previous studies utilizing similar variations of acid/based-hydrolysis extraction have only relied on pre-published protocols from studies which were proposed in the 1930s, and it

should be noted that these initial studies were performed with the primary purpose of obtaining pure sporopollenin, rather than obtaining microcapsules for use in microencapsulation. For the purpose of producing microcapsules for use in microencapsulation applications, the results of these studies indicate that base-hydrolysis is a redundant step and that the majority of protein removal is achieved by acid-hydrolysis alone. Additionally, it was observed that acid-hydrolysis conditions can be significantly reduced from existing published protocols. In particular, excessive acid-hydrolysis duration leads to decreased microstructural integrity as indicated by increasing S-SMC fracturing and fragmentation. Therefore, overall, this study highlights that optimal S-SMCs can be obtained by significantly reduced processing requirements, as compared to existing literature. This is of significant importance to the application of S-SMC extraction with regards to microencapsulation. However, also significant from a materials science perspective is the observation that excessive acid-hydrolysis conditions leads to decreases in mechanical robustness, as literature on the acid extraction of SMDCs suggests that sporopollenin is largely impervious to harsh acid-hydrolysis conditions. Whereas, the observations from this initial thesis study also open the way for further studies to determine how sporopollenin is altered and weakened by acidolysis.

These efforts to review the generality of various acid/base-hydrolysis protocols for extraction of angiosperm derived pollen was also the first of its kind study looking at the efficacy of various extraction protocols and each processing step. There exist a range of studies looking at various pollen species, however, there is also much variability in the base and acid types used without any comparative study clearly indicating the effect of different hydrolysis media. For the purpose of extracting S-SMCs from angiosperm derived pollen sources, the results of these studies indicate that base-hydrolysis can completely destabilize the pollen morphology, and that different acids and enzymatic treatments produce S-SMCs of variable quality and cleanliness. Additionally, after determining that phosphoric acid (H_3PO_4) achieves more effective cytoplasmic removal and cleaner microcapsules surfaces, it was identified that acid-hydrolysis duration can be limited to only 5 hours, which is significantly less than many existing protocols. Importantly, this study also

provided the first clear protocol and data set for analysis of protein residues utilizing matrix assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS) analysis. The standard for protein removal efficacy in the field of S-SMC extraction relies on the quantification of nitrogen content by elemental analysis. At this time there exists only one other study which indicates the use of a more sensitive technique, such as MALDI-TOF MS. However, this study provides no data, nor provides the protocol used for analysis. Due to effective protein removal being crucial for the production of non-allergenic microcapsules for human-centered applications, these results are an important step to developing a more robust and standardized protocol for analysis of protein residues in S-SMCs.

These efforts to systematically explore loading mechanisms, washing requirements, loading distributions, and the theoretical maximum loading for BSA was the first of its kind study based on the use of S-SMCs. There exist a number of studies exploring the potential of loading various compounds within S-SMCs, however, these typically only highlight loading efficiencies and depict single or small groups of ideally loaded full particles, and rarely address the issues of surface cleanliness in the calculations of loading efficiencies. For the purpose of more fully understanding the dynamics and limitations of existing loading protocols, the results of compound loading studies, with BSA as a representative macromolecule, indicate that loading of aqueous solutions within S-SMCs is driven by air bubble collapse, and that assisted loading protocols, such as vacuum loading, are required to ensure complete loading. Additionally, multiple washing steps are typically required to ensure adequate removal of residual surface adhered loaded compounds. Based on the resulting standard optimized loading and washing protocols, utilizing various loading solution concentrations, it was possible to show that loading distributions result in many particles remaining empty and average volumetric loading efficiencies of between 13 and 29 vol%. From the obtained experimental loading efficiency wt% data and vol% data, it was possible for the first time to estimate a theoretical maximum loading efficiency, assuming every particle was fully loaded. Overall, this study provides important insights to ensure compound loading protocol optimization and to provide a foundation for increasing

practical loading efficiencies within S-SMCs and other hollow microcapsule systems.

These efforts to utilize BSA-loaded S-SMCs extracted from *H. annuus* to develop a multiparticulate targeted delivery system to achieve targeted delivery to the digestive tract, was the first of its kind study utilizing S-SMCs. There exist a small number of studies briefly exploring compound-loaded S-SMCs with various forms of coencapsulants for the purpose of controlled release, however, none of these studies have clearly elucidated the release dynamics of compound release or achieved drug delivery systems with release profiles ideally suited to targeted intestinal delivery. For the purpose of exploring the potential applications of utilizing S-SMCs in the delivery of therapeutic compounds, the results of these compound release studies from S-SMCs indicate that hydrophilic compounds, such as BSA, release quickly by diffusion upon hydration in both acidic and pH neutral environments. Additionally, through pelleting and enteric coating of BSA-loaded S-SMCs, it is possible to develop multiparticulate delivery systems with release dynamics suitable for targeted delivery of therapeutic compounds to the intestinal tract. Further, to highlight to overall robustness of *H. annuus* S-SMCs, it was shown that overall morphology and microstructural integrity was maintained throughout the loading, washing, pelleting, coating, and release processes.

Chapter 2

Background & Literature Review

At this time, nature's abundant sources of renewable microcapsules have been largely unexplored with regards to microencapsulation applications. This chapter provides an overview of the basic spore structure, of the biopolymer, sporopollenin, which is one of nature's unsung biomaterial superheroes, of existing S-SMC extraction methods, of S-SMC compound loading, and of S-SMC compound release. Further, there is provided a critical discussion of the existing studies and the current gaps in scientific knowledge, which have provided the motivation for this research. Finally, the relevance of this research and the major contributions to the field of S-SMC extraction and application are highlighted.

2.1. Overview

2.1.1. Pollen Structure

Pollen grains and spores are nature's solution to the microencapsulation and protection of the sensitive genetic material critical for the propagation of a plant or microbial species [1]. Cytoplasmic contents located in the central cavity of this natural microcapsule are protected against harsh environmental conditions by a cell wall referred to as a sporoderm [2]. The sporoderm typically comprises two primary layers the outer layer, exine, and internal layer, intine. Each of the exine and intine may be further subdivided into various layers and structures [3]. Typically, the exine comprises a remarkably physicochemically robust biopolymer known as sporopollenin, and the intine comprises the polysaccharides, cellulose, hemicellulose, and pectin [4]. The sporoderm is porous and permeable to moisture so as to allow for varying degrees of the hydration and dehydration of the internal cytoplasmic contents [5]. The permeability of the exine is due to one or both of nanochannels on the scale of 10s to 100s of nm and apertures on the scale of μm [6]. The permeability of the intine is due to the hydrophilic nature and polymeric mesh size of crosslinked polysaccharides. Often, the sporoderm has a layer of primarily lipidic compounds adhered to the outer surface, typically referred to as pollenkitt [7]. The pollenkitt layer aids in the pollen adhering to other surfaces [8,9] and has been observed to increase the permeability of the sporoderm [10].

Pollens and spores are involved in the sexual reproductive processes of higher plants and the asexual reproduction of mosses, ferns, and some algae [11]. Many bacteria and fungi also utilize spores in various stages of their lifecycles [12,13]. There is significant variability in the morphology of pollen and spores, with many comprising only a basic spherical structure, yet others exhibiting complex microstructural features or multicompartmental forms [14]. Pollen and spore size ranges are from 1s to 100s of μm , with many plant-based pollen falling in the range of 20 to 80 μm . Broadly speaking, the majority of pollens and spores can be divided into those dispersed by wind and those

dispersed by insects or animals [15]. Wind pollination is referred to as anemophily, and anemophilous plants typically produce pollen in huge quantities, and which is light and non-sticky so that it may be transported by air currents. Insect aided pollination and animal aided pollination are referred to as entomophily and zoophily, respectively. Entomophilous and zoophilous plants typically produce pollen in smaller quantities than observed in anemophilous plants, and the pollen is usually sticky and may exhibit protrusions, such as spikes, to aid in adhering to the insect or animal transporter.

Figure 2.1 Schematic depicting general basic structure of pollen. A sporoderm shell surrounds a cytoplasmic interior, with the sporoderm comprising an outer exine and inner intine. The exine typically comprises a robust biopolymer, sporopollenin. The intine typically comprises a combination of cellulosic compounds, such as, cellulose, hemicellulose, and pectin. The surface of many pollen species is coated with a thin layer of primarily lipidic compounds, collectively referred to as pollenkitt.

2.1.2. Sporopollenin Overview

There exists a diverse range of robust natural polymers in the plant world, with some of the more widely studied polymers being, cellulose, cutin, cutan, suberin, algaenan, lignin, lignan, and tannin. These polymers can typically be categorized into polysaccharides (cellulose), polyesters of fatty acids (cutin, cutan)[16], polyesters of glycerol and fatty acids (suberin)[17], polyesters and polyethers of fatty acids (algaenan) [18], and polyphenols (lignin, lignan, tannin)[19]. Of particular interest are the polymers cutan, lignin,

algaenan, and sporopollenin, due to their high physicochemical resilience. Cutan is from the cuticle of some plants, lignin is found in woods, and algaenan is produced in the sporoderm walls of some algae. Generally, these polymers predominately comprise either aliphatic or phenolic monomers. However, sporopollenin is the only plant-based polymer that comprises large proportions of both aliphatic and phenolic moieties [16].

There exist two comprehensive and important literature sources providing a broad overview of the background research relating to pollen, spores, and sporopollenin. In 1974, the book 'Pollen: Biology, Biochemistry, and Management,' written by Stanley & Linskens [4], was published as a comprehensive resource on all things relating to pollen. This provides an important overview of the biological process of sporopollenin deposition and sporopollenin biochemistry as was understood in the early 1970s. Points of note from this text are: 1) sporopollenin has an elemental composition of C, H, O, with a C/H ratio of approximately 1/1.6, examples being, pine pollen $C_{90}H_{158}O_{44}$, rye pollen $C_{90}H_{134}O_{31}$, and *Lilium henryi* pollen $C_{90}H_{134}O_{36}$; 2) the dry weight of sporopollenin, with respect to pollen weight, for gymnosperms is ~ 24%, and for angiosperms is ~ 5%; and 3) pollen species show variable degradation rates in biologically active soils. These observations highlight the variability which exists between sporopollenin from various plant species. In 1990 the book 'Microspores: Evolution and Ontogeny,' edited by Blackmore and Knox [11], provided a more updated overview of spore and pollen research. Chapter 10, 'Exine biochemistry,' by D. Southworth, provides an important overview of sporopollenin isolation methods and sporopollenin degradation properties. It claims that sporopollenin can be isolated using: 1) acetolysis; 2) solvent, base, acid processing; and 3) enzymatic processing. Also, sporopollenin is reported to be insoluble in non-oxidizing acids, dilute bases, and be resistant to hydrolytic enzymes. The only compounds reported to dissolve sporopollenin are 2-aminoethanol, 3-animopropanol, 2,2'2"-nitriltriethanol, and 4-methylmorpholine-N-oxide. Degradation has been observed in fused potassium hydroxide, and in oxidizing mixtures such as hypochlorite/hydrochloric acid, potassium dichromate/sulphuric acid, hydrogen peroxide/sulphuric acid, and ozone. An understanding of the

chemical robustness of sporopollenin is relevant to the diversity of capsule extraction protocols.

Figure 2.2 Hypothetical simplified structure of a sporopollenin building block. The substituents R can either be linked by ether bonds or, in the case of the two cinnamic acids, also through ester bridges (adapted from van Bergen et al. [16] and Barrier et al. [20]) NOTE: Some important confirmed characteristics and observations have not been taken into account: 1) methyl and hydroxyl groups have been neglected; and 2) the proportion of oxygen has largely been under evaluated.

To develop a clear picture of the current view of the chemical structure of sporopollenin it is important to review the history of sporopollenin research. The earliest work, from the 1930s, suggested that sporopollenin was related to terpenoids, and in the 1960s carotenoids [20]. Although, in the 1960s an opposing view was proposed wherein sporopollenin comprised lipidic and lignin-like components [20]. In the late 1980s FTIR (Fourier Transform Infrared spectroscopy) [21] and solid-state NMR (Nuclear Magnetic Resonance) [22,23] studies went on to support the lipidic view of sporopollenin. However, other studies from the late 1980s and early 1990s identified the presence of oxygenated aromatic units in sporopollenin [24-27]. Initially, based on these results, researchers proposed either the existence of two completely different types of sporopollenin, or that sporopollenin may consist of a polymeric structure comprising both aromatic and aliphatic components. However, the consensus was that the core structure was likely to

be made of aliphatic monomers. In 2004, van Bergen et al. considered the current research and proposed a structure for the aliphatic backbone cross-linked with aromatic side chains (Figure 1)[16]. It is now accepted that the backbone structure is common between all species and that sporopollenin species variation is due to differences in the cross-linking side chains [20].

The existence of the aliphatic backbone was proposed in 1988 from FTIR spectra and was supported separately in the same year through the use of solid-state NMR. In 1995, a subsequent study supported the 1988 hypothesis of a lipidic backbone [28]. Then, in 1996, tracer studies with a ^{14}C -tagged precursor showed that oleic acid (C18:1) was incorporated into algal sporopollenin [29]. In 1999, studies with FTIR and GC-MS (Gas Chromatography–Mass Spectrometry), suggested that sporopollenin is a polymer comprising unsaturated fatty acids, with linoleic (C18:2) and linolenic (C18:3) acids being precursors, also, it was proposed that backbone monomers were linked by ether bonds [30]. In 2000 and 2002, FTIR spectra were also interpreted to suggest that sporopollenin had an ether-bonded backbone [31,32]. In 2007, a study utilizing thermochemolysis and pyrolysis with GC-MS, and incorporating micro-FTIR, detected saturated linear carboxylic acids (in the range C8 to C22) and unsaturated linear acids (C16 and C18) [33]. This study also detected the cinnamic acids, p-coumaric acid and ferulic acid, as well as additional phenolic compounds. The presence of both linear carboxylic acids and unsaturated linear acids, along with aromatic compounds reinforces the view of sporopollenin comprising an aliphatic backbone cross-linked with aromatic side chains.

Strong evidence for the existence of aromatic compounds in sporopollenin was produced in the late 1980s [24-27]. Mass spectrometry, IR spectroscopy, UV spectroscopy, thin-layer chromatography (TLC), and high-pressure liquid chromatography (HPLC) were used in several studies to analyze the extracts and degradation products of a number of sporopollenin species. These results are also supported by the more recent 2007 study by Watson et al. [33] as mentioned above. The main observations included the presence of a range of phenolic compounds with p-coumaric acid and ferulic

acid being dominant. A 1995 study of sporopollenin solubilized in 2-aminoethanol and analyzed by various spectrographic methods supported the previous observations [28]. Also, in the early 1990s, a series of convincing tracer experiments were conducted showing that p-coumaric acid was integrated into the exine and that phenylalanine is an important precursor in sporopollenin biosynthesis [27,34]. It was further shown that the aromatic ring, and potentially the entire phenylpropane unit, was incorporated into the final polymer matrix. In 2003, solution NMR was used to show that phenols (cinnamic acids) were present in sporopollenin solubilized in piperidine [35]. Overall, there is strong evidence that sporopollenin contains aromatic compounds in the form of cinnamic acids, primarily p-coumaric acid and ferulic acid.

There have been a several studies from the 1960s to 1980s regarding sporopollenin degradation products obtained by potash fusion (molten potassium hydroxide (KOH)) and saponification (KOH hydrolysis). These studies were carried out with similar protocols, and analysis was conducted using HPLC and/or TLC. The early studies from the 1960s and 1970s explored potash fusion only [36-39]. These studies produced similar results for a range of sporopollenin species and identified several phenolic degradation products. Later, in the 1980s, the degradation of *Corylus avellana* [25] and *Pinus mugo* [24] (wing) sporopollenin was explored in separate studies using both potash fusion and saponification with 6 M KOH at 100 °C. Potash fusion resulted in the detection of p-hydroxybenzoic acid, which was also seen from the studies carried out in the 1960s and 1970s. However, from saponification, both p-coumaric acid and ferulic acid were present in large amounts. In control studies p-coumaric acid and ferulic acid were shown to be stable under the same saponification processing conditions. Potash fusion is a very harsh chemical process and it is proposed that the resulting p-hydroxybenzoic acid is the degradation product of the naturally occurring p-coumaric acid and ferulic acid. These results suggest that sporopollenin saponification with KOH leads to the extraction of the aromatic cross-linking units from the sporopollenin biopolymer matrix. However, some phenolic degradation products have still been detected even after sporopollenin has been isolated with harsh

saponification processing. Therefore, either there are additional diverse aromatic compounds present, which are resistant to saponification, or the aromatic compound removal process occurs gradually.

2.1.3. S-SMC Extraction Methods

To obtain hollow microcapsules from pollen and spores it is necessary to remove non-sporoderm constituents, such as, pollenkitt and cytoplasmic materials. One of the major concerns when utilizing pollen-based microcapsules relates to the potentially allergenic properties of pollen. It is important to note that allergenic reactions associated with pollen and most plant-based compounds are related to the presence of various proteinaceous compounds [40,41]. Within pollen, there are typically proteins present in the cytoplasmic constituents [42]. Therefore, various methods have been developed to achieve effective removal of cytoplasm. Additionally, there has been some interest in the extraction of pure sporopollenin exine capsules (SECs), which also requires the removal of the cellulosic intine materials [24,43]. Much of the existing literature regarding the extraction of pollen microcapsules uses the terminology SECs [44-56], however, many of the extraction protocols used are likely to produce capsules with varying degrees of residual cellulosic intine materials [20,57-60], and it may be more accurate to use the more general term sporopollenin sporoderm microcapsule (S-SMC), which can also encompass other sources of sporoderm microcapsule, such as algaenan sporoderm microcapsule (A-SMC) from algae, etc.

Overall, the various methods of S-SMC extraction, which have developed over the years, may be categorized into four main categories: acetolysis; acid & base hydrolysis; 4-methylmorpholine N-oxide monohydrate (MMNO) treatments; and physical treatments. Acetolysis is a popular method to extract S-SMCs involving the use of acetic anhydride and concentrated sulphuric acid [61-64]. Although commonly used in the preparation of pollen samples in biological and materials analysis, studies suggest that it may not always reliably remove all traces of cellulosic materials [65,66]. Additionally, acetolysis is known to be extremely corrosive and to react violently with

Figure 2.3 Example of conventional acid/base-hydrolysis extraction method as utilized in literature [80] and comparison with an optimized extraction protocol resulting from studies outlined in Chapter 4.

water [67]. Acid and base hydrolysis is the most common method used in literature relating to S-SMC extraction [20,44-59,68,69]. Solvents are used to remove surface bound lipidic compounds, base-hydrolysis is used to remove cytoplasmic materials, and acid-hydrolysis is used to remove remaining cytoplasmic and cellulosic materials. Various solvents, bases, and acids have been shown to exhibit varying degrees of efficacy with varying hydrolysis temperatures and durations. MMNO treatments for S-SMC isolation are somewhat less common than acetolysis and acid/base-hydrolysis [70-76]. MMNO is commonly used in to dissolve cellulosic materials and other polysaccharides [77,78]. Some studies suggest that MMNO treatment leads to

weakening and limited dissolution of sporopollenin exines in some pollen species. Physical treatments primarily involve probe sonication or autoclaving with a sucrose solution [79]. These result in separation of the protoplast from the sporoderm or separation between the exine, intine, and protoplast. A major disadvantage of physical treatments is that they typically lead to significant damage and fracturing of the sporoderm/exine, and therefore are of limited use in obtaining S-SMCs for microencapsulation applications. However, one advantage of physical treatments is that they can allow for the isolation of pure exine sporopollenin for use in characterization studies, whereas other chemical extraction methods lead to varying kinds of polymer modification of the sporopollenin material. Based on the majority of the literature relating to S-SMC extraction for microencapsulation applications, studies in this thesis have focused on exploration of acid/base-hydrolysis methods. A comparison of a standard protocol to an optimized protocol, being one of the results from the initial thesis study outlined in chapter 4, can be seen in **Figure 2.3**.

A wide range of spores and pollens have been studied for the production of S-SMCs by various acid/base-hydrolysis protocols. Research into acid/base-hydrolysis extraction of S-SMCs began in the 1920s [81-84] and continued sporadically over the following decades with a significant resurgence of interest in recent years. Initially, it was determined that cytoplasmic contents of spores and pollen could be removed by treatment with organic solvents and boiling in alkaline solutions. However, the remaining capsule structure still contained the cellulosic intine layer. Early studies into the isolation of S-SMCs were primarily to obtain pure sporopollenin for studying the chemical and physical properties of this material. Therefore, in order to remove the cellulosic intine, researchers explored the use of prolonged acidic hydrolysis processing using hydrochloric acid, hot sulphuric acid, or hot phosphoric acid over a several day period. However, both hydrochloric acid and sulphuric acid have been shown to leave residual chlorine and sulphur after processing [20]. Phosphoric acid came to be viewed as the best option for the isolation of S-SMCs as no phosphorus residues were observed in the resulting sporopollenin material [20]. One potential drawback of phosphoric acid was

brought to light in the late 1990s when careful analysis with ^{13}C -NMR showed that traces of cellulose were still present in sporopollenin material which had been obtained by hot phosphoric acid acidolysis treatment for several weeks [57]. However, although some cellulosic material remains, phosphoric acid is widely used in the food and pharmaceutical industries and is still considered to provide the best balance between intine removal and the production of S-SMCs which may be considered safe for human consumption [20].

2.1.4. S-SMC Compound Loading

Microencapsulation is one of the major potential applications for plant-based microcapsules such as S-SMCs. Therefore, after S-SMC extraction, it is important to explore the various methods used for compound loading into, and mechanisms of compound release from S-SMCs. The main loading methods in the literature relating to S-SMCs are passive encapsulation, centrifugation encapsulation, compression encapsulation, vacuum encapsulation, and micro-reactor encapsulation [20,57-59].

Passive encapsulation is the simplest reported method and involves mixing the S-SMCs with a solution containing the compound to be loaded, and may be accompanied by vortexing and/or incubation. It is proposed that loading is achieved by the loading solution diffusing through the pores in the sporoderm to fill the internal cavity [6,85-88], although the exact mechanisms of passive loading have not been fully studied. However, passive encapsulation has been shown to be effective with both aqueous and oil-based compound loading systems [20,45,89]. Centrifugation encapsulation is a modified form of passive loading whereby S-SMCs and the loading solution are mixed and centrifuged so as to achieve improved loading efficiencies. However, there is only a single study which explores this loading protocol [45]. Compression encapsulation involves pressing the S-SMCs in a pelleting press and then exposing the pellet to the loading solution. The elastic properties of the S-SMCs allow for the flattened capsules to return to their pre-pressing morphology [90,91]. The stored potential energy of the compressed capsules results in a low-pressure differential between the inside and outside of the S-

SMCs drawing the loading solution through the pores into the central cavity [20,58,87]. Vacuum loading is the most widely reported method for the loading of S-SMCs [44-56]. The loading solution and the S-SMCs are mixed and exposed to a low pressure for some duration so as to achieve improved loading. As the air is drawn out of the internal cavity due to the lower external pressure the loading solution is drawn into the central cavity [87]. Micro-reactor encapsulation involves synthesizing target compounds within the S-SMCs. A first compound (A) is loaded within the S-SMCs, followed by exposure to a second compound (B), the reaction between compounds A and B result in the formation of a third compound (C) [55]. Micro-reactor encapsulation may be used to achieve loading of compounds with high-melting points and/or low solubility.

Figure 2.4 Schematic of common S-SMC loading techniques and loading mechanisms applied to natural *L. clavatum* spores. A) Spore microstructure depicting uniform ridges distributed on the surface with natural sporoplasm constituents contained inside. B) Natural spores suspended in a macromolecule-containing solution for the uptake of macromolecules, the enlarged inset depicts macromolecule entry via nanochannels located within the *L. clavatum* microstructure. C) Spores encapsulating macromolecules indicated as green along with the natural sporoplasm constituents. D) Passive macromolecule loading technique involving the incubation of natural spores in the aqueous macromolecule solution. E) Compression technique involving the compression of a dry spore powder and incubating the resulting spore tablet in the

macromolecule solution for the uptake of macromolecules by the spores. F) Vacuum loading technique involving the application of a vacuum to a suspension containing natural spores and macromolecules. Adapted with permission from [87].

2.1.5. Microcapsule Compound Release

Microencapsulation is an important technology for ensuring the stability and controlled delivery of compounds in a wide range of industrial applications. However, after compounds have been successfully loaded within a microcapsules system, it is necessary for them to release under appropriate conditions. The two primary mechanisms of compound release from S-SMCs are solvent extraction and sporoderm degradation [58]. Additionally, coencapsulation of loaded S-SMCs with various secondary coencapsulants is an important method of tailoring release profiles and achieving targeted delivery [51]. Solvent extraction of compounds from S-SMCs is the most common method to achieve compound release. Typically, encapsulated water soluble compounds, which have been dried and stabilized, will release from within S-SMCs by diffusion through cavity wall pores when hydrated by exposure to aqueous environments. Encapsulated oils and oil soluble compounds have also been shown to release from S-SMCs upon exposure to appropriate lipophilic solvents in a manner similar to hydrophilic compounds and aqueous solution exposure [45]. Sporoderm degradation compound release is an alternative method to solvent extraction and involves the breaking down of the S-SMC wall structure. Although the sporoderm and sporopollenin exine in particular are known to be highly chemically stable, it has been proposed that the body may have mechanisms to achieve S-SMC degradation based on enzymatic hydrolysis [49,54,56,92]. Studies by Lorch *et al.*, have presented compelling evidence of S-SMC degradation upon exposure to human blood plasma *in vitro* [54]. However, comprehensive studies and a clear understanding of the mechanisms driving such degradation are yet to be achieved. Coencapsulation of compound loaded S-SMCs with secondary coencapsulants involves the further processing and coating of loaded S-SMCs with materials so as to achieve greater control over the release properties of the loaded compounds. There exist a wide range of secondary coencapsulants

commonly used in microencapsulation applications, which provide the potential to manipulate release rates and tailor release to specific environmental conditions, such as pH, temperature, etc. [93-96].

2.2. Relevance of Thesis Work

2.2.1. S-SMC Extraction – *L. clavatum*

Microcapsules extracted from *L. clavatum* spores by acid/base hydrolysis methods are the most commonly studied S-SMCs for a wide range of microencapsulation applications [44-56]. However, there are significant variations in acid/base extraction protocols, which are loosely based on studies and observations from the 1920s and 1930s. At this time, there exist no studies systematically analyzing the efficacy of various S-SMC extraction steps so as to provide a clear baseline for developing an optimal extraction protocol for producing high quality microcapsules. Basic acid/base extraction protocols involve: 1) defatting; 2) base-hydrolysis; 3) acid-hydrolysis; 4) washing; and 5) drying [58]. Defatting, typically utilizes exposure to organic solvents, at temperatures up to 50 °C, for up to 12 hours, to remove surface adhered lipidic compounds [80]. Base-hydrolysis, typically utilizes potassium hydroxide (KOH) at temperatures of up to 120 °C, for up to 12 hours to remove cytoplasmic and proteinaceous materials [80]. Acid-hydrolysis, typically utilizes phosphoric acid (H₃PO₄) at temperatures up to 180 °C, for up to 7 days to remove cellulosic intine materials [80]. Washing, typically utilizes water, solvents, acids, and bases to remove all remaining non-exine material and chemical residues. Drying, typically involves convection drying followed by vacuum oven drying to remove any residual moisture to achieve stable weight. Overall these steps require very long duration and extremely harsh chemical processing to achieve S-SMCs, and it is unclear as to the actual efficacy and necessity of each step to achieve the goal of useful microcapsules for microencapsulation applications. The studies in this thesis were chosen so as to systematically explore each processing step under milder conditions, with regards to reduced duration and processing temperature, as also suggested in the literature.

This is the first comprehensive study to address the apparently arbitrary variability in reported S-SMC extraction protocols so as to determine, what are the minimum processing conditions necessary to produce useful S-SMCs for microencapsulation applications. This study has identified that base-hydrolysis is redundant, and that it is necessary to perform acid-hydrolysis at significantly reduced processing temperatures and durations to produce microcapsules with greater structural integrity. These results provide a baseline for engineering extraction protocols for S-SMC extraction and represent an important contribution to ongoing research into the preparation and application of S-SMCs. For the first time, researchers can reliably produce S-SMCs on a significantly reduced time scale, with safer processing conditions, and still be confident that the capsules they are producing are of high quality. Capsules produced by this protocol can therefore be used for exploring a wide range of applications in drug delivery, food products, and personal care formulations.

2.2.1. S-SMC Extraction – *H. annuus*

Currently less is known about the potential for S-SMC extraction from pollen and spores of species other than *L. clavatum*. Various studies discuss sporopollenin extraction from the pollen of a number of plant species, such as pine pollen [24,26,85,86] and cattail pollen [31,32,35,43,97], due to their relative abundance and ease of obtaining in large quantities at high purity. However, these studies have typically been focused on isolating sporopollenin for studies relating to chemical composition and material properties, rather than for extracting S-SMCs for potential use in microencapsulation applications. Therefore, issues such as process optimization and microcapsule integrity have typically been overlooked and not discussed. At this time few studies exist with a focus upon acid/base hydrolysis extraction of intact pollen-based microcapsules from non-*L. clavatum* sources, such as, *B. campestris* L. [98], *Zea mays* [68], and *Pinus taeda* [69]. Within sporopollenin literature it is widely accepted that sporopollenin is a family of similar biopolymers [11], wherein there are significant physicochemical differences which result in varying levels of resilience to processing conditions [20,97,99]. Additionally,

various plant species produce pollen with intriguing microstructural features which may be advantageous to defining specific applications, yet it is unclear whether it is possible to extract intact S-SMCs from various pollens with high levels of structural ornamentation.

Pollens from flowering plants may provide one major source of S-SMCs with surface ornamentation in the form of spikes. In the process of pollination, flowering plants typically rely on insects or animals to carry its pollen from one flower to another. Many flowering plants therefore produce pollens with spikey surfaces so as to provide better adherence to the insect or animal carrier [15]. Due to the limited research exploring S-SMC extraction from flower-based pollen sources, this study explores the generality of the standard acid/base extraction protocols commonly cited in sporopollenin literature and applies them to sunflower (*H. annuus*) pollen. These efforts represent the first study dedicated to applying standard S-SMC extraction protocols to non-*L. clavatum* pollen sources with a focus on process optimization and microcapsule integrity with the goal of obtaining microcapsules suitable for use in microencapsulation applications. It has been identified that base-hydrolysis destabilizes the morphology of *H. annuus* pollen and that acid-hydrolysis with H_3PO_4 for only 5 hours can obtain intact and useful S-SMCs devoid of any residual and potentially allergenic proteins. Overall, this study represents an important contribution to expanding the potential of S-SMC extraction from pollen and spores to other plant species and furthering the usefulness of standard S-SMC extraction protocols and the potential applications thereof.

2.2.3. S-SMC Loading & Controlled Release

There is a need to understand the fundamental mechanisms of loading and release which have been commonly employed in various S-SMC application-based studies. Various methods of compound loading have been employed in S-SMC loading studies as described above, however, the basic mechanisms underlying these loading protocols have yet to be clearly elucidated. Additionally, although loading efficiencies have been determined

for a wide range of compounds [20,45,80,89] it is unclear as to the actual compound loading distributions across large numbers of capsules. Only single or small groups of loaded particles have been presented in published studies and typically these depict particles being fully loaded [45]. However, during extensive preliminary studies following established loading protocols, it became clear that the loading efficiency values presented in many existing studies, represented many S-SMCs being only partially full or completely empty.

Further, no existing studies clearly depict S-SMCs after post-loading washing to remove surface adhered loading compounds. Therefore, it is not possible to rely on many existing loading and washing protocols or loading efficiency data, as it is unclear whether these protocols are achieving effective removal of surface adhered loading compounds, or whether the loading efficiency data presented is only the portion of compound loaded within S-SMCs, or if these values also include residual compounds adhered to the surface of unclean capsules. Therefore, to overcome this significant lack of data, fundamental studies are required to clearly indicate loading efficiencies of various compounds after various washing protocols, accompanied by detailed morphological analysis of single and multiple particles to indicate the degree of residual loading compound which is adhered to the particle surface.

The lack of clear data regarding loading distributions across large numbers of S-SMCs, as well as the washing requirements to effectively remove all non-loaded compound, raises several important issues to be addressed in this field of research. First, what are the maximum practical loading efficiencies which can be achieved based on standard loading methods and effective removal of surface adhered compounds. Second, what are the loading distributions typically observed with realistically achievable loading efficiencies. Third, what are the theoretical maximum loading efficiencies assuming every S-SMC is completely full. By gaining deeper insights into these issues it is possible to begin to understand the limitations of current protocols and to explore means to improve on existing loading methods.

2.3. PhD in context of literature

The topic of S-SMCs represents a field of study which has significant potential for industrial applications, yet there are limited detailed systematic studies with regards to capsule extraction, loading, and release. The results of this thesis have provided important fundamental insights into several key topics: 1) a robust analysis of the most commonly cited S-SMC extraction protocol, with significant improvements toward producing high quality capsules, balancing adequate protein removal with increased structural integrity; 2) a robust analysis of extracting S-SMCs from flower-based pollen sources so as to ensure adequate protein removal and that functional microstructural features remain intact; and 3) systematic analysis of the mechanisms driving compound loading and release, leading to insights into loading distributions, washing requirements, and theoretical loading limits, as well the utilization of S-SMCs for targeted delivery to the intestinal tract. These studies provide an important body of work to facilitate further progress in the development of plant-based microcapsules for a wide range of applications.

Figure 2.5 Trends in publications relating to S-SMCs for 2007 to 2017, comparing total publication numbers with the keywords “sporopollenin” and “microcapsule”, with total publication numbers with the keyword “sporopollenin” only.

It should be noted that in recent years there has been a large increase in publications relating to S-SMCs. From assessing publication numbers from Google Scholar it can be seen that the publication numbers from 2007-2017 using the keywords “sporopollenin” and “microcapsule” increased from 10 in

2007 to 23 in 2015, then jumped suddenly to 51 and 60, in 2016 and 2017, respectively (Figure 2.5). Publication numbers with the keyword “sporopollenin” only were approximately 20 times higher for the same period yet did not exhibit any noticeable change in publication trend for 2016 and 2017. Overall, this indicates that interest is growing for research of sporopollenin as a more application based material, in the form of microcapsules, and the relevance of this thesis work appears to be very timely.

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Chapter 3

Experimental Methodology

To further the field of S-SMC extraction and application, a series of studies based on SDCM extraction, S-SMC compound loading, and S-SMC compound release were pursued. The effect of various acid/base-hydrolysis S-SMC extraction steps for L. clavatum spores as the most commonly explored raw material, and for H. annuus pollen as a representative angiosperm derived raw material source were explored. Then, the mechanisms driving S-SMC compound loading upon the loading of BSA as a representative macromolecule, and the distribution of the loaded compound over a large sample population were explored. Finally, the processes of S-SMC compound release and applied standard pelleting and enteric coating methodologies to develop a multiparticulate targeted delivery system suitable for targeted delivery to the digestive tract were explored. This chapter provides a clear justification for the materials and methods selected for these studies, as well as a detailed summary of all protocols utilized and an overview of key characterization principles and techniques.

3.1. Rationale for selection of methods and materials

3.1.1. S-SMC extraction

Two separate S-SMC extraction studies were performed, the first was acid/base-hydrolysis extraction of *L. clavatum* spores, and the second was acid/base-hydrolysis extraction of *H. annuus* pollen. The first of these studies was defined based on acid/base-hydrolysis extraction of *L. clavatum* spores being the most common extraction method and raw material source cited in the literature [1-13]. The second of these studies, utilizing *H. annuus* pollen, was defined based on the goal of determining the generality and variability of commonly cited acid/base-hydrolysis extraction protocols on an angiosperm derived pollen source with potentially functional surface microstructure.

For the first S-SMC extraction study, *L. clavatum* spores were utilized, and acid/base-hydrolysis extraction parameters were defined after careful revision of various existing protocols. In the literature it is proposed that *L. clavatum* has been widely studied due to it being widely available in industrial quantities at reasonable cost [2,14]. Traditionally, raw *L. clavatum* spores have been used in fireworks [15] and have some history of being used in powder lubricant applications [16]. The *L. clavatum* used in this study was obtained from Sigma-Aldrich (Singapore). The process of acid/base-hydrolysis extraction was based on standard protocols [1-13], with an initial organic solvent defatting step, followed by base-hydrolysis, acid-hydrolysis, washing, and drying. The organic solvent defatting is to remove surface adhered lipidic compounds from the raw pollen or spores. Acetone treatment at 50°C for 6 hours is the most commonly used defatting protocol and is the protocol utilized in this thesis [1-13]. Base-hydrolysis is implemented to remove cytoplasmic compounds and may be performed by either sodium hydroxide (NaOH) or KOH. Processing parameters commonly used are aqueous base (NaOH or KOH) concentrations of 6 % (w/v), with stirring at temperatures up to 120°C for 6 to 12 hours, and may be repeated multiple times. Based on common literature conventions, it was determined to use 6 % KOH at 70°C for 6 hours,

Figure 3.1 Visual depiction of primary steps used in optimized acid/base-hydrolysis extraction protocol from first S-SMC study in Chapter 4. (A) Untreated spores. (B) Spore defatting using a refluxing set-up at 50 °C for 6 hr. (C) Solvent filtration and collection of defatted spores. (D) Drying of defatted spores at room temperature. (E) Spore acidolysis in polytetrafluoroethylene (PTFE) flask using 85% (v/v) phosphoric acid at 70 °C for 30 hr. (F) Acid filtration using vacuum filtration set- up. (G) S-SMC washing using a series of water, acid, base, and solvents washing steps. (H) S-SMC drying using vacuum oven at 60 °C and 1 mbar vacuum for 10 hr. (I) Intact, clean, and dried S-SMCs as a final product.

and repeat this process for a total of two times. Acid-hydrolysis is implemented to remove cellulosic compounds and may be performed with a

wide range of acids, such as hydrochloric acid (HCl), phosphoric acid (H₃PO₄), sulphuric acid (H₂SO₄), or hydrofluoric acid (HF). Processing parameters commonly used are aqueous acid solutions of high concentration, with stirring at temperatures up to 180°C for up to 7 days, depending on acids used. Based on common literature conventions, it was determined to use 85 % H₃PO₄ at 70°C for up to 120 hours, with sample analysis at various hydrolysis durations to explore the effect of processing duration. A mild temperature of 70°C is desirable as it makes the process safer to conduct with common laboratory equipment. Washing is implemented to remove residual lipidic, cytoplasmic, and cellulosic debris, as well as processing compounds. Washing is typically achieved with multiple water, organic solvent, HCl, and NaOH washing steps. Based on common literature conventions, it was determined to use water (×5), acetone (×1), 2M HCl (×1), 2M NaOH (×1), water (×5), acetone (×1), and ethanol (×1), for a total of 15 washing steps. Drying is commonly achieved by convection drying for up to 72 hours, followed by vacuum oven drying to remove residual moisture and achieve stable weight of extracted S-SMCs. A visual depiction of the primary steps used in optimized acid/base-hydrolysis extraction protocol from first S-SMC study in Chapter 4 is shown in **Figure 3.1**.

For the second S-SMC extraction study, *H. annuus* pollen was utilized, and acid/base-hydrolysis extraction parameters were defined after careful revision of various existing protocols. *H. annuus* pollen was selected as a representative angiosperm derived pollen for determining generality of acid/base-hydrolysis extraction protocols. Sunflowers (*H. annuus*) are grown on a large scale for their seeds and therefore sunflowers may also be a viable source of large scale pollen harvesting. Additionally, *H. annuus* pollen has an interesting surface tethered-spike morphology which may provide additional functionality with regards to binding, and the presence of the spikes allowed us to more rigorously determine the stability of the sporoderm when undergoing extraction processes. *H. annuus* was obtained from Greer Labs (Lenoir, NC, USA) as defatted pollen. The process of acid/base-hydrolysis extraction protocols explored were based on standard protocols, base-hydrolysis, acid-hydrolysis, and additional enzymatic extractions were explored and compared

for efficacy. Base-hydrolysis was implemented as a standard S-SMC extraction step. Acid-hydrolysis was implemented with HCl treatment for 48 hours as defined in the limited existing literature for angiosperm pollen extraction [17]. Further exploration of HCL based extraction involved treatment for varying hydrolysis durations and with the implementation of various rigorous washing protocols as commonly used in other acid/base-hydrolysis extraction protocols. Additional, enzymatic incubation with proteases was explored in addition to HCl acid-hydrolysis, so as to remove residual proteinaceous compounds and debris [18]. Finally, acid-hydrolysis with H_3PO_4 was implemented as a method shown to provide good results in existing literature, as well as the initial studies with *L. clavatum* spore extraction. Drying was based on standard convection drying followed by vacuum oven drying protocols.

3.1.2. S-SMC compound loading

Compound loading was explored based on several pre-existing loading methods defined in existing literature. Passive loading, and assisted loading with vortexing and vacuum loading were chosen as the most fundamental and representative S-SMC loading methodologies. A wide range of compounds have been explored for their loading potential in S-SMCs. Hydrophilic compounds have typically received more attention, and a wide range of proteinaceous compounds have been explored. The loading of proteins within S-SMCs is promising due to passive and vacuum loading methods allowing for loading protocols which avoid harsh chemicals or conditions which are potentially harmful to delicate proteins. For these studies of compound loading in S-SMCs, bovine serum albumin (BSA) was selected as a commonly studied representative macromolecule. Fluorescein isothiocyanate conjugated BSA (FITC-BSA) was utilized for fluorescence microscopy related loading dynamics and loading distribution. A visual depiction of the primary steps used in vacuum-loading protocol from first S-SMC study in Chapter 4 is shown in **Figure 3.2**.

Figure 3.2 Visual depiction of primary steps used in vacuum-loading protocol from first S-SMC study in Chapter 4. (A) Intact, clean, and dried S-SMCs as a final product. (B) Mixing of S-SMCs and BSA solution. (C) Homogenization of S-SMCs and BSA solution. (D) Vacuum loading of BSA into S-SMCs. (E) BSA-loaded S-SMCs.

3.1.3. S-SMC tableting & coating

To utilize BSA-loaded S-SMCs extracted from *H. annuus* to develop a multiparticulate targeted delivery system to achieve targeted delivery to the digestive tract, standard pelleting and enteric coating methodologies were explored. Pelleting by application of a hydraulic press is common in the field of drug delivery, and therefore it was determined to apply pelleting to S-SMCs for the first time. Enteric coating with various natural and synthetic coencapsulants is also common in the field of drug delivery, and it was determined to apply a standard enteric coating system for which there exists a wide body of reliable literature. As an enteric coating system, eudragit was utilized as a controlled release polymer with appropriate binders and solvents.

The eudragit used in these studies offers stable coating systems in low pH conditions, and dissolves upon exposure to pH neutral conditions allowing compound release. With enteric coated and pelleted BSA-loaded S-SMCs it was possible to determine release profiles *in vitro* by standard methods for analysis of targeted intestinal delivery.

3.2. Sample Preparation

3.2.1. Materials

Chapter 4. L. clavatum S-SMC extraction

Natural *L. clavatum* spores (S-type) [19], bovine serum albumin (BSA), FITC-conjugated BSA, and other solvents were purchased from Sigma-Aldrich (Singapore). Phosphoric acid (85 % w/v) and hydrochloric acid were procured from Merck (Singapore). Polystyrene microspheres ($50 \pm 1 \mu\text{m}$) were purchased from Thermoscientific (Santa Clara, CA, USA). Vectashield (H-1000) medium was purchased from Vector labs (Burlingame, CA, USA) and Sticky-slides, D 263 M Schott glass, No.1.5H ($170 \mu\text{m}$, $25 \text{ mm} \times 75 \text{ mm}$) unsterile glass slides were purchased from Ibidi GmbH (Munich, Bavaria, Germany). Commercial *Lycopodium* S-SMCs (L-type) [19] were purchased from Polysciences, Inc. (Warrington, PA, USA).

Chapter 5. H. annuus S-SMC extraction

Defatted sunflower pollen grains (*Helianthus annuus* L.) were procured from Greer labs (Lenoir, NC, USA). Potassium hydroxide, hydrochloric acid, Tween-20, phosphoric acid (85% w/v), reagent grade salts and solvents were procured from Sigma-Aldrich (Singapore). Polystyrene microspheres ($50 \pm 1 \mu\text{m}$) were purchased from Thermoscientific (Santa Clara, CA, USA). Vectashield (H-1000) medium was procured from Vector labs (Burlingame, CA, USA) and sticky-slides, D 263 M Schott glass, No. 1.5H ($25 \text{ mm} \times 75 \text{ mm}$) unsterile were procured from Ibidi GmbH (Munich, Bavaria, Germany). Milli-

Q water (Millipore Corp., MA, USA) with a resistivity of 18 MU cm was used in all the experiments.

Chapter 6. H. annuus S-SMC loading & controlled release

Bovine serum albumin (BSA), FITC-conjugated BSA, hydrochloric acid, sodium hydroxide, ethanol, and acetone were purchased from Sigma-Aldrich (Singapore). Defatted sunflower (*Helianthus annuus L.*) pollen grains were purchased from Greer Labs (Lenoir, NC, USA). Vectashield (H-1000) was purchased from Vector labs (Burlingame, CA, USA). Sticky-slides, D 263 M Schott glass, No.1.5H (170 µm, 25 mm x 75 mm) unsterile were purchased from Ibidi GmbH (Munich, Bavaria, Germany). Milli-Q water was used in all experiments. Eudragit L100 was purchased from Evonik Industries (Essen, North Rhine-Westphalia, Germany). Perfluoroalkoxy polymer (PFA) flasks were purchased from Vitlab (Grossostheim, Bavaria, Germany). A stainless steel pellet press die (13 mm) was purchased from Specac (Orpington, Kent, UK).

3.2.2. S-SMC extraction

Chapter 4. L. clavatum S-SMC extraction

For the first S-SMC extraction study based on acid/base-hydrolysis of *L. clavatum* spores the following protocol was implemented. S-SMCs were extracted by four main chemical processes: defatting, alkaline lysis, acidolysis, and serial washing followed by drying. Natural *L. clavatum* spores (100 g) were suspended in acetone (500 ml) in a round bottomed flask fitted with a glass condenser, and were refluxed at 50 °C for 6 h under gentle stirring. The defatted spores were collected by filtration under vacuum and air dried in a glass dish for 12 h. The dried samples were then refluxed (70 °C) in aqueous 6% (w/v) potassium hydroxide solution (500 ml) with gentle stirring for 6 h. The samples were collected by filtration and washed using MilliQ (MQ) water (2 × 500 ml) before resuming the alkaline lysis for another 6 h using fresh potassium hydroxide solution (500 ml). After the 12 h of alkaline lysis, the S-

SMCs were collected by centrifuging at 4500 rpm and washed using hot MQ water (5×500 ml) to neutralize the alkaline residues. After each wash the suspension was filtered using vacuum filtration. The S-SMCs were then washed twice using hot ethanol (2×500 ml) and dried overnight at 25 °C. The resultant S-SMCs were subjected to acidolysis by suspending capsules in 85% (v/v) phosphoric acid (500 ml) and stirred under gentle reflux at 70 °C for up to 120 h. The phosphoric acid was replenished after 60 h. At each stage of the treatment, 15 ml of the suspension was collected for characterizations. After acidolysis, the S-SMCs were washed by a series of washing steps involving hot water (5×800 ml), hot acetone (600 ml), hot 2M hydrochloric acid (600 ml), hot 2M sodium hydroxide (600 ml), hot water (5×800 ml), hot acetone (600 ml), and hot ethanol (600 ml), and finally the resulting S-SMCs were collected by vacuum filtration. The washed S-SMCs were transferred to a clean glass dish and air-dried for 12 h. The drying was completed in a vacuum hot plate oven (Memmert, Schwabach, Germany) at 60 °C at 1 mBar for 8 h and the dried S-SMCs were then stored in a dry cabinet until characterization.

Chapter 5. H. annuus S-SMC extraction

For the second S-SMC extraction study based on acid/base-hydrolysis of *H. annuus* pollen the following protocol was implemented. The natural sunflower pollen grains were collected from the Czech Republic by Greer labs (Lenoir, NC, USA) and were harvested by vacuum and then allowed to water set. The cleaning of pollen grains to remove plant debris was performed by mechanical vibration under vacuum. The cleaned pollen grains were chemically washed using acetone at room temperature and defatted by washing with American Chemical Society (ACS) grade ethyl ether and supplied as defatted sunflower pollen grains. In the current extraction process, defatted pollen grains were used as the starting material to produce sunflower S-SMCs. The sunflower S-SMCs were extracted by three different approaches. (1) Alkaline lysis: in this process, defatted sunflower pollen grains (20 g) were suspended in a round bottom perfluoroalkoxy (PFA) flask fitted with a glass condenser. Alkali (6% potassium hydroxide, 100 ml) was added to the flask and gently mixed to form a homogeneous suspension and heated at 80 °C for

12 hours under gentle stirring (replenished with fresh alkali after 6 hours). The sporopollenin after alkaline lysis was collected by centrifugation at 4500 rpm for 5 min and washed twice using hot water (50 ml), hot ethanol (50 ml) and dried at 60 °C for 8 hours. (2) Acidolysis using 6 M hydrochloric acid (HCl): defatted sunflower pollen grains (2 g) were suspended in 15 ml 6 M HCl and heated at 70 °C under gentle stirring for 48 hours, HCl was removed after 24 hours by filtration and replenished with fresh 6 M HCl. Collected S-SMCs were washed using Milli-Q water and ethanol by gentle stirring and dried in an oven at 60 °C for 8 hours. In order to reduce HCl acidolysis processing time, the heating time was reduced to 10 hours, 20 hours and 30 hours and extensive washing was implemented using hot water (5 x 50 ml), hot acetone (2 x 50 ml), hot 2 M hydrochloric acid (50 ml), hot water (5 x 50 ml), hot acetone (50 ml) and hot ethanol (2 x 50 ml). S-SMCs were dried at 60 °C for 8 h and stored in a dry cabinet until further characterization. To remove surface-adhered proteinaceous materials, sunflower S-SMCs after HCl-acidolysis (10 h) were washed with trypsin (0.25%) or Tween-20 (2%) separately for 24 hours and further washed using sodium chloride (1%) and water. Collected S-SMCs were dried in an oven at 60 °C for 8 hours. (3) Acidolysis using phosphoric acid (85% v/v): defatted sunflower pollen grains (2 g) were suspended in phosphoric acid (H_3PO_4 15 ml) and mixed gently to form a homogeneous suspension. The pollen grains were heated at 70 °C under gentle stirring for 10 hours. The S-SMCs were collected by filtration and washed extensively by using hot water (5 × 50 ml), hot acetone (2 × 50 ml), hot 2 M hydrochloric acid (50 ml), hot water (5 × 50 ml), hot acetone (50 ml) and hot ethanol (2 × 50 ml). S-SMCs were dried at 60 °C for 8 hours and stored in a dry cabinet at 25 °C until further characterization.

3.2.3. S-SMC compound loading

Chapter 4. L. clavatum S-SMC extraction

For compound loading in the first S-SMC extraction study based on acid/base-hydrolysis of *L. clavatum* spores the following protocol was implemented. Biomacromolecule loading into S-SMCs was performed using a

vacuum loading technique based on a previous protocol [20]. Due to variations in the absorption properties of untreated spores and S-SMCs, 150 mg of untreated spores were suspended in 0.6 ml of 125 mg/ml aqueous BSA solution, and 150 mg S-SMCs were suspended in 1.2 ml of 125 mg/ml aqueous BSA solution in polypropylene tubes to form a homogeneous suspension and then mixed for 10 min using a vortex mixer (IKA, Staufen, Baden-Württemberg, Germany). The tubes were transferred to a freeze dryer (Labconco, Kansas City, MO, USA) and a vacuum was applied at 1 mbar for 2 h. The tubes were collected and the loaded S-SMCs were washed using 2 ml water then centrifuged to remove residual BSA, this washing step was repeated for a total of two washes. The S-SMCs were then placed in a freezer at $-70\text{ }^{\circ}\text{C}$ for 30 min before freeze-drying for 24 h. The resultant S-SMCs were stored at $-20\text{ }^{\circ}\text{C}$ until characterization. Placebo capsules were prepared with the same procedure, except BSA, and also stored at $-20\text{ }^{\circ}\text{C}$. In order to determine the localization of BSA within the S-SMCs, FITC-conjugated BSA was encapsulated in S-SMCs using the same procedure as the above-mentioned vacuum loading technique. Encapsulation efficiencies of BSA loading were determined by the following protocol. 5 mg of BSA-loaded S-SMCs were suspended in 1.4 mL of phosphate buffered saline (PBS), then vortexed for 5 min, and probe sonicated for 3 cycles of 10 s (40% amplitude). The solution was filtered to collect the extracted BSA using a $0.45\mu\text{m}$ polyethersulfone (PES) syringe filter (Agilent, Santa Clara, CA, USA). The absorbance values were measured at 280nm (Boeco-S220, Hamburg, Hamburg, Germany) using a placebo extract as a blank and the amount of BSA in the S-SMCs was calculated using a BSA standard curve.

Chapter 6. H. annuus S-SMC loading & controlled release

For studies of compound loading in S-SMCs extracted from *H. annuus* pollen, the following protocol was implemented. BSA loading into sunflower S-SMCs was performed using a vacuum loading technique [20]. 150 mg of S-SMCs was suspended in 1.2 mL of aqueous BSA solution at one of three different BSA-loading proportions (23, 37.5, 54.5 wt%) in polypropylene tubes to form a homogeneous suspension and then mixed for 10 min using a vortex

mixer (IKA, Staufen, Baden-Württemberg, Germany). The samples were then lyophilized for 12 h. The tubes were collected and the loaded S-SMCs were washed using 2 mL water then centrifuged to remove residual BSA, this washing step was repeated for a total of two washes. The BSA-loaded S-SMCs were frozen at $-80\text{ }^{\circ}\text{C}$ for 30 min and lyophilized for 24 h. The resultant sunflower S-SMCs were stored at $-20\text{ }^{\circ}\text{C}$. Placebo S-SMCs were prepared and stored following the same procedure. In order to determine the localization of BSA within the sunflower S-SMCs, FITC-conjugated BSA was encapsulated in S-SMCs using the same procedure as the above-mentioned vacuum loading technique. Encapsulation efficiencies of BSA loading were determined by the following protocol. Five mg of BSA-loaded S-SMCs were added to 1.4 mL of phosphate buffered saline (PBS), vortexed for 5 min, and then probe sonicated for three cycles of 10 s (40% amplitude) [20]. The extracted BSA was collected by passing the solution through a $0.45\text{ }\mu\text{m}$ PES syringe filter (Agilent, Santa Clara, CA, USA) with the BSA extraction step repeated for a total of two times. The absorbance values were measured at 280 nm (Boeco-S220, Hamburg, Hamburg, Germany) using a placebo extract as a blank and the BSA in the S-SMCs was quantified using a BSA standard curve.

3.2.4. S-SMC tableting and coating

Chapter 6. H. annuus S-SMC loading & controlled release

For studies of tableting, enteric coating, and controlled release in S-SMCs extracted from *H. annuus* pollen, the following protocols were implemented. Three kinds of tablets were prepared. (1) Tablets comprising BSA-loaded sunflower S-SMCs (150 mg per tablet) with a BSA-loading proportion of 54.5 wt%, which were tableted and enteric coated. (2) Control tablets with no S-SMCs, comprising a dry mix of 33 mg of BSA and 117 mg of Eudragit-L 100, equivalent to a BSA-loading efficiency of 22.3% as used in the BSA-loaded sunflower S-SMC tablets. Wherein, the BSA and Eudragit were first passed through a $50\text{ }\mu\text{m}$ sieve to ensure uniformity and vortexed together for 5 min to blend, then 150 mg of the blend was tableted and enteric coated. (3) Control tablets comprising BSA-loaded sunflower S-SMCs (150

mg per tablet) with a BSA-loading proportion of 54.5 wt%, which were tableted and not enteric coated. For tableting, either 150 mg of BSA-loaded sunflower S-SMCs (150 mg per tablet), or 150 mg of the BSA/Eudragit dry mix, were loaded in a 13 mm pellet press then compressed with a 5 ton load for 20 s (cross-sectional area of 132.75 mm² with compression pressure of 370 MPa). For enteric coating, tablets were dip-coated in a solution of 15% (w/v) Eudragit-L 100 in a mixture of isopropyl alcohol:acetone (92:8, v/v) with triethyl citrate as a plasticizer (15%, w/v). Dipping duration was 5 s followed by drying at room temperature. This coating step was repeated for a total of three coatings and followed by drying under vacuum for 2 h at room temperature. The dimensions of the tablets were recorded before and after tableting.

3.2.5. S-SMC compound release

Chapter 6. H. annuus S-SMC loading & controlled release

For *in vitro* drug release in simulated gastric fluid and simulated intestinal fluid the following protocol was implemented. *In vitro* release of BSA from sunflower S-SMCs was performed by suspending 5 mg of BSA-loaded S-SMCs in 1.4 mL of pH 1.2 SGF (0.1 M HCl) or SIF (PBS, pH 7.4) [20]. The mixture was incubated in an orbital shaker incubator (LM-450D, Yihder, Xinbei City, Taiwan) at 37 °C at 50 rpm. Supernatants were collected at various time intervals by centrifugation of samples at 9391 rcf (10 000 rpm) for 30 s, whereupon fresh release media were added to the samples. Supernatants were filtered using PES filters. Absorbance values were measured at 280 nm using a placebo extract as a blank and the released BSA was quantified using a BSA standard curve. To simulate gastrointestinal conditions in the case of the tablets, the BSA release was performed for 2 h in SGF followed by 8 h in SIF. The tablets were added to 20 mL of release media then incubated at 37 °C at 50 rpm in an orbital shaker incubator. At various time points, 1 mL of release media was collected followed by the addition of fresh release media. The released BSA was quantified by UV/Vis as described in section 3.3.6.

For fluorescence intensity analysis of release dynamics, images were collected with CLSM, and all image processing and data collection was performed using ImageJ. Ten regions were selected at points inside FITC-BSA loaded S-SMCs indicating fluorescence from FITC-BSA, and ten regions were selected at points outside the FITC-BSA loaded S-SMCs. Green channel images from a representative S-SMC FITC-BSA release data set were selected at time points of 2, 5, 10, 15, 30 and 60 min, and converted to 8-bit grayscale. Total image intensity values were collected for each region inside and outside the FITC-BSA loaded S-SMCs and averaged with standard deviations ($n = 10$). Data values, both inside and outside the FITC-BSA loaded S-SMCs, were normalized to the maximum fluorescence intensity of inside FITC-BSA loaded S-SMCs at 2 min and plotted. For the “BSA Release Comparison”, the “Inner S-SMC” data was inverted to represent “BSA Release (%)”, resulting in a “Normalized Fluorescence (%)” of 100% equating to the lowest amount of “BSA release” at 2 min, and the lowest value of “Normalized Fluorescence (%)” was equated to 100% “BSA release (%)”. However, the “BSA Release data” had begun from 0 min, whereas the “Fluorescence data” had begun at 2 min (due to experimental setup limitations), with uncertainty regarding how much fluorescence had dropped in the initial 2 min. Therefore, to more accurately compare the trends in release profiles, the “Fluorescence data” was assumed to have the same value of “BSA Release (%)” at 2 min and scaled accordingly.

3.3. Characterization – Principles and Analysis

3.3.1. Elemental Analysis (Carbon, Hydrogen, Nitrogen - CHN)

Elemental analysis (EA) is an analytical chemistry method used to determine the elemental composition of a material (**Figure 3.3**). It allows users to ascertain the chemical structure and purity of a material. It is regarded as one of the fastest and cheapest ways to ascertain sample purity. In S-SMC extraction literature, carbon, hydrogen, nitrogen (CHN) elemental analysis is used to determine the efficacy of protein removal.

EA is performed by combustion, wherein samples are subjected to a high combustion temperature of above 900 °C in the presence of oxygen such that gaseous products like carbon dioxide, water, and nitric oxide are produced. These gases are then separated using selective trap columns and pass through a thermal conductivity detector (TCD). When column effluent enters the cell, TCD detected changes, usually a decrease when compared to the reference or carrier gas, in thermal conductivity and a signal is produced and peaks are integrated by Vario EL software to determined weight % content of carbon, hydrogen, and nitrogen present in samples.

Figure 3.3 Schematic of elemental analysis system structure depicting basic operational principle. Sample loading, sample combustion, gas separation, detection, and analysis [21].

Experimental protocol: The protocol used for EA of pollen and S-SMC samples is as follows. Prior to elemental analysis, samples were dried in a vacuum oven to remove excess moisture. Calibration was carried out using acetanilide as a standard. Analysis was carried out using helium as a carrier gas. Samples (5 mg) were weighed out using Mettler Toledo UMT2 Micro Balance (Mettler-Toledo International Inc, Zürich, Kanton Zürich, Switzerland), enclosed in 12 × 6 × 6 mm tin boats and analyzed using a Vario EL III CHNS Elemental Analyser (Elementar Analysensysteme GmbH, Langenselbold, Hesse, Germany). Final protein concentration in unprocessed and processed samples was measured using percent nitrogen with an assumed

multiplication factor of 6.25 to convert weight percent of nitrogen to weight percent of protein.

3.3.2. Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry

MALDI-TOF MS (Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry) is used to determine the mass of compounds present in a wide range of extracts (**Figure 3.4**). Mass spectrometry (MS) requires the formation of positive or negatively charged molecules which may be accelerated and manipulated by electric and magnetic fields. MS systems provide data based on mass-to-charge (m/z) ratio, and therefore, it is ideal if molecules have only a single charge, so as to ensure greater ease in mass measurements and data analysis. To obtain charged molecules for analysis, MS may be divided into hard and soft types. Hard types of MS expose compounds to harsh conditions and often excessively damage or ionize molecules to be measured. Soft types of MS often require more complex preparation protocols and are limited in the compounds and sample types which may be analyzed.

MALDI is a versatile soft MS technique, utilizing laser ionization of samples to obtain charged molecules. Due to the laser power being inadequate to ionize most molecules directly, it is necessary to incorporate the use of a matrix compound. The matrix compound facilitates the transfer of photon energy to ionize the sample molecules. Ultraviolet (UV) light is absorbed by the matrix and converted to heat energy. Ionization by MALDI typically results in singly charged molecules for analysis with minimal molecular fragmentation. Upon ionization, molecules are desorbed from the sample stage and accelerated in an electric field. A Time-of-Flight (TOF) mass spectrometer is used to determine the mass-to-charge ratio of the molecule based on the time from laser pulse to the detection of ionized molecules on the detector.

Matrix selection is an important factor in obtaining good results in MALDI analysis. If small molecules (< 1kDa) are the target of detection, the laser power can be lower and it is better to use a appropriate matrix which functions optimally at lower power, such as 2,5-dihydroxybenzoic acid (DHB). For mid-sized molecules (1 - 10 kDa), α -cyano-4-hydroxycinnamic acid (α -CHCA) is more appropriate, and for large molecules (10+ kDa), 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid). Additionally, it is important that both the compounds of interest and the matrix are solubilized in similar solvents so as to ensure co-crystallization and efficient transfer of the ionization energy to the analyte compounds.

Figure 3.4 Schematic of matrix assisted laser desorption/ionization-time of flight mass spectrometer structure depicting basic operational principle [22].

Experimental protocol: The protocol used for analysis of protein residues in S-SMCs extracted from *H. annuus* pollen is as follows. MALDI-TOF mass spectrometry was performed with ground and ethanol extracted pollen and S-SMCs. 5 mg of pollen or S-SMCs was ground with a mortar and pestle, then suspended in 0.5 mL of ethanol, followed by vortexing for 2 min.

0.7 μL of the suspension was added to a MALDI plate followed by addition of 1 μL of an α -cyano-4-hydroxycinnamic acid matrix solution. After drying, samples were analyzed in a Shimadzu Biotech Axima Performance MALDI-TOF system, in linear mode, with a power of 90, and 100 profiles per run. All samples were run in triplicate and data were processed with Gaussian smoothing (100), and baselined (100).

3.3.3. Dynamic Imaging Particle Analysis

Dynamic imaging particle analysis (DIPA), is based on an optical microscope imaging system with glass flow cell and allows for analysis of the number and morphology of microparticles in solution (**Figure 3.5**). The camera collects raw image files whereupon the software identifies, extracts, and stores single particle images for later analysis. Image processing provides a powerful means to categorize particles and extract a wide range of analysis parameters based on 100s to 1000s of particles.

There are a wide range of imaging configurations possible and the appropriate imaging setup is typically based upon the size of sample particles. The optical imaging system is based on a standard optical microscope and current commercial systems allow for objective lenses up to $\times 20$. Flow cells typically range from 50 μm to 500 μm and to ensure optimal focus of particles upon imaging it is important to correlate appropriate objective lens magnification to flow cell thickness. As the objective lens magnification increases the focal plan becomes thinner, such that if a $\times 20$ lens is used with a 500 μm flow cell, the majority of particles imaged will be out of focus.

Other important setup parameters include flow rates, imaging rates, lighting settings, and particle detection sensitivity. To obtain reliable particle count data and avoid duplication of particles captured it is important to ensure that the flow rate and imaging rates are set to collect unique raw image frames with no image frame overlap. Additionally, when dealing with transparent, semi-transparent, or particles with similar coloration to the background it is important to optimize the lighting settings and particle detection sensitivity.

Figure 3.5 Schematic of dynamic imaging particle analysis structure depicting basic operational principle. Images are collected of samples passing through the flow cell [23].

Once data has been obtained it is possible to perform analysis for a wide range of parameters based on image processing software. Typical outputs obtained include particle size, particle aspect ratio, particle circularity, and particle edge gradient distributions, as well as particles/ml. All of these parameters can be explored based on selecting a specific particle size range for example, which allows for separation of data for particles of interest and debris present in the suspension. To ensure system accuracy, a polystyrene microsphere standard suspension is typically analyzed prior to sample runs to ensure precise system focus, size analysis, and particle counts. ‘Edge gradient’ is of particular importance to indicate that other micromeritic data is reliable. ‘Edge gradient’ refers to the transition of dark to light that occurs at the edge of a particle. In the case of dark/opaque particles, this provides a means to indicate how well-focused the particles appear, as well-focused particles have a sharp well-defined edge and therefore higher edge gradient, whereas poorly-focused particles have a poorly-defined edge and therefore lower edge gradient.

The inclusion of this data indicates that the particles used for analysis of other morphological properties were well-focused and therefore the data is expected to be more precise.

Figure 3.6 Dynamic imaging particle analysis (DIPA): Polystyrene microspheres as a standard ($50 \pm 1 \mu\text{m}$). Plots are representative graphs of (A) diameter, (B) circularity, (C) aspect ratio, and (D) edge gradient, obtained by the spline curve fitting of histogram data from 1000 well-focused particle images after triplicate measurements ($n=3$). (E) Representative microsphere images from DIPA.

Experimental protocol: The DIPA protocol implemented throughout the S-SMC extraction and loading studies was as follows. DIPA by FlowCam[®]: The benchtop system (FlowCamVS, Fluid Imaging Technologies, Scarborough, Maine, USA) was equipped with a 200 μm flow cell (FC-200), and a 20X magnification lens (Olympus[®], Shinjuku, Tokyo, Japan). The system was flushed with 1 mL deionized water (Millipore, Singapore) at a flow rate of 0.5 ml/min and flow cell cleanliness was visually inspected before each sample run. Untreated spores, S-SMCs, and BSA-loaded S-SMCs solutions with a concentration of 2 mg/ml were primed manually into the flow cell (a pre-run volume of 0.5 mL) and were analyzed with a flow rate of 0.1 ml/min and a camera rate of 14 frames/s leading to a sampling efficiency of approximately 12.2 %. A minimum of 10,000 particles were fixed as the particle count for each measurement and three separate measurements were performed. Data analysis was carried out using ~ 1000 well-focused particles

obtained from the raw data by segregating based on edge gradient. The instrument was calibrated using polystyrene microspheres ($50 \pm 1 \mu\text{m}$) and representative microsphere data is depicted as histograms with Gaussian curves fitted thereto. All specific values are reported with standard deviations (**Figure 3.6**).

3.3.4. Scanning Electron Microscopy

Scanning electron microscopy (SEM) is commonly used to observe the morphology or the surface of a specimen (**Figure 3.7**). Signals are produced, collected, and amplified when a specimen is irradiated with a fine electron beam, also known as an electron probe, in a vacuum chamber. The signals are produced due to interactions between the incident electrons and the constituent atoms in the specimen. These signals are in the form of secondary electrons, back scattered electrons, Auger electrons, and X-rays (characteristic, continuous and fluorescent). A SEM micrograph is generated from the signals collected by the secondary electron detector.

SEM is made up of an electron optical system that produces a fine electron beam, a specimen stage on which the specimen is placed, a secondary electron detector that collects secondary electrons from the specimen, an image display unit, and an operation system to perform various operations. The main components of the electron optical system are the electron gun, condenser lens, objective lens, and scanning coil. The condenser lens allows a user to control the electron probe diameter and current. The objective lens is used for focusing. Parameters to consider in order to obtain high quality SEM images are accelerating voltage, probe current, and probe diameter. Specimens should also be dried thoroughly to prevent charging effects that result in an image with extremely bright patches.

Figure 3.7 Schematic of scanning electron microscope structure depicting basic operational principle. Images are built up line by line as electron beam scans over the specimen [24].

Experimental protocol: The scanning electron microscopy protocol implemented throughout the S-SMC extraction and loading studies was as follows. An FESEM 7600F (JEOL, Akishima, Tokyo, Japan) was used for SEM image processing. Cross section samples were prepared by mounting intact spores or S-SMCs on conductive carbon adhesive tape, followed by immersion in liquid nitrogen for 30 s. A steel blade (No. 10 round edge carbon steel scalpel blade, RS Components, Singapore) was used to slice across the particles several times. The cut lines were followed during SEM until a suitable cracked particle was found. All samples were coated with gold at a thickness of 10 nm using JFC-1600 (JEOL, Akishima, Tokyo, Japan) (20 mA, 60 sec). Images were recorded with an acceleration voltage of 5.00 kV at different magnifications to observe morphological changes before and after each S-SMC extraction processing step.

3.3.5. Confocal Laser Scanning Microscopy (CLSM)

Confocal laser scanning microscopy (CLSM) allows for cross-sectional imaging and three-dimensional 3D reconstructions of samples (**Figure 3.8**). A pinhole system is implemented to exclude out-of-focus light, resulting in depth selectivity when imaging. Scanning microscopy is used to scan pixels and lines along the x- and y-axis of the focal plane, and to build a two-dimensional (2D) image based on a single slice through the sample. By varying the depth of the focal plane along the z-axis for each slice, it is possible to obtain multiple slices in the form of a z-stack of images, which can then be reconstructed to form a 3D image.

Fluorescence images are obtained by the use of excitation lasers of specific wavelength frequencies which stimulate materials to fluoresce across a range of emission wavelengths. Typically, CLSM analysis allows for fluorescence imaging across a wavelength range of typically 400 – 800 nm. Various laser types are passed through excitation filters used to provide excitation light in blue, green, and red wavelength regions. The energy of photons incident upon sample materials is used to excite electrons in outer atomic shells to higher energy states, then upon spontaneous collapse, these excited electrons drop back to a lower more stable energy state releasing an emission photon in the process. The light passes through emission filters of specific wavelength ranges and is received upon photomultiplier tubes for detection.

With regards to obtaining high quality images, it should be noted that each 2D slice does actually comprise a slice thickness and therefore does in fact represent a 3D section of the sample. The thickness of the slice is dependent on the size of the pinhole, with a smaller pinhole providing a thinner slice thickness and a sharper 2D image. Conversely, as the pinhole diameter is increased, the thickness of the slice also increases. Slice thicknesses may range from 10's of μm at lower magnification to 100's of nm at high magnification. Although a thin slice is desirable to obtain higher quality sharp edges in images, there are multiple limiting factors to consider. If the goal is to

obtain a single 2D cross-sectional type slice then the primary consideration is to ensure that the pinhole is large enough to allow adequate light to pass therethrough for imaging.

Figure 3.8 Schematic of confocal laser scanning microscope structure depicting operational principle. Images are built up line by line as electron beam scans over the specimen in x-axis and y-axis directions, and it is possible to create 3D images by scanning of multiple focal planes in a z-axis direction [25].

When using specific fluorescent molecules the light emission intensity of the molecules is typically adequate to allow for a small pinhole and thin slice thickness. However, when imaging samples with an intrinsic autofluorescence derived from the stimulation of natural compounds present in the sample, the light emission intensity of the molecules is typically weaker and requires a larger pinhole and greater slice thickness.

Experimental protocol: The confocal laser scanning microscopy (CLSM) protocol implemented throughout the S-SMC extraction, compound loading, and compound release studies was as follows. CLSM analysis was performed using a Carl Zeiss LSM710 (Oberkochen, Baden-Württemberg, Germany) confocal microscope equipped with three spectral

reflection/fluorescence detection channels, six laser lines (405/458/488/514/561/633 nm), and connected to a Z1 inverted microscope (Carl Zeiss, Oberkochen, Baden-Württemberg, Germany). Images were collected under the following conditions: laser excitation lines 405 nm (6%), 488 nm (6%), and 561 nm (9%), with DIC using an EC Plan-Neofluar 100 × 1.3 oil objective M27 lens. Fluorescence data from untreated spores, S-SMCs, and BSA-loaded S-SMCs was collected in photomultiplier tubes equipped with the following emission filters; 416–477, 498–550, and 572–620. The laser scan speed was set at 67 sec per each phase (1024 × 1024: 84.94 μm² sizes) and plane mode scanning was performed with a pixel dwell of 12.6 μs.

3.3.6. UV/Vis Spectrophotometry

Ultraviolet-visible spectrophotometry (UV/Vis) is often used in analytical chemistry to quantify analytes (**Figure 3.9**), such as highly conjugated organic compounds and biological macromolecules. In the UV/Vis region of the electromagnetic spectrum, atoms and molecules absorb energy and transit to the excited state from the ground state. Samples for UV/Vis are typically solutions that are placed in a quartz glass cuvette with an internal width of 1 cm (path length). This technique makes use of the Beer-Lambert law wherein the absorbance of a solution is directly proportional to the concentration of the absorbing species in the solution at a fixed path length.

The spectrophotometer measures the intensity of light passing through a sample and compares it to the intensity of light before it passes through the sample and this ratio, called transmittance (%T) is recorded. Absorbance (A) is calculated in the machine using the equation: $A = -\log (\%T / 100\%)$. A concentration of a solution is accurately determined more using a calibration curve or standard curve.

Figure 3.9 Schematic of ultraviolet-visible spectrophotometer depicting operational principle. Varying wavelengths of monochromatic light are passed through a sample and transmittance is measured [26].

Experimental protocol: The UV/Vis spectrophotometry protocol implemented throughout the S-SMC extraction and loading studies was as follows. 5 mg of BSA-loaded S-SMCs were suspended in 1.4 mL of PBS, then vortexed for 5 min, and probe sonicated for 3 cycles of 10 s (40% amplitude). The solution was filtered to collect the extracted BSA using a 0.45 μm PES syringe filter (Agilent, Santa Clara, CA, USA). The absorbance values were measured at 280 nm (Boeco-S220, Hamburg, Hamburg, Germany) using a placebo extract as a blank and the amount of BSA in the S-SMCs was calculated using a BSA standard curve. A BSA standard curve was generated by measuring the absorbance of BSA solutions of known concentrations (1000 $\mu\text{g} / \text{mL}$, 800 $\mu\text{g} / \text{mL}$, 600 $\mu\text{g} / \text{mL}$, 400 $\mu\text{g} / \text{mL}$ and 200 $\mu\text{g} / \text{mL}$). BSA solutions of known concentrations were made using a stock solution of 1000 $\mu\text{g} / \text{mL}$ which was serial diluted to make solutions of 800 $\mu\text{g} / \text{mL}$, 600 $\mu\text{g} / \text{mL}$, 400 $\mu\text{g} / \text{mL}$, and 200 $\mu\text{g} / \text{mL}$. All solutions were prepared in a volumetric flask to ensure accuracy.

3.3.7. Statistical Analysis

Measurements were conducted in triplicate unless explicitly indicated and the results are expressed as mean $\pm$ standard deviation (SD) of the mean. Statistical analysis of data was performed based on two-tailed t -tests, with $P < 0.05$ being considered to be statistically significant.

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Chapter 4

L. clavatum* S-SMC extraction

Microcapsules extracted from L. clavatum spores by acid/base hydrolysis methods are the most commonly studied S-SMCs for a wide range of microencapsulation applications. However, there are significant variations in acid/base extraction protocols and no studies systematically analyzing the efficacy of various S-SMC extraction steps. This chapter outlines the exploration of the most commonly used S-SMC extraction protocol so as to build a greater understanding of S-SMC extraction and to provide a clear baseline for developing an optimal extraction protocol for producing high quality microcapsules. Through analysis, refinement, and optimization, protocol efficiency is significantly improved while still producing S-SMCs suitable for use in microencapsulation applications.

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4.1. Introduction

Natural capsules obtained from plant spores and pollens are drawing significant attention for diverse microencapsulation applications [1-17]. Through various processes [7,9,18-20], it is possible to obtain empty exine capsules devoid of cytoplasmic material, proteins, and intine layer [2,12,21]. The resulting sporoderm microcapsules (S-SMCs) exhibit a high degree of structural and chemical stability [22-27], as well as species-specific uniformity with regards to size distribution, morphology, and micromeritic properties, offering an intriguing alternative to existing synthetic encapsulants [7,9,13].

Plant spores from the species *Lycopodium clavatum* (**Figure 4.1A**) have become the most widely studied single source of S-SMCs, and have a long history of use as a natural powder lubricant [28], a base for cosmetics [28], and in herbal medicine [29-33]. It is proposed that this is primarily due to its availability, low cost, and chemical robustness [9]. The exine layer of *L. clavatum* is more resilient to acid and alkali treatment than spores and pollen of many other species [2]. After processing, the resulting S-SMCs retain their intricate microridge structures and high morphological uniformity while providing a large internal cavity for encapsulation [7] (Figure 4.1A). Recent studies of *L. clavatum* S-SMCs as an encapsulant have shown high loading efficiencies with drugs [10,13], vaccines [11], proteins [7,14], cells [8], oils [5-7,9], and food supplements [5,15], in comparison to conventional encapsulation materials [7]. There are also reports of *L. clavatum* S-SMC encapsulation providing taste-masking [6,10] and antioxidant protection [12].

This chapter provides a systematic analysis of each S-SMC extraction step commonly used for *L. clavatum* spores and shows that it is possible to significantly reduce the processing temperatures and durations (Figure 4.1B). Finally, it was determined that alkaline lysis treatment can be completely removed and still produce S-SMCs of equivalent quality. Further, to demonstrate the functionality of S-SMCs extracted from this protocol, they were used for the encapsulation of bovine serum albumin (BSA) as a model

system to determine the potential loading efficiency, and a high degree of loading was observed.

Figure 4.1 Process of extracting sporoderm microcapsules (S-SMCs) from spores. (A) Schematic of plant spores and S-SMCs, (i) *L. clavatum* in its natural form, (ii) Spores containing sporoplasmic organelles, and (iii) S-SMC after removal of sporoplasmic organelles and biomolecules. (B) Flowchart of more eco-friendly processes to translate plant spores into S-SMCs.

4.2. Experimental Methods

Materials

Materials were used as defined in Chapter 3.2.1.

Extraction of Sporoderm microcapsules (S-SMCs)

S-SMCs were extracted by four main chemical processes: defatting, alkaline lysis, acidolysis, and serial washing followed by drying as defined in Chapter 3.2.2.

Surface Morphology Evaluation by Scanning Electron Microscopy (SEM)

SEM was performed as defined in Chapter 3.3.4.

Elemental CHN Analysis

Elemental analysis was performed as defined in Chapter 3.3.1.

Dynamic Image Particle Analysis (DIPA)

DIPA was performed as defined in Chapter 3.3.3.

Confocal Laser Scanning Microscopy (CLSM) Analysis

CLSM was performed as defined in Chapter 3.3.5.

Biomacromolecule Encapsulation by Vacuum Loading Technique

Biomacromolecule loading into S-SMCs was performed as defined in Chapter 3.2.3.

Encapsulation Efficiency

Encapsulation efficiency evaluation was performed as defined in Chapter 3.2.3.

Statistical analysis

Statistical analysis of data was performed as defined in Chapter 3.3.7.

4.3. Principle Outcomes

Several *L. clavatum* S-SMC extraction processes have been developed involving acetolysis, mild enzymatic chemical treatments, or aggressive prolonged non-oxidative reagents [4-11,34-36]. However, acetone defatting, KOH reflux, and phosphoric acid reflux is preferred due to its convenience and utilization of non-toxic and inexpensive reagents [7].

4.3.1. Analysis of S-SMC acid/base hydrolysis extraction

Morphology of S-SMCs during extraction

Natural *L. clavatum* spores (**Figure 4.2A**) were characterized with SEM to reveal a uniform morphology and size distribution ($31.0 \pm 2.2 \mu\text{m}$) with reticulate web-like microridges and a tripartite structure as observed in numerous previous studies [4-10]. The internal cavity of the natural spores appears predominantly hollow with sporoplasmic material forming clusters of cellular organelles and biomolecules [11]. Defatted spores from acetone refluxing exhibit no obvious changes in external spore morphology and microstructures, however, the sporoplasmic contents appeared to have been disrupted and partially removed (Figure 4.2B). The effect of alkaline lysis with 6 % KOH was examined at both 6 and 12 h. SEM imaging revealed that neither showed obvious changes in the external S-SMC morphology and microstructures. Nevertheless, the SEM cross-section showed a significant reduction in the sporoplasmic debris. However, on the inner surface of the S-SMC, there appears a thin ($< 1 \mu\text{m}$) partially wrinkled secondary layer which may be attributed to the cellulosic intine layer [21]. The spore intine layer is reported to be undamaged by alkaline lysis and requires acidolysis to remove [2,6,7].

Conventionally, acidolysis has been performed with phosphoric acid at temperatures up to 180°C for up to 7 days (168 h) [1-11,15]. In order to optimize this process, studies were undertaken with S-SMC acidolysis by phosphoric acid reflux at 70°C at different time points for up to 120 h. After

acid refluxing for 5 to 30 h (**Figure 4.3**), there are no obvious changes in external S-SMC morphology and microstructures. However, for all of the 5 to 30 h treated S-SMCs, the thin wrinkled intine layer observed after KOH reflux appears to have been removed and the inner surfaces of the capsules are smooth. Based on this data alone, it appears that the acid reflux temperature be reduced from 180°C to 70°C, and the duration can be reduced from 168 h to a window of 5 to 30 h. Under these optimized conditions, it is possible to

Figure 4.2 Characterization of *L. clavatum* spores during different stages of pre-acid treatment by scanning electron microscopy. (A) Untreated *L. clavatum* spores are intact with uniform size, well defined microstructure, and cross section indicating sporoplasmic cellular organelles and biomolecules. (B) Defatted spores at different magnifications are intact and the cross section reveals sporoplasmic biomolecules. (C) and (D) respectively show S-SMCs after 6 h and 12 h alkaline lysis indicating intact capsules with the cross section revealing residual proteinaceous debris and the cellulosic intine layer.

Figure 4.3 Scanning electron microscopic images of sporoderm microcapsules (S-SMCs) during different stages of acidolysis using 85 % (v/v) phosphoric acid. (A) S-SMCs after 5 h acidolysis indicating an intact, well defined microstructure with a clean empty inner cavity. (B), (C), and (D) respectively indicate empty, intact S-SMCs after acidolysis for 10 h, 20 h, and 30 h durations at different magnifications.

produce S-SMCs which appear to be devoid of the secondary intine layer. The prolonged acidolysis, for 60, 90, and 120 h treatments, resulted in no further visible changes in external or internal S-SMC morphology and microstructures (**Figure 4.4**). For comparison, SEM images of commercially available *Lycopodium* S-SMCs (L-type), produced by standard extraction methods, indicate heavy damage and significant fragmentation (Figure 4.4D).

Figure 4.4 Characterization of sporoderm microcapsules (S-SMCs) after prolonged acidolysis by scanning electron microscopy. (A) S-SMCs after 60 h acidolysis indicating an intact, well defined microstructure with a clean empty inner cavity. (B) and (C) respectively indicate S-SMCs after acidolysis for 90 h and 120 h. (D) Commercial L-type *Lycopodium* S-SMCs at different magnifications.

Protein content of S-SMCs during extraction

Further, compositional analysis was performed to confirm whether the S-SMCs are adequately devoid of proteins. To compare the protein content of the obtained S-SMCs with those reported in the existing literature, elemental (CHN) analysis of untreated spores and S-SMCs at each stage of the utilized protocol was performed. It is well established that the only major component of plant materials which contain nitrogen is proteins, and that the average nitrogen content of protein in plant materials is considered to be 16% [2].

Based on this, the protein content of untreated spores was determined to be 8 % (**Figure 4.5**), which is in agreement with existing studies [2,6,7,11].

Figure 4.5 Protein content in sporoderm microcapsules (S-SMCs) at different stages of treatment. The protein content is obtained from CHN elemental analysis data and is a measure of sporoplasm constituents in untreated spores and treated S-SMCs. Data represented is an average of triplicate measurements with standard deviation (n = 3).

Acetone refluxing had no significant effect on protein content. Based on statistical analysis of the data ($p < 0.001$), alkaline lysis resulted in small but statistically significant reductions in protein content, with 6 h and 12 h treatments yielding ~ 7 % and ~ 6 % total protein, respectively. Acidolysis resulted in the largest overall reduction in protein, to less than 2 %, even after only the first 5 hours of treatment. Over 10, 20, and 30 h acid reflux, there is a progressive decrease until total protein content reaches ~ 1 %, corresponding to a nitrogen content of 0.15 %. Beyond 30 h there is no significant reduction in nitrogen content. These results of 0.15 % nitrogen content are in line with several well-executed recent studies of S-SMC encapsulation [2,6,7,11] as well as commercially available S-SMCs (**Table 4.1**). Of note, the nitrogen content for S-SMCs produced commercially or presented in existing literature were

typically obtained from S-SMCs which had undergone harsh extraction processes of up to 7 days (168 h) at 180°C [11]. However, this study indicates that the severity of acidolysis processing can be significantly reduced and that acidolysis is the largest contributor to the removal of proteinaceous S-SMC components.

Table 4.1 Sporoderm microcapsules (S-SMCs): CHN composition

Treatment conditions	Carbon (%)	Hydrogen (%)	Nitrogen (%)
Untreated	65.4 ± 0.3	9.5 ± 0.1	1.30 ± 0.0
Acetone-6 h	65.6 ± 0.1	9.3 ± 0.0	1.33 ± 0.0
Alkaline lysis-6 h	62.8 ± 0.3	8.9 ± 0.0	1.10 ± 0.0
Alkaline lysis-12 h	62.5 ± 0.1	8.9 ± 0.0	0.91 ± 0.0
Acidolysis-5 h	61.5 ± 0.2	8.2 ± 0.0	0.22 ± 0.0
Acidolysis-10 h	61.8 ± 0.1	8.1 ± 0.0	0.21 ± 0.0
Acidolysis-20 h	62.9 ± 0.3	8.4 ± 0.0	0.19 ± 0.0
Acidolysis-30 h	63.6 ± 0.1	8.6 ± 0.0	0.15 ± 0.0
Acidolysis-60 h	62.1 ± 0.2	8.0 ± 0.1	0.15 ± 0.0
Acidolysis-90 h	62.1 ± 0.6	8.0 ± 0.1	0.13 ± 0.0
Acidolysis-120 h	61.1 ± 1.4	8.2 ± 0.1	0.13 ± 0.0
Commercial	65.6 ± 0.3	6.1 ± 0.1	0.18 ± 0.0

Micromeritic properties of S-SMCs during extraction

In order to produce high quality S-SMCs for use in microencapsulation and large-scale industrial applications, it is important to confirm the uniformity of S-SMC micromeritic properties. Towards this, a systematic characterization of untreated spores and S-SMCs was performed at each stage of processing using high-throughput dynamic image particle analysis (DIPA) techniques to analyze the micromeritic properties of 1000 particles.

Figure 4.6 Characterization of sporoderm microcapsules (S-SMCs) at various stages of treatment using dynamic imaging particle analysis (DIPA). Micromeritic properties of S-SMCs are displayed as column (A) Pre-acid treatment, column (B) Acid treatment using 85 % (v/v) phosphoric acid at 70°C, and column (C) Intact S-SMCs from 22 to 38 μm in diameter after acidolysis. Plots are representative graphs of diameter, circularity, aspect ratio, and edge gradient, obtained by the spline curve fitting of histogram data from 1000 well-focused particle images after triplicate measurements ($n=3$).

The DIPA data indicates that the average diameter of intact natural untreated *L. clavatum* spores is $31.0 \pm 2.2 \mu\text{m}$ (**Figure 4.6A**). This is in agreement with reported results obtained by laser diffraction, which identified

a median diameter of 30.6 μm and a very narrow size distribution [37]. The bimodal distribution observed throughout the S-SMC diameter data indicates that the majority of the particles in the samples are either intact particles (24 - 36 μm), or debris (1 - 7 μm). Natural untreated spores show a small portion of debris, which are attributed to small amounts of surface particulate matter being released during immersion in water during sample preparation.

The defatted spores from acetone reflux show minimal difference in diameter from that of untreated spores, however, defatted pores exhibit a notable increase in the debris population in the 1 - 7 μm range. The SEM observations indicate that defatted S-SMCs (Figure 4.2B) are intact. Therefore, the observed increase in debris is attributed to additional surface particulate matter being released due to long exposure to acetone. After successive alkaline reflux steps, the portion of 1 - 7 μm debris returns to a level similar to untreated spores, and continues to decrease with the second alkaline lysis treatment (Figure 4.6A). Overall, circularity and aspect ratio distributions show similar trends and indicate minimal changes in the uniformity of particle morphology throughout the pre-acid phase.

Acid treatment shows a reduction of intact particle diameter from $31.0 \pm 2.2 \mu\text{m}$, for natural untreated spores, to $\sim 27.5 \pm 2.2 \mu\text{m}$. The debris population drops significantly for 5, 10, and 20 h treatments but progressively increases from the 30 h treatment. In addition, after 60, 90, and 120 h treatments, there is a significant increase in larger S-SMC fragments (Figure 4.6B, diameter insert) in the diameter range of 7 - 22 μm . The progressive increase in both the small debris and larger fragments strongly suggests that the structural integrity of the S-SMCs is deteriorating with prolonged acidolysis. The circularity and aspect ratio distributions of the acid treatment data (Figure 4.6B) both show clear shifts towards lower values when acidolysis treatment is prolonged beyond 30 h. These shifts indicate decreases in the overall uniformity of particles. In the case of acid-treated intact S-SMCs (Figure 4.6C), both of the $\sim 3.5 \mu\text{m}$ decrease in average particle diameter and the uniformity of intact S-SMC morphology is shown to be relatively stable (a complete collection of DIPA images of intact S-SMCs are provided in **Figure**

4.7 and comparative DIPA data from commercially available S-SMCs is provided in **Figure 4.8**). Taken together, S-SMC structural integrity decreases with prolonged acidolysis and begins to cause significant fragmentation of the S-SMCs beyond 30 h treatment durations.

Figure 4.7 Representative DIPA images of intact sporoderm microcapsules (S-SMCs) at various stages of treatment.

Figure 4.8 Dynamic imaging particle analysis (DIPA): Commercial sporoderm microcapsules (S-SMCs). Plots are representative graphs of (A) diameter, (B) circularity, (C) aspect ratio, and (D) edge gradient, obtained by the spline curve fitting of histogram data from 1000 well-focused particle images after triplicate measurements ($n=3$). (E) Representative S-SMC images from DIPA.

Figure 4.9 Sporoderm microcapsule (S-SMC) fragmentation analysis based on dynamic imaging particle analysis (DIPA) at various stages of processing. (A) Fragment count from 1000 well-focused particle images based on particles in the size range of 7 to 22 μm. (B) Representative DIPA images of particles in the size range of 7 to 22 μm from natural untreated spores. (C) Representative DIPA images of particles in the size range of 7 to 22 μm after 120 h acidolysis treatment. Data represented is an average of triplicate measurements with standard deviation (n = 3). (scale bar is 20 μm).

Fragmentation of S-SMCs during extraction

Fragmentation of S-SMCs was analyzed further by a precise quantification of particles which could be clearly observed to be large portions of the S-SMC walls. A particle diameter range of 7 to 22 μm was chosen for analysis, as smaller particles may be the result of the web-like reticulum wall (muri) fragmentation only. The natural untreated spores exhibited ~ 24

fragments (of 1000 particles) (**Figure 4.9A**), which are attributed to plant-based debris (Figure 4.9B) rather than spore fragmentation. The number of fragments progressively drops during the pre-acid treatment to less than 10 fragments, and is expected to be due to degradation of the plant-based debris from the natural untreated spores. With acid treatment, a gradual increase to ~ 29 fragments can be seen within the first 30 h. Prolonged acidolysis results in a substantial jump to ~ 95 fragments after 60 h and then a steady increase to ~ 138 fragments (or 13.8% of total particles) after 120 h. A representative collection of fragment images after 120 h treatment is presented in Figure 4.9C. These results suggest that S-SMC structural integrity is progressively reduced with increasing acidolysis treatment times. Therefore, to reduce fracturing and fragmentation, and obtain the highest quality S-SMCs, it is important to minimize the duration of acidolysis. In this study, 30 h appears to be the maximum treatment time permissible to avoid significant reductions in S-SMC structural integrity.

4.3.2. Acid hydrolysis only extraction

As an extension of the initial study objectives, the potential for S-SMC extraction excluding the use of alkaline solutions was further explored. It has been common practice to use alkaline treatments to remove proteins from *L. clavatum* S-SMCs [1-11]. However, upon examination of the S-SMC protein content data (Figure 4.5), acid processing demonstrated a much greater effect on total protein removal than the alkali treatment steps. The data indicates that phosphoric acid treatment for 30 h produces S-SMCs with optimal characteristics. After 30 h treatment, the S-SMC proteinaceous nitrogen content was reduced to 0.1 % and the S-SMC fragmentation was minimal. Therefore, the next objective was to determine if it is possible to produce S-SMCs of similar quality using acetone refluxing and acid refluxing only. A new 20 g batch of 30 h acidolysis-only S-SMCs were prepared using all the same conditions as in the initial study except for the alkaline lysis treatment steps.

Figure 4.10 Characterization of 30 h acidolysis-only sporoderm microcapsules (S-SMCs) using scanning electron microscopy (SEM), dynamic imaging particle analysis (DIPA), and confocal laser scanning microscopy (CLSM). (A) SEM images indicating an intact, well defined microstructure with a clean empty inner cavity. (B) Micromeritic properties of BSA-loaded S-SMCs. Plots are representative graphs of diameter, circularity, and aspect ratio, obtained by the spline curve fitting of histogram data from 1000 well-focused particle images after triplicate measurements ($n=3$).

The SEM results of acidolysis-only S-SMCs indicate no obvious variations in external or internal S-SMC morphology and microstructures (**Figure 4.10A**) when compared to the previous 30 h S-SMCs (**Figure 4.3D**). However, DIPA analysis reveals that the new acidolysis-only S-SMCs have a diameter 1.5 μm larger (32.5 ± 2.7 μm) than untreated spores and 4.0 μm larger than the conventional 30 h S-SMCs (**Figure 4.10B**). All other parameters reveal minimal variation in S-SMC morphology. Overall, the only significant morphological difference between S-SMCs produced with or without alkaline lysis is that the utilization of acidolysis alone produces S-SMCs which are ~ 4 μm greater in diameter. However, perhaps of most importance, the 30 h acidolysis-only S-SMCs have a residual nitrogen content of only 0.1 %, which is comparable to values discussed above (0.15 % for the previously extracted conventional 30 h S-SMCs). Through the removal of the alkaline reflux processing step, the production of S-SMCs becomes substantially more eco-friendly and it becomes more viable to explore industrial-scale applications for this emerging technology.

4.3.3. Macromolecular loading of S-SMCs

In order to demonstrate the effectiveness of the obtained S-SMCs for use in microencapsulation applications, a study to compare the loading efficiency of natural untreated spores with the obtained S-SMCs was undertaken. To explore the potential encapsulation efficiency of the 30 h S-SMCs, bovine serum albumin (BSA) was utilized as a well-studied model protein [38-40] and encapsulation was achieved utilizing a modified vacuum loading technique [2,5-7]. **Figure 4.11** depicts the process of encapsulating BSA within S-SMCs. Briefly, empty S-SMCs are suspended in an aqueous solution of BSA, and with the application of a vacuum, the internal cavity pressure is altered to forcefully draw the BSA solution into the empty capsules (Figure 4.11A) [41]. Nanochannels (dia. 15 - 20 nm), which have been previously identified in the exine walls of *L. clavatum* [42], allow the solution to enter into the large internal cavity (Figure 4.11B-C). After loading, the capsules were washed to remove residual BSA from the outer surface, and the BSA-loaded S-SMCs were dried in a freeze-drier to yield a free-flowing powder (Figure 4.11D).

Figure 4.11 Schematic of microencapsulation of bovine serum albumin (BSA) into intact sporoderm microcapsules (S-SMCs). (A) BSA loading by incubating S-SMCs in aqueous BSA solution under vacuum. (B) Uptake of BSA through nanochannels in S-SMC walls. (C) and (D) respectively represent a S-SMC during the loading process and a finally prepared BSA-loaded S-SMC.

Loading efficiencies & observation

The results of loading efficiencies (**Table 4.2**) indicate a 2.49 times greater loading by weight for the 30 h S-SMCs in comparison to untreated spores with a statistical significance of $p < 0.001$. The 30 h S-SMCs had a

loading of 0.734 ± 0.013 g of BSA per 1 g of S-SMCs, whereas, the natural untreated spores had a loading of 0.295 ± 0.015 g of BSA per 1 g of untreated spores.

Table 4.2 Encapsulation of Bovine serum albumin into S-SMCs

Material	BSA loading (125 mg ml ⁻¹)	BSA in 5 mg S-SMCs (mg)	Amount of BSA loaded (g g ⁻¹ of S-SMCs)
Untreated spores	0.6 ml	1.47 ± 0.06	0.295 ± 0.013
S-SMCs	1.2 ml	3.67 ± 0.08	0.734 ± 0.015

To confirm the cleanliness of the capsules and the effectiveness of encapsulation, confocal laser scanning microscopy (CLSM) was performed for untreated spores as well as 30 h S-SMCs before and after macromolecule loading (**Figure 4.12**). To observe the localization of loaded BSA, FITC-BSA was encapsulated into 30 h S-SMCs following the same protocol as mentioned above. FITC fluoresces in the green light spectrum (excitation: 490 nm, emission: 525 nm) [43] and it is known that plant spores also fluoresce across a broad spectrum of excitation wavelengths [44-46]. To ensure that no overlap was observed in the emission spectrums of the FITC-BSA and untreated spores or S-SMCs, the CLSM settings were calibrated to produce no visible emissions in the green spectrum for either the untreated spores or the 30 h S-SMCs. After calibration, all scan results shown in Figure 4.12 were obtained using the same green spectrum excitation and emission collection settings. The results for untreated spores indicate no autofluorescence in the green channel. However, for the blue and red channels it is clear that the internal spore cavity contains a collection of cellular organelles similar to those observed in the SEM cross section (Figure 4.2A) and SEM data from existing studies [11]. It should be noted that the existence of these organelles appears to contradict previous studies [2,6,7] wherein CLSM images of *L. clavatum* spores depict that the entire internal cavity of natural spores exhibit autofluorescence. However, this observation may be due to species-specific variations dependent on material source. The 30 h S-SMCs show a similar morphology to untreated spores, but with an empty internal cavity. After loading with FITC-BSA, the S-SMCs

exhibit near complete loading of the internal cavity and minimal binding of BSA to the reticulate ridge structures of the S-SMC surface. It is important to acknowledge that during the loading of many common encapsulation materials, some portion of the loaded compound may bind to the external surface of the encapsulant [38-40]. However, the CLSM data clearly depicts minimal surface binding of BSA from the encapsulation process utilized in this study. **Figure 4.13** depicts a series of Z-stack CLSM images for FITC-BSA loaded 30 h acidolysis-only S-SMCs.

Figure 4.12 Confocal laser scanning microscopy (CLSM) analysis of sporoderm microcapsules (S-SMCs) before and after treatment and BSA encapsulation. CLSM images in the first row are unprocessed *L. clavatum* spores indicating autofluorescence due to sporoplasmic cellular organelles and biomolecules. The second row indicates intact S-SMCs after 30 h acidolysis treatment with a clean and intact large inner cavity, and the third row indicates FITC-BSA loaded into 30 h acidolysis treated S-SMCs. (Scale bars are 10 μ m).

Figure 4.13 Confocal laser scanning microscopy (CLSM) Z-stack analysis of 30 h acidolysis-only S-SMCs loaded with FITC-BSA.

Morphology & micromeritic properties of compound loaded S-SMCs

Size uniformity and morphological characteristics are important factors for evaluating the quality of a successful encapsulant. Therefore, SEM and DIPA were utilized to investigate the stability of S-SMC micromeritics throughout the loading process. From the SEM images in **Figure 4.14A**, the loaded S-SMCs exhibit no obvious changes in morphology. Although there appears to be some external binding of BSA to some of the S-SMCs, careful inspection of the single loaded S-SMC reveals no significant BSA binding to the microridge surface structure. This is in agreement with the CLSM observation discussed above. The DIPA data indicates that there are no significant changes in morphology before and after loading with respect to diameter, circularity, and aspect ratio (Figure 4.14B). The edge gradient data indicates some variation in the optical focus of the particle images used for the quantification of other parameters, although it appears to have had no influence on the analysis of the other parameters. Overall, the process of the vacuum loading of BSA used in this study has had no influence on the uniformity of S-SMC micromeritic properties.

Figure 4.14 Characterization of BSA-loaded sporoderm microcapsules (S-SMCs) using scanning electron microscopy (SEM) and dynamic imaging particle analysis (DIPA). (A) SEM images of BSA-loaded S-SMCs. (B) Micromeritic properties of BSA-loaded S-SMCs. Plots are representative graphs of diameter, circularity, aspect ratio, and edge gradient, obtained by the spline curve fitting of histogram data from 1000 well-focused particle images after triplicate measurements ($n = 3$).

4.4. Conclusions

This study demonstrates that it is possible to produce higher quality S-SMCs from *L. clavatum* spores while also achieving a significant streamlining of the conventional extraction protocol. By reducing the acidolysis time to 30 h and reflux temperature to 70°C, as well as the complete removal of the alkaline

lysis step, the environmental impact of S-SMC processing can be reduced and S-SMCs with higher structural integrity are obtained. The extracted S-SMCs retain the well-defined microstructure of natural untreated spores with effective removal of cytoplasmic and cellulosic contents. Importantly, the analysis of the micrometric properties of large samples of S-SMCs, at various stages of chemical treatment, highlights the detrimental effect of prolonged acidolysis and indicates progressive reductions in structural integrity in the form of increased S-SMC fragmentation. The high encapsulation efficiency of the resulting S-SMCs, which may be achieved by simple loading techniques, goes on to highlight the versatile nature of these capsules for microencapsulation applications. Taken together, these results are important for the large-scale industrial production of S-SMCs and stimulating greater interest in the potential applications of this field of research. This systematic study of S-SMC extraction from *L. clavatum* spores may lead the way for further exploration of new S-SMCs extracted from a wide variety of plant species.

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Chapter 5

H. annuus* S-SMC extraction

*The potential diversity of raw material sources for S-SMC extraction represents significant opportunities for the fields of microencapsulation and biopolymers. Various plant species produce pollen or spores with intriguing microstructural features which may be advantageous to defining specific applications, yet it is unclear whether it is possible to extract intact S-SMCs with high levels of structural ornamentation from genetically diverse pollen sources. This chapter outlines the application of commonly used S-SMC extraction protocols with sunflower (*H. annuus*) pollen, as a representative angiosperm-derived pollen, to compare the efficacy of various acid/base-hydrolysis extraction methods and determine the optimal S-SMC extraction method for application to angiosperm-derived pollen.*

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5.1. Introduction

Natural microcapsules produced from plant spores and pollen grains have received increasing attention in recent years due to their broad utility as drug delivery vehicles, cell encapsulating scaffolds and biotemplates for advanced materials synthesis [1-6]. Conventional manufacture of polymeric microcapsules is costly and difficult, especially to obtain microcapsules with a uniform size distribution and large inner cavity [7-10]. There is wide interest in producing microcapsules through alternative strategies, and pollen-based microcapsules offer a natural solution that is readily available in abundant quantities [1,11-13]. Pollen constituents are mainly protected by a cellulose-rich cell wall called the intine, and a resistant outer wall composed largely of sporopollenin called the exine. Exine morphology varies significantly between pollen species, with highly unique and reproducible 3D architectures and morphologies. Importantly, the exine also provides environmental protection for successful pollination, motivating its extraction for use as natural microcapsules which are Generally Recognized as Safe by the US Food and Drug Administration [14,15]. Sporoderm microcapsules (S-SMCs) produced from spores and pollen grains are resistant to extreme temperatures, pressures, and harsh chemical treatments as well as are devoid of allergenic materials [1,14]. In order to extract S-SMCs, a simple chemical process is highly desirable to remove all interior and surface pollen constituents without altering the native microstructure of the exine capsule [12]. Several attempts have reported the extraction of S-SMCs from various plant spores and pollen grains by complex multiple steps including alkaline lysis, acidolysis, and enzymatic processes [1,2,13,16]. While various degrees of success have been achieved in obtaining intact S-SMCs without pollen inner constituents, in all cases, the process is time consuming and requires harsh reflux conditions which are particularly challenging when working with highly ornamented 3D microstructures. The primary requirement of the extraction process is to achieve empty S-SMCs without appreciable damage to intrinsic exine surface morphology. In this regard, sunflower pollen stands out due to its highly ordered surface spike ornamentation and monodisperse size distribution, which

motivate the development of novel 3D materials and drug delivery platforms [1,4,17].

Figure 5.1 One-pot process to extract intact, clean, and conserved distinct morphological sunflower sporoderm microcapsules (S-SMCs); Schematic shows (A) and (B) Harvesting of natural sunflower pollen grains and processing to remove plant-based debris. (C). Scanning electron microscopic images of natural pollen with different magnifications. (D) S-SMC extraction processes involving alkaline lysis with KOH, acidolysis with HCl or phosphoric acid to remove all pollen constituents. (E) Washing steps using organic solvents and dilute acids to remove residual pollen materials to yield clean, intact S-SMCs. (F) Scanning electron microscopic images of S-SMCs after complete processing at various magnifications. (G) Different one-pot process approaches used to extract intact sunflower S-SMCs.

To address this issue, the objective of the current study is to develop a simple chemical scheme to extract preserved S-SMCs from sunflower pollen. In particular, a one-pot scheme to extract sunflower S-SMCs by a simple chemical process is reported, and involves acidolysis using phosphoric acid, resulting in the first successful extraction of sunflower S-SMCs which retain highly ornamented microstructures. The processed S-SMCs were rigorously characterized by CHN elemental analysis, scanning electron microscopy (SEM), confocal laser scanning microscopy (CLSM) and dynamic imaging particle analysis (DIPA). The reported method represents a dramatic improvement over existing strategies and builds on chemical knowledge about pollen extraction in order to establish a general scheme that can be broadly applied.

Figure 5.1 depicts the one-pot extraction process for sunflower S-SMCs. The sunflower pollen grains are readily obtained from field harvests and are separated from miscellaneous plant debris (Figure 5.1A-B). Sunflower pollen possesses a unique microstructure with a uniform size distribution ($37 \pm 0.21 \mu\text{m}$) and surface decorated with spikes surrounded by pores as imaged by SEM (Figure 5.1C). The pollen grains are processed under gentle reflux conditions to remove all pollen constituents and washed extensively with a series of aqueous and organic solvents to remove residual pollen constituents (Figure 5.1D-E). The SEM images indicate that sunflower S-SMCs retain the intrinsic microstructure and have a uniform size distribution ($30 \pm 0.4 \mu\text{m}$) (Figure 5.1F). The first sunflower S-SMC extraction protocols were strategized based on alkaline lysis with 6 % potassium hydroxide (KOH) and acidolysis using 6 M hydrochloric acid (HCl) at 70°C (Figure 5.1G) [18,19]. The representative SEM images of sunflower pollen grains before and after alkaline lysis and HCl acidolysis are presented in **Figure 5.2**.

5.2. Experimental Methods

Materials

Materials were used as defined in Chapter 3.2.1.

Extraction of sunflower sporoderm microcapsules (S-SMCs)

S-SMCs were extracted as defined in Chapter 3.2.2.

Elemental analysis

Elemental analysis was performed as defined in Chapter 3.3.1.

Dynamic image particle analysis (DIPA)

DIPA was performed as defined in Chapter 3.3.3.

Confocal laser scanning microscopy analysis

CLSM was performed as defined in Chapter 3.3.5.

Surface morphology evaluation by scanning electron microscopy (SEM)

SEM was performed as defined in Chapter 3.3.4.

Matrix-assisted laser desorption/ionization - time of flight (MALDI-TOF) Mass Spectrometry (MS)

MALDI-TOF MS was performed as defined in Chapter 3.3.2.

Statistical analysis

Statistical analysis of data was performed as defined in Chapter 3.3.7.

5.3. Principle Outcomes

5.3.1. Acid/Base hydrolysis extraction exploration

Morphology of S-SMCs from standard extraction protocols

Natural sunflower pollen grains possess well-organized spikes on the pollen surface and cross-section analysis reveals the presence of pollen constituents inside the large inner cavity (Figure 5.2A). Interestingly, the pollen microstructure is destabilized by alkaline lysis resulting in the loss of the unique microstructure (Figure 5.2B). The structural destabilization of sunflower pollen indicates that alkaline lysis is detrimental to retaining unique pollen microstructures. On the other hand, acidolysis using HCl was able to retain the microstructure, yet extracted debris still remained on the S-SMC surface coats (Figure 5.2C). The alkaline lysis destabilization of the sporoderm morphology may be due to the cleaving of ester bonds within sporopollenin in a process of saponification. It is known that there are variations in sporopollenin chemistry between species, and it seems that sunflower pollen may be more heavily ester-bound rather than ether-bound leading to greater susceptibility to alkaline lysis.

Morphology of S-SMCs during HCl hydrolysis extraction optimization

In order to achieve empty, clean S-SMCs devoid of all pollen constituents, the effect of HCl acidolysis duration with a cleaning process using a series of organic solvents and dilute acids was examined. **Figure 5.3A-C** show the SEM images of sunflower S-SMCs after 10 h, 20 h and 30 h HCl acidolysis. The washing steps improved the cleanliness of capsules with less pollen debris and the S-SMCs are morphologically intact. However, cross-section SEM images reveal residual pollen constituents inside the S-SMC cavity. It is crucial to remove all pollen constituents from sunflower S-SMCs to create a large inner cavity devoid of allergenic proteins. Therefore, additional washing steps were incorporated using trypsin and Tween-20 [20]. Figure 5.3D demonstrates that trypsin incubation induced breakage of S-SMCs

spikes although there was a relatively clean inner cavity. With Tween-20 washing, the sunflower S-SMCs still retained pollen debris on the surface, indicating that residual pollen debris may include a cellulosic material along with pollen proteins (Figure 5.3E) [14,21].

Figure 5.2 Scanning electron microscopic images of sunflower pollen grains and sporoderm microcapsules (S-SMCs) obtained from different extraction processes: (A) Natural sunflower pollen grains intact with uniform size and well-defined spikes, pores and cross-section reveal pollen constituents. (B) S-SMCs after alkali (6 % potassium hydroxide) treatment (12 h). (C) S-SMCs after acidolysis using hydrochloric acid (6 M) process for 48 h.

Morphology & micromeritic properties of S-SMCs from H_3PO_4 extraction

As phosphoric acid is more efficient in removal of cellulose materials from spores and pollen [1,13,21], acidolysis treatment of sunflower pollen by using phosphoric acid (85 % v/v) as a one-pot process was next investigated. The SEM images of sunflower S-SMCs after acidolysis using phosphoric acid showed intact, clean S-SMCs (**Figure 5.4A**). It is noteworthy that the produced S-SMCs resemble the surface ornamentation similar to native sunflower pollen.

In order to confirm the removal of pollen constituents from S-SMCs, the sunflower pollen grains were analyzed before and after the one-pot process by CLSM. It is well known that natural pollen grains exhibit autofluorescence due to their inner constituents [22,23]. The sunflower pollen exhibit strong autofluorescence before one-pot process (Figure 5.4B) while, in marked contrast, no autofluorescence was observed inside the S-SMCs after acidolysis with phosphoric acid, suggesting successful removal of inner constituents.

Figure 5.3 Scanning electron microscopic images of sunflower sporoderm microcapsules (S-SMCs) after acidolysis using hydrochloric acid and extensive washing for different time points: (A) 10 h. (B) 20 h. (C) 30 h. (D) Trypsin washed S-SMCs, (E) Tween-20 washed S-SMCs.

Figure 5.4 Sunflower sporoderm microcapsules (S-SMCs) produced after one-pot process using phosphoric acid: (A) Scanning electron microscopic images of S-SMCs after acidolysis at different magnifications and a cross-section image. (B) Confocal microscopy analysis of natural sunflower pollen grains before and after one-pot processing: CLSM images in first row are natural pollen grains before processing and indicate autofluorescence due to the presence of terpenoid, phenolic, and carotenoid molecules. Second row indicates clean S-SMCs with complete removal of pollen constituents. (Scale bars are 10 μm).

The CLSM data also support that the one-pot acidolysis process produces intact capsules, albeit somewhat smaller than the unprocessed samples due to removal of pollen cellular constituents [14]. The reduction in S-SMC size is mainly due to loss of hydration resulting from complete removal of cytoplasmic materials [24]. Interestingly, in nature, pollen grains change shape, volume and structure due to pollen hydration, dehydration and rehydration as part of biological processes involved in plant reproduction [24,25].

In order to verify the reduction in particle size due to phosphoric acid acidolysis, high-throughput particle characterization of untreated pollen and treated S-SMCs by using the dynamic imaging particle analysis (DIPA)

technique was also performed. The results indicate that the average diameter of untreated sunflower pollen is $37 \pm 0.2 \mu\text{m}$ with a narrow size distribution (**Figure 5.5A**). Upon phosphoric acid acidolysis, the S-SMC average size is reduced to $30 \pm 0.4 \mu\text{m}$ supporting the CLSM data that shows a reduction in S-SMC diameter after removal of pollen constituents (**Table 5.1**) [24-26]. DIPA images of untreated sunflower pollen reveal a unique microstructure with surface ornamentation consisting of spikes (Figure 5.5B). After one-pot processing with phosphoric acid acidolysis, the sunflower S-SMCs retained the native microstructure without any damage to the S-SMC structure (Figure 5.5C). Taken together, the data supports that the one-pot process yields S-SMCs with uniform size and similar structural ornamentation as natural sunflower pollen grains. Based on the current understanding of sporopollenin, two main options for particle shrinkage after acid-extraction are possible: 1) the acid processing alters the sporopollenin chemistry causing shrinkage in the polymer; or 2) the sporopollenin shell is known to be elastic in nature, being able to shrink and swell with desiccation and hydration, and after the removal of the cytoplasmic contents the shell may shrink to a size it would have if there were no outward pressure of the contained cytoplasmic contents. Overall, it is likely that both factors are contributing to shrinkage in particle size.

Figure 5.5 Characterization of sunflower sporoderm microcapsules (S-SMCs) after one-pot extraction process. (A) Frequency histogram of particle diameter (μm) for 1000 untreated pollen and S-SMCs. Optical micrographs of (B) untreated pollen grains and (C) S-SMCs after one-pot acidolysis using phosphoric acid.

Table 5.1 DIPA data of sunflower sporoderm microcapsules ^a

Sunflower pollen	Diameter (μm)
Defatted	37 ± 0.2
Capsules ^b	30 ± 0.4

^aDynamic imaging particle analysis performed in triplicate and reported are average with standard deviation (n= 3).

^bSporoderm microcapsules prepared using extraction process with phosphoric acid for 10 h (2 g batch scale).

Protein content of S-SMCs from various extraction protocols

Finally, the amount of residual protein present is investigated for sunflower S-SMCs obtained using different processing schemes by performing CHN elemental analysis before and after S-SMC extraction. It is well known that proteinaceous nitrogen is one of the major components of plant materials [16] and untreated sunflower pollen contains 32 wt% of proteins (**Figure 5.6**). The acidolysis process using HCl for 48 h yielded 14 wt% residual protein. The residual protein content remained nearly unchanged between 10 wt% and 14 wt% with varying duration of HCl acidolysis treatment time and extensive washing. Strikingly, acidolysis using phosphoric acid produced sunflower S-SMCs with significantly ($p < 0.01$) low protein content (4 wt%) compared to S-SMCs produced by acidolysis using HCl (see CHN data in **Table 5.2**). During early studies on sporopollenin isolation, it was observed that phosphoric acid achieved better removal of the cellulosic intine material [16]. Therefore, HCl may be less effective at breaking down the intine wall layer and allowing for exposure and complete removal of the proteinaceous cytoplasmic contents.

Figure 5.6 Protein content in sunflower sporoderm microcapsules (S-SMCs) produced by different chemical processes from CHN elemental analysis. The protein content is a measure of pollen protein constituents in unprocessed and processed S-SMCs and determined by using the percent nitrogen from elemental analysis with a multiplication factor of 6.25 to convert percent nitrogen into percent protein. Data represented is average of triplicate measurements with standard deviation ($n = 3$).

5.3.2. H₃PO₄ hydrolysis S-SMC extraction optimization and analysis

Morphology & protein content of S-SMCs during H₃PO₄ extraction

Based on the determination that phosphoric acid extraction provides the best S-SMC extraction results, further optimization of the S-SMC extraction was undertaken by conducting acidolysis with phosphoric acid (85 % v/v) at four different durations, 1, 5, 10, and 20 h. Comprehensive SEM analysis indicated that all acidolysis treatment durations produced a clean inner cavity and intact external microstructure (**Figure 5.7**, **Figure 5.8**). CLSM analysis also showed that H₃PO₄ acidolysis achieved effective removal of internal cytoplasmic contents, indicated by an absence of autofluorescence in the

internal cavity following acidolysis processing (Figure 5.7C, **Figure 5.9**). The reduced size of processed S-SMCs is attributed to the removal of sunflower pollen cytoplasmic constituents (SEM or CLSM) [27]. The efficacy of protein removal was also characterized by elemental (CHN) analysis; the quantity of nitrogen in plant materials is assumed to be proportional to protein content [1,3,6,12,27-29]. A comparative analysis of the protein removal efficacy, based on % nitrogen content, indicated that 5 h of H₃PO₄ treatment resulted in the maximum possible protein removal (Figure 5.7 A, **Figure 5.10**, and **Table 5.3**).

Table 5.2 Sunflower sporoderm microcapsules: CHN composition^a

Process conditions	Carbon (%)	Hydrogen (%)	Nitrogen (%)
Defatted ^b	48.8 ± 0.2	7.6 ± 0.2	5.2 ± 0.0
HCl ^c acidolysis-48 h	64.6 ± 0.5	7.3 ± 0.4	2.2 ± 0.2
HCl acidolysis-10 h with extensive washing	62.0 ± 0.1	6.9 ± 0.5	2.2 ± 0.0
HCl acidolysis-20 h with extensive washing	63.5 ± 0.2	7.3 ± 0.1	1.6 ± 0.0
HCl acidolysis-30 h with extensive washing	64.6 ± 0.7	7.1 ± 0.5	1.6 ± 0.0
H ₃ PO ₄ ^d acidolysis-10 h with extensive washing	61.5 ± 0.3	7.9 ± 0.4	0.7 ± 0.0

^a CHN analysis performed in triplicate and reported are average value with standard deviation. ^b Defatted pollen grains. ^c 6 M Hydrochloric acid. ^d 85 % (v/v) Phosphoric acid.

Acid hydrolysis of proteins is common in food processing to produce acid protein hydrolysates. Medium temperature, short duration, strong acid processing produces peptides from proteins, which are typically in the 1 kDa to 20 kDa range [27]. Mass spectrometry, MALDI-TOF, was performed with ground and ethanol extracted samples. Analysis of defatted pollen indicated

multiple peaks present in the 2500 m/z to 4500 m/z range, suggesting the presence of large biomacromolecules. However, these peaks were absent in all of the 1, 5, 10, and 20 h acid-processed S-SMCs (**Figure 5.11**), supporting the effective removal of proteinaceous compounds as observed by elemental analysis. The MALDI-TOF analysis strongly suggests that the determination of 0.7 % nitrogen from elemental analysis is the result of analytical error and/or residual amino acids resulting from acidolysis of proteins [27].

Figure 5.7 Optimized sunflower pollen sporoderm microcapsule (S-SMC) extraction: (A) Comparative analysis of protein removal efficacy following H_3PO_4 acidolysis, based on % nitrogen content; data is presented as the average of triplicate measurements with standard deviation ($n = 3$); (B) Scanning electron microscope cross-section images of a defatted sunflower pollen grain and S-SMC following 5 h H_3PO_4 acidolysis; and (C) Confocal microscopy analysis of a defatted sunflower pollen grain and S-SMC following 5 h H_3PO_4 acidolysis.

Figure 5.8 Scanning electron microscope images of sunflower sporoderm microcapsules (S-SMCs) following different durations of H_3PO_4 acidolysis: (A) without acidolysis; (B) 1 h; (C) 5 h; (D) 10 h; (E) 20 h.

Figure 5.9 Confocal microscopy analysis of sunflower sporoderm microcapsules (S-SMCs) following varying durations of H_3PO_4 acidolysis. (Scale bars 10 μm).

Figure 5.10 Residual protein content in sunflower sporoderm microcapsules (S-SMCs) following different periods of H_3PO_4 acidolysis, as measured with CHN elemental analysis. Data is presented as the average of triplicate measurements with standard deviation ($n = 3$).

Table 5.3 Sunflower sporoderm microcapsules (S-SMCs): CHN composition^{a)}

Treatment conditions	Carbon [%]	Hydrogen [%]	Nitrogen [%]
Defatted	48.8 ± 0.2	7.6 ± 0.2	5.2 ± 0.0
H_3PO_4 acidolysis 1 h	60.4 ± 0.0	8.0 ± 0.0	1.1 ± 0.0
H_3PO_4 acidolysis 5 h	61.5 ± 0.7	7.5 ± 0.6	0.7 ± 0.0
H_3PO_4 acidolysis 10 h	62.1 ± 0.6	7.5 ± 0.5	0.6 ± 0.0
H_3PO_4 acidolysis 20 h	61.8 ± 1.4	6.8 ± 1.6	0.6 ± 0.0

^{a)}CHN analysis performed in triplicate and reported as average values with standard deviation

Figure 5.11 Large molecule content analysis by MALDI-TOF for sunflower pollen and sporoderm microcapsules (S-SMCs) based on sample grinding and ethanol extracts: (A) Mass-to-charge ratio spectrum for pollen, S-SMCs, and MALDI matrix (CHCA) from 1000 m/z to 20000 m/z ; (B) Major mass-to-charge ratio peaks for S-SMCs; (C) Major mass-to-charge ratio peaks for pollen; and (D) Relative peaks heights of major pollen peaks, CHCA peaks, and major S-SMC peaks. Data is presented as representative of triplicate measurements.

Micromeritic properties of S-SMCs during extraction

For morphological analysis, the size of sunflower pollen grains was characterized by dynamic imaging particle analysis (DIPA) (**Figure 5.12**) following different acidolysis durations. DIPA data imply that the S-SMC extraction resulted in the reduction of intact particle diameter, from 37 ± 1.5 μm for natural pollen grains to 30 ± 1 μm for 5, 10, and 20 h processed S-SMCs (**Figure 5.12**, **Table 5.4**), respectively. This supports the reduction in particle size observed by other techniques including SEM and CLSM. Moreover, the micromeritic analysis regarding circularity and aspect ratio of S-SMCs processed at different periods exhibited a near to circular shape with no significant difference between processed S-SMCs and native pollen (**Figure 5.12**). The DIPA analysis indicates uniform morphology and intactness of S-SMC architecture in comparison to native pollen grains (**Figure 5.13**). Overall, based on the intactness of the pollen microstructure, the cleanliness of the internal cavity, and the efficacy of proteinaceous nitrogen removal, it was determined that 5 h H_3PO_4 treatment is adequate to produce S-SMCs from sunflower pollen.

Table 5.4 Equivalent spherical diameter of sunflower S-SMCs^{a)}

Sunflower	Equivalent spherical diameter (ESD) [μm]
Defatted pollen grains	37.0 ± 1.5
H_3PO_4 acidolysis 1 h	27.8 ± 1.3
H_3PO_4 acidolysis 5 h	30.2 ± 1.1
H_3PO_4 acidolysis 10 h	30.3 ± 0.8
H_3PO_4 acidolysis 20 h	30.3 ± 0.8

^{a)}DIPA analysis performed in triplicate and reported as average values with standard deviation

Figure 5.12 Characterization of sunflower sporoderm microcapsules (S-SMCs) before and after extraction using phosphoric acid at varying time points: (A) Phosphoric acid treated S-SMCs; (B) Only intact S-SMCs.

Figure 5.13 Optical micrographs of sunflower pollen grains and S-SMCs produced by varying periods of H_3PO_4 acidolysis.

5.4. Conclusions

In summary, a one-pot process was developed and optimized in order to extract intact, clean sunflower S-SMCs without altering the native microstructure and surface ornamentation. Indeed, the newly developed process achieves the twin goals to preserve the S-SMC microstructure and remove a high fraction of protein constituents, as desired for therapeutic applications. On the other hand, the data also support that the S-SMC microstructure is damaged by alkaline lysis, which is a long established step in S-SMC extraction. This discovery underscores the potential significance of simplified processing schemes that not only reduce processing time but also improve the final product. By contrast, this study shows for the first time that unique, intrinsic microstructure of pollen is retained after removal of all pollen

constituents by simple one-pot acidolysis process using phosphoric acid. This one-pot process provides access to further explore the wide range of available natural pollen grains with narrow size distributions. Sunflower S-SMCs offer a unique microstructure with a large inner cavity for diverse microencapsulation applications including drug delivery, cosmetics, and food technology.

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Chapter 6

H. annuus* S-SMC loading & controlled release

Microencapsulation is one of the major potential applications for plant-based microcapsules such as S-SMCs. Therefore, after S-SMC extraction, it is important to explore compound loading into S-SMCs, mechanisms of compound release from S-SMCs, and the means to release compounds under desired conditions. This chapter outlines analysis of standard S-SMC loading protocols to clearly elucidate loading mechanisms, requirements, results, and the potential of compound loading within S-SMCs. Additionally, standard controlled release methodologies are applied for the purpose of developing a multiparticulate targeted delivery system to achieve targeted delivery appropriate for the delivery of sensitive therapeutic compounds to the digestive tract.

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6.1. Introduction

The microencapsulation of drugs have been extensively studied over the past few decades, and major achievements had been made; nevertheless, a long-standing, remaining challenge is the rapid and low-cost manufacturing of microcapsules with uniform size, porosity, and architecture, particularly for oral delivery of therapeutic proteins [1,2]. Sporoderm microcapsules (S-SMCs), which are extracted from natural pollen, provide an attractive, natural, and robust drug delivery system, due to their striking structural uniformity, mechanical and chemical robustness, monodisperse size and porosity, large inner cavity, and abundant availability. In nature, sporopollenin forms the outermost layer of pollen grains, and is responsible for the safe and protective transportation of the enclosed reproductive genetic materials under diverse environmental conditions [3,4]. Moreover, pollen and S-SMCs have been explored for their nutritional and therapeutic effects, as well as the encapsulation of drugs, proteins, vaccines, and oils [4-12]. These features and particularly the S-SMCs' inherent physicochemical robustness make them an excellent candidate for encapsulating therapeutic proteins for oral delivery with controlled release [3,4,7,8].

In recent years, therapeutic proteins have emerged as an important treatment option, and they represent a massive share of the pharmaceuticals market [13-17]. The administration of therapeutic proteins largely involves injections [14,18], which suffer from low patient compliance and are often painful [2,19]; on the other hand, oral delivery is a convenient and painless administration route and is preferred by patients [2,18]. Nevertheless, due to the harsh environment of the stomach, only negligible amounts of unprotected therapeutic proteins typically reach the intended delivery site, and the oral delivery of proteins is therefore limited [2,18,20-22]. Various delivery systems have been explored to overcome this challenge, including the use of copolymers as an enteric coating [13,19,22-25]. In particular enteric coating using a copolymer of methacrylic acid and methyl methacrylate (Eudragit), which is insoluble in acidic pH, has been shown to provide protection in low pH conditions [25]. Although progress has been made, oral delivery of

proteins is still lacking a cost effective and efficient encapsulation process [2,20].

Figure 6.1 Schematic diagram showing the development of tableted sporoderm microcapsules (S-SMCs) for intestinal protein delivery.

So far, S-SMCs have been explored for the encapsulation of proteins and it is suggested that their mechanical and chemical durability may facilitate the oral delivery of proteins [5,26,27]. Interestingly, enzymes encapsulated into *Lycopodium clavatum* S-SMCs have been shown to retain their functionality following their release from the S-SMCs, thus implying the protective nature of S-SMCs toward encapsulated proteins [5]. Sporopollenin, is a complex biopolymer which forms the outer shell structure of S-SMCs and is known to exhibit morphological stability under harsh chemical processing, such as acetolysis, acidolysis, and exposure to solvents. In previous chapters, an eco-friendly streamlined extraction process was introduced for obtaining S-SMCs from natural *Lycopodium clavatum* (*L. clavatum*) spores and natural sunflower (*H. annuus*) pollen grains while retaining their unique microstructure [3,28];

thus contributing to the potential of using pollen as the basis of a cost effective microencapsulation system.

Herein, the potential of S-SMCs for drug delivery applications is expanded on by conducting the optimization of S-SMC loading, and controlled release. The results provide significant insights into the mechanisms and potential of compound loading and drug delivery utilizing S-SMCs or other porous microcapsule systems. S-SMCs were characterized in different stages of the encapsulation and tableting processes to study their morphological stability. The loading kinetics were characterized, and key factors affecting the loading process were identified. Insights that were gained from mechanistic evaluation were then applied to explore the practical and theoretical potential of compound loading. Finally, it was demonstrated that tableted BSA-loaded S-SMCs with an enteric coating (Eudragit L100) provide an effective method for the oral delivery of therapeutic proteins. Overall, the schematic diagram in **Figure 6.1** outlines the transition of sunflower pollen into S-SMCs, BSA loading into S-SMCs, and tableting with enteric coating for intestinal protein delivery.

6.2. Experimental Methods

Materials

Materials were used as defined in Chapter 3.2.1.

Dynamic image particle analysis (DIPA)

DIPA was performed as defined in Chapter 3.3.3.

Confocal laser scanning microscopy (CLSM) analysis

CLSM was performed as defined in Chapter 3.3.5. For fluorescence intensity analysis of FITC-BSA loading into S-SMCs, green channel CLSM images were used from the mid region of the S-SMCs. Only whole S-SMCs

were utilized for fluorescence analysis. The total fluorescence intensity values for the first 50 S-SMCs of each sample set were selected, i.e. FITC-BSA-loading proportions of 23 wt.%, 37.5 wt.%, and 54 wt.%. The S-SMC fluorescence intensity values for all 150 S-SMCs were normalized, with 1.0 being the highest value out of all 150 S-SMCs. The normalized S-SMC fluorescence data was used for further statistical analysis and sample set comparisons. Assuming that an S-SMC with a normalized fluorescence intensity value of 1.0 is equivalent to having a loading of 100 vol.%, then the average S-SMC fluorescence intensity values of each sample set can be correlated to an equivalent loading vol.% estimation.

Surface morphology evaluation by scanning electron microscopy (SEM)

SEM was performed as defined in Chapter 3.3.4.

BSA loading into sunflower S-SMCs - mechanistic model

The BSA loading into S-SMCs (radius a_0 , thickness d , and porosity ε) is modeled by considering the dissolution of the air bubble inside the S-SMC (bubble radius a and volume $V = 4/3\pi a^3$). The model is based on well-established concepts in fluid mechanics, and extensive treatments of these principles have been reported [29-31].

The change of the bubble volume in time is governed by the mass balance equation:

$$c \frac{dV}{dt} = -\varepsilon A_0 J, \quad (1)$$

where $c = p_\infty/RT$ is the air mass density inside the bubble, R is the universal gas constant, T is the temperature, $p_\infty = 10^5 \text{ kg m}^{-1}\text{s}^{-2}$ is the ambient pressure, $A_0 = 4\pi a_0^2$ is the inner surface area of the capsule, and J is the air diffusive mass flux in solution, whose resistance is assumed to be concentrated over the S-SMC shell:

$$J = \frac{D}{d}(c_0 - c_\infty). \quad (2)$$

Here D is the diffusivity of the air in the liquid, $c_0 = p_0/RTk_H$ is the saturated air mass density in the liquid at the air - liquid interface, $c_\infty = p_\infty/RTk_H$ is the saturated air mass density in the liquid outside the capsule, k_H is the Henry constant, and $p_0 = p_\infty + \Delta p$ is the pressure inside the bubble, where the difference to the pressure outside the bubble is due to the gas - liquid surface tension, σ , according to Laplace's law:

$$\Delta p = \frac{2\sigma}{a}. \quad (3)$$

Noting that dV/dt is negative and combining all relations, results in the following equation for the change of the radius of the dissolving gas bubble:

$$\frac{da}{dt} = -\frac{\sigma \varepsilon A_0 D}{2\pi d a^3 p_\infty k_H}, \quad (4)$$

which is solved by:

$$a = a_0 \left(1 - \left[\frac{t}{\tau}\right]\right)^{\frac{1}{4}}, \quad (5)$$

where the dissolution time scale is given by:

$$\tau = \frac{da_0^2 p_\infty k_H}{8\sigma \varepsilon D}. \quad (6)$$

Equation 5 and Equation 6 provide the rate of liquid loading into the S-SMCs, where it has been recognized that the loading is equivalent to the dissolution of the encapsulated gas bubbles. The model assumes that the dissolution is driven by surface tension, which pushes the air out of the bubble into the surrounding liquid. Although not all material constants are known, which are involved in Equation 6, Equation 5 has been qualitatively compared

to experimental BSA-loading data. In Figure 6.3D below, it is shown that the measured radii of encapsulated gas bubbles follow a one-fourth power law, as predicted by Equation 5, which validates the proposed mechanism of liquid loading in the S-SMCs, as a surface tension driven dissolution process.

Bovine serum albumin (BSA) encapsulation into sunflower S-SMCs

BSA loading into sunflower S-SMCs was performed as defined in Chapter 3.2.3.

Encapsulation efficiency

Encapsulation efficiency evaluation was performed as defined in Chapter 3.2.3.

Tableting and enteric coating

Tableting and enteric coating was performed as defined in Chapter 3.2.4.

In vitro drug release in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)

In vitro release of BSA from sunflower S-SMCs was performed as defined in Chapter 3.2.5.

Statistical analysis

Statistical analysis of data was performed as defined in Chapter 3.3.7.

6.3. Principle Outcomes

6.3.1. Mechanistic evaluation of protein loading

To ensure an industrially applicable loading protocol it is important to achieve optimal loading with minimal processing requirements. However, due to the limited quantity of literature regarding S-SMCs for use in microencapsulation, there is limited understanding regarding the fundamental loading mechanisms of these porous microcapsules. Therefore, the initial goal was to determine how it is possible to completely fill all S-SMCs with the loading solution, and then to understand the processes driving complete loading.

Real-time compound loading observations

To explore the loading phenomenon, CLSM analysis of S-SMCs loaded by modified passive loading, vortex mixing, and vacuum loading methods was performed so as to retain the S-SMCs in a wet FITC-BSA solution environment (**Figure 6.2**). Passive loading was performed by real time CLSM imaging of S-SMCs submerged in FITC-BSA solution for up to 60 min, whereas vortex mixed and vacuum loaded samples were analyzed after processing. Passive loading observations indicated that overall, FITC-BSA loading inside S-SMC cavities increases with time. Interestingly, a time lag prior to the loading of most S-SMCs was detected; this is attributed to the surface tension existing between air pockets within the S-SMCs and the FITC-BSA solution. Additionally, a significant proportion of S-SMCs were not loaded, even after 60 min. CLSM analysis revealed that once an S-SMC began to load, full S-SMC loading was achieved in approximately 15 min. Loading assisted by vortex mixing improved the loading efficacy, compared with passive loading; nonetheless, the application of a vacuum was necessary for complete particle loading. The observed loading of FITC-BSA solution under vacuum may be attributed to the creation of a negative pressure differential occurring when the air is evacuated from the inner cavity of the S-SMCs [3,4].

Based on these mechanistic loading observations vacuum loading was utilized for the remainder of the study.

Mechanistic loading model

To further clarify the forces driving loading, a mathematical model for liquid loading into S-SMCs was derived (6.2.5. BSA loading into sunflower S-SMCs - mechanistic model). The model factors in the native sunflower microstructure and assumed correlation factors, and can be related to the surface tension driven dissolution of the gas bubbles encapsulated in the S-SMCs. This model was compared with the experimental size of the shrinking air bubbles encapsulated in the S-SMCs, which were

Figure 6.2 Confocal laser scanning microscopy of BSA loaded sunflower S-SMCs based on passive and assisted loading. The second row depicts an enlarged view of a representative S-SMC indicating the evacuation of air from the S-SMC inner cavity. (Scale bars 10 μ m).

measured by analysis of real time CLSM images (**Figure 6.3**). A representative time evolution of a bubble radius is shown in Figure 6.3 A. Interestingly, in this case, the loading (shrinking air bubble) does not start immediately after submerging the S-SMCs into the BSA solution. Instead there is a lag time before loading takes place. In the representative bubble dissolution example shown in Figure 6.3 A, the lag time equals $t_0 = 19$ min and the subsequent loading time equals $\tau = 11.7$ min. The lag time may be caused by the bubble initially blocking the S-SMC's micron-sized apertures, thus reducing the liquid flow into the S-SMC. Upon bubble shrinkage, the micron-sized apertures unblock, thus leading to an accelerated liquid flow into the S-SMC cavity.

Figure 6.3 Liquid loading into S-SMCs, measured by tracking encapsulated air bubbles in S-SMCs using confocal microscopy: (A) a representative time evolution of a bubble radius; (B) a comparative lag time (t_0) distribution for the 45 S-SMCs that showed loading; (C) a comparative loading time (τ) distribution for the 45 S-SMCs that showed loading; and (D) a comparison of Equation 7 and experimental data for 10 S-SMCs.

Analysis of three sets of 56 confocal microscopy images (5 min to 60 min) presented 91 S-SMCs. Out of these 91 S-SMCs, the loading process was

clearly observed for 45 S-SMCs (49 %); and for each of those the lag time t_0 and the loading time τ were determined. Calculated mean and standard deviation values were $t_0 = 14 \pm 12$ min and $\tau = 13 \pm 9$ min (Figure 6.3B-C). Notably, 26 S-SMCs (29 %) did not show any loading during the 1 h observation period, while 20 S-SMCs (22 %), were already loaded within the 5 min following the submerging in BSA solution before the first microscopy image was taken. These S-SMCs were not included in the analysis.

Figure 6.4 Scanning electron microscopic images of BSA-loaded sunflower sporoderm microcapsules (S-SMCs) with varying BSA-loading proportions: (A) 23 wt.%; (B) 37.5 wt.%; and (C) 54.5 wt.%. (Scale bars 10 μ m).

Next, the experimental data was compared to the fluid transport model, which is derived in section 6.2.5.. The model assumes that the surface tension pushes the air out of the bubble into the surrounding liquid. Based on the mass balance equation, it was derived that the bubble radius decreases in time

according to a one fourth power law and then from experimental observations (Figure 6.3A) a lag time t_0 was introduced to produce **Equation 7**:

$$a = a_0 \left(1 - \left[\frac{t-t_0}{\tau} \right] \right)^{\frac{1}{4}}. \quad (7)$$

In Figure 6.3D, Equation 7 was compared to experimental data by plotting data for 10 S-SMCs on $[(t - t_0)/\tau, (a/a_0)^4]$ -coordinates. The data follow a straight line [Equation 7], which confirms that the model captures the dynamics of liquid loading into S-SMCs, which is a surface tension driven dissolution process.

6.3.2. Loading optimization and analysis

To optimize loading and elucidate the loading distribution of dried compounds within S-SMCs, BSA loading into sunflower S-SMCs was evaluated for three different initial BSA-loading proportions, 23 wt.%, 37.5 wt.%, and 54.5 wt.%. The loading process involves applying a vacuum to the S-SMC-BSA suspension leading to a reduction in the internal cavity pressure, which in turn applies a force drawing the BSA solution into the hollow S-SMCs [26,27].

Morphology & micromeritic properties of compound loaded S-SMCs

To evaluate the stability of the S-SMC morphology following the BSA loading and washing processes, and to ensure that external S-SMC surfaces were free of residual BSA, SEM analysis of the loaded S-SMCs was performed for all three BSA-loading portions (**Figure 6.4**). All S-SMCs exhibited similar structural and morphological features of well-defined spikes with intact uniform sized S-SMCs. Notably, the sunflower S-SMC surfaces were free of any residual BSA, indicating the encapsulation of BSA within the S-SMC inner cavities. To further confirm the stability of the micromeritic properties of BSA-loaded sunflower S-SMCs following BSA loading, DIPA analysis of the S-SMCs before and after loading was conducted (**Figure 6.5**). The analysis of

54.5 wt.% BSA-loaded S-SMCs indicated that there were no significant changes in S-SMC microstructure with respect to diameter, circularity, and aspect ratio. Overall, these data imply that the native microstructure of the sunflower S-SMCs enabled the loading of the model protein within the S-SMCs without disrupting their morphological characteristics.

Figure 6.5 Micromeritic properties of BSA-loaded sunflower S-SMCs: (A) diameter (μm); (B) circularity; (C) aspect ratio; for 1000 sunflower S-SMCs prior and following BSA-loading. The data obtained by the spline curve fitting of histogram data from triplicate batches ($n = 3$).

Table 6.1 Efficiency of Bovine Serum Albumin (BSA) encapsulation into S-SMCs

Sunflower S-SMCs:BSA [mg]	BSA-loading proportions ^{a)} [wt.%]	BSA-loading efficiency [wt.%]	BSA encapsulation efficiency [%]
150:45	23.0	9.4 ± 0.7	40.6 ± 2.9
150:90	37.5	15.8 ± 1.9	42.2 ± 4.8
150:180	54.5	22.3 ± 1.5	40.8 ± 2.5

^{a)} Loading is based on total initial weight of S-SMCs and BSA. Values represent the mean of three batches ($n=3$) with standard deviations.

Loading efficiencies & distributions

The loading and encapsulation efficiency for varying BSA proportions are summarized in **Table 6.1**. The efficiency was significantly enhanced with the increase in the BSA-loading portions; a BSA-loading proportion of 54.5 wt.% provided a loading efficiency of 22.3 ± 1.5 wt.% ($p < 0.05$), compared

with BSA-loading proportions of 23 wt.% and 37.5 wt.%, which resulted in lower loading efficiencies, at 9.4 ± 0.7 and 15.8 ± 1.9 wt.%, respectively.

Figure 6.6 Confocal laser scanning microscopy (CLSM) analysis of FITC-BSA-loaded sunflower S-SMCs: (A) unloaded and FITC-BSA loaded S-SMCs; (B) 3D images constructed using z-stacks illustrate the inner cavity of the S-SMCs; (C) diagram depicting the average BSA loading based on fluorescence intensity per S-SMC, vol.%; and (D) comparative analysis of BSA-loading fraction, BSA : S-SMC, and S-SMC average loading, vol.%, with estimation of theoretical maximum loading. (Scale bars 10 μ m).

To quantify and further evaluate the loading process, CLSM images of S-SMCs following FITC-BSA loading were obtained and compared with empty S-SMCs (**Figure 6.6**, **Figure 6.7**, and **Figure 6.8**). The CLSM settings were calibrated to produce no visible S-SMC autofluorescence emissions in the green spectrum. Empty S-SMCs did not exhibit any green fluorescence, thus confirming empty, clean, intact S-SMCs. S-SMCs following FITC-BSA loading demonstrated an increase in the fluorescence intensity trend with the

increase of FITC-BSA-loading proportion (Figure 6.6A, Figure 6.8). To confirm the intracavity loading, CLSM z-stack images (Figure 6.7) were used to construct 3D images (Figure 6.6B), which demonstrated that the green fluorescence originated from the inner cavity and not the S-SMCs surface. Additionally, all loaded S-SMCs, with varying FITC-BSA proportions, exhibited an intact, clean microstructure.

Figure 6.7 Confocal laser scanning microscopy (CLSM) Z-stack analysis of sunflower S-SMCs before and after FITC-BSA loading at three different BSA-loading proportions.

Figure 6.8 Confocal laser scanning microscopy (CLSM) of sunflower S-SMCs before and after FITC-BSA loading at three different BSA-loading proportions: (A) Unloaded S-SMCs, and FITC-BSA loading at three different loadings; (B) Quantification of fluorescence intensity from FITC-BSA loaded S-SMCs at three different BSA-loading proportions.

The FITC-BSA volume loading was quantified by the fluorescence intensity per S-SMC. By normalizing the average fluorescence intensities, with a value of 1 being the particle with the greatest fluorescence intensity value and appearing to be full (Figure 6.8), vol.% loading values (Figure 6.6C) were calculated. The vol.% loading values increase with increases in initial FITC-BSA loading proportion. However, to accurately compare the correlation between vol.% loading estimations and BSA-loading efficiency wt.% values, it is necessary to convert the BSA-loading efficiency wt.% values obtained above to BSA : S-SMC loading fractions (Figure 6.6D). There is a close correlation between increases in BSA : S-SMC loading fractions and vol.% loading estimations, which provides validation for the encapsulation and analysis processes.

Theoretical maximum loading efficiencies

It is possible to estimate what the theoretical maximum BSA-loading efficiency wt.% would be if all S-SMCs were 100% full after loading and washing (100 vol.% loading) by comparing the fluorescence intensity vol.% loading estimations with BSA : S-SMC loading fractions. For each of the three sets of loading data, 23 wt.%, 37.5 wt.%, and 54.5 wt.%, it is possible to calculate a multiplication factor to determine a theoretical maximum BSA : S-SMC loading fraction equivalent to 100 vol.% loading (**Table 6.2**). Therefore, based on all three sets of data, a theoretical maximum BSA : S-SMC loading fraction is 0.870 ± 0.089 (Figure 6.6D), which relates to a theoretical maximum BSA-loading efficiency of 46.4 ± 2.5 wt.%. It is important to note that this estimation would require all S-SMCs to be 100 vol.% full after loading and washing, which is practically implausible due to some portion of BSA leaching during the washing and handling processes. However, having a robust estimation for the potential maximum loading of S-SMCs provides very useful insight into any future loading optimization work in the field of microencapsulation utilizing S-SMCs or other porous microcapsule systems.

The protein loading data and theoretical maximum loading potential is in line with several other sources of data for protein loading in S-SMCs. There

is data for various protein loading in various S-SMCs [3,32], with most of the S-SMCs being extracted from *Lycopodium clavatum*. However, it should be noted that some data is for protein loading with washing to remove surface adhered/unloaded protein, and some data is for protein loading without additional washing. Without additional washing, loading efficiencies range from 2.1 to 74.5 %, with much of the data being around 50.0 %, which is very close to the estimated theoretical maximum of 46.4 %. However, it was observed that for some studies, with loaded particle images, there appeared to be surface adhered protein residues [26]. With additional washing, loading efficiencies range from 5.0 to 30.2 %, with much of the data being around 16.6 %, which is what we have observed in this study and which is in line with this studies protein loading results and estimated theoretical maximum.

Table 6.2 Estimation of S-SMC theoretical maximum BSA-loading efficiency^{a)}

BSA Loading Achieved			Multiplication factor required to achieve 100% full SDMCs	Theoretical Maximum BSA Loading	
BSA loading achieved [wt. %]	BSA weight proportion to SDMCs [BSA : SDMCs]	BSA loading achieved [vol. %]		BSA weight proportion to SDMCs [BSA : SDMCs]	Theoretical max. BSA loading [wt. %]
9.4	0.104	12.7	x 7.9	0.817	45.0
15.8	0.188	22.9	x 4.4	0.819	45.0
22.3	0.287	29.5	x 3.4	0.973	49.3
			Average	0.870 ± 0.089	46.4 ± 2.5

^{a)}Loading analysis based on data from triplicate batches

6.3.3. S-SMC Protein Release Analysis

Analysis of BSA release from BSA-loaded S-SMCs was analyzed by release studies and CLSM fluorescence intensity analysis (**Figure 6.9**). A BSA-loading proportion of 54.5 wt.% was used based on the loading optimization and analysis conducted in section 6.3.2.. To investigate the

release profile from BSA-loaded S-SMCs, release experiments were performed in pH 1.2 simulated gastric fluid (SGF), and pH 7.4 simulated intestinal fluid (SIF), for up to 60 min (Figure 6.9A-B). High BSA release rates were observed, of up to 90% in the initial 15 min, and complete release was recorded within 60 min in both SGF and SIF. The observed rapid BSA release is attributed to the passage of molecules through the nanopores and micron-sized apertures in the surface of S-SMCs. A similar release profile was reported from natural sunflower (*H. annuus*) pollen grains and *L. clavatum* S-SMCs in SGF and SIF [26,27].

For the CLSM fluorescence intensity analysis of FITC-BSA release (Figure 6.9), it should first be noted that the overall fluorescence intensity observed in SGF (pH 1.2) is less than that observed in SIF (pH 7.4) due to the fluorescence quenching of FITC in acidic conditions.^[30] However, by intensity analysis in imageJ, with normalization of the data from 2 min to 60 min, it is possible to accurately compare trends within each system. Additionally, the large standard deviations in the inner S-SMC data are due to significant variations in initial intensity values and are reinforced by differences in rates of release.

The data, for release in SIF (Figure 6.9A), indicates that the inner S-SMC fluorescence intensity drops to $49.5 \pm 16.8\%$ within 15 min, and $41.6 \pm 11.4\%$ within 30 min, relative to 2 min, and remains stable until 60 min. The stabilization of the inner S-SMC region at $\sim 40\%$ may be attributed to residual amounts of FITC-BSA remaining in the S-SMCs. The outer S-SMC fluorescence intensity rises from $12.3 \pm 0.9\%$ at 2 min, to $28.7 \pm 0.9\%$ at 60 min as FITC-BSA is released into the surrounding media. It is assumed that the stabilization of inner S-SMC fluorescence intensity correlates to near complete release of BSA. Comparing the inverse of the inner S-SMC fluorescence intensity data between 100% and 41.6%, to the BSA release data, over 2 min to 60 min, the trends are shown to be similar. In the 5 to 10 min region, the fluorescence intensity data indicates slightly slower release than in the BSA release data, however, this may be attributed to the differences in experimental conditions used to collect the data. CLSM analysis comprised a

static system at room temperature ($\sim 25^\circ\text{C}$), however, BSA release analysis comprised gentle shaking (50 rpm) at 37°C .

Figure 6.9 *In vitro* release of BSA from S-SMCs, comparing BSA release study data and fluorescence intensity analysis data: (A) BSA-loaded S-SMCs in simulated gastric fluid, pH 1.2; (B) BSA-loaded S-SMCs in simulated intestinal fluid, pH 7.4. BSA release profile data is presented as the average of triplicate measurements with standard deviation ($n = 3$). Fluorescence intensity analysis data is presented as analysis of 10 regions ($n = 10$).

The data, for release in SGF (Figure 6.9B), indicates that the inner S-SMC fluorescence intensity drops to $42.3 \pm 22.1\%$ within 15 min, relative to 2 min, and remains stable until 60 min. The stabilization of the inner S-SMC region, also at $\sim 40\%$, may be attributed to residual amounts of FITC-BSA remaining in the S-SMCs. The outer S-SMC fluorescence intensity rises from $19.3 \pm 1.2\%$ at 2 min to $55.9 \pm 1.9\%$ at 60 min as FITC-BSA is released into the surrounding media. The trends of release based on S-SMC fluorescence intensity data and the BSA release data, are also similar, wherein subtle variations may be attributed to the differences in experimental conditions.

The porosity of the pollen shell is dominated by 3 micron-sized apertures (~ 1 to $3\ \mu\text{m}$), and numerous nano-sized pores (~ 20 to $200\ \text{nm}$). However, it should be noted that only the 1 to $3\ \mu\text{m}$ apertures are not completely covered by the cellulosic intine layer. Therefore, it is proposed that the apertures penetrating the entire shell are responsible for driving compound release.

6.3.4. Tableted S-SMC formulation for intestinal protein release

Oral delivery of therapeutic proteins is still a remaining challenge. In particular, the controlled release of a multiple-units dosage, i.e. microparticle pellets, is attracting growing attention due to having advantages over more common single unit formulations [33,34]. In order to achieve intestinal delivery of the model protein loaded into S-SMCs, the S-SMCs were tableted and enteric coated. The tablets used for enteric coating contained $149.4 \pm 0.3\ \text{mg}$ of BSA-loaded S-SMCs, with a consistent tablet diameter ($12.92 \pm 0.00\ \text{mm}$) and thickness (1.10 ± 0.01) (**Table 6.3**). To avoid premature release in gastric conditions, the enteric coating was performed using Eudragit L100. Eudragit L100 (EL100) is insoluble under acidic conditions and therefore provides a stable coating within the stomach. Preliminary exploration of enteric coating indicated that $15\ \%$ (w/v) EL100 with $15\ \%$ (w/v) triethyl citrate as a plasticizer provided the most promising formulation for further investigation.

Table 6.3 Details of tableted BSA-loaded S-SMCs ^{a)}

Tableted formulation	Weight of tablet [mg]	Diameter of tablet [mm]	Thickness of tablet [mm]
Before coat	149.4 ± 0.30	12.92 ± 0.00	1.10 ± 0.01
After coat	169.4 ± 0.85	13.00 ± 0.01	1.49 ± 0.03

^{a)} S-SMC tablets prepared using 13 mm stainless steel press die punch and results represent the mean of three batches (n=3) with standard deviations.

Morphological analysis of tableted S-SMCs

To evaluate surface morphology and cross-sectional topography of tableted S-SMCs, SEM images were obtained before and after the coating process (**Figure 6.10**). The surface roughness of the tableted S-SMCs is the result of the intact and robust S-SMC microstructure after compression (Figure 6.10A). The cross-sectional SEM image revealed an intact S-SMC microstructure with native spike morphology, without any breakages; this implied that S-SMCs were present as discrete particles in the tablet (Figure 6.10B). Moreover, although compressed, the fundamental S-SMC architecture was retained even following compression, without any pharma excipients, indicating the robust microstructure of sunflower S-SMCs.

Analysis of tablet morphology and S-SMC particle counts provides further insight into the tableted S-SMC structure. Dynamic image particle analysis (DIPA) measurements indicate $1.83 \pm 0.17 \times 10^5$ S-SMCs per mg. A BSA-loaded S-SMC loading efficiency of 22.3% results in 116.6 mg of S-SMCs per 150 mg tablet, correlating to 2.14×10^7 S-SMCs per tablet. With a pre-coating tablet volume of $1.44 \times 10^{11} \mu\text{m}^3$, each S-SMC comprises $6.75 \times 10^3 \mu\text{m}^3$, and based on a compressed S-SMC diameter of 30.0 μm (Figure 7A), the thickness estimation of a compressed S-SMC is 9.6 μm . Therefore, each 150 mg tablet comprises ~ 430.7 S-SMCs across the tablet diameter, and 115.1 S-SMCs across the tablet thickness. Regarding BSA distribution throughout the tablet, a uniform distribution may be assumed with an average S-SMC

volume loading of 29%, correlating to an S-SMC loading efficiency of 22.3% (Figure 6.6 and Figure 6.8).

Figure 6.10 Scanning electron microscopic images of tableted BSA-loaded S-SMCs with and without 15 % (w/v) Eudragit L100: (A) surface prior to enteric coating; (B) cross-section prior to enteric coating, arrow indicates the cross-sectional direction; (C) surface following enteric coating; and (D) cross-section following enteric coating, arrows indicate the enteric coating layer.

Following the enteric coating, the surface of the tableted S-SMCs was smoother (Figure 6.10C), compared with tableted S-SMCs only (Figure 6.10A), thus implying the formation of a continuous protective layer. The cross-sectional image of enteric coated tableted S-SMCs confirmed the formation of a 40-50 μm coating layer on the surface of the S-SMCs (Figure 6.10D).

Overall, the morphological analysis of the tablets confirmed the presence of intact BSA-loaded S-SMCs, and a well-defined layer of enteric coating.

Analysis and optimization of controlled release

It is crucial for intestinal targeted delivery of proteins to ensure that the protein is not exposed to harsh gastric conditions. The release profile of the tableted BSA-loaded S-SMCs following the enteric coating was measured *in vitro* in SGF and SIF (**Figure 6.11**). The release profile exhibited a controlled release of the protein, with no release in SGF for up to 2 h, and a complete release in SIF, 100%, within 8 h. These data indicate the suitability of the tablet formulation to protect encapsulated proteins in gastric conditions and to target a significant portion of the intestinal area [13,14].

Figure 6.11 *In vitro* release of BSA from enteric coated S-SMC tablets and controls. Tablet release profiles are based on incubation of 2 h in simulated gastric fluid, pH 1.2, and 8 h in simulated intestinal fluid, pH 7.4. Data is presented as the average of triplicate measurements with standard deviation (n = 3).

Release control studies were conducted with enteric coated tablets with no S-SMCs, as well as with BSA-loaded S-SMC tablets without enteric coating (Figure 6.11), and further control tablet details are provided in supporting information (**Table 6.4**). The enteric coated tablets with no S-SMCs, utilized

Eudragit L100 as a dry matrix [35] and contained the same proportion of BSA as in the study tablets. The results indicate that the tablets are stable up to 6 hours, 2 hours in SGF + 4 hours in SIF, with $2.6 \pm 1.2\%$ release. Then, after 8 hours in SIF there is a total BSA release of $31.3 \pm 1.9\%$. However, even at 30 hours there is only $54.2 \pm 9.7\%$ release, suggesting that this system is sub-optimal for targeted intestinal delivery. The BSA-loaded S-SMC tablets without enteric coating, exhibit $39.9 \pm 19.1\%$ in 2 hours in SGF and a total of $92.8 \pm 15.6\%$ with an additional 1 hour in SIF, suggesting that this system is unstable and not appropriate for targeted intestinal delivery.

Table 6.4 Details of control study tablets ^{a)}

Tableted formulation	Weight of tablet [mg]	Diameter of tablet [mm]	Thickness of tablet [mm]
No S-SMCs -before coat	149.8 ± 2.2	12.92 ± 0.01	0.95 ± 0.01
No S-SMCs - after coat	175.5 ± 0.7	12.99 ± 0.01	1.32 ± 0.04
BSA S-SMCs - no coat	150.1 ± 1.3	12.92 ± 0.01	1.08 ± 0.01

^{a)} Tablets prepared using 13 mm stainless steel press die punch and results represent the mean of three batches (n=3) with standard deviations.

The observed extended release profile of the BSA-loaded S-SMC enteric coated tablets in SIF conditions is attributed to tablet fragmentation into compact aggregates of S-SMCs. It is proposed that, as the enteric coating slowly erodes, the S-SMC tablet starts disintegrating as larger compact aggregates of BSA-loaded S-SMCs. Hence, these tableted S-SMC formulations are expected to spread uniformly throughout the gastrointestinal tract (GIT), thereby avoiding the rapid release of a therapeutic protein or drugs at a single location. Distributed release of multiple smaller units in the GIT helps reduce the potential of drug toxicity and also results in more uniform absorption. In order to investigate the microstructural changes of tableted S-SMCs after *in vitro* release, SEM analysis was performed. The SEM analysis (**Figure 6.12**) indicated discrete microparticles with intact surface topography and structure

similar to native S-SMCs, confirming the robust nature of sunflower S-SMCs and highlighting their potential for use as a microparticle-based delivery system.

Figure 6.12 Scanning electron microscopic images of sunflower S-SMCs following their *in vitro* release SGF and SIF. (Scale bars 10 μm).

6.4. Conclusions

S-SMCs provide a highly compelling platform for microencapsulation and drug delivery, particularly for the oral administration of therapeutic proteins. These studies have led to the successful development of an effective and low cost controlled delivery system for proteins. The vacuum-loading BSA encapsulation and S-SMC tableting methods utilized are straightforward mechanical processes which do not require the use of additional reagents, organic solvents, or high shear forces, which can damage proteins. Additionally, this study demonstrated insights into protein loading mechanisms through real time and post processing CLSM observations of FITC-BSA-loaded S-SMCs. For the first time, a detailed investigation of protein loading and distribution in BSA-loaded-S-SMC batches was revealed with the assistance of CLSM and mathematical modeling. The validity of these observations was supported by a high correlation between CLSM fluorescence intensity analysis and experimental quantification of BSA loading. It was shown that the evacuation of air from S-SMC internal cavities through S-SMC surface nanopores and micron-sized apertures drives the BSA-loading process. Additionally, these studies have led to a robust estimation of the maximum

potential loading of these S-SMCs, therefore providing a useful indicator of practical loading efficacy in this and future studies utilizing S-SMCs or other porous microcapsule systems.

Finally, the tableting of BSA-loaded S-SMCs and enteric coating using Eudragit L100 produced stable multiparticulate tablets for oral delivery. The enteric coated tablets exhibited successful inhibition of BSA release in simulated gastric fluid for 2 h and allowed for complete release within 8 h in simulated intestinal fluid. The S-SMCs size and microstructure were preserved throughout the loading, tableting, and release processes. These results taken together imply that the S-SMCs may protect the encapsulated protein up to its release at the targeted site. Furthermore, the S-SMCs ability to retain their morphology and porosity, following tableting and *in vitro* release, provides the benefit of a unified rapid release of the protein from the S-SMCs upon tablet disintegration at the desired site. The release profile was modulated with polymers, thus also highlighting the potential flexibility of S-SMCs as a delivery system. To conclude, these studies have demonstrated that S-SMCs provide an effective and versatile drug formulation platform enabling intestinal targeted release of orally-delivered protein therapeutics.

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Chapter 7

Future Outlook

Further exploration of pollen, spores, and algae, as solutions for microencapsulation applications, offers a wide range of potential opportunities. The studies outlined in this thesis represent important progress and contributions to the field of S-SMC extraction and application. However, this work represents only a small contribution toward future studies in this area. This chapter provides a comprehensive overview of key applications and key project examples which may be pursued in this field. The potential use of various pollen, spores, and algae is proposed. Microencapsulation as a key research project is discussed, with two key example applications, of microencapsulation for functional foods, and microencapsulation for herbal therapeutics, being outlined. Novel polymeric biomaterials as a key research project is discussed, with two key example applications, of cosmetics and personal care, and novel polymeric biomaterials, being outlined. Finally, the highlights and conclusions of this research are summarized.

7.1. Introduction - S-SMCs from Pollen, Spores, & Algae

Many pollen, spores, and algae [1], as well as some fungi [2] and bacteria [3] comprise a highly physicochemically robust biopolymer, sporopollenin, as an outer layer protective coating of the sporoderm. Sporopollenin is a related group of biopolymers with variations between species. Within the wide range of pollen from various pollen producing plant species there is a wide range of morphologically and biochemically distinct pollen types. This diversity provides huge opportunities for both microencapsulation and potential composite material development. Plant spores from plant species which reproduce by asexual reproduction have been shown to have significant biochemical variations and physicochemical properties compared to pollen from flowering plant species [4]. Some fungal spores also comprise sporopollenin, although there is relatively little research into which fungal species produce sporopollenin or sporopollenin-like compounds [2]. Importantly, the exploration of the biochemical properties of fungal spore outer coatings has implications beyond the potential for microencapsulation, and may have a significant impact on human health and agriculture. Some algae are known to contain sporopollenin in the outer layer [5,6], such as several species of the blue-green algae, chlorella, which has been widely explored as a source of nutrients and biofuels, as well as its health promoting properties. Due to its rapid growth and homogeneity, algae provide a compelling source of large-scale sporopollenin and/or microcapsule production.

Pollen and other sources of sporopollenin have a number of advantages as naturally sourced biomaterials. Many are biocompatible and pollen has no limiting regulations for use as an ingredient for human consumption [7,8]. Pollen and spores have been shown to be useful for microencapsulation, either in their natural form or after processing to create hollow non-allergenic microspheres, S-SMCs [9-11]. The outer wall of these sporoderms comprises an extremely physicochemically robust biopolymer, sporopollenin, and pollen sporopollenin can be processed to incorporate various functional groups for

binding to diverse materials [12-17]. Pollen sporopollenin and various composites can be used produce gels or bioplastic-like sheeting [18].

Pollen offers an underutilized material for a range of industrial applications. In particular, both natural pollen and extracted pollen capsules can be utilized as a microencapsulant, and the high degree of microstructural uniformity is ideal for a wide range of oral, topical, and agricultural applications.

Figure 7.1 Overview of the potential for exploring pollen, spores, and algae as solutions to microencapsulation, novel composite biomaterials, and the implications for other and related areas of research and industry.

Various forms of chemical processing allows for the production of biocompatible pollen gels and soft composites, which may be used in pure or composite systems [4,19]. Natural biopolymer gels may have applications in personal care and cosmetics products, as well as in the food industry.

Additionally, pollen composites may have applications in a wide range of novel disposable consumer products.

Greater understanding of sporoderm physicochemical properties and processing methods has implications for fields as diverse as biofuel production. Some species of algae have been widely studied as a source of renewable biofuel production and appropriate modification of the protective sporopollenin coating may allow for easier biofuel extraction [20-24]. Additionally, utilization of the waste sporopollenin material as a byproduct of biofuel extraction may provide a natural biopolymer feedstock and may significantly increase the financial viability of algal biofuel production.

Overall, pollen, spores, and algae are all sources of raw materials which have been largely overlooked in materials science research and industry. It is important to highlight and explore the true potential of these materials and to stimulate greater interest in furthering both fundamental research and applied research. Sporoderms of many of nature's microcapsules may be put to use as next generation solutions to existing scientific and industrial challenges, and provide an inexhaustible source of environmentally friendly and biocompatible materials.

7.2. S-SMCs for Microencapsulation

Microencapsulation is the most obvious application for pollen, spores, and algae, and the biocompatible nature of these natural or extracted microcapsules may be applied to a wide range of industries currently utilizing microencapsulation. One of the primary purposes of microencapsulation is to protect sensitive compounds from environmentally induced degradation [25-29]. In the food industry, processed foods and drinks comprise a wide range of compounds, such as, flavors, aromas, and other volatile bioactives. Microencapsulation offers an attractive solution to developing novel products or adding extended functionality and shelf life to existing products. It is important to note that pollen has a long history of use as a food ingredient around the world and in many cultures, and even in its raw form can be added

to most existing foods as a flour-like additive providing increased nutrient density and other health benefits [30-35].

Figure 7.2 Overview of microencapsulation as a key research project. There exists potential for use in diverse industrial fields, and clear research goals and objectives which may be pursued.

The health food and food supplement industries represent a huge market sector requiring increased product stabilization and demanding novel and improved functional natural ingredients [36]. In particular, many of the health benefits attributed to health foods and supplements are derived from sensitive bioactive compounds, and it is these bioactive compounds which need to be protected from degradation so as to retain efficacy [37-39]. On a similar note, medicinal therapeutics, either in traditional medicines or single molecule therapeutics, require encapsulation and delivery systems which protect sensitive compounds from degradation so as to remain active. Pollen capsules are nature's solution to biomolecule protection and the biological role

of pollen is to protect sensitive genetic material from exposure to harsh environmental conditions.

The cosmetics and personal care product industries are another huge market sector which requires increased product stabilization and which is constantly seeking new materials to gain a competitive edge through improved product functionality or unique product branding [40,41]. Both natural cosmetics and personal care products rely on incorporating high quality bioactive ingredients or compounds with specific desirable properties. Pollen provides protection to sensitive genetic material through several mechanisms, with oxidation protection and UV light protection characteristics offering functionality which may be exploited in various cosmetics and personal care products [42,43]. Additionally, sporoderm materials have been shown to bind with environment pollutants, such as heavy metals, and may provide a functional material for use in developing topological detoxification treatments [44-46].

Even within agricultural industries natural microcapsules from pollen, spores, and algae may be developed to play a significant role. Biologically active & potent pesticides, herbicides, and fertilizers are used in agriculture and require methods to modify stability & release rates [47]. Pollen particles naturally undergo dehydration & hydration processes to absorb & release compounds in relation to their biological role. After release of active compounds, pollen-based microcapsules may be integrated naturally into the local ecosystem without becoming a new source of environmental pollution.

There are several key factors driving ongoing and future pollen, spore, and algal materials science research. Importantly, in most countries pollen has no limiting regulations for use as an ingredient for human consumption [7,8]. Both raw pollens and extracted SMDs provide potential microencapsulation systems. Existing research into microencapsulation with raw pollen and S-SMDs has indicated that they exhibit protection against oxidation and UV degradation of encapsulated compounds [19], as well as provide taste-masking effects [48]. Although, compounds loaded into pollen capsules are protected

from degradation and can be released quickly, it is also possible to apply various methods of co-encapsulation to provide a more controlled release profile under specific environmental conditions.

Key goals for future pollen, spore, and algal microencapsulation research should incorporate analysis of compound stability, taste and odor masking, development of topically applied formulations, and compound release properties. The stability of volatile bioactive compounds is very important for applications in foods, supplements, herbal therapeutics, personal care products, and agricultural products. The potential of taste and odor masking may be particularly relevant for unpleasant tasting functional foods, health supplements, and herbal therapeutics. The ability to incorporate pollen based microencapsulation systems in topical pollen-based creams is of great significance for developing novel cosmetics and personal care products. Release characteristics need to be better understood for a wide range of compounds and natural microcapsule systems, especially the kind of long term, slow release that may be required for delivery of a range of commonly used natural pesticides, herbicides, and fertilizers in indoor or outdoor agricultural settings.

There is a diverse range of specific projects which may be explored so as to utilize pollen, spores, or algae. With regards to the food industry, it would be of significant interest to develop a flour-like food additive containing bioactive functional food ingredients for use in processed foods. An ideal functional food system would be a ‘flour’ with standardized properties, which could be incorporated into existing industrial food processing systems as a flour substitute, and which comprises a mixture of microparticles carrying a range of functional ingredients. With regards to food supplements and herbal therapeutics, it would also be desirable to develop a natural flour-like system comprising a range of bioactive compounds which provides taste-masking effects and which achieves targeted release in various regions of the digestive tract. With regards to cosmetics and personal care products, it would be desirable to develop a range of topical creams which releases bioactive compounds upon application to the skin and which may utilize the natural

functionality of sporoderm microcapsules, such as anti-oxidant effects, UV protection, and the binding and detoxification of environmental pollutants. With regards to agricultural products, it would be desirable to develop a range of natural agricultural products for controlled delivery of fertilizers, as well as pest and weed control. Such an agricultural delivery system may utilize the natural dehydration and hydration properties inherent in many pollen species so as to rehydrate and release active compounds upon exposure to adequate environmental moisture.

7.2.1. S-SMCs for Microencapsulation in Functional Foods

Functional foods represent an important developing area of the food industry and rely on the incorporation and delivery of specific bioactive compounds. Microencapsulation is an important technology for the development of novel functional foods and the development of new natural microencapsulant systems is important for this growing market [49,50]. An ideal functional food microencapsulant would provide a flour-like system with a high degree of homogeneity and which contains a range of stable bioactive compounds [51]. Pollen, spores, and algae provide a compelling solution as a completely natural and healthy food-based microencapsulant for the development of a standardized flour-like functional food ingredient.

Pollen microcapsules provide a number of attractive properties such as taste masking, product stability, ease of loading, and the potential for unique product branding. With regards to taste masking, many bioactives, which may be useful as functional food ingredients, have a strong, unpleasant taste and/or odor [52,53]. Microencapsulation is a standard industry solution to mask tastes and odor. Pollen has the potential to be utilized as a natural robust microencapsulant and has the added benefit that it is already a food product commonly available for human consumption in health food stores and a wide range of existing products [7,8]. With regards to product stability, many active food-based compounds are sensitive to environmental conditions and degradation. Microencapsulation is a standard industry method to prolong product stability and the shelf life of processed foods. Pollen has the inherent

UV protective and oxidative protection capacities required of an effective microencapsulant. With regards to ease of loading, pollen naturally absorbs water in preparation for plant sexual reproduction. Microencapsulation with pollen allows for water or alcohol extracts to be easily absorbed into the internal cavity without the need for specialized equipment. Microencapsulation with SMDCs may utilize simple loading protocols, such as passive incubation, compression, or the application of a vacuum, to achieve high levels of compound loading [10,11]. With regards to unique product branding, the food and supplement market is very competitive, and product differentiation can be challenging. Pollen may be used to develop a range of novel products utilizing pre-extracted herbal compounds encapsulated with synergistic pollen species.

KEY APPLICATION

Microencapsulation for Functional Foods

<div style="background-color: #d9ead3; padding: 5px; margin-bottom: 10px;"> Natural Food-Based Microencapsulant <p>Microencapsulation is a key technology for the development of healthful functional food products. Pollen provides a completely natural and healthy food-based microencapsulant for the development of a standardized flour-like functional food ingredient.</p> </div> <div style="display: flex; align-items: flex-start;"> <div> Taste Masking <p>Functional food ingredients have a strong & unpleasant taste & odor. Microencapsulation is a standard industry solution to mask tastes & odor. Pollen can function as a natural & robust microencapsulant and is already a common food product.</p> </div> </div> <div style="display: flex; align-items: flex-start;"> <div> Product Stability <p>Many active food-based compounds are sensitive to environmental conditions & degradation. Microencapsulation is a standard industry method to prolong product stability. Pollen has UV protective and oxidative protection capacities.</p> </div> </div> <div style="display: flex; align-items: flex-start;"> <div> Ease of Loading <p>Pollen naturally absorbs water in preparation for plant fertilization. Microencapsulation with pollen allows for water or alcohol extracts to be easily absorbed into the internal cavity without the need for specialized equipment.</p> </div> </div> <div style="display: flex; align-items: flex-start;"> <div> Unique Product Branding <p>The food and supplement market is very competitive. Pollen may be used to develop a range of novel products utilizing pre-extracted herbal compounds encapsulated with synergistic pollen species.</p> </div> </div>	<div style="background-color: #d9ead3; padding: 5px; margin-bottom: 10px;"> EXAMPLE - Uniform Dry-Powder Product Line </div> <div style="display: flex;"> <div style="flex: 1;"> Regulation-free Microencapsulant <ul style="list-style-type: none"> • "though sold in many health food stores, pollen is not considered an additive.....does not have to comply with special standards" Larkin (1984) • The US FDA opinion on the safety of pollen is that pollen particles can be regarded as GRAS (Generally Recognized as Safe). </div> </div> <div style="display: flex;"> <div style="flex: 1;"> Flour-like Product Characteristics <ul style="list-style-type: none"> • It doesn't have to be milled before using it to upgrade or extend regular flour. • Very fine like flour, about 100 mesh or finer. • Flavor ranges widely from nutty, to bitter, to neutral, however, it typically adds more color than flavor. • A very high protein flour replacement. </div> </div> <div style="display: flex;"> <div style="flex: 1;"> Add to Existing Products <ul style="list-style-type: none"> • Traditionally used in food preparation around the world. • Fennel & dill pollen spice is used in Tuscany. • Substituted for flour in all kinds of breads, biscuits, cakes, and cookies in the Americas. • Blended into drinks or added to various desserts in Asia. • There are recipes for stews to meatloaf, from soup to cinnamon rolls, from casseroles to puddings. </div> </div>
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Figure 7.3 Overview of microencapsulation for functional foods as a key application example. As a natural food-based microencapsulant, taste masking, improved product stability, ease of loading, and unique product branding are some advantages which may be obtained. A uniform dry-powder product line is a good example of a specific potential application of microencapsulation for functional foods.

An attractive pollen-based system for use in the functional food industry would involve the creation of a uniform dry-powder product line. Importantly, pollen can be viewed as a regulation-free microencapsulant, “though sold in many health food stores, pollen is not considered an additive.....does not have to comply with special standards” Larkin (1984) [7]. Additionally, the United States Food and Drug Administration (U.S. FDA) opinion on the safety of pollen ingestion is that pollen particles can be regarded as GRAS (Generally Recognized as Safe) [8]. The lack of regulation over pollen in food is a significant advantage over many other new microencapsulant additives, which may be required to undergo safety evaluation and approval. As an additional benefit, pollen and S-SMCs inherently have flour-like product characteristics in their natural form. Pollen, spores, and algae don’t have to be milled before adding to existing food formulations so as to upgrade or extend regular flour portions. Pollen is naturally a very fine like flour, with a size of approximately 100 mesh or finer. When considering the use of natural pollens, pollen flavor ranges widely from nutty, to bitter, to neutral. However, pollen usually more significantly affects color than flavor. Another potential benefit of utilizing natural pollens in food is the high protein content found in pollen, resulting in it being used as an effective high protein flour replacement in numerous recipes. On the topic of adding pollen or S-SMCs to existing food products, it is important to note that a wide range of pollens are traditionally used in food preparation around the world. Fennel & dill pollen spice is used in Tuscany [54]. Recipes exist for substituting various kind of pollen species for flour in all kinds of breads, biscuits, cakes, and cookies in the Americas [55]. Pollens are commonly blended into drinks or added to various desserts in Asia [56]. Globally, there are recipes for a very diverse range of commonly consumed foods, such as, from stews to meatloaf, from soup to cinnamon rolls, and from casseroles to puddings [57]. The long standing traditions of adding pollens to foods highlights the viability of utilizing this promising microencapsulation system for developing a wide range of food products.

With regards to specific experiments that would need to be undertaken for developing various food-based applications, it has been shown that oils can

be loaded into S-SMCs, however, there exist no comprehensive loading optimization studies, loading distribution studies, or studies for final product development. Based on furthering the techniques outlined in this thesis it would be good to: explore the loading of room temperature solid oils into the S-SMC cavity, such as cocoa butter; add sensitive phytochemicals into the loaded solid oil matrix; study the release dynamics of phytochemicals from solid oil matrices; and develop a food product to explore the stability of oil loaded S-SMCs during food processing.

7.2.2. S-SMCs for Microencapsulation in Herbal Therapeutics

Herbal therapeutics also represents an important developing area of the food, supplements, and wellness industries, and relies on the incorporation and delivery of specific bioactive compounds. Microencapsulation is an important technology for the development of novel herbal therapeutics and the development of new natural microencapsulant systems is important for this growing market [58]. An ideal herbal therapeutics microencapsulant would provide a standardized powdered product range with a high degree of homogeneity which may be utilized to contain various stable bioactive compounds. Pollen, spores, and algae provide a compelling solution as a completely natural and healthy food-based microencapsulant for the development of a standardized powdered herbal therapeutics product range.

Many herbal therapeutics have a strong and unpleasant taste and odor, for which pollen-based microencapsulation could provide effective taste and odor masking [52]. Herbal therapeutics relies on the efficacy of plant-based compounds which are typically sensitive to environmental conditions and degradation. The UV protective and oxidation protection capacities of pollen-based capsules provide an attractive solution to ensuring the stability of sensitive bioactive compounds. To ensure compound stability it is also important that compound loading processes are benign to the compounds being loaded [51]. Raw pollens naturally absorb water and may uptake molecules as part of the plant reproductive cycle, and S-SMCs have been shown to facilitate loading by the use of gentle passive- or assisted-loading protocols. This is

highly appropriate for many existing herbal therapeutics as aqueous or alcohol extracts may be encapsulated within pollen-based microencapsulation systems without the need for specialized equipment [10,11]. Additionally, the unique product branding potential of pollen-based microencapsulation systems may provide a novel and unique product branding opportunity for a new range of products in the herbal medicine or supplement markets. With regards to raw pollens, it may be possible to develop a range of novel products utilizing pre-extracted herbal compounds encapsulated with pollen species already shown to have synergistic health benefits within traditional medicine disciplines.

KEY APPLICATION

Microencapsulation for Herbal Therapeutics

Natural Food-Based Microencapsulant

Microencapsulation is a key technology for the development of modern herbal therapeutics products. Pollen provides a completely natural and healthy food-based microencapsulant for the development of a standardized powdered herbal therapeutics products range.

Taste & Odor Masking

Many herbal therapeutics have a strong & unpleasant taste & odor. Microencapsulation is a standard industry solution to mask tastes & odor. Pollen can function as a natural & robust microencapsulant and is already a common food product.

Product Stability

Many therapeutic plant-based compounds are sensitive to environmental conditions & degradation. Microencapsulation is a standard industry method to prolong product stability. Pollen has UV protective and oxidative protection capacities.

Ease of Loading

Pollen naturally absorbs water in preparation for plant fertilization. Microencapsulation with pollen allows for water or alcohol extracts to be easily absorbed into the internal cavity without the need for specialized equipment.

Unique Product Branding

The herbal medicine and supplement market is very competitive. Pollen may be used to develop a range of novel products utilizing pre-extracted herbal compounds encapsulated with synergistic pollen species.

EXAMPLES - Microencapsulant Options

Pine Pollen Based Formulations

- Androgenic, Aphrodisiac, Kidney tonic, Liver tonic, Heart tonic, Spleen tonic, Lung tonic.
- Anabolic endocrine activity, healthy & high libido, rejuvenates the brain, hair, bones & endocrine system, liver regeneration & bile secretion, cardiovascular endurance & lowers cholesterol, muscles & increased metabolism.
- Boosts the immune system & beautifies the skin.

Cattail Pollen Based Formulations

- Promote blood clotting, lower blood pressure, reduce cardiac load, increase blood flow, enhance tolerance to anoxia, reduce myocardial ischemic lesions.
- Excitatory effect on isolated uterine. Enhance the motility of isolated intestine
- Lower levels of lipids like cholesterol & triglyceride.
- Anti-inflammatory, choleric, diuretic, analgesic, and anti-asthmatic effects. Resists ischemia reperfusion injury.

Bee Collected Pollen Based Formulations

- Improving endurance & vitality, Extending longevity, Recovery from chronic illness, Reducing cravings & addictions, Regulating the intestines, Building blood.
- Preventing infectious diseases, i.e. cold & flu.
- Helping overcome retardation and other developmental problems in children.
- Radiation protection & anti-cancer qualities.
- Rich in proteins, free amino acids, and vitamins, including B-complex and folic acid.

Figure 7.4 Overview of microencapsulation for herbal therapeutics as a key application example. As a natural food-based microencapsulant, taste masking, improved product stability, ease of loading, and unique product branding are some advantages which may be obtained. A range of herbal therapeutics encapsulated within synergistic pollen species is a good example of a specific potential application of microencapsulation for herbal therapeutics.

Several key examples of raw pollen-based microencapsulation systems which deliver herbal therapeutics with synergistic effects to the pollen used for delivery, include, pine pollen based formulations, cattail pollen based formulations, and bee-collected pollen based formulations.

- Pine pollen is widely used within traditional medicines and herbal therapeutics for a wide range of effects, many of which relate to men's health issues [59,60]. A list of potential benefits of pine pollen and potentially synergistic herbal therapeutics includes [61]: androgenic; aphrodisiac; kidney tonic; liver tonic; heart tonic; spleen tonic; and lung tonic effects. Pine pollen is often utilized to: boost anabolic endocrine activity; support a healthy & high libido; rejuvenate the brain, hair, bones & endocrine system; promote liver regeneration & bile secretion; improve cardiovascular endurance & lower cholesterol; and support muscles & increased metabolism. Additionally, pine pollen has been used to boost the immune system and beautify the skin. This wide array of potential benefits could be exploited to develop a huge range of potentially synergistic herbal therapeutics products based on pine pollen as a microencapsulant.
- Cattail pollen as a therapeutic is often associated for treatment of blood related issues and is commonly utilized for women's health issues [62,63]. Cattail pollen based formulations could be directed toward: promoting blood clotting; lowering blood pressure; reducing cardiac load; increasing blood flow; enhancing tolerance to anoxia; and reducing myocardial ischemic lesions. Cattail is believed to: provide an excitatory effect on isolated uterine; enhance the motility of isolated intestine; lower levels of lipids like cholesterol and triglycerides; and provide anti-inflammatory, choleric, diuretic, analgesic, and anti-asthmatic effects.
- Bee collected pollen is very commonly found in herbal supplement stores and is widely used in traditional herbal therapeutics. Bee collected pollen based formulations could be directed to [64,65]: improving endurance and vitality; extending longevity; recovering from chronic illness; reducing cravings and addictions; regulating the

intestines; and building blood. Bee collected pollen has been linked with: preventing infectious diseases, i.e. cold and flu; helping overcome retardation and other developmental problems in children; and providing radiation protection and anti-cancer qualities. Additionally, bee collected pollens are known to be rich in proteins, free amino acids, and vitamins, including B-complex and folic acid. Overall, synergistic pollen-based microencapsulation systems have significant potential for developing a wide range of novel herbal therapeutics products.

7.3. S-SMCs as Novel Polymeric Biomaterials

There are significant opportunities for exploring new applications of sporopollenin as a novel polymeric biomaterial. There is limited understanding and applications of sporopollenin derived from pollen, spores, and algae, however, the data which does exist provides compelling insights into potential applications. Natural and synthetic biopolymers are utilized in a wide range of products and applications, such as foods, coatings and packaging, disposable products, medical implants, and electroactive plastics. In particular processed foods incorporate a vast range of biocompatible composites to achieve desired ingredient stability and texture properties [66]. New gel based materials derived from processed and functionalized pollen or pollen composites may be used as biocompatible food ingredients. In the field of environmentally friendly coatings and packagings, there is an ongoing need for novel materials which exhibit mechanical robustness and exhibit good chemical stability. Processed and functionalized sporopollenin or sporopollenin composites derived from the extracted outer pollen wall polymers may be incorporated into pollen-based sheeting and films to offer novel environmentally friendly solutions from natural materials [18]. Similarly, disposable consumer products make up a huge market sector in the modern age, and the development of biodegradable consumer products is an important contribution to solving the growing issues of pollution and environmental waste. Pollen polymer composites may be developed to provide a robust biocompatible and environmentally friendly consumer product material. Medical implants and devices provide another potential application for novel biocompatible polymers.

Robust biocompatible polymers are important for the development of next generation medical implants and devices [67]. The pollen polymer composite structure inherent in S-SMCs has been shown to be both biocompatible and exhibit resistance to degradation in physiological conditions. Electroactive plastics are also an area of massive growth with the fields of materials science and electronic devices [68]. Next generation consumer electronics require the use of a diverse range of new electroactive polymer composites and integrating environmentally friendly naturally derived materials is of significant interest. Sporopollenin-based polymer composites may be processed and functionalized to produce a wide range of electrically conductive materials.

There are several key factors driving ongoing and future pollen, spore, and algal research with regards to the development of novel polymeric biomaterials. Primarily, the inherent nature of sporoderms provides an attractive combination of robust polymers. The outer wall exine of most pollen, spores, and some algae is comprised of an extremely chemically and mechanically robust family of biopolymers, known as sporopollenin [1]. The inner wall intine of pollens is typically comprised of several commonly occurring cellulosic compounds. Importantly, pollen and particularly pollen sporoderms are comprised of fundamentally biocompatible compounds and in most countries there are very few or no limiting regulations for using pollen as an ingredient for human consumption and therefore these materials may be viewed as safe for human centered technologies [7,8]. Various multidisciplinary studies suggest that pollen sporopollenin and cellulose composites may be processed and functionalized to produce composite gels or bioplastic-like sheetings and coatings [18]. The existing literature is clear that sporopollenin is a complex biopolymer which can be processed to incorporate various functional groups which may in turn be utilized for binding to a diverse range of pre-existing industrial materials.

Key goals for future pollen, spore, and algal novel polymeric biopolymer research should incorporate food science, packaging and coatings, forming and biodegradation, stability and physiological immune responses, and inherent electromagnetic properties. There is significant potential for

developing pollen-based gels for use in processed foods. The potential for incorporating sporopollenin into polymer composites may be particularly relevant for biopolymer coatings and packaging. Additionally, the physicochemical properties of sporopollenin composites may be found to be well suited for use in producing disposable consumer products [18]. Comprehensive exploration of pollen sporoderm composite stability and physiological immune responses to sporopollenin composite materials will be key to the development of pollen-based composites for new medical devices and implants. A greater understanding of the electrical and magnetic properties of various functionalized electroactive sporopollenin composites may highlight their use in developing next generation eco-friendly and biocompatible electrical devices.

Figure 7.5 Overview of novel polymeric biomaterials as a key research project. There exists potential for use in diverse industrial fields, and clear research goals and objectives which may be pursued.

There is a diverse range of specific projects which may be explored so as to utilize pollen, spores, or algae. With regards to the food industry, it would be of significant interest to develop a gel-based food product which incorporates pollen gel as a primary ingredient. This may require sporoderm extraction followed by further processing and functionalization to allow for integration with existing food-based binders. Such a pollen-based gel could provide the opportunity for new product ranges with inherent detoxification potential [5]. With regards to packagings and coatings, it would be desirable to develop a spray-on pollen-based environmentally resistant coating. Processed and functionalized sporoderms may be mixed with compounds facilitating inter-particle binding to result in a robust protective coating with inherent UV protective and chemical resistant properties [1,42]. With regards to disposable consumer products, it would be desirable to develop a robust sporopollenin-cellulose composite which may be molded into a wide range of forms, such as eating utensils, plates, and containers. Sporopollenin and cellulosic compounds have a natural affinity for one another as indicated by their presence in the sporoderm [69]. With further research it may be possible to elucidate and exploit the mechanisms of sporopollenin-cellulose interactions. With regards to biomedical implants, it would be desirable to further explore the stability of pollen-derived composites within physiological conditions, and understand the degree of immune responses of the body toward sporopollenin. Humans and animals are exposed to pollen, spores, and algae in various manners throughout their entire lifetime, however, very little is understood regarding the bodies response to the presence of the sporoderm as a biopolymer complex [30]. With regards to developing electroactive pollen-based composites, it is important to gain greater insight into the electric and magnetic properties inherent in sporopollenin. Once the resistive and capacitive properties are better understood it may be possible to integrate a wide range of electrically conductive materials and develop novel composites for applications such as flexible, wearable biocompatible electronics [13].

7.3.1. S-SMCs for Cosmetics & Personal Care

Cosmetics and personal care products represents an important application of novel polymeric biomaterials. Overall, there is a global trend in the cosmetics and personal care industries to move away from the use of synthetic chemicals [70,71]. Pollen, spores, and algae provide a compelling new source of multi-functional natural materials for use in a wide range of products. The intrinsic properties of pollen particles allow for the development of novel products, i.e. UV absorbance, toxin binding, skin nourishment, and microencapsulation [11,42,46,65].

Figure 7.6 Overview of cosmetics and personal care as a key application example. As a multi-functional natural material, UV protection, detoxification, improved product stability, and unique product branding are some advantages which may be obtained. A range of potential products, such as, novel sunscreens, novel skin detoxification creams, and functional microbeads, are good examples of specific potential cosmetics and personal care applications.

Pollen-based materials provide a number of attractive properties such as UV protection, detoxification, product stability, and the potential for unique product branding. With regards to UV protection, there is a growing demand for natural and biocompatible products which provide robust UV protection [72]. It is well established that the sporopollenin in pollen has the potential for use as a sunscreen due to UV absorbing compounds present in the pollen wall which are naturally present to protect internal genetic material from damage [42]. With regards to heavy metal and environmental pollutant detoxification, skin cleansing and detoxification are becoming more relevant in modern urbanized lifestyles where people come into contact with hundreds of industrial chemicals on a daily basis [73]. The outer shell material of pollen has been proven to bind with heavy metals and other synthetic chemicals that may accumulate on and in human bodies, and these properties may be exploited to develop topical formulations which aid in the binding and removal of potentially harmful compounds which accumulate on skin. With regards to product stability, many bioactive beauty industry compounds are sensitive to environmental conditions and degradation [50]. As discussed extensively above, utilizing pollen or extracted S-SMCs as a microencapsulant system may significantly prolong product stability due to the inherent UV protective and oxidative protection capacities. With regards to unique product branding, these days the global cosmetics and personal care markets are very competitive. Pollen derived materials may be used to develop a range of novel products utilizing both pollen & pre-extracted herbal compounds with synergistic efficacy.

In particular, there are several clear potential applications for pollen and extracted S-SMCs, first, in developing a new sunscreen product range, second, in developing a skin detoxification product range, and third, the development of a functional microbead system which may have applicability to a wide range of products as a replacement for problematic synthetic microbeads.

- As a sunscreen it is important to remember that a key role of the pollen outer shell is to protect the sensitive genetic material from UV damage [42,74]. Currently, most modern sunscreens use either chemical or

physical barriers, however, there are major concerns about toxic byproducts formed with UV absorption [75]. Conveniently, pollen particles or pollen fragments may be obtained in the same size range as common UV blocking compounds such as titanium oxide powders often used in physical barrier type sunscreens. Additionally, if utilizing raw pollens, it is important to note their biocompatibility and that the rich nutrient profile of the internal cytoplasmic materials may also nourish the skin [65].

- As a skin detoxification system, it may be possible to develop a product range which utilizes the inherent chelating properties of the outer shell so as to bind with heavy metals and synthetic toxins. It is important to note that sporopollenin has been used in mining and waste water treatment for extracting heavy metals [76]. Additionally, sporopollenin is widely used as a food supplement for clearing heavy metals and toxins from the body [5,65]. Therefore, the development of lotions comprising pollen particles or pollen fragments may bind with heavy metals and toxins in the skin and allow for their removal upon washing.
- As functional microbeads, it may be possible to develop a product to replace synthetic microbeads, which are being phase-out globally, due to being a major environmental pollutant [77]. For many years, tiny microbeads have been used as fillers and texturizers in a wide range of personal care products. Currently, there is a global trend to ban plastic microbeads due to their contribution to environmental pollution, and many major countries have already implemented bans, or are in the process of passing legislation to phase out microbeads [78]. Pollen-based particles offer a safe natural alternative to synthetic plastics. Additionally, pollen-based particles may also be used for microencapsulating flavors, aromas, or bioactive compounds to provide functional effects.

7.3.2. S-SMCs for Novel Composite Biomaterials

Novel composite biomaterials represent a huge multidisciplinary area of research and industrial applications. The introduction of a novel multi-functional range of natural materials such as the sporoderms constituents from pollen, spores, and algae represents a vast opportunity for both research and industry. Overall, there is growing global interest in the development and use of biomaterials for more diverse applications [79,80]. In particular, the pollen biopolymer, sporopollenin, is an extremely robust natural material, and due to improved and diverse processing techniques it is possible to form various novel composite biomaterials.

Figure 7.7 Overview of novel composite biomaterials as a key application example. As a multi-functional natural material, chemical resistance, UV protection, biocompatibility, and mechanical robustness are some advantages which may be obtained. A range of potential products, such as, natural chemically resistant biopolymers, composite biopolymer sheets, and soft and flexible electronics, are good examples of specific potential novel composite biomaterials applications.

Pollen-based materials provide a number of attractive properties, such as, chemical resistance, UV protection, biocompatibility, and mechanical robustness [1,30,81]. With regards to chemical resistance, some biomaterials inherently lack the chemical robustness of synthetic materials. However, the pollen outer shell comprises a highly robust biopolymer, sporopollenin, which is known to exhibit exceptional stability when exposed to acids, bases, and solvents. Incorporating pollen-based materials into existing composites may improve the overall chemical resistance of the system. With regards to UV protection, material degradation due to exposure to UV light is a problem encountered in the development of many products [82]. Pollen naturally has UV absorbing compounds present in the pollen wall to protect internal genetic material from damage, and may provided improved UV stability to pollen-based composites [18,42]. With regards to biocompatibility, biocompatible materials represent a very important category of materials in the modern age, due to their potential application in a wide range of biomedical products. Pollen has a long history of use as a food, and comes in contact with the entire human race through inhalation and skin exposure [30]. It is important to note that the sporoderm and particularly, the outer shell material, sporopollenin, has no known allergenicity issues. The allergenicity typically associated with some species of pollen is related to sensitive bioactive proteins, which may be effectively removed during most standard sporoderm extraction processes [83]. With regards to mechanical robustness, mechanical stability is a key consideration in the development of all products. The pollen outer shell material, sporopollenin, has been shown to be flexible and highly robust when exposed to high-pressure compression [81,84]. These properties may be imparted to pollen-based composites and provide significant opportunities for developing novel composite materials.

In particular, there are several clear potential applications for pollen and extracted S-SMCs, first, in developing natural chemically-resistant biopolymer composites, second, in developing composite biopolymer sheets for diverse applications, and third, the development of soft and flexible electroactive composites for use in a wide range of standard and wearable electronics devices.

- As a natural chemically-resistant biopolymer composite, there is a high demand for safe and reliable chemically resistant materials which provided adequate protection against environmental and industrial chemicals [79]. Most modern protective materials are derived from synthetic/petrochemical-based compounds. The pollen biopolymer, sporopollenin, is known to be highly resistant to environmental conditions and chemical exposure [1]. Through processing and functionalization it may be possible to produce sporopollenin composites in the form of sheets and coatings [18].
- As composite biopolymer sheets, there exist a wide range of potential applications, however, many biopolymers lack adequate chemical and mechanical robustness [79]. Research into suitable processing methods may allow for the incorporation of the pollen biopolymer, sporopollenin, into a wide range of composite sheets. Through further addition of various natural or synthetic fibrous materials, such as, cellulose, silk, carbon nanotubes, graphene, etc., it may be possible to further tune the properties of these composite biopolymer sheets for specific applications [13,18].
- As soft and flexible electronics, there exist diverse applications which may lead to the development of various next generation devices and appliances [68]. A greater understanding of the electrical and magnetic properties of sporopollenin combined with processing and functionalization methods may allow for the development of novel gels or sheets suitable for various electronics applications. Through further incorporation of electrically conductive compounds, such as, iron, copper, carbon nano-tubes, graphene, etc., it may be possible to create robust and electrically conductive composite soft materials [13,18].

7.4. Conclusions

Pollen, spores, and algae sources, which comprise robust outer coatings of sporopollenin, hold significant potential for a wide range of fundamental and application based studies, both in academia and industry. Sporopollenin

represents a compelling source of little understood biopolymers which may become fundamental in developing a wide range of novel products or adding improved functionality to existing products. However, there is needed much fundamental research into the basic extraction, characterization, and utilization of plant-based sporoderms before significant progress can be made. Thus far, the efforts and results outlined in this thesis have contributed to the field by providing a greater understanding of fundamental sporoderm extraction methods and insight into utilizing S-SMCs for microencapsulation applications.

Analysis of S-SMC acid/base extraction protocols have yielded important information for continued research in this field. The simplifying of commonly cited S-SMC extraction protocols has ensured that other researchers can enter this field of study with greater certainty and clarity, and has provided a foundation for improving the industrial applicability of S-SMC extraction. The determination that phosphoric acid hydrolysis extraction at sub-100 °C temperatures produces viable and mechanically robust S-SMCs, allows for improved safety as well as reduced energy and infrastructure requirements for S-SMC extraction. Additionally, clarifying that S-SMC extraction processing times need to be limited to 5 to 30 hours ensures that future researchers can produce high quality capsules on a favorable time scale. Most importantly, highlighting the potential for extracting high-quality viable S-SMCs from genetically diverse material sources, allows for a vast expansion in the applicability of this field of study.

Analysis of S-SMC loading mechanisms and loading dynamics with a common representative macromolecule has yielded important information for the utilization of SMDs for microencapsulation applications. The understanding that loading into hollow core-shell microspheres is driven by the collapse of air bubbles trapped within capsules, highlights that assisted loading methods, such as vacuum loading may be required for comprehensive particle loading. Additionally, clarifying that loading distributions of compounds across a large sample population include a significant number of empty or partially empty capsules, highlights that it may be possible to refine loading and washing protocols further to achieve greater loading potential. Importantly,

these studies were able to provide robust estimations for theoretical maximum loading efficiencies and provide a benchmark for the comparison of practical loading efficiencies.

Analysis of release dynamics, and the development of a practical controlled release S-SMC-based multiparticulate drug delivery system for the delivery of sensitive macromolecules to the digestive tract, is a significant contribution to exploring the viability of S-SMCs in microencapsulation and compound delivery. Real-time observations of compound release from S-SMCs in simulated gastric and simulated intestinal conditions, were highly correlated with standard drug release profile analysis methodologies, and highlighted the diffusion driven release of compounds upon hydration by aqueous media. Through the application of standard drug delivery methodologies, it was possible to produce an enteric-coated BSA-loaded S-SMC pellet, which exhibited an ideal release profile for the release of therapeutic compounds in the intestinal tract.

Overall, these studies have provided a significant contribution to the furthering of fundamental and applied research into the properties and utilization of S-SMCs for microencapsulation applications and more. This is an exciting field of research which will continue to receive increased attention in the coming years and may lead to the development of a wide range of novel products and eventually develop into a prominent area of materials science research.

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Appendix

Research Publications

Work related to S-SMC extraction and applications

1. Wang H, **Potroz MG**, Jackman JA, Khezri B, Marić T, Cho N-J, Pumera M. "Bioinspired Spiky Micromotors Based on Sporopollenin Exine Capsules." *Advanced Functional Materials* (2017), In Press.
2. Prabhakar AK, Lai HY, **Potroz MG**, Corliss MK, Park JH, Mundargi RC, Cho D, Bang S-I**, Cho N-J**, "Chemical Processing Strategies to Obtain Sporopollenin Exine Capsules from Multi-Compartmental Pine Pollen," *Journal of Industrial Engineering* (2017), In Press.
3. **Potroz MG**, Mundargi RC, Gillissen JJ, Tan E-L, Meker S, Park JH, Jung H, Park S, Cho D, Bang S-I**, Cho N-J**, "Plant-based hollow microcapsules for oral delivery applications: Towards optimized loading and controlled release," *Advanced Functional Materials* (2017), 1700270.
4. Wang L, Jackman JA, Tan E-L, Park JH, **Potroz MG**, Hwang ET, Cho N-J, "High-Performance, Multifunctional Electronic Skin Sensor Incorporating Natural Microcapsule Actuators," *Nano Energy* 36 (2017), 38 - 45.
5. **Potroz MG**, Mundargi RC, Park JH, Tan E-L, Cho N-J. "Extraction of Plant-Based Capsules for Microencapsulation Applications" *JoVE (Journal of Visualized Experiments)* 117 (2016): e54768-e54768.
6. Mundargi RC, **Potroz MG**, Park JH, Seo JE, Tan E-L, Lee JH, Cho N-J. "Eco-friendly streamlined process for sporopollenin exine capsule extraction." *Scientific Reports* 6 (2016): 19960.
7. Mundargi RC, **Potroz MG**, Park JH, Seo J, Lee JH, Cho N-J. "Extraction of sporopollenin exine capsules from sunflower pollen grains." *RSC Advances* 6, no. 20 (2016): 16533-16539.

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Other topics

1. **Potroz MG***, Brown M*, The S-W, Cho N-J, "Natural Products for the Treatment of Chlamydiae Infections," *Microorganisms* 4.4 (2016): 39. Special Issue on Chlamydia-like Bacteria: Evolution, Pathogenicity, Diagnostics and Treatment.
2. Belling JN*, Jackman JA*, Avsar SY, Park JH, Wang Y, **Potroz MG**, Ferhan AR, Weiss PS, Cho NJ, "Stealth Immune Properties of Graphene Oxide Enabled by Surface-Bound Complement Factor H," *ACS Nano* (2016).
3. Mundargi RC, **Potroz MG**, Park SH, Shirahama H, Lee JH, Seo J, Cho N-J. "Natural Sunflower Pollen as a Drug Delivery Vehicle." *Small* 12.9 (2016): 1167-1173.
4. Mundargi RC, **Potroz MG**, Park SH, Park JH, Shirahama H, Lee JH, Seo J, Cho N-J. "Lycopodium Spores: A Naturally Manufactured, Superrobust Biomaterial for Drug Delivery." *Advanced Functional Materials* 26.4 (2016): 487-497.
5. **Potroz MG**, Cho NJ. "Natural Products for the Treatment of Trachoma and Chlamydia trachomatis." *Molecules* 20, 20.3 (2015): 4180-4203. Special Issue on Natural Products as Leads or Drugs against Neglected Tropical Diseases.

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Acknowledgements in published research

1. Wang L, Jackman JA, Ng WB, Cho N-J. "Flexible, Graphene-Coated Biocomposite for Highly Sensitive, Real-Time Molecular Detection." *Advanced Functional Materials* 26.47 (2016): 8623-8630.
2. Wang L, Ng WB, Jackman JA, Cho N-J. "Graphene-Functionalized Natural Microcapsules: Modular Building Blocks for Ultrahigh Sensitivity Bioelectronic Platforms." *Advanced Functional Materials* 26.13 (2016): 2097-2103

Patents

Work related to S-SMC extraction and applications

1. N.J. Cho, **M.G. Potroz**, R.C. Mundargi, J.H. Park, J.A. Jackman. Microencapsulation of compounds into natural spores and pollen grains. WO2017010945 A1.