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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**SURFACE MODIFICATION OF
PLANT-BASED MICROPARTICLES FOR
COLLOIDAL SCIENCE AND
CELLULAR ADHESION APPLICATIONS**

TAN EE-LIN

**SCHOOL OF MATERIALS SCIENCE AND ENGINEERING
2018**

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TAN EE-LIN

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

A thesis submitted to the Nanyang Technological
University in partial fulfilment of the requirement for the
degree of Master of Engineering

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.

05/04/2018

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Date

A handwritten signature in black ink, appearing to be 'GL' followed by a horizontal line.

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Tan Ee-Lin

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Authorship Attribution Statement

This thesis contains material from a paper published in the following peer-reviewed journal where I was the first author.

Chapter 4 is published as E.-L. Tan, M. G. Potroz, G. Ferracci, J. A. Jackman, H. Jung, L. Wang, N.-J. Cho. Light-Induced Surface Modification of Natural Plant Microparticles: Toward Colloidal Science and Cellular Adhesion Applications. *Adv. Funct. Mater.* 2018, 1707568. DOI: 10.1002/adfm.201707568.

The contributions of the co-authors are as follows:

- A/Prof. Cho provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts. The manuscript was revised by Dr. Potroz and Dr. Jackman.
- I co-designed the study with Dr. Potroz.
- I performed all the laboratory work at the School of Materials Science and Engineering.
- Tissue culture experiments were done by Ms. Ferracci.
- Ms. Jung, Ms. Wang and I did scanning electron microscopy at the Facility for Analysis, Characterization, Testing and Simulation.

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Summary

Pollen microparticles and sporopollenin - sporoderm microcapsules (S-SMCs) provide a compelling all-natural solution for use in a wide range of oil/water emulsion-type applications. To enhance the utility of pollen microparticles and S-SMCs, surface modification was shown to be a mechanism for altering surface chemistry and thereby tuning microparticle wetting properties, emulsification characteristics, and particle/cell adhesion. This project explored the extraction and surface modification of discrete pollen particles from bee-collected pollen granules and the extraction of S-SMCs from pollen particles. Characterization of the ultraviolet-ozone (UV-O) surface modified particles (pollen and S-SMCs) by X-ray photoelectron spectroscopy (XPS) and attenuated total reflectance Fourier transform infrared (ATR-FTIR) to give insight to the UV-O modifications to sporopollenin (both native and acid extracted). This project will also be exploring the possible applications for such microparticles as functional microbeads. UV-O treatment is shown to increase the proportion of surface elemental oxygen and C=O bonds, leading to enhanced particle dispersion properties, control over Pickering emulsion characteristics, and increased particle/cell binding affinities. Looking forward, bee-collected pollen is widely available, competitively priced, and is considered regulation-free for oral consumption due to a long history of use as a food and medicine, with extensive literature purporting numerous health benefits. Beyond facilitating the utilization of pollen and S-SMCs as a functional microparticle system, a greater understanding of UV-O surface modification of sporopollenin also provides insights into the inherent UV protective properties of pollen shells, and provides insights into the use of pollen sporopollenin for exploring climate change through fossil records.

Table Captions

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Table 4.1 Bond-type binding energies and proportions for carbon (C1s) and oxygen (O1s) binding.

Table 5.1. Bond-type binding energies and proportions for carbon (C1s) and oxygen (O1s) binding.

Figure Captions

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Figure 5.3 Surface chemistry analysis of untreated and ultraviolet-ozone (UV-O) treated sporopollenin sporoderm microcapsules (S-SMCs): (a) XPS quantification of atomic carbon and oxygen; (b) XPS quantification of proportional shifts in major oxygen binding groups; (c) XPS quantification of overall oxygen binding distributions; (d) XPS narrow scan carbon (C1s) peak of untreated and UV-O treated S-SMCs; (e) ATR-FTIR spectra and difference analysis of untreated and UV-O treated S-SMCs; and (f) normalized peak height ratio analysis of key peaks of interest from ATR-FTIR spectra.

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Figure 5.5 Influence of ultraviolet-ozone (UV-O) treatment on oil loading of sporopollenin sporoderm microcapsules (S-SMCs): (a) CLSM cross-sectional images of an oil/water system with homogenization, and floating oil-loaded S-

SMCs isolated after system stabilization; (b) quantification of the proportion of fully oil-loaded floating S-SMCs; (c) schematic representation of the proposed mechanisms of oil loading and system stabilization; (d) false-color SEM images of dried oil-loaded S-SMCs highlighting clean particle surface and representative oil loading (original uncolored images can be found in **Figure S11, Chapter 5 Appendix**). Scale bars: (a) and (d) = 10 μm .

Figure 5.6 Influence of ultraviolet-ozone (UV-O) treatment on sporopollenin sporoderm microcapsule (S-SMC)/cell interactions with Huh-7.5 liver hepatocytes: (a) CLSM images of S-SMC and cell distributions and coverage areas; (b) quantification of S-SMC and cell coverage area proportions; (c) CLSM and false-color SEM images of representative S-SMC/cell spheroid and network structures (original uncolored images can be found in **Figure S16, Chapter 5 Appendix**). Scale bars: (a) 100 μm .

Abbreviations

2D	Two Dimensional
3D	Three Dimensional
AES	Auger Electron Spectroscopy
ATR-FTIR	Attenuated Reflectance Fourier Transform Infrared
CLSM	Confocal Laser Scanning Microscopy
CVD	Chemical Vapour Deposition
DI	Deionized
DIPA	Dynamic Imaging Particle Analysis
EA	Elemental Analysis
ECM	Extracellular Matrix
FC	Flow Cell
FTIR	Fourier Transform Infrared
GCMS	Gas Chromatography–Mass Spectrometry
HPLC	High-Pressure Liquid Chromatography
ISS/SIMS	Ion Scattering Spectroscopy/Secondary Ion Mass Spectroscopy
MMNO	4-methylmorpholine N-oxide monohydrate
MQ	Milli-Q
MS	Mass Spectrometry
NaOH	Sodium Hydroxide
NMR	Nuclear Magnetic Resonance Spectroscopy
PAA	Polyacrylic Acid
PDEAEMA	Poly(2-(Diethylamino)Ethyl) Methacrylate
PDMS	Polydimethylsiloxane
PE	Polyethylene
PEG	Polyethylene Glycol
PEEK	Polyether Ether Ketone
PMMA	Polymethyl Methacrylate
POEGMA	Polyoligoethylene Glycol Methacrylate
SECs	Sporopollenin Exine Capsules
S-SMCs	Sporopollenin – sporoderm microcapsules

USA	United States of America
USD	United States Dollar
UV-O	Ultraviolet-Ozone
XPS	X-ray Photoelectron Spectroscopy

Chapter 1

Introduction

Bee-collected pollen is widely available because 90 % of the plant kingdom is made up of flowering plants, also known as angiosperms. Nature's microcapsule, pollen particles are the inspiration for the development, synthesis and research of microparticles yet there is a lack of utilization of such particles for various applications that microparticles are dominating. Synthetic microparticles possess similar traits to pollen particles such as micrometer size range, and size uniformity, interesting topology. Pollen is made of a robust biopolymer called sporopollenin which has been shown to have antioxidant properties, heavy metal binding properties, and biocompatibility. The following will be carried out to explore the potential use of nature's microparticles as functional microbeads. The extraction of pollen particles and sporopollenin sporoderm microcapsules (S-SMCs) from bee-collect pollen, surface modify these microparticles using ultraviolet-ozone (UV-O), characterization of the surface chemical due to treatment, and the investigation of the effect on emulsification characteristics and particle/cell adhesion properties. Findings from this project are UV-O treatment resulted in changes in the surface chemistry of pollen and S-SMCs and the changes in hydrophobicity and surface chemistry affect emulsification characteristics and particle/cell adhesion properties. Overall, this chapter contains the introduction, the hypotheses, the objectives, the scope, the dissertation overview, the findings and the outcomes/originality of this project.

1.1. Hypothesis/Problem Statement

The overall hypothesis of this project is to explore utilization of pollen and S-SMCs as functional microparticle system. Microparticle research has been conducted for years but the use of microparticles in the industry has increased recently and market trends indicates microparticle usage will continue to grow [1]. Pollen is a natural microcapsule, nature's solution for the protection of delicate genetic information is the source of inspiration for the synthesis and research in man-made microparticles, microspheres or microcapsules [2, 3]. With attractive factors, such as biocompatibility, size uniformity and being a renewable resource, pollen and S-SMCs present a huge potential in microparticle technology [4, 5]. Due to the lack of understanding regarding the surface chemistry of pollen and S-SMCs, functionalization of pollen and S-SMCs for potential applications may be difficult. Therefore, the exploration and better understanding of the surface chemistry of pollen and S-SMCs more interest would be generated for the use pollen and S-SMCs in the industry as functional microparticles. In line with such goals, it is hypothesized that:

- Surface chemistry of pollen and S-SMCs can be modified using UV-O treatment
- UV-O can allow for the tuning of the wetting properties, the emulsification characteristics, and the particle/cell adhesion properties.

1.2. Objectives and Scope

Based on the defined hypotheses in the previous section, the scope of this project was defined. Due to the great variety of bee collected pollen available in the market, it is not possible or practical to study many types of pollen in this project. Therefore, this project focused on using *C. sinensis* bee-collected pollen as the raw material. In addition, the relatively low allergenicity of *C. sinensis* pollen, the availability of the pollen, the reasonable price of the bee pollen, and the lack of research on *C. sinensis* pollen particles makes *C. sinensis* the ideal candidate [6, 7]. The pollen particles and S-SMCs to be studied will be

extracted from *C. sinensis* bee pollen. There are two main categories of surface modification – dry and wet processes [8]. Examples of wet processes are silanization and polymer grafting. Examples of dry processes are flame treatment, corona treatment and UV-O treatment. UV-O treatment is simple to set up, easy to use, and is cheap. Hence, UV-O was chosen as the mechanism to modulate the surface properties of pollen particles and S-SMCs. From the scope defined, the objectives of this project are as follow:

1. To surface modify defatted *C. sinensis* pollen particles using UV-O, to characterize if physical or chemical changes occurred using various techniques and to explore the potential of surface modified pollen particles as functional microbeads.
2. To surface modify acid extracted *C. sinensis* S-SMCs using UV-O, to characterize if physical or chemical changes occurred using various techniques and to explore the potential of surface modified S-SMCs as functional microbeads.

1.3. Dissertation Overview

This study began with literature review of various topics related to bee collected pollen or bee pollen granules. Topics such as the pollen structure, the biopolymer known as sporopollenin that makes up the exine shell of pollen particles, the protocol for defatting bee pollen, the surface modification methods available, the types of emulsions and the behaviour of cells towards microparticles or hydrophilic surfaces.

The protocol for defatting bee pollen granules made used of common solvents such as acetone, water and diethyl ether. Extraction of S-SMCs was done based on recent publications on the topic. Extraction protocol was adapted from various studies done by Mundargi, *et al.* where the team used acid hydrolysis to extract S-SMCs [9]. The protocol has been shown to work for many types of pollen [9-11]. Morphology of the material, pollen particles and S-SMCs were determined with various characterization methods such as dynamic imaging

particle analysis (DIPA), scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). The removal of cytoplasmic content was ascertained by elemental analysis.

Ultraviolet-ozone treatment was selected as the method for surface modification because of its commercial availability, ease of use and its relatively low operation cost [12]. UV-O treatment of pollen particles and S-SMCs were carried out for various durations. Scanning electron microscopy was carried out to determine if UV-O treatment resulted in physical changes to the morphology of pollen particles and S-SMCs. Chemical characterization methods such as attenuated total reflection Fourier-transform infrared spectroscopy (ATR-FTIR) and x-ray photoelectron spectroscopy (XPS) were performed. ATR-FTIR and XPS were performed on particles and microcapsules that were untreated and treated to elucidate the chemical changes after UV-O treatment. Peak height ratio analysis was done for ATR-FTIR data to show the changes with treatment time. Peak fitting was done for XPS data to elucidate how the oxygen moieties on sporopollenin change with treatment.

Potential application for pollen particles and S-SMCs that were exposed to UV-O were explored in the form of Pickering emulsions, cocoa butter loading and in the study of cell adhesion to such particles or microcapsules. Pickering emulsion protocol was adapted from recent publications. Height of the water, oil and emulsion layer was monitored and the structure of the Pickering emulsion was observed using the stereomicroscope and the CLSM. Cocoa butter loading was carried out with S-SMCs and not pollen particles because pollen particles were not hollow and there was no space for the loading of cocoa butter. Cocoa butter loading into S-SMCs was done using emulsification method where warm cocoa butter was emulsified in water with S-SMCs present. Cocoa butter filled S-SMCs were separated with separating funnel. Characterization of cocoa butter loaded S-SMCs was done using SEM and CLSM. For the cell adhesion study pollen particles and S-SMCs that were untreated and treated were incubated with liver hepatocarcinoma cells in 24-well plates in culture media for 24 h and loose particles or microcapsules were removed by gently washing with media. DIPA and bright field microscopy

were done to make sure washing protocol was appropriate. Observation of cell adhering to pollen particles and S-SMCs were done using SEM and CLSM.

1.4. Findings and Outcomes/Originality

The findings and outcomes of this project are as follow:

- It is possible to tune the hydrophobicity of pollen and S-SMCs. The contact angle measurements would decrease as the UV-O treatment time increase.
- From surface sensitive characterization techniques such as X-ray photoelectron spectroscopy (XPS) and attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR), insights to the chemical changes due to UV-O treatment could be gained. Based on the existing literature of UV-O or oxygen plasma treatment of polymers, the oxygen moieties should increase with UV-O treatment.
- The changes in wettability and surface chemistry of pollen particles and S-SMCs will result in change in emulsification characteristics and particle/cell adhesion properties.
- The insights gained from Pickering emulsion resulted in the possibility of loading cocoa butter into the S-SMCs such that the microcapsules are mostly filled, have clean surface, discrete and the sample is like dry powder.

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Chapter 2

Background & Literature Review

Literature review on various topics was conducted before the motivations and the experimental protocols were proposed in this thesis. The topics reviewed are bee pollen, pollen, sporopollenin, defatting of bee pollen, extraction of S-SMCs, UV-O treatment, Pickering emulsions, cocoa butter oil loading into S-SMCs, and cell adhesion to pollen particles or S-SMCs. This chapter highlights that pollen particles and S-SMCs are largely unexplored. Overall, this chapter provides the background and current knowledge on the topics to be explored in this thesis and the gaps in the current scientific knowledge that have resulted in the motivations for this study.

2.1. Pollen Overview

2.1.1. General Pollen Structure

Pollen particles are nature's microcapsules or microparticles that are used by plants for the safe storage of their genetic material during pollination. Pollen is made up of a hard outer shell or exine made of a biopolymer called sporopollenin and an inner shell or intine made of pectin, cellulose, hemicellulose and polysaccharides [1]. These two shells combined is the sporoderm [2]. The important genetic material is protected by the sporoderm. Pollenkitt or the pollen coat is a layer of adhesive material that is composed of lipids that surrounds the pollen. Pollenkitt are usually produced by the anthers of flowering plants or angiosperms so that pollen grains can stick to bugs that help pollination [3].

Pollen particles are monodispersed for a species and the sizes range from 6 to 100 μm [4]. Pollen particles also possess various surface morphologies having different number of apertures and different sexine sculpturing [5]. For example, *Zea mays* (corn) pollen has a relatively smooth surface and a single aperture whereas *Helianthus annuus* (sunflower) pollen has spiky surface and three apertures. The purpose of pollen is to protect the genetic material during pollination. Due to the importance of this task, sporopollenin is a robust material that possess ultraviolet light shielding properties, antioxidant properties, and chemical resistance [6]. Some studies have utilized natural pollen grains as templates due to the unique and interesting hierarchal structure and monodispersity of the pollen grains [7-9]. Other studies have also explored using pollen grains from lycopodium, sunflower and pine as microcapsules for drug delivery [10-12]. However, there is a lack of literature on the use of *C. sinensis* pollen grains such applications.

Figure 2.1 Schematic of basic structure of pollen. The important genetic material is encapsulated by the both exine and intine. The exine is made of a robust biopolymer called sporopollenin. The intine is cellulosic in nature comprise of pectin, cellulose and hemicellulose. The intine and exine make up the sporoderm. The sporoderm is usually covered with pollenkitt which is lipidic in nature and act as a natural adhesive in pollination process.

2.1.2. Sporopollenin as Key Material of Pollen

Sporopollenin is a robust complex biopolymer that can be found in pollen, spores, algae and some bacteria [13]. Sporopollenin has been reported to be resistant to non-oxidizing acids, dilute bases, hydrolytic enzymes. Reagents that can dissolve sporopollenin are reported to be 2-aminoethanol, 3-animopropanol, 2,2',2''-nitrilotriethanol, and 4-methylmorpholine-N-oxide [14-17]. Several studies reported that reagents such as fused potassium hydroxide, ozone, and hydrogen peroxide and sulphuric acid mixture can degrade sporopollenin [13, 18, 19]. The elemental composition of sporopollenin is carbon, hydrogen and oxygen. Sporopollenin from different species have slightly different sporopollenin [13, 20] but the carbon:hydrogen ratio is 1:1.6 is consistent. For example, sporopollenin from lily, pine, and rye has chemical formulae of $C_{90}H_{134}O_{36}$, $C_{90}H_{158}O_{44}$, and $C_{90}H_{134}O_{31}$ respectively [18].

The earliest chemical structure for sporopollenin was proposed in the 1930s that terpenoids which is a class of naturally occurring that is similar to terpenes that are produced by plants to ward off herbivores [18]. And in the 1960s sporopollenin was proposed to be composed of carotenoids or are lipidic in

nature and have compounds similar to lignin [18]. Studies done later with characterization techniques like FTIR, nuclear magnetic resonance spectroscopy (NMR), high-performance liquid chromatography (HPLC), gas chromatography–mass spectrometry (GCMS) and XPS have shown sporopollenin to be made up of lipidic backbone with aromatic side chains such as the cinnamic acids, *p*-coumaric acid and ferulic acid [21-27]. These studies were mostly done with sporopollenin extracted from lycopodium, pine and cattail.

Figure 2.2 Simplified structure of sporopollenin oligomer. This is a hypothesized structure that is widely accepted by researchers who studied sporopollenin. The substituent R groups can ether or ester bonded. Note that in this structure the methyl groups, hydroxyl groups and number oxygen has been under represented for simplification [6].

2.1.3. Bee Collected Pollen

Bee collected pollen also known as bee pollen, approximately 4 to 5 mm, are agglutination of pollen grains made by bees during collection or packaging. Pollen grains are agglutinated by nectar, waxes, lipids and pollenkitt [28, 29]. Bee pollen is harvested by farmers or bee keepers by the installation of pollen mesh to trap bee pollen when bees enter bee boxes. Pollen that comes from this source are usually from flowering plants or angiosperms. Bee pollen can be classified into two categories, monofloral or heterofloral. Monofloral bee pollen

is made up of pollen grains from a single plant whereas heterofloral bee pollen is made up of pollen grains from many plants [30].

Bee pollen are harvested and sold commercially due to consumption as functional foods and medicine [31-33]. Due to growing consumer awareness about health and functional foods, the consumer demand for natural foods with health benefits or herbal therapeutics is growing. The health foods industry, particularly the functional foods market and the herbal supplement market are expected to reach USD 64.6 billion and USD 87.7 billion by 2022 respectively [34]. Bee pollen are produced in many countries. Countries that produce and export bee pollen are Spain, Australia, Brazil, Argentina, etc. China is the biggest producer, exporter and consumer of bee pollen [35].

Bee pollen are consumed as functional foods because it contains numerous nutrients, vitamins and minerals [36, 37]. Numerous studies have reported that bee pollen contains phenolic acids, kaempferol, palmitic, linoleic acids, calcium, sodium, potassium, and vitamin B, C and E [38, 39]. Bee pollen has been studied in apitherapy which is a branch of alternative medicine that mainly uses honey bee products such as honey, pollen, bee venom, etc. for treatment. Bee pollen has also been consumed in ancient times and have been referred to as the dust of life. Uses in traditional medicine has also been reported in several sources. Bee pollen are mainly used in alternative medicine due to its therapeutic qualities. Studies in the field of apitherapy especially has reported therapeutic qualities such as anti-inflammatory properties, anti-microbial properties, etc. [40-42]. It has been reported that bee pollen can help in healing burns, gastritis, and anemia [30, 40]. Although there is numerous research done on the liquid or lipidic extracts of bee pollen [43-45], there are no reports of the extraction pollen grains from bee pollen to be used as raw material for materials science applications.

2.2. Sporopollenin Based Microparticles Overview

2.2.1. Defatting of Bee Pollen Granules

Literature review revealed the lack of literature on the topic of defatting of bee pollen granules to produce loose pollen particles. This is because most research conducted on bee pollen has focused on investigating the therapeutic effects of bee pollen or of the extracts of bee pollen specifically the lipids and other nutrients on bee pollen. Studies investigating the polyphenols, lipids or flavonoids on bee pollen have used either ethyl ether, hexane, ethanol or acetone for defatting. Other research that studies the allergenicity of bee pollen extracts have also used similar organic solvents [29, 41, 43, 44]. Various sources – patents and research papers have suggested that the use of any other volatile organic solvent for defatting are also suitable [30, 36, 38, 40, 46-49]. Although there is numerous research done on the liquid or lipidic extracts of bee pollen, there are no reports of the extraction pollen grains from bee pollen to be used as raw material for materials science applications.

2.2.2. Extraction of Sporopollenin Based Microcapsules

Due to the varying chemical structure of sporopollenin from different plants there are many types of extraction method [6, 20, 50-53]. The first method to isolate sporopollenin from pollen grains was proposed in 1930s by Zetzsche *et al.* where pollen are defatted with organic solvents such as acetone, ethanol or diethyl ether and then boiled in alkaline solutions made up of sodium hydroxide or potassium hydroxide [18, 54]. This process successfully remove the cytoplasmic content and produce pollen shells that retain the original shape of pollen but the shells consist of both sporopollenin exine and cellulosic intine. Further study by Zetzsche *et al.* showed that removal of intine is possible [55, 56]. Following the treatment with hydrochloric acid or sulphuric acid or phosphoric acid over several days removes intine. In the 1960s, another method was proposed. This method is known as acetolysis which utilizes an acetic anhydride and sulphuric acid mixture which involves a single step that removes both cytoplasmic content and intine yielding sporopollenin exine capsules

(SECs) [57-60]. However, this method is known to cause serious fragmentation and the protocol is dangerous. Acetolysis is known to be very corrosive and very reactive. Other methods of isolating sporopollenin have also been explored such as autoclaving, ozone treatment and enzymatic extraction [18, 25, 61]. Recently, there has been some interest utilizing SECs for various application such as microencapsulation, drug delivery, sensors, heavy metal binding, etc. [62-66]. However, there may still be some cellulosic material (intine) left with the extraction procedures used so the term sporopollenin sporoderm microcapsules (S-SMCs) is much more suitable when referring to those microcapsules.

Sporopollenin with its interesting properties have been studied by many. The interesting properties sporopollenin based microparticles or microcapsules possess are monodispersity, unique and interesting hierarchal structure, antioxidant properties, heavy metal binding capabilities, taste masking ability, etc. [18, 64, 67-69]. Studies on S-SMCs conducted so far are for application in drug delivery, taste masking, applications in biosensors, etc. [6, 8, 18, 51, 62-64, 68]. S-SMCs have been extracted from different sources such as lycopodium, cattail, sunflower, rape seed flower, ragweed, pine, etc. but there are no reports on S-SMCs extracted from *C. sinensis* pollen [50-53, 70, 71]. Also, many studies on sporopollenin are done utilizing acetolysis extracted SECs or acid extracted S-SMCs which many have noted is quite different from native sporopollenin from pollen grains.

2.3. Potential Treatments for Sporopollenin Based Microparticles Overview

2.3.1. Ultraviolet-Ozone Surface Modification

Surface modification is a branch of material science that mainly studies the surface of materials. The surface of materials is an important interface this is because surfaces make up a large percentage of a material and interacts with the environment around the material. The ability to control the surface properties of a material is important in certain field of study such as in research on

biomaterials, semiconductors, aerospace, textile, construction, etc. [72-75]. Surface modification can affect the reactive properties, adhesive properties, catalytic properties, etc. of materials of various classes such as metals, biopolymer, polymers and ceramics. There are many surface modification techniques and can be categorized into wet or dry surface modification. The wet methods usually involve the manipulation of surface properties through the use of chemical reagents [76]. In these cases, the surface is functionalized with chitosan, polyethylene glycol (PEG) or undergo chemical oxidation or coating. Dry methods are preferable because it does not require much preparation of the material and can be used on a variety of material. Examples of dry methods are chemical vapour deposition (CVD), plasma treatment, ion implantation, UV-O treatment, etc. [77, 78]. Surface engineering or modification is an important aspect of biomaterials especially for implants where the surface properties determine the product is biocompatible, resistant to corrosion, thrombogenicity, etc. [77-79].

Ultraviolet-ozone (UV-O) treatment is a dry surface modification method that is relatively cheap, simple and easy to use method used. UV light has been known since 1970s to be able to decompose organic molecules. The importance of the presence of oxygen or ozone was discovered when researchers found out that polymers do not degrade when placed under UV in a nitrogen atmosphere [80]. There are various parameters in UV-O treatment such as the wavelengths of light emitted by the UV source, the distance between the sample and UV source, presence of excess oxygen supplied by oxygen gas inlet, etc. [80]. Examples of characterization methods employed to determine if surface has been modified by UV-O are XPS, FTIR, Auger electron spectroscopy (AES) and ion scattering spectroscopy/secondary ion mass spectroscopy (ISS/SIMS) [80]. In the industry UV-O is used in the manufacture of quartz crystal, the manufacture of solar panels, water treatment and sterilization in biological research. There is numerous research done on polymer metallization that reported UV-O treatment resulted in better adhesion between polymer and metals. Studies have been conducted on various polymers like polystyrene, polydimethylsiloxane (PDMS), polyethylene (PE), etc. to determine whether surface properties are affected by UV-O [81-86]. The results are that treatment reduced contact angle

and increase the surface oxygen content thereby improving wettability and cellular attachment [87-89]. Surface modification by UV-O has been explored relatively more extensively for synthetic polymers compared to natural polymers like collagen, chitosan, or sporopollenin [73, 85, 88]. Surface modification by dry methods that has been reported thus far are done by Bormashenko *et al.* where lycopodium spores were surface modified by oxygen plasma [90]. There is also limited surface chemistry characterization done on *C. sinensis* derived sporopollenin.

2.4. Potential Applications Sporopollenin Based Microparticles Overview

2.4.1. Pickering Emulsion

Pickering emulsions are also known as particle stabilized emulsions are emulsions stabilized when the microparticles or nanoparticles used adsorb on the interface between the two phases (oil and water) or when the particles form a structural barrier that separates the individual droplets when large number of particles are present. This type of emulsion uses solid particles unlike conventional emulsifiers which are small molecules of surfactants or biopolymers such as protein molecules. Conventional emulsifiers are amphiphilic nature and can adsorb on the interface forming an interfacial layer [91, 92]. Pickering emulsions are considered to relatively more thermal stable than conventional emulsions because of the high desorption energy required to disrupt the emulsion and the solid particles used are unlikely to denature. These emulsions are more biocompatible and less cytotoxic because surfactants are not used [91].

Figure 2.3 Schematic of a classic emulsion and Pickering emulsion. Both emulsions are oil in water (o/w) emulsion. In classic emulsion, oil (or water) droplets are stabilized by molecular surfactants in water (or oil) continuous phase whereas in Pickering emulsion particles are the stabilizers [93].

Although Pickering emulsion was discovered around the year 1907, not many studies have been conducted compared to conventional emulsions. However, in the recent year there is growing interest in this field, particularly in the possible utilization of such emulsion in food applications [94]. In food applications, the types of solid particles used are biopolymer based such as cellulose, chitin, starch particles, gelatin, whey protein particulates and soy protein particulates [91, 94, 95]. The emulsifier in Pickering emulsions can range from spheres to crystals or fibers. Various studies done on the topic of food grade Pickering emulsions have reported that the particles used have reduced oxidation due to the inherent antioxidant properties of the particles [91, 92].

There are a few variables that affect the stability of Pickering emulsions. Examples of variables that affect Pickering emulsions are the amphiphilicity of the particles used, the composition of the emulsion phase. There are various ways of tuning the amphiphilicity of the particles. Examples of methods explored are functionalizing the particle surface, polymer coating. Functionalizing of particle surface with small molecules such as amines, palmitic acid and oleic acid [92]. Amines which are hydrophobic are attached to the particles by non-covalent adsorption and the amphiphilicity of particles is modulated by the concentration of amines used [91, 92]. The grafting of polymer chains that are either hydrophilic or hydrophobic onto extremely hydrophobic or hydrophilic particles have also been reported to be useful. Examples of polymers that have been grafted are poly(oligoethylene glycol methacrylate) (POEGMA) and poly(2-

(diethylamino)ethyl methacrylate (PDEAEMA) [92, 96]. Polymer coatings on particles have also been reported to be able to tune the amphiphilicity of particles. Particles with coatings made of polymers such as polyacrylic acid (PAA), polyethylenimine (PEI) and others have been explored [97-99]. Only a few research has explored pollen particles or S-SMCs in Pickering emulsions and there are none that explore the used of UV-O surface modified S-SMCs in Pickering emulsions.

2.4.2. Oil Loading into S-SMCs

The frequently proposed application of SECs or S-SMCs is drug delivery where an active compound is loaded into the cavity of sporopollenin derived microcapsules that can be delivered orally or through other means. The active compound could be one that is soluble in either water or organic solvents like ethanol [6]. Examples of loaded material are bovine serum albumin, fluorouracil, Nile red and Evans blue [6, 63, 68]. Oils, waxes and solid fats have also been explored for encapsulation in such microcapsules. Loading of such material would take place at elevated temperature to allow the oil or fats to flow or mix with the microcapsules. Examples of oils that have been encapsulated are fish oil, rape seed oil and sunflower oil. Examples of solid lipids that have been encapsulated are cocoa butter, beeswax or palm wax. There are three common used methods of encapsulation – passive loading, compression loading and vacuum loading [10, 100]. In these methods, the SECs or S-SMCs are immersed in the solution or liquid containing the active compound for a few hours and then dried which are. Several papers have reported of the need to rinse or wash the dried sample because observation under the SEM revealed that there are active compounds adhered to the outer walls of the microcapsules [63, 71]. Loading of oils or waxes through the common methods proposed by Barrier, *et al.* results in microcapsules that have oil on the surface, poorly loaded and are usually agglomerates. Preliminary trials to replicate cocoa butter oil loading protocol from Barrier, *et al.* with *C. sinensis* S-SMCs was not promising [68]. CLSM image of preliminary samples show that only a few S-SMCs were filled and there are oil on the exterior of the S-SMCs.

Figure 2.4 CLSM image of cocoa butter oil loading in *C. sinensis* S-SMCs. Cocoa butter is stained with Nile red and fluoresces red while S-SMCs show blue auto fluorescence.

2.4.3. Cell Adhesion to Pollen / S-SMCs Particles Study

Cell adhesion to are studied because it is an important aspect to determine if a material is suitable to be used in biomedical applications. Material substrates can be made of metals, ceramics and polymers [77]. Polymers dominate the field of tissue engineering because of its relatively low cost, its range of mechanical properties, its transparency and its ability to be tuned to achieve various physico-chemical properties. Usually unmodified polymers are not suitable for the tissue engineering applications and will require surface modification. Surface modification can be in the form of plasma treatment, laser treatment, grafting, UV-O treatment, etc. The modulation of surface properties, for example, can affect the affinity to proteins affecting resulting in antifouling ability or attachment of extracellular matrix (ECM) [73, 75]. The surface modification of the polymers have been studied and in particular surface modification by UV-O treatment has been conducted on polymers like polystyrene, polycarbonate, polymethyl methacrylate (PMMA), polyether ether ketone (PEEK), etc. [77, 101]. But there is few research on the UV-O surface modification of natural polymers like chitosan, collagen or cellulose [65, 75, 87, 89]. Papers reported that UV-O treatment of natural polymer substrate

enhanced cell adhesion. However, there are no studies on the effect of UV-O of sporopollenin on cell adhesion.

2.5. Relevance of Thesis Work

The literature review revealed some outstanding questions or literature gaps about pollen particles or S-SMCs. The outstanding questions or literature gaps are as follows. Numerous research done on the liquid or lipidic extracts of bee pollen but there are no reports of the extraction pollen grains from bee pollen to be used as raw material for materials science applications. Pollen from other species of plants like lycopodium, sunflower and pine have been utilized in studies but there is lack of literature on the use of *C. sinensis* pollen grains. Many studies were done on extracted sporopollenin in the form of acid extracted S-SMCs and acetolyzed SECs that have been noted to be different from native sporopollenin in the form of pollen grains. Given the wide range of pollen species which far exceeds those tested so far, there remains significant potential to explore different types of pollen. Therefore, study of *C. sinensis* pollen or S-SMCs was proposed. Surface modification of pollen or SECs or S-SMCs by wet processes have been explored but the use of dry processes has not been explored extensively for pollen or S-SMCs. The effects of dry surface modification such as plasma treatment, UV-O, etc. on synthetic polymers are extensively studied and there are few reports on natural polymers. The investigation of the change in the surface chemistry due to surface modification methods have been performed with XPS, FTIR, AES, etc. Thus far, there has been one case of dry surface modification in the form of plasma treatment done on sporopollenin. Modulation of the hydrophobicity of lycopodium spores has been reported to be possible with the use of plasma treatment. However, the surface chemistry changes have not been investigated in that paper. Therefore, UV-O treatment of pollen and S-SMCs and the investigation of the effects of UV-O on surface were was proposed for this project. There is renewed and growing interest in Pickering emulsions and only a few research has explored pollen particles or S-SMCs in Pickering emulsions. There are none that explore the used of UV-O surface modified S-SMCs in Pickering emulsions. Although there is few research on the topic of UV-O effects on natural polymers, it has

been reported that UV-O result in increased cell adhesion of cells to UV-O treated natural polymer substrates such as chitosan and collagen. Hence, investigation on the effects of UV-O pollen particles and S-SMCs on Pickering emulsion and cell adhesion was proposed to explore the potential of pollen particles and S-SMCs as functional microbeads.

2.6. M. Eng in Context of Literature

The study of S-SMCs could be of significant interest and potential for various industrial applications with the push towards the usage of more naturally derived materials by the companies and consumer market. This thesis provided several important insights:

- The possibility of extracting loose pollen particles from bee pollen granules.
- The extraction of S-SMCs from *C. sinensis* pollen.
- UV-O treatment is effective in modulating the wetting properties of pollen grains and S-SMCs and how UV-O results in various potential applications in colloidal science and tissue engineering.
- Systematic analysis of the chemical changes that occur with UV-O treatment.

This thesis provides a collection of studies that deepen the understanding of sporopollenin chemistry and help facilitate further exploration or utilization of sporopollenin microcapsules for various applications.

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Chapter 3

Experimental Methodology

To explore the potential of nature's microparticles as functional microbeads, a series of experiments were carried out. Pollen particles were extracted from C. sinensis bee pollen using defatting procedure. S-SMCs were extracted from pollen particles. The modulation of surface properties was conducted using UV-O treatment. To determine if the surface properties changed with varying treatment time various characterization techniques such as elemental analysis, dynamic imaging particle analysis, scanning electron microscopy, contact angle measurements, x-ray photoelectron spectroscopy, attenuated total reflection Fourier-transformed infrared spectroscopy, stereomicroscopy, confocal laser scanning microscopy and bright field microscopy were employed. Emulsification characteristics, oil loading and pollen particles and S-SMCs/cell adhesion properties were studied to explore the potential of the pollen particles and S-SMCs as functional microbeads. Overall, this chapter aims to justify the materials and methods used in this thesis, provides a detailed summary of the protocols used for sample preparation, and the principles of the characterization techniques used.

3.1. Rationale for Selection of Methods and Materials

3.1.1. *Camellia sinensis* Bee Pollen Granules and Defatting

C. sinensis also known in lay terms as the tea plant, not to be confused as tea tree (*Melaleuca alternifolia*) is widely farmed in tea plantations and its leaf buds are harvested for making tea. In tea farms bee hives are placed so that bees can pollinate the tea plants and bees pick up *C. sinensis* pollen and pack them into pellets or granules known as bee pollen. With the growth of health awareness and move towards natural products many people know of the health benefits of bee pollen and bee pollen are commercially available as health supplements in health food stores. Therefore, large quantities of monospecies bee pollen can be obtained relatively easily. This study was done using *C. sinensis* bee pollen granules because of the relative ease of procuring large quantities of bee pollen granules that have few impurities.

For this study, literature survey about pollen or sporopollenin was conducted and it was gathered that most researchers are working with *Lycopodium clavatum* spores, or sporopollenin exine capsules (SECs) extracted via acetolysis or acid hydrolysis. The reason for the preference is the commercial availability and the reasonable price of *L. clavatum* spores and SECs. Few researchers are exploring the use of natural pollen or sporopollenin in its native form. Most choose to focus on the SECs which were utilized as microcapsules for delivery of compounds. The literature survey also revealed that few studies have been done using *C. sinensis* pollen as the raw material. Therefore, this study focused using *C. sinensis* bee pollen granules as the raw material.

3.1.2. Extraction of S-SMCs from *C. sinensis* pollen

From the literature review conducted, many researchers have explored the extraction of sporopollenin sporoderm microcapsules from various plant pollen or plant spores with many focusing on lycopodium, cattail and pine. Others plant pollen that have been explored for S-SMC extraction are sunflower pollen, corn pollen, date palm pollen, etc. [1-3]. However, there has been no studies to

explore the extraction of S-SMCs from *C. sinensis* pollen. Even though *C. sinensis* pollen are commercially available due to the global tea industry. It is still important to explore S-SMCs extraction from different pollen source because the established protocol might not be suitable for certain pollen or some pollen require longer treatment. Tweaking of the established protocol might be required because studies in the field of sporopollenin have shown that sporopollenin from different species vary slightly in terms of its composition [4].

3.1.3. UV-O Surface Modification

Literature survey showed that many researchers have used chemical methods or routes to modify the surface of natural pollen or acid extracted SECs and only a few studies have been done utilizing physical surface modification methods. It was reported in a study that oxygen plasma, a physical surface modification technique, can turn hydrophobic *L. clavatum* spores hydrophilic and allow it to suspend in water easily. UV-O is another commonly used physical surface modification technique employed commercially to alter the hydrophobicity of a material. Due to its wide spread commercial use and relatively cheap set up, this study would like to explore if surface modification by UV-O would have the similar effects. There is no literature on the effects of UV-O treatment on pollen particles and S-SMCs.

3.2. Sample Preparation

3.2.1. Materials

Camellia sinensis bee pollen granules were purchased from Xi'an Yuensun Biological Technology Company Limited (China). Acetone was obtained from Aik Moh Paints & Chemicals Pte Ltd (Singapore). Diethyl ether, absolute ethanol and hydrochloric acid were procured from Merck Millipore Corporation (USA). Phosphoric acid, sodium hydroxide, acetanilide standard, isopropyl myristate, sodium chloride, Nile red and glutaraldehyde solution were purchased from Sigma-Aldrich Pte Ltd (USA). Nylon mesh was purchased

from ELKO Filtering Co. LLC (USA). Duke polystyrene microsphere standards ($50 \pm 1 \mu\text{m}$), Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum, 1% penicillin-streptomycin, LIVE/DEAD™ Viability/Cytotoxicity Kit, for mammalian cells and flat bottom Nunclon Delta 24-well plates were purchased from Thermo Scientific Pte Ltd (USA). Human hepatocellular carcinoma cells (Huh 7.5) was purchased from Apath (USA). Tin weighing boats were obtained from Elementar Vario EL III (Germany). Duke polystyrene microsphere standards ($50 \pm 1 \mu\text{m}$) were procured from Thermo Scientific Pte Ltd (USA). Cocoa butter was purchased from Phoon Huat Pte Ltd (Singapore).

3.2.2. Defatting of Bee Pollen Granules

The following defatting protocol was implemented throughout this study. The protocol involved a series of washing, heating and drying steps. *C. sinensis* bee pollen granules (250 g) were suspended in acetone (500 ml) and refluxed in a round bottom flask at 50 °C and 220 rpm for 3 h. After that, acetone was decanted and 1 L of warm (50 °C) Milli-Q (MQ) water was added to the sample, mixed and stirred for approximately 10 min. The sample and water mixture was passed through a 150 μm nylon mesh to remove sand and other contaminants. Water was removed from resulting filtrate using a ceramic filter funnel and a vacuum pump. Next, sample was mixed with another 1 L of 50 °C Milli-Q water and filtered. The resulting sample was suspended in 500 ml of acetone and refluxed for 3 h at 50 °C and 220 rpm. After that acetone was removed and defatted pollen was transferred to a petri dish and left to dry in the fume hood. After defatting with acetone, 20 g of dry sample was mixed with 250 ml of diethyl ether with constant stirring at 300 rpm at room temperature for 2 h. This was done twice and each time fresh diethyl ether was used. Removal of diethyl ether was done by using a ceramic filter funnel and a vacuum pump. After washing with diethyl ether twice, sample was added to another 500 ml of diethyl ether and left to stir at 300 rpm overnight at room temperature. Diethyl ether was removed and sample was transferred to a petri dish and left to dry in the fume hood. Dry, loose and defatted pollen particles were then used for Ultraviolet-Ozone (UV-O) treatment and cell adhesion to pollen particles

experiments. Further characterization with various techniques such as dynamic imaging particle analysis (DIPA), scanning electron microscopy (SEM), contact angle measurements, X-ray photoelectron spectroscopy (XPS), and attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) was also done with defatted *C. sinensis* pollen.

3.2.3. Extraction of S-SMCs

The following S-SMCs extraction protocol was implemented throughout this study. The protocol involved acid hydrolysis followed by a series of washing and ended with drying of the extracted material. Defatted *C. sinensis* pollen particles (60 g) were refluxed (70 °C, 220 rpm, 1 h, 3 h, 5 h and 10 h) with phosphoric acid (85% (w/v), 600 ml) in a round bottom flask. Phosphoric acid was removed with the aid of vacuum filtration and extracted S-SMCs were washed with DI water (500 ml, 50 °C, 5 times), acetone (500 ml, 50 °C, 2 times), sodium hydroxide solution (2M, 500 ml, 50 °C), hydrochloric acid solution (2M, 500 ml, 50 °C), DI water (500 ml, 50 °C, 5 times), acetone (500 ml, 50 °C), ethanol (500 ml, 50 °C, 2 times) and DI water (500 ml, 50 °C). Washing was done with stirring using a spatula. Extracted S-SMCs were characterized by DIPA, SEM and elemental analysis.

3.2.4. Ultraviolet-Ozone Treatment

The following Ultraviolet-Ozone (UV-O) treatment protocol was implemented throughout this study. A thin layer of defatted *C. sinensis* pollen, approximately 50 mg was spread evenly on a 90 x 15 mm plastic disposable petri dish and exposed to UV-O using a benchtop PSD Series UV-O cleaner (Novascan, USA). Samples were exposed for various duration ranging from 0, 15, 30, 60 and 120 min. Samples were prepared fresh for any characterization. Contact angle measurements were done to observed if UV-O treatment resulted in a gradual decrease in contact angle or hydrophobicity. Refer to section 3.3.4 Contact Angle Measurements for detailed contact angle measurements protocol. The surface morphology of samples was characterized using scanning electron microscopy (SEM) at various magnifications to determine if any contact angle

changes were due to surface roughness. To determine if the surface chemistry was altered with vary degrees of exposure to UV-O samples were analyzed with X-ray photoelectron spectroscopy (XPS) and Attenuated Total Reflection Fourier-Transform Infrared Spectroscopy (ATR-FTIR). Detailed protocol can be found in section 3.3.3. Scanning Electron Microscopy, section 3.3.5. X-ray Photoelectron Spectroscopy and section 3.3.6. Attenuated Total Reflection Fourier-Transform Infrared Spectroscopy. Samples were also used in cell adhesion to pollen particles study to determine if more pollen particles were adhered to cells if the particles were treated with UV-O. S-SMCs were exposed to UV-O following the same protocol except for different durations (0, 1, 5, 15 and 30 min) and characterized with SEM, ATR-FTIR and XPS.

3.2.5. Pickering Emulsion

Chapter 4. UV-O Surface Modification of Natural Plant Microparticles

Pickering emulsions were made to determine whether surface chemical changes that was the result of UV-O treatment will change the way particles interact with oil and water. For the emulsions, the water phase is made of 10 mM sodium chloride and the oil or hydrocarbon phase is made of isopropyl myristate (IPM). Firstly, 250 mg of 0 min UV-O pollen was added to 5 ml of IPM, vortexed for 1 min and then sonicated using a probe sonicator with a probe tip diameter of 30 mm at 20 kHz, 10 W for 2 min. Water phase was first transferred to a glass vial (20 ml) and the oil-particle mixture was added. Water phase, oil phase and particle system was then agitated using a IKA Ultra Turrax T18 rotor stator mixer (IKA Works GmbH & Co. KG, Germany) with a 1.0 cm dispersing head operating at 19 000 rpm for 2 min. This protocol was repeated for the 15 and 120 min UV-O pollen.

Chapter 5. UV-O Surface Modification of S-SMCs

Pickering emulsions were made using S-SMCs (0, 1, and 30 min UV-O). Suspensions of S-SMCs (100 mg) in isopropyl myristate (5 ml) were made with the aid of a probe sonicator (probe tip diameter of 30 mm, 20 kHz, 10 W, 2 min), then added to a glass vial (20 ml) containing sodium chloride solution (10 mM, 5 ml). Water phase, oil phase, and particle system was then vortexed

(speed 10, 5 min). For imaging purposes to differentiate between oil and water, the oil phase was dyed with Nile red (0.3 mg/ml). Heights of the emulsion, oil, and water layer were recorded initially and after one week.

The height of the oil layer, water layer, emulsion layer and the total height were monitored and recorded. For imaging purposes to differentiate between oil and water, the oil phase was dyed with Nile red. Concentration of Nile red used was 0.3 mg/ml. A few samples of the emulsion were removed from the glass vial, placed on a glass slide and then imaged using a stereomicroscope equipped with a digital camera. Refer to section 3.3.7. Stereomicroscopy for detailed protocol for stereomicroscopy of samples.

3.2.6. Cocoa Butter Oil Loading into S-SMCs

S-SMCs (250 mg) were added to cocoa butter (5 ml, 40 °C), vortexed (speed 10, 1 min), and then transferred to a vial containing DI water (5 ml, 40 °C) and homogenized using a IKA Ultra Turrax T18 rotorstator mixer (IKA Works GmbH & Co. KG, Germany) (1.0 cm dispersing head, 19 000 rpm, 2 min). Resulting emulsion (1 ml) was transferred using a disposable dropper to a separating funnel (100 ml) and left to stand in the fridge (10 °C, 30 min) for cocoa butter to harden. Hot water (50 ml, 90 °C) was added to the separating funnel and left to stand (25 °C, 12 h) for S-SMCs to settle. Sedimented S-SMCs were collected and analyzed. A second extraction was performed for collection and isolation of floating S-SMCs.

3.2.7. Cell Adhesion to Pollen Particles and S-SMCs Study

Chapter 4. UV-O Surface Modification of Natural Plant Microparticles

Cell adhesion to pollen particles study was carried out using the following protocol. The human hepatocellular carcinoma cells (Huh 7.5 cells) used in this study was thawed and then allowed to culture in a flask with culture media in the incubator at 37 °C for 2 days. Culture media used was made up of Dulbecco's Modified Eagle Medium (DMEM) 1x, 10% fetal bovine serum and 1% penicillin-streptomycin. Once the appropriate number of cells are in the

flask, cells were then transferred to the 24-well tissue culture plates at a seeding concentration of 100 000 cells/well with 1 ml of culture media. Cells were incubated for 24 h to allow for attachment to culture plate. After 24 h, the culture media is removed and 1 ml of fresh culture media containing approximately 50 000 pollen particles (1 mg of pollen contains approximately 31 204 pollen particles) were added and left to incubate for 24 h. Pollen particles used were 0 min UV-O and 120 min UV-O *C. sinensis* pollen. For sterilization, 30 mg of each type pollen particles were incubated in diethyl ether overnight and dried with the aid of vacuum oven at 25 °C at 100 mbar till stable weight. After 24 h, 1 ml of media was collected from the wells and each well was washed with 1 ml of culture media for 2 times. Washings were collected and mixed with 1 mL media that was collected. These culture media mainly contained pollen particles that were the cells did not attach to. Solution was centrifuged at 5000 ×g, 25 °C for 5 min to collect the pollen particles. Pollen particles sediment were collected and dynamic imaging particle analysis (DIPA) was used to quantify the amount of pollen particles that were washed off and the number of particles attached were calculated. Refer to section 3.3.2. Dynamic Imaging Particle Analysis for detailed protocol for DIPA. Separate wells were prepared for both 0 min UV-O and 120 min UV-O samples. The same protocol was followed but additional step of staining of cytoplasm with calcein-acetoxymethyl (calcein-AM) from the Live/Dead Cell Viability/Cytotoxicity kit for mammalian cells was included. Staining was done with 4 μM calcein-AM and incubated for 1 h at 37 °C. This was done to allow for differentiation of cells from pollen particles when confocal laser scanning microscopy (CLSM) was carried out to visualize how the cells are attaching to the pollen particles. Refer to section 3.3.8. Confocal Laser Scanning Microscopy for detailed protocol for CLSM. Separate samples for scanning electron microscopy (SEM) characterization following the same protocol were prepared with the additional step of cell fixation included at the end. Samples were fixed with 4% glutaraldehyde solution for 40 min at 25 °C and dehydrated for a series of ethanol solution of increasing concentrations (50%, 75%, 95%, and 100%) for 15 min each and dried using a critical point dryer. Refer to

section 3.3.3. Scanning Electron Microscopy for detailed protocol for SEM of samples.

Chapter 5. UV-O Surface Modification of S-SMCs

Sterilized S-SMC particles in media were incubated with human hepatocyte carcinoma cells. Cells (160000 cells per well) were seeded in 24-well tissue culture plate (37°C, 5% CO₂, 24 h) with culture media (1 ml) made of Dulbecco's Modified Eagle Medium (1x), fetal bovine serum (10%), and penicillin-streptomycin (1%). Culture media was removed and S-SMC particles (40 000) were added to each well (1 ml, 0.16 mg/ml) and incubated (37 °C, 5% CO₂, 24 h). Culture media was collected from wells and samples were washed with culture media (1 ml, 3 times). Unbound S-SMC particles were collected from supernatants by centrifugation (5000 × g, 25 °C, 5 min). Characterization techniques were used as

3.3. Characterization – Principles and Analysis

3.3.1. Elemental Analysis

Elemental analysis (EA) is used by analytical chemist to determine the composition and the purity of a compound. This method of analysis is rapid and relatively cheap. Its principles are quite simple. In EA, the sample (of fixed or known weight) is combusted at high temperature (1200 °C) in the presence of oxygen to ensure that complete combustion takes place. Combustion of sample will produce gaseous products such as carbon dioxide, water vapour and nitric oxides which are separated by selective trap columns and are passed through a thermal conductivity detector (TCD). Thermal conductivity will be compared to the reference gas and changes will result in a signal produced and these peaks will be integrated by Vario EL software to give users the carbon, hydrogen and nitrogen content present in the sample in terms of weight%.

Experimental protocol: The elemental analysis protocol implemented throughout this study was as follows. Acid extracted S-SMCs were dried (5 mg) and analyzed by CHN elemental analysis (Elementar Vario EL III, Germany) to

ascertain removal of nitrogen and estimate protein removal. Acetanilide (5 mg) was analyzed to calibrate the machine before the sample run.

3.3.2. Dynamic Imaging Particle Analysis

Dynamic imaging particle analysis (DIPA) system comprises of a pump, a glass flow cell and an optical microscope that enables users to analyze a solution for the large number of micro sized particles and their morphology (**Figure 3.1**). The camera set up opposite the light source take images of flow cell and from the raw image files collected the software identifies, extracts, and stores single particle images for later analysis. Image processing software available allows users to analyze large number of particles ranging from hundreds to thousands of particles and thereby allowing them to extract a wide range of data based on a wide range of parameters.

Figure 3.1 Schematic of dynamic imaging particle analysis setup. Setup is simple and like a basic light microscope. Particles that passes through the flow cell will be imaged [5].

Due to the catering to different industries and users a wide range of imaging settings are available. Usually settings are chosen based on the size of the particles in the sample. The imaging system like an optical bright field

microscope and the maximum magnification the system allows for is $\times 20$. There are also a wide range of flow cells available to cater to possible sizes of particles that can be tested and their sizes range from 50 μm to 500 μm . Users should account for the thickness and the magnification of the objective lens to ensure that optimal focus for imaging. This is because as the magnification of the objective lens increases the dimension of the focal plane decreases causing most images of the particles bring out of focus if one decides to use a 500 μm flow cell together with a $\times 20$ objective lens. Therefore, resulting in a less efficient data collection process.

Other settings that can be manipulated are camera settings, particle detection sensitivity, flow rates and imaging rates. Ensuring that the flow rate and the imaging rate are adjusted to collect only unique raw image frames with no image frame overlap is vital because these two parameters usually prevent particle image duplication and allow users ensure that the particle count data obtained is reliable.

Sample preparation is also important in ensuring that the data collection is efficient and reliable. Concentration of the sample is important. Concentrated sample usually results in not enough images of single particles collected while diluted sample usually results in longer sample run time. It is also important to use the appropriate flow cell to prevent clog up in the flow cell so data collected to mostly comprise of small particles resulting in non-representative data presented.

Analysis for a wide range of parameters can be done using an image processing program can be done after the data collection has been completed. Information such as the distributions of particle circularity, aspect ratio, edge gradient, size and the number of particles present in solution. Analysis can be done such that debris and particles of interests are separated if users input a specific size range. Calibration runs conducted with commercial polystyrene microspheres standard is usually done prior to sample runs to ensure that system focus, size analysis and particle counts are reliable and accurate.

Experimental protocol: The DIPA protocol implemented in this study was as follows. DIPA was carried out using FlowCam®: The benchtop system (FlowCamVS, Fluid Imaging Technologies, Maine, USA) equipped with a 200 μm flow cell (FC-200), and a 4X magnification lens (Olympus®, Japan). Before calibration runs, the system was flushed consecutively with 3 ml of Milli-Q water (Millipore, Singapore), 3 ml of 0.22 μm syringe filtered 20 % Hellmanex™ III detergent solution at a flow rate of 1.5 ml/min to ensure cleanliness of the flow cell. Defatted *C. sinensis* pollen powder was added to an appropriate amount of Milli-Q water such that the final concentration of the suspension was 2 mg/ml. Calibration was done using $50 \pm 1 \mu\text{m}$ commercial polystyrene microspheres standard. Representative microsphere data are plotted as histograms with Gaussian model (**Figure 3.2**). After calibration, system was flushed and sample runs were conducted. Samples are analyzed with an imaging rate of 14 frames/s and a flow rate of 0.1 ml/min with a sampling efficiency of 12.2 %. The particle count was fixed at 10,000 particles for each run and three separate runs were conducted. Data analysis was performed with 1000 well-focused particles obtained from the 10,000 particle images. Segregation of well-focused particles are done based on edge gradient values. The same protocol was used for the analysis of S-SMCs.

Figure 3.2 DIPA of polystyrene microspheres standard. Polystyrene microspheres are $50 \pm 1 \mu\text{m}$. Plots obtained are representative data of 1000 well-focused particles in

triplicate measurements – (A) diameter, (B) circularity, (C) aspect ratio and (D) edge gradient. (E) Representative images of polystyrene microspheres obtained from DIPA.

3.3.3. Scanning Electron Microscopy

Scanning electron microscopy (SEM) is a useful characterization technique commonly used by researchers to take image and to yield various information about samples depending on the mode or detector used. The detection of secondary electrons expelled from the excitation atoms on the surface of samples by the electron beam to generate a SEM micrograph is often used. Signals such as the secondary electrons, backscattered electrons, Auger electrons and the characteristic X-rays that are generated from the interaction of a fine electron beam or probe and the atoms on the sample surface are collected by specific detectors and amplified under a high vacuum environment. Depending on the signal collected a secondary electron image (SEI), backscattered electron image (BEI) or an energy-dispersive X-ray (EDX) spectrum can be obtained.

Figure 3.3 Schematic of scanning electron microscope. Secondary electrons are generated by the sample when probed with electron beam. A secondary electron

detector collects secondary electron signals and change the signals to pixels and hence an image of the sample is generated [6].

Main parts of SEM are the electron optics system that produces the electron beam that acts as the fine electron probe, the specimen stage which the sample stub is secured to in the high vacuum chamber, the detectors for detecting various signals, the display unit which is usually a computer that also allows users to control the SEM. Usually three detectors – the secondary electron detector, the backscattered electron detector and the X-ray detector are installed in the SEM chamber. The microscope column houses the electron optics system and the system is made up of an electron gun, an anode, condenser lens, deflection coils and objective lens. These works together to allow users to control the diameter and the current of the electron probe. Image quality can be affected by parameters like accelerating voltage, probe current, probe diameter, objective aperture and working distance. Sample preparation is very important. If sample is not dried or coated enough the resulting image collected will have bright patches due to the accumulation of charges in semi dry areas. Extra care should be taken when preparing biological samples to prevent deformation of specimen and the precipitation of impurity from the dehydration process and the fixing process respectively.

Experimental protocol: The scanning electron microscopy protocol implemented throughout this study was as follows. Samples for SEM imaging were dried under fume hood overnight. A thin layer of powder was spread on self-adhesive carbon tape and coated with gold using JEOL JFC-1600 Auto Fine Coater (JEOL Ltd, Japan) for 85 s at 20 mA under vacuum. Images were taken with JEOL JSM-7600F Field Emission Scanning Electron Microscope (JEOL Ltd, Japan) at various magnifications – 500 X and 2500 X for intact particles and 50 000 X for viewing of pollen or S-SMCs topology before and after UV-O treatment. SEM was also done on 0 and 120 min UV-O pollen samples that underwent the cell adhesion to pollen particles experiment to obtain images of cells adhering to pollen particles. These samples were fixed with 4% glutaraldehyde and dehydrated with increasing concentrations of ethanol and dried with the freeze dryer. The same was done for 0 and 30 min

UV-O S-SMCs samples that underwent the cell adhesion study. After fixing and dehydration, samples were cut out from the polystyrene 24-well plate using a heated blade and taped onto SEM stub using self-adhesive carbon tape. To prevent the accumulation of charges or electrons on sample surface copper tape was taped on the sides of polystyrene substrate. Cross sections of pollen and S-SMCs samples were prepared by freeze fracturing of the particles or microcapsules. Sample was deposited on carbon tape affixed on a piece of aluminum foil and immersed in liquid nitrogen for 1 min. Once sample is removed from liquid nitrogen, it is cut or fractured using a surgical blade. After freeze fracturing, sample is dried in the fume hood and freeze dryer.

3.3.4. Contact Angle Measurements

The most common instrument used to detect contact angle telescope goniometer. The basic components of this instrument are the telescope, the light source, the Teflon needle attached by a tube to a syringe containing the liquid usually water and the stage on which the sample is placed. The sessile drop profile method is often used by researchers. Other methods of measuring contact angle include the pendant drop technique, the dynamic sessile drop method and the dynamic Wilhelmy method. Measurements in the sessile mode are taken when the tangent se, droplet of liquid is deposited in the surface, the high-resolution camera captures the profile and the software on the computer analyzes the image for the contact angle.

Figure 3.4 Schematic of contact angle goniometer. Microliter drop of water is usually dispensed by a motor controlled syringe with a micro needle. Images of water droplet profile are taken and contact angles are measured by software in sessile mode [7].

Experimental protocol: The contact angle measurements protocol implemented throughout this study was as follows. A thin layer of pollen particles or S-SMCs was spread out on self-adhesive carbon tape on a glass slide. A 2 μL bead of Milli-Q water was slowly lowered onto the layer of pollen particles or S-SMCs. The contact angle was measured using Attension Theta Optical Tensiometer (Biolin Scientific Holding AB, Sweden) with OneAttension 1.0 software. Measurements were taken using the following settings: 0.7 X magnification and 20 s at 12 FPS. Contact angle measurements were done on 0, 15, 30, 60 and 120 min UV-O pollen samples. Contact angle measurements were done on 0, 1, 5, 15 and 30 min UV-O S-SMCs samples.

3.3.5. X-ray Photoelectron Spectroscopy

X-ray photoelectron spectroscopy also known as electron spectroscopy for chemical analysis (ESCA) is a quantitative spectroscopic technique that is usually used to obtain information about the surface of a material. Information that can be obtained with the use of this technique include the chemical state,

empirical state, the chemical composition and the electronic state. XPS spectra is collected in an ultrahigh vacuum (UHV) environment by irradiating samples with X-rays while measuring the kinetic energy of the electrons that are excited and expelled from the atomic shell. XPS can be used to analyze a wide range of material such as metal alloys, polymers, ceramic, semiconductors, plant material and many more.

The main components of XPS are an X-ray source, a UHV pump connected to the UHV chamber, an electron detector and a magnetic shield. Commonly used X-ray source is the monochromatic aluminum K- α source. During analysis, X-ray is irradiated on the sample surface and when an electron in an atom or molecule gains enough energy it is ejected from the valence band with a certain amount of kinetic energy. Users can gain information about the material because the kinetic energy of the expelled electron depends on the binding energy of the electron and the binding energy depends on which orbital it is expelled from, the chemical environment of the atom it is expelled from and the element from which the electron came from. Due to the surface sensitivity, users should be extremely careful to not touch the surface of the sample when preparing or transporting the sample for analysis.

Figure 3.5 Schematic of XPS. X-rays from the source excites the electrons present on the surface of sample and excited electrons are expelled. Expelled electrons are detected and signals are generated. Energy required to expel an electron from the outer shell of an atom or molecule depends on the binding environment. Depending on the modes used a wide or narrow scan spectrum is generated [8].

Experimental protocol: The X-ray photoelectron spectroscopy protocol implemented throughout this study was as follows. Pollen particles or S-SMCs were dried using a freeze drier overnight before UV-O treatment. Pollen particles or S-SMCs were deposited on carbon tape on a 5 mm by 5 mm silicon wafer. Samples were analyzed using AXIS Supra (XPS) surface analysis instrument (Kratos Analytical Ltd, United Kingdom) equipped with a monochromatic Al/Mg X-ray source operated at 225 W and pressure of 2×10^{-9} mbar. Spectra were obtained using aluminum anode (Al $K\alpha = 1491.600$ eV) and charge neutralization. Acquisition of wide and narrow scan spectra pass energies of 160 and 20 eV were used respectively.

3.3.6. Attenuated Total Reflection Fourier-Transform Infrared Spectroscopy

Infrared spectroscopy, is a technique to determine the infrared absorption or emission spectrum of a material upon irradiation with an infrared red laser. Analysis may be performed over a wide spectral range, typically from ~ 4000 to 4000 cm^{-1} , with high resolution, typically 1 cm^{-1} . Absorption/emission spectra indicate the amount of light absorbed by molecules within the sample material. The mid-infrared spectrum, ~ 4000 to 4000 cm^{-1} , can be used to study the fundamental rotational and vibrational structure of molecules. Molecules may vibrate in many ways or 'modes', with complex molecules producing many peaks during IR spectroscopy. Common forms of molecular vibration/rotation associated with IR analysis are as follows.

Figure 3.6 Different modes of molecular vibrations that produces different peaks in FTIR spectrum. Examples of molecular vibrations are symmetric stretching, asymmetric stretching, scissoring, rocking, wagging and twisting [9].

Fourier transform infrared spectroscopy (FTIR), involves irradiating the sample multiple times with a broadband light source with slight variations in the combination of frequencies, then processing the collected data by Fourier transform to determine the absorbance at each wavelength. A Michelson

interferometer is used to produce the slight variations in the combination of irradiation frequencies and produce data in the form of interferograms. The interferograms are then converted to a spectrum by Fourier transformation. Analysis of the absorption/emission spectra provides insights into the molecules present within the material.

Common methods for FTIR sample preparation include incorporation of the sample material into a potassium bromide pellet, or by pressing the sample against a crystal as in attenuated total reflectance (ATR) spectroscopy. ATR-FTIR is typically considered an easier method to analyze solid material samples. The sample material is simply pressed against a crystal, whereupon IR radiation is passed through the crystal in such a way that the IR light interacts with the surface of the sample material to a penetration depth of only a few microns.

Figure 3.7 Schematic of ATR-FTIR. Sample is placed on crystal, incident infrared beam from source is reflected because the refractive index of the crystal is greater than that of sample and reflected infrared beam is collected and spectrum is generated. Depending on the ATR-FTIR model, ATR crystal can be changed and the depth of penetration can be changed [10].

Currently, there exists numerous literature sources which have utilized FTIR for the analysis of sporopollenin, for natural unprocessed pollens and spores, as well as for pollens and spores which have undergone various forms of processing and degradation. Peak assignments utilized in this study are derived from the literature sources listed in **Table 3.1**.

Table 3.1 FTIR peak assignments of sporopollenin from various sources [11, 12].

Wavenumber (cm^{-1})	Band assignment	Interpretation
3300	νOH	Hydroxyl
2925	νasCHn	Aliphatic, lipids and sporopollenin
2850	νsCHn	Aliphatic, lipids and sporopollenin
1740	$\nu\text{C} = \text{O}$	Lipids
1710	$\nu\text{C} = \text{O}$	Carboxyl, sporopollenin
1650	$\nu\text{C} = \text{O}$	Amide I, proteins
1600	$\nu\text{C} = \text{C}$	Aromatic, sporopollenin
1550	δNH , $\nu\text{C-N}$	Amide II, proteins
1510	$\nu\text{C} = \text{C}$	Aromatic, sporopollenin
1460	δCH_2	Aliphatic, lipids
1440	$\delta\text{C-H}$	Aromatic, proteins and sporopollenin
1265	δNH , $\nu\text{C-N}$	Amide III, proteins
1160	$\nu\text{C-H}$	Aromatic, sporopollenin
1030	$\nu\text{C-H}$	Aromatic, sporopollenin
853	$\delta\text{C-H}$	Aromatic, proteins and sporopollenin
817	$\delta\text{C-H}$	Aromatic, sporopollenin

Experimental protocol: The attenuated total reflection Fourier-transform infrared spectroscopy protocol implemented throughout this study was as follows. Absorbance spectrum was obtained using Perkin Elmer Frontier FT-IR spectrometer with a universal ATR sampling accessory. A layer of sample was placed on the diamond/ZnSe crystal of the FTIR ATR accessory and sample holder was lowered gently onto sample to ensure that sample has good contact with the crystal. Samples was scanned from 4000 cm^{-1} to 600 cm^{-1} , with 16 scan accumulations, at resolution of 32 cm^{-1} and data interval of 1 cm^{-1} . Spectra were backgrounded, baselined and smoothed using Spectrum v10.5 program. Data gathered were normalized to 1028 cm^{-1} peak and plotted in Origin. To allow for the ease of comparing different spectra difference plots relative to 0 min UV-O sample were made. Peak heights of specific functional groups were extracted and ratios between functional group peak heights were

calculated. All box plots was done in R version 3.1.0 (R Core Team, 2014) program using modified R code adapted from paper done by Jardine *et al.*, 2015 [11]. ATR-FTIR was performed on UV-O pollen particles and S-SMCs samples.

3.3.7. Stereomicroscopy

Stereo microscope is an optical microscope that is usually used to observe samples at low magnification. This microscope is also known as the stereoscopic or dissecting microscope. The sample view under this microscope has a three-dimensional (3D) or stereo effect due to the presence of prisms bending light to give a depth of view. It is usually used in the manufacturing sector for inspection of parts and quality control. Stereo microscope is made up of prisms, zoom lens, and objective lens.

Stereo microscope is unlike the Bright-field microscope because it utilizes reflected light instead of transmitted light. Also unlike Bright-field microscope it has two separate objectives lens and eye-pieces resulting in two separate optical paths that provides the left and right eye with different viewing angles resulting in a stereo view of the sample. When users use stereo microscope to view very small samples illumination might be required. This is because at high magnification the images are often quite dim. Illumination can be provided by some setup with a bulb or a mirror located under a transparent stage. This light source is not focused at all unlike in the Bright-field microscope which uses condenser lens to focus light. Also with the right setup, fluorescent samples can also be observed.

Figure 3.8 Schematic of a stereomicroscope showing the basic working principles. Two sets of lens are used, one for each eye, providing different viewing angles resulting in a stereo view of the sample [13].

Experimental protocol: The stereomicroscope protocol implemented throughout this study was as follows. Stereomicroscope images were obtained using the Nikon SMZ1000 Zoom Stereomicroscope (Nikon Instruments Inc., USA) equipped with LV-TV adapter connected to a digital camera and linked to NIS-Elements F Microscope Imaging Software Version 4.0 (Nikon Instruments Inc., USA). Images of the Pickering emulsion droplet were taken at magnifications of 3X and 8X with backlighting.

3.3.8. Confocal Laser Scanning Microscopy (CLSM)

Confocal laser scanning microscopy utilizes optical sectioning to allow users to obtain cross-sections of sample and obtain three-dimensional (3D) visualization by reconstructing the cross-sections. This is because unlike conventional optical microscope system it is based on this system uses laser which penetrates the sample much more compared to light. CLSM is made up of the laser, the beam splitter, the scanner, the objective lens, the Z-control, the pinhole and the photomultiplier tube.

CLSM allows users obtain a 3D reconstruction of the sample because of the incorporation of the Z-control. Usually a two-dimensional (2D) image is obtained by scanning the laser along the x-axis and y-axis of the focal plane but by scanning multiple 2D planes or slices with slightly varying focal depth and then reconstructing the stack of slices a 3D image can be developed. Quality of images or 3D reconstructions are affected by parameters such as the pinhole size, number of slice, slice thickness, scan speed and scan time. Note that the sample needs to be fluorescent to be imaged by this technique. This is because the image is generated from the photons that were expelled from the fluorophores by the laser beam excitation. Users can label or tag fluorophores to their sample if the sample does contain fluorophores naturally. If the auto fluorescence of the sample is not enough to generate a good image, then labelling with commercial fluorophores is necessary.

Figure 3.9 Schematic of CLSM showing the laser pathways. Unlike light microscope CLSM uses laser for its optics. Image is generated from the photons that were expelled from the fluorophores by the laser beam excitation. Note that sample needs to contain fluorophores [14].

Experimental protocol: The confocal laser scanning microscopy (CLSM) protocol implemented throughout this study was as follows. CLSM images were obtained using Carl Zeiss LSM710 (Carl Zeiss Microscopy GmbH, Germany) confocal microscope equipped with three spectral reflection/fluorescence detection channels, six laser lines (405/458/488/514/561/633 nm) connected to a computer with the ZEN (Blue Edition) software for image acquisition, processing and analysis. Images were taken at a scan speed of 6 and a pixel averaging of 2, 12 slices with a Z-stack thickness of 4 μm with a 2 μm interval with bidirectional laser scanning. Z-stack slices were reconstructed using ZEN and 3D image was obtained. CLSM was performed on pollen particles and S-SMCs samples.

3.3.9. Bright Field Microscopy

Bright field microscopy is a simple and easy to use technique that is widely used by biologist to observe specimens. It consists of the eyepiece, objective lens, condenser lens and the light source. In this technique, light produced by the light source passes through a diaphragm, passes through the condenser lens, the slide with the sample, the objective lens and the transmitted light travels through the eye piece to the camera so an image can be seen. The diaphragm controls the amount of light exposure on the sample. The condenser lens focuses the light beam. Specimen is placed perpendicular to the light path. The objective lens helps magnifies the sample.

Figure 3.10 Schematic of bright field microscope showing the basic light pathways through the lens [15].

Experimental protocol: The bright field microscopy protocol implemented throughout this study was as follows. Bright field microscope images and

videos were obtained using Nikon Eclipse Ti-E Inverted Microscope System (Nikon Instruments Inc., USA) linked up with NIS-Elements AR Microscope Imaging Software Version 4.0 (Nikon Instruments Inc., USA). Videos of unbound pollen particles or S-SMCs being washing away in the 24-well plate were recorded using the software at 5FPS for 1 min. Images were extracted from the video using Fiji image processing package.

3.3.10. Statistical Analysis

Triplicate DIPA data of defatted pollen particles and S-SMCs, contact angle measurements and XPS data were collected and the results are expressed as mean $\pm$ standard deviation (SD) of the mean. Six separate sets of ATR-FTIR data were collected, peak height data were recorded in an excel sheet and then feed into R version 3.1.0 (R Core Team, 2014) program using modified R code adapted from paper done by Jardine et al., 2015 to output a series of box and whiskers plots [11]. Statistical analysis of data was performed based on two-tailed *t*-tests, with $P < 0.05$ being statistically significant.

3.4. Strengths and Limitations of Methodological Approach

3.4.1. Elemental Analysis

The strengths of elemental analysis are that it allows to users to analyze large number of organic samples, up to 50 samples at a time and that it can be both qualitative and quantitative method. It is also a simple and relatively inexpensive technique for one to get the elemental composition of a sample. From the data obtained from this technique chemist would be able to determine the chemical formula and purity of the compound synthesized. Depending on the model, the machine can be automated.

One of the limitations of this methods is that users are limited to organic compounds or elements such as carbon, hydrogen, nitrogen and chlorine. It requires more amount of the sample tested compared to more modern techniques like nuclear magnetic resonance and mass spectrometry. This

technique limits the user to dry, solid samples whereas other techniques such as mass spectrometry allows users to analyze liquid or aqueous samples.

3.4.2. Dynamic Imaging Particle Analysis

The strengths of DIPA are that it allows the users to characterize large number of particles with various parameters, the minimum required sample volume is about 300 μL , run time for analysis is quite short – approximately 2 min for each sample and the setup is not very complex. Due to the flow present in this technique possible sedimentation and clumping of particles could be alleviated.

The limitation of this technique is that different setup – objective lens and flow cell used the will determine if the instrument was capable distinguish translucent particles accurately. Another limitation is that a certain particle concentration is required to get determine the particle counts per volume accurately so users are required to spend some time and use some samples to optimize the concentration. Imaging particles in an opaque solution might be difficult or inaccurate because image taken by the camera in DIPA might not be good quality as the setup such that the light source is opposite the camera. Also, depending on how users process data, results might defer therefore care and thought must be used when processing and presenting DIPA data.

3.4.3. Scanning Electron Microscopy

The strengths of SEM are that minimal sample preparation is required, allows user to get topographical details of sample, with the right detectors installed various information can be yielded and data can be obtained relatively quickly.

The limitations of this technique are the setup is costly, require regular maintenance, size and types of samples might be limited (SEM chambers are small and are usually maintained in a moderately high vacuum environment, so aqueous samples cannot be imaged), and special training is required to prepare

samples and operate an SEM. Mastering of SEM might take a relatively long time compared to other techniques.

3.4.4. Contact Angle Measurements

The strengths of direct optical method of measuring contact angle are that it is simple and easy to use, does not require large substrate and require small amount of liquid for testing.

The limitation of this technique is the high possibility of inconsistent data due to the small volume of testing liquid and small substrate required. This is because if there are contaminants in either liquid or on the substrate contact angle can vary greatly. Another limitation is that for small angles the direct optical method is not reliable because of the uncertainty pertaining to the assignment of the tangent line when the profile of the droplet of liquid is almost flat.

3.4.5. X-ray Photoelectron Spectroscopy

The strengths of XPS is that it is a highly surface sensitive method. It can detect surface chemistry changes of more than 0.1 atomic percent and can analyze about 3 to 10 nm of a material. It allows for the chemical state identification of the material tested. It is a quantitative analysis. Another advantage of XPS is that it is applicable for a wide range of materials such as polymers, glass, metals and paper. It is also a non-destructive analysis method.

The limitation of the technique is that due to the high sensitivity any contaminants that are on the surface can result in great variability in the data collected. Therefore, users must be extremely careful during sample preparation and transportation for analysis. Another limitation is that peak fitting or deconvolution can be difficult if users do not know much about the material analyzed or if users do not have prior experience is peak fitting. Peak fitting can also be quite subjective if there are not much literature containing XPS data of the sample. One of the limitation of XPS is that sample must be compatible at

ultrahigh vacuum (UHV) conditions therefore type of sample analyzed are limited to solids.

3.4.6. Attenuated Total Reflection Fourier-Transform Infrared Spectroscopy

The strengths of ATR-FTIR are minimal sample preparation is required compared to transmission FT-IR, data collection is relatively easy and fast, it can analyze opaque samples or samples with high absorption coefficient. ATR-FTIR can analyze the surface of a material. It is a non-destructive analysis technique.

The limitation of the technique is users must ensure that there is good optical contact between the sample and the ATR crystal. To ensure there is good contact with the crystal users can use Perkin Elmer Frontier FT-IR spectrometer with a universal ATR sampling accessory that comes with a force gauge to ensure that when testing separate samples have adequate and similar contact with the crystal. Good contact is especially important when testing solid samples. Although ATR-FTIR is a surface characterization technique users should note that it penetrates several wavelengths into the material and this results in the IR signal penetrating several micrometers into the material. Typical penetration depth ranges from 0.5 to 10 μm depending on material and experimental parameters.

3.4.7. Stereomicroscopy

The strength of stereo microscope is that it can be used to view large objects and opaque or thick samples because it uses reflected light. It allows users to have 3D viewing effect so that inspection of specimens is more accurate. It has a relatively simple setup and is relatively cheap compared to other forms of microscopy such as confocal laser scanning microscopy, scanning electron microscopy or transmission electron microscopy.

The limitation of this microscope is that it is unable to provide very high magnifications of samples. The stereo microscope only able to provide magnifications of up to 300X whereas the highest magnification for the compound Bright-field microscope is 1000X.

3.4.8. Confocal Laser Scanning Microscopy

The strength of CLSM is that it can be used for quantitative analysis if calibration with the specific fluorophore used was done. CLSM require minimal sample preparation. If parameters such as pin hole size, number of slices, slice thickness, scan speed and averaging are selected properly high quality and resolution three-dimensional (3D) images can be collected. It allows users to see into the capsules and look at the amount of loading achieved if users are interested in using microcapsules for drug loading.

The limitation of this technique is if appropriate parameters for imaging are not selected the image or data collected could be an underestimation or overestimation of the actual data. CLSM cannot be used to image or quantify very thick samples due to the attenuation of the laser and limited penetration of the sample. CLSM can be quite dangerous due to the use of laser and license and special training might be required for certain countries like Singapore. CLSM setup is expensive and require regular maintenance.

3.4.9. Bright Field Microscopy

The strength of bright field microscopy is that the setup and maintenance is relatively simple and easy. The microscope is easy and safe to use, does not require special training and samples do not need to be stained or tagged with fluorophore to be seen. Damage to sample is almost impossible in this microscopy compared to electron microscopy.

The limitations of this microscopy are that it requires strong light source at higher magnifications, it is not able to image thick or opaque samples and

samples with low contrast such as cells cannot be imaged properly without additional step of staining [16].

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Chapter 4

UV-O Surface Modification of Natural Plant Microparticles

Pollen microparticles provide a compelling all-natural solution for use in a wide range of oil/water emulsion-type applications. However, to enhance the utility of pollen microparticles, surface modification is shown to be a mechanism for altering surface chemistry and thereby tuning microparticle wetting properties, emulsification characteristics, and particle/cell adhesion. Herein, the exploration, the extraction and surface modification of discrete pollen particles from bee-collected pollen granules. Ultraviolet-ozone (UV- O) treatment is shown to increase the proportion of surface elemental oxygen and C=O bonds, leading to enhanced particle dispersion properties, control over Pickering emulsion characteristics, and increased particle/cell binding affinities. Looking forward, bee-collected pollen is widely available, competitively priced, and is considered regulation-free for oral consumption due to a long history of use as a food and medicine, with extensive literature purporting numerous health benefits. Beyond facilitating the utilization of pollen as a functional microparticle system, a greater understanding of UV- O surface modification of pollen also provides insights into the inherent UV protective properties of pollen shells, and provides insights into the use of pollen sporopollenin for exploring climate change through fossil records.

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4.1. Introduction

Novel natural materials for formulating microparticle-based Pickering emulsions are of significant interest for a wide range of industrial applications [1-12]. Pollen particles represent an ideal source of natural microparticles for use in developing microparticle stabilized Pickering emulsion systems. Pollen exhibit numerous desirable properties for functional emulsion applications, such as particle monodispersity [13], physicochemical robustness [14], amphiphilicity [15], biocompatibility [16], and complex surface chemistry for potential functionalization and compound binding [17-20]. In general, there is growing interest in utilizing pollen for a wide range of applications, such as the extraction of pollen shells for microencapsulation and drug-delivery applications [21-28]. Although, sporoderm microcapsules (S-SMCs) have been shown to offer a wide range of potential benefits, they typically rely on harsh chemical extraction protocols, and are limited by requiring additional regulatory approval for use in oral delivery applications. Whereas, natural pollens have the advantages of requiring minimal processing, and are considered to be regulation-free for human consumption and topical applications [29, 30].

Pollen represents an ideal microparticle for developing natural Pickering emulsion systems for use in a wide range of applications. Firstly, pollen exhibits both hydrophobic and hydrophilic properties with a high degree of particle uniformity and monodispersity [31]. Secondly, bee-collected pollens are available in industrial-scale quantities and are competitively priced to other natural materials which may be used as microparticles, such as, celluloses, waxes, etc. [32, 33]. Thirdly, bee-collected pollens have a long history of use as a food and medicine in a wide range of cultures with extensive published research supporting numerous health benefits, and are widely available in health food stores [34-36]. Overall, discrete pollen particles extracted from bee-collected pollens present an ideal source of microparticles for stabilizing oil and water systems in applications as diverse as foods, therapeutics, cosmetics, and

paints, etc., while overcoming potential regulatory hurdles associated with the ingestion and topical application of many other highly processed compounds. In particular, previous studies have highlighted the potential for compound loading directly into natural pollens [37, 38], and thereby, discrete pollen particles may provide a key platform technology for enhancing and modernizing the extensive field of traditional herbal therapeutics [39, 40].

Understanding and tuning the interfacial properties of discrete pollen particles is crucial to ensure the effective utilization of pollen for developing functional oil / water based formulations. Bee-collected pollens are bound into millimeter-sized granules by a complex mixture of bee salivary gland secretions, nectars, and pollenkitt on the pollen surface [41]. During the process of extracting discrete pollen particles, water soluble and lipidic compounds are removed, exposing the native pollen outer-shell biopolymer, sporopollenin. Sporopollenin is generally considered to be an amphiphilic complex biopolymer [15, 31], with variations in composition between plant species [42, 43]. Acid-extracted and natural sporopollenin-based plant spores from *Lycopodium clavatum* have been shown to stabilize oil and water emulsion systems, and the stabilization mechanism has been attributed to the Janus structure of the particles [15, 31]. However, Pickering emulsion studies have yet to be undertaken with pollen, and to further develop the utility of pollen-based microparticle-stabilized Pickering emulsion formulations, a facile means to tune the wetting potential and/or surface chemistry of pollen is of significant interest. Plasma treatment of *L. clavatum* spores has been shown to enhance wetting and dispersion properties in pure aqueous environments [44]. However, ultraviolet-ozone (UV-O) treatment is a more easily implementable surface modification process, which also leads to enhanced surface wetting of polymers [45], and potential enhancements in polymer/cell adhesion [46, 47].

Herein, we explored the extraction and UV-O surface modification of bee-collected *Camellia sinensis* pollen for developing Pickering emulsion-based formulations and enhancing pollen/cell adhesion properties. *C. sinensis* is a species of plant used for the production of tea, and has been shown to exhibit numerous health benefits and is used in numerous well-being products [48].

Washing and defatting of raw bee-collected pollen granules were conducted to obtain discrete pollen particles. Pollen morphological properties were analyzed to determine the degree of particle purity and monodispersity. UV-O treatment of defatted-pollen was conducted and the basic wetting properties of UV-O treated pollen were analyzed. Analysis of surface elemental composition and chemical binding were conducted to elucidate the effect of UV-O treatment. Properties of pollen particle aqueous suspensions and Pickering emulsions were explored with both untreated and UV-O treated pollen. Finally, pollen/cell adhesion properties were explored with both untreated and UV-O treated pollen and Huh 7.5 liver hepatocarcinoma cells (**Figure 4.1**).

Figure 4.1 Schematic diagram showing pollen extraction and surface modification, resulting in tuning of wetting properties, emulsification potential, and particle/cell adhesion: (a) Extraction of discrete pollen particles from bee-collected pollen granules; (b) Ultraviolet-ozone (UV-O) treatment of defatted pollen particles resulting in the opening of aromatic rings with the formation of $C=O$ bonds and increased overall oxygen content; (c) Tuning of wetting and aqueous suspension properties; (d) Tuning of microparticle Pickering emulsion properties; (e) Tuning of particle/cell adhesion for pollen binding with liver hepatocarcinoma cells.

4.2. Experimental Methods

4.2.1. Materials

Materials were used as defined in Chapter 3.2.1.

4.2.2. Defatting of Bee Pollen Granules

Defatting of bee pollen granules protocol was as defined in Chapter 3.2.2.

4.2.5. Ultraviolet-Ozone (UV-O) Treatment

UV-O treatment protocol was as defined in Chapter 3.2.4.

4.2.6. Contact Angle Measurements

Contact angle measurements were performed as defined in Chapter 3.3.4.

4.2.7. X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy was performed as defined in Chapter 3.3.5.

4.2.8. Attenuated Total Reflection Fourier-Transform Infrared Spectroscopy

ATR-FTIR was performed as defined in Chapter 3.3.6.

4.2.9. Dynamic Image Particle Analysis

DIPA was performed as defined in Chapter 3.3.2.

4.2.10. Pickering Emulsions

Pickering emulsions were prepared using protocol as defined in Chapter 3.2.5.

4.2.12. Pollen Particles/Cell Adhesion Study

Pollen Particles/ cell adhesion study was done using protocol as defined in Chapter 3.2.7.

4.2.13. CLSM Analysis

CLSM analysis was performed as defined in Chapter 3.3.8.

4.2.14. Bright Field Microscopy

Bright Field Microscopy was performed as defined in Chapter 3.3.9.

4.2.15. Surface Morphology Evaluation by Scanning Electron Microscopy

SEM was performed as defined in Chapter 3.3.3.

4.2.16. Evaluation of Pickering Emulsion by Stereomicroscopy

Stereomicroscopy was performed as defined in Chapter 3.3.7.

4.2.17. Statistical Analysis

Statistical analysis was performed as defined in Chapter 3.3.10.

4.3. Principle Outcomes

4.3.1. Extraction of Pollen from *C. sinensis* Bee Pollen Granules

Defatting and washing of bee pollen granules removed various surface adhered residues and produced free-flowing discrete particles. Bee pollen defatting was carried out using water, acetone, diethyl ether, and filtration through 150 μm mesh to remove surface adhered organic materials and dust or other particulate

matter that may be present in the raw bee pollen (**Figure S1, Chapter 4 Appendix**). After the defatting and drying processes, bee pollen pellets produced loose pollen powder, with a weight yield of $42.6 \pm 0.9 \%$, and changed in color from light orange to pale yellow (**Figure 4.2a; Figure S2, Chapter 4 Appendix**). The change in color may be attributed to the removal of surface wax and nectar [49]. Dynamic imaging particle analysis (DIPA) indicated that the resulting loose defatted *C. sinensis* pollen powder exhibited $99.3 \pm 0.5 \%$ purity of pollen species, was highly monodispersed with a particle size of $36.2 \pm 1.8 \mu\text{m}$ (**Figure S3, Chapter 4 Appendix**), and comprised 31204 ± 3390 particles / mg with a particle weight of $32.0 \pm 3.5 \text{ ng}$.

4.3.2. Ultraviolet-Ozone (UV-O) Exposure Effect on Pollen Surface Chemistry

Surface Morphology and Wetting Properties

UV-O treatment of defatted pollen resulted in smoothing of the pollen surface and increased pollen wetting. Defatted pollen samples were treated with UV-O for 15, 30, 60 and 120 min. From SEM images collected, UV-O treatment did not result in significant morphological changes of pollen (**Figure 4.2b; Figure S4, Chapter 4 Appendix**), however, surface smoothing was observed at the nano-scale (**Figure 4.2c**). Contact angle measurements indicated that the contact angle decreases with increasing exposure to UV-O but stabilizes beyond 30 min treatment (**Figure 4.2d**), with 0, 15, 30, 60, and 120 min UV-O resulting in contact angles of 128.4 ± 4.3 , 87.2 ± 1.1 , 58.8 ± 4.1 , 51.1 ± 1.5 , and $55.0 \pm 4.4^\circ$, respectively. The lack of changes in macroscale pollen morphology suggests that the reduction of the contact angle with UV-O treatment may be primarily attributed to modification of surface chemistry.

Figure 4.2 Defatted pollen extraction and ultraviolet-ozone (UV-O) treatment: (a) Photographs, stereo-micrographs, and scanning electron micrographs (SEM) of bee pollen granules and defatted pollen particles; (b) SEM images of untreated and UV-O treated pollen particles; (c) SEM images of untreated and UV-O treated pollen surfaces; and (d) Optical images of water droplet formation during contact measurements of untreated and UV-O treated pollen. Scale bars: (b) = 10 μ m; (c) = 100 nm; (d) = 0.5 mm.

Pollen Surface Elemental Composition and Binding Profiles

Surface chemistry analysis of untreated and UV-O treated pollen indicated increases in the proportion of elemental oxygen attributable to increases in the

proportion of C=O binding. Wide scan XP-spectra highlight the presence of oxygen (O1s) and carbon (C1s) peaks, with an increase in the O1s peak and a decrease in the C1s peak between 0 min and 120 min UV-O treatment (**Figure 4.3a; Figure S5, Chapter 4 Appendix**). Overall, the total proportion of elemental oxygen increased from 0 min to 120 min UV-O, with 0, 15, 30, 60, 120 min UV-O treatment resulting in an atomic concentration of 20.7 ± 0.4 , 21.7 ± 0.1 , 23.0 ± 0.1 , 24.9 ± 0.1 , and 28.0 ± 0.3 %, respectively (**Figure 4.3b**). The proportion of elemental carbon decreased relative to the increases in oxygen.

Table 4.1 Bond-type binding energies and proportions for carbon (C1s) and oxygen (O1s) binding.

Carbon (C1s)				Oxygen (O1s)			
Bond type	Binding energy (eV)	0 min (%)	120 min (%)	Bond type	Binding energy (eV)	0 min (%)	120 min (%)
CC / CH	284.9	43.0 ± 6.9	40.2 ± 6.6				
COR	286.3	37.3 ± 7.9	31.5 ± 3.6	COR	532.7	47.7 ± 7.2	55.5 ± 0.5
C=O	288.0	13.3 ± 2.2	25.6 ± 3.4	C=O	531.6	21.0 ± 4.5	32.8 ± 0.9
COOR	289.0	6.1 ± 1.7	2.6 ± 0.5	COOR	534.1	31.3 ± 4.1	11.7 ± 0.7

Figure 4.3 XPS analysis of untreated and ultraviolet-ozone (UV-O) treated pollen: (a) Wide-scan XP-spectra of untreated and 120 min UV-O treated pollen; (b) Carbon and oxygen elemental concentration of untreated and UV-O treated pollen; (c) Peak-fitting for chemical binding of carbon (C1s) and oxygen (O1s) peaks for untreated and 120 min UV-O treated pollen; (d) Shifts in oxygen binding proportions with 120 min UV-O treatment; and (e) Total oxygen binding distributions for untreated and 120 min UV-O treated pollen.

Peak fitting analysis of C1s and O1s peaks for untreated and 120 min UV-O treated pollen was used to examine the shifts in proportions of carbon and oxygen bond types. For the carbon binding (C1s) peak, COOR, C=O, COR, and C-C binding were assigned to binding energies of 289.0, 288.0, 286.3, and

284.9 eV [50], respectively (**Table 4.1, Figure 4.3c; Figure S6, Chapter 4 Appendix**), and UV-O treatment resulted in decreases in COOR binding, increases in C=O binding, and minimal changes in COR and CC binding. For the oxygen binding (O1s) peak, COOR, COR, and C=O binding were assigned to binding energies of 534.1, 532.7, and 531.6 eV [50], respectively, and UV-O treatment resulted in decreases in COOR binding, increases in C=O binding, and minimal changes in COR binding. Collectively, shifts in the proportion of oxygen binding were similar for both C1s and O1s binding, with an ~ 57 % decrease in COOR bonds, an ~ 2 % proportional increase for COR bonds, and an ~ 78 % increase in C=O bonds (**Figure 4.3d**). Relating increases in total oxygen % and changes in oxygen binding proportions it is possible to depict total oxygen binding distributions (**Figure 4.3e**). At 0 min UV-O, the total oxygen content of the surface is 20.7 ± 0.4 %, with COR, C=O, and COOR binding proportions of 12.5 ± 2.3 %, 4.9 ± 0.9 %, and 4.6 ± 0.8 %, respectively. Whereas, at 120 min UV-O, the total oxygen content of the surface is 28.0 ± 0.3 %, with COR, C=O, and COOR binding proportions of 15.1 ± 0.9 %, 10.6 ± 0.9 %, and 2.3 ± 0.8 %, respectively. The data indicates that overall, UV-O induced oxidative damage results in minimal changes in COR bonds (12.5 to 15.1 %), doubling of C=O bonds (4.9 to 10.6 %), and halving of COOR bonds (4.6 to 2.3 %).

Overall Trends in Surface Chemistry

ATR-FTIR analysis of the UV-O treated pollen was performed to obtain a more complete understanding of surface chemistry changes, and provided verification of the observations made from XPS analysis. Major peaks in untreated (0 min) pollen were assigned from previous FTIR studies on sporopollenin, with peak attributions being hydroxyl (3300 cm^{-1}), aliphatic (2925 cm^{-1}), carbonyl (1670 cm^{-1}) aromatics (1515 cm^{-1} , 1425 cm^{-1}), and COR (1028 cm^{-1}) (**Figure 4.4a; Figure S7a, Chapter 4 Appendix**) [51, 52]. Subtracting UV-O treated spectra from untreated spectra suggested reductions in absorption for most major peaks of interest, and highlighted a relatively large increase in the shoulder peak at 1718 cm^{-1} (**Figure 4.4b**), which may be attributed to C=O [52] and correlates to the increased C=O binding observed from the XPS analysis.

Peak height ratio analysis was performed on major peaks of interest and indicated that the broad strong 1028 cm^{-1} peak may be utilized as a relatively stable reference peak. All major peaks decrease or remain stable relative to the 1028 cm^{-1} peak as UV-O treatment duration is increased. Broad strong peaks in the $1300 - 900\text{ cm}^{-1}$ region can be typically attributed to COR bonds [53, 54]. The XPS analysis data indicates that COR surface bonds are stable, and surface COR bonds may be attribute to the presence of ether or ester linkages in sporopollenin [50, 51, 55]. However, ATR-FTIR analysis penetrates the sample surface 0.5 to $5+ \mu\text{m}$ depending on wavelength and angle of incidence [56], and this can be expected to penetrate through the entire sporopollenin outer shell to the cellulosic intine [38]. In pollen, major FTIR peaks around 1028 cm^{-1} have also been shown to relate to COR binding in cellulosic compounds, which may be attributable to the inner sporoderm layer (intine) [56] and therefore resistant to surface modification by UV-O treatment. Due to the stability of the surface and intine COR bonds, subsequent peak height ratio analyses were performed relative to the 1028 cm^{-1} peak height.

Figure 4.4 ATR-FTIR characterization of untreated and ultraviolet-ozone (UV-O) treated pollen: (a) ATR-FTIR spectra of untreated and UV-O treated pollen; (b) ATR-FTIR spectra of UV-O treated pollen relative to untreated pollen; and (c) Peak-height ratio analysis of untreated and UV-O treated pollen for major peaks of interest.

Overall, peak height ratio analysis indicates that most bond types are reduced with increasing UV-O treatment, with the most significant reductions in aromatic ring ν C=C bonds, and significant increases in ν C=O bonds (**Figure 4.4c; Figure S7b, Chapter 4 Appendix**). Normalized peak height ratio values from 120 min UV-O exposure for hydroxyl (ν OH), aliphatic (ν asCHn), ketone (ν C=O), ester / carboxylic (ν C=O), aromatic (ν C=C), and aromatic (δ C-H), were 0.93 ± 0.02 , 0.95 ± 0.02 , 1.35 ± 0.02 , 0.93 ± 0.02 , 0.85 ± 0.03 , and 0.93 ± 0.02 , respectively. The decrease in absorbance of the aromatics may be attributed to UV-O treatment cleaving aromatic ring C=C bonds [57, 58]

present in cinnamic acids in the sporopollenin biopolymer [51]. Studies from the ozonation of *p*-coumaric acid molecules, a primary constituent of sporopollenin, proposes aromatic ring cleavage resulting in C=O groups attached to various remaining organic compound structures [59]. In sporopollenin, cinnamic acids act as crosslinking side-chains and are bound within the copolymer structure, therefore cleaving of aromatic rings within the cinnamic acids would be expected to produce an exposed C=O group attached within the copolymer, in a ketone-like formation. Correspondingly, our data indicates that the primary surface chemistry modification from UV-O treatment of pollen, is the cleaving of aromatic rings resulting in the formation of C=O functional groups which may be associated with ketone bonds.

It should be noted that the FTIR peak assignment of various carbonyl groups in sporopollenin is challenging due to variation, complexity, and uncertainty in sporopollenin chemistry. However, the XPS data from this study indicates an increase in C=O and a decrease COOR, and the FTIR data indicates an increase in carbonyls at 1718 cm⁻¹ and a decrease in carbonyls at 1670 cm⁻¹, therefore, the data suggests that 1718 cm⁻¹ (C=O) may be attributed to ketones, and 1670 cm⁻¹ (COOR) may be attributed to either esters or carboxylic acids.

4.3.3. Applications of UV-Ozone Surface Modified Pollen

Aqueous Suspensions and Pickering Emulsions

UV-O treating of pollen reduced particle clustering in water and allowed for tuning of Pickering emulsion properties. Decreasing particle hydrophobicity reduced the proportion of large clusters of particles, with the number of 90+ μm clusters being significantly ($p = **$) reduced after 120 min UV-O. Overall, after 120 min UV-O, the relative proportion of 30-50 μm, 50-90 μm, 90-150 μm, and 150+ μm particle clusters, was 1.22 ± 0.14 , 0.88 ± 0.15 , 0.47 ± 0.15 , and 0.10 ± 0.04 , respectively (**Figure 4.5a; Figure S8, Chapter 4 Appendix**), with cluster sizes representing particle counts of approximately, 1, 2 to 6, 7 to 20, and 21+ particles (**Figure 4.5b; Figure S9, Chapter 4 Appendix**). The reduction in

particle clustering, for clusters greater than 6 particles, may be attributed to improved particle wetting and thus improved affinity to water.

Analysis of oil and water systems stabilized by untreated and UV-O treated pollen indicated that pollen can be used to form microparticle stabilized Pickering emulsions, and that UV-O treatment allowed for tuning of emulsion properties. Emulsions were formed with isopropyl myristate (IPM) as the oil phase and deionized (DI) water as the aqueous phase, along with 0, 15, and 120 min UV-O treated pollen, and were allowed to stabilize for one week (**Figure S10, Chapter 4 Appendix**). Overall, emulsions formed with 0, 15, and 120 min UV-O treated pollen, resulted in oil fractions (f_{oil}) of 0.33 ± 0.05 , 0.11 ± 0.03 , and 0.51 ± 0.08 , and aqueous fractions (f_{aq}) of 0.94 ± 0.05 , 0.92 ± 0.01 , and 0.79 ± 0.08 , respectively (**Figure 4.5c**). The addition of a lipophilic dye (Nile red) to the emulsions indicated that all emulsions comprised oil droplets in an aqueous continuous phase (o/w) (**Figure S11, Chapter 4 Appendix**).

Figure 4.5 Aqueous suspension and Pickering emulsion properties of untreated and ultraviolet-ozone (UV-O) treated pollen: (a) Comparison of pollen particle cluster proportions for untreated and 120 min UV-O treated pollen; (b) Optical microscope image depicting examples of cluster size; (c) Diagrammatic depiction of emulsion composition, for untreated and UV-O treated pollen; (d) Stereo-microscope images of pollen particles on Nile red stained oil droplets in emulsions comprising untreated and UV-O treated pollen; (e) Confocal laser scanning microscopy (CLSM) images of pollen particle stabilized Pickering emulsions incorporating a hydrophobic dye, Nile red, with untreated and UV-O treated pollen particles; and (f) Stereo-microscope images of pollen particle stabilized Pickering emulsions incorporating a hydrophobic dye, Nile red, with untreated and UV-O treated pollen particles. Scale bars: (b) (d) (e) = 100 μm ; (f) = 500 μm .

The degree of UV-O treatment influences the pollen particle to oil droplet interaction dynamics. Prolonged UV-O treatment duration appeared to reduce the number of pollen particles per unit area adhering to the oil droplets (**Figure 4.5d; Figure S11, Chapter 4 Appendix**). Further, CLSM imaging of emulsion samples indicated that for both 0 min and 120 min samples, excess pollen particles settled to the bottom of the sample, whereas with the 15 min sample there were few settled particles (**Figure 4.5e; Figure S12, Chapter 4 Appendix**). For the 0 min sample, the settled pollen is typically bound to the oil phase on the glass slide surface, suggesting that the untreated pollen has strong affinity to the oil and that as excess pollen particles settle they draw oil to the bottom of the emulsion. For the 15 min and 120 min samples there is no observed pollen/oil binding on the glass slide surface, suggesting that these systems may be more stable. The 15 min sample results in minimal excess settled particles, indicating an appropriate oil to particle ratio. Whereas, the 120 min sample has numerous excess settled particles, indicating that it may be possible to reduce the proportion of particles necessary to stabilize the emulsion.

Overall, both untreated and UV-O treated pollen were able to form Pickering emulsions with varying properties, with reductions in oil droplet size (**Figure 4.5f**). Untreated (0 min) pollen formed emulsions with a yield of 36.4 ± 5.4 % of initial oil + water, comprising ~ 92 % oil and ~ 8 % water, resulting in an oil:water proportion of 11.0:1, and oil droplets of ~ 1.0 to 3.0 mm diameter. Pollen treated for 15 min produced emulsions with an increased overall yield of 48.8 ± 11.1 % of initial oil + water, comprising ~ 91 % oil and ~ 9 % water, resulting in an oil:water proportion of 10.5:1, and oil droplets of ~ 0.6 to 1.3 mm diameter. Whereas, pollen treated for 120 min produced emulsions with an overall yield of 34.7 ± 4.9 % of initial oil + water, but with an increased proportion of water, comprising ~ 70 % oil and ~ 30 % water, resulting in an oil:water proportion of 2.4:1, and oil droplets of ~ 0.2 to 0.8 mm diameter. Therefore, both of untreated and UV-O treated pollen are effective in stabilizing emulsions, and may be utilized depending on desired application. Although, it should be noted that prolonged UV-O treatment of pollen facilitates greater water uptake into the emulsion.

Pollen/Cell Binding Enhancement

UV-O treating of pollen enhanced cell binding to pollen grains indicating that it is possible to tune cell-pollen affinity and interactions. Cells (Huh 7.5, liver hepatocytes) were seeded and incubated for 24 h to ensure a stable sub-layer for pollen adhesion. Pollen grains were added, and the pollen/cell system was incubated for a further 24 h to determine whether cells would adhere to untreated and/or UV-O treated pollen while sitting atop them. Both untreated and UV-O treated pollen resulted in some proportion of pollen becoming adhered to the stable cell sub-layer. During washing to quantify pollen binding, live microscopy imaging indicated that the poorly-bound pollen particles were removed while the well-bound pollen remained attached (**Figure 4.6a**). Quantification of the removed pollen by DIPA indicated that the proportion of bound pollen increased with UV-O treatment, with 120 min UV-O resulting in 2.6 ± 0.2 times more pollen being bound (**Figure 4.6b**). Confocal scanning laser microscopy (CLSM) analysis suggests that cells incubated with UV-O treated pollen had a greater tendency to proliferate around the base of the pollen (**Figure 4.6c; Figure S13 and S14, Chapter 4 Appendix**). Cells are depicted in green due to immunostaining (Alexa Fluor 488 labeled phalloidin), whereas pollen are shown in blue due to autofluorescence. Samples were analyzed with CLSM while still in cell-culture media and without any washing to remove unbound pollen. Typically, there are more bright green regions surrounding the pollen particles when the pollen has been UV-O treated, which may be attributed to a thicker layer of cells surrounding the base of well-bound pollen. Analysis of CLSM z-stack slices indicates that cells typically formed a 5 to 10 μm base-layer of cells, but grew to a total height of 15 to 20 μm when surrounding UV-O treated pollen particles (**Figure S14, Chapter 4 Appendix**). Imaging with scanning electron microscopy (SEM), provides direct observations of cell proliferation and attachment around the base of well-bound pollen. Cell growth can be seen up to the lower third of the pollen ($\sim 12 \mu\text{m}$), and cells appear to adhere to the pollen surface (**Figure 4.6d; Figure S15, Chapter 4 Appendix**). Fibrils originating from the cells were observed on the surface of pollen at the boundary of the cell coverage, suggesting that the

process of cell attachment to pollen is led by the spreading of actin filopodia filaments [60].

Overall, enhancement of pollen / cell adhesion suggests that UV-O treatment of pollen produces functional microparticles which are suitable for use in internal and topical applications, and may be better suited to drug / compound delivery applications. Although, UV-O has been shown to enhance polymer / cell adhesion to other cell types, such as, stem cells [46, 61], and ovarian cells [62], the use of hepatocarcinoma cells in this study suggests potential benefits to developing topical formulations for treating various cancers, such as basal-cell skin cancer, squamous-cell skin cancer, and melanoma.

4.4. Conclusions

Surface modification of pollen derived from bee-collected *C. sinensis* pollen granules may be utilized to control surface chemistry, enhance wetting and dispersion properties, tune Pickering emulsion properties, and improve pollen/cell adhesion. High purity, monodisperse pollen particles may be obtained from bee-collected pollen granules through washing and defatting. Pollen particles are known to be predominantly hydrophobic, however, UV-O treatment results in enhanced surface wetting. The resulting oxidative damage leads to altered pollen surface chemistry, increasing the proportion of surface oxygen with significant increases in C=O binding, yet reducing the proportions of most other chemical bonds, with the most significant reductions in C=C binding. Decreases in C=C binding with concomitant increases in C=O binding may be attributed to cleaving of aromatic ring structures present in the pollen shell biopolymer, with oxygen forming new bonds with the exposed carbon.

Figure 4.6 Pollen/Cell binding properties for untreated and ultraviolet-ozone (UV-O) treated pollen binding with Huh 7.5 liver hepatocytes: (a) Optical micrographs of pollen/cell binding for poorly-bound pollen and well-bound pollen before and after washing; (b) Relative pollen binding for untreated and UV-O treated pollen showing data for multiple measurements of multiple batches; (c) Confocal scanning microscopy (CLSM) 3D z-stack reconstructions for untreated and UV-O treated pollen; and (d) Colored scanning electron microscopy (SEM) images of pollen/cell binding for well-bound UV-O treated pollen (original uncolored images can be found in **Figure S11, Chapter 5 Appendix**). Scale bars: (a) (c) = 20 μ m.

Control over pollen surface chemistry provides a means for enhancing the utility of pollen in a wide range of applications. The enhancement of surface wetting from increased C=O binding correlates with improved dispersion properties by reducing particle clustering. Further, UV-O treating of pollen is shown to modify the properties of microparticle stabilized Pickering emulsions, leading to shifts in emulsion stability, emulsion yields, proportions of oil-to-water, proportions of pollen particles required, and oil droplet size. The ability to tune emulsion properties through the simple process of UV-O treatment expands the potential applications for utilizing pollen in natural consumer products. Finally, UV-O treatment is shown to enhance the binding affinity of pollen and liver hepatocarcinoma cells. Pollen particles are known to be biocompatible, however, this is the first study to highlight and enhance pollen / cell binding affinities, and an awareness of pollen / cell binding has potential implications for a wide range of fields, from biology and pollination, to natural product development and drug delivery.

Looking forward, pollen particles offer an attractive material for developing natural oil/water based products for natural foods, cosmetics, and herbal therapeutics. Herein, we have highlighted the potential of surface modification through UV-O treatment to tune the interfacial properties of pollen and thereby exhibit greater control over system properties. These observations open the way for ongoing fundamental and applied research in this field, and expand the potential of pollen as an important source of regulation-free natural functional microparticles.

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Chapter 5

UV-O Surface Modification of S-SMCs

Readily extracted from plant pollen grains, S-SMCs are abundant in quantity, produced renewably, possess a wide range of species-specific ornamental and shape architectures, and are highly monodisperse and uniform for each individual species. UV-O allows for the tuning of S-SMC interfacial properties resulting in wetting properties ranging from hydrophobic to superhydrophilic, and programmable function for colloidal science and cellular applications. Even though the microparticles were initially predominantly hydrophobic due to the lipidic/aromatic nature of sporopollenin. Stable Pickering emulsions are achieved using S-SMCs with short duration UV-O treatment, while incorporation of superhydrophilic S-SMCs into oil/water systems provides a novel means to produce, and isolate, fully oil-loaded microparticles. Furthermore, it is shown that human cells adhere to S-SMCs acting as tissue seeds, with the controllable formation of either 3D cell spheroids or network structures dictated by the interfacial properties of the particles employed. Collectively, light-induced modification of S-SMCs provides a simple and effective strategy to modulate functional properties, with broad implications across colloidal science, microencapsulation, drug delivery, and cellular applications.

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5.1. Introduction

There is growing interest in utilizing hollow plant-based sporoderm microparticles, which are extracted from pollen and other sporomorphs, for materials science and biotechnology applications [1-14]. Sporopollenin sporoderm microcapsules (S-SMCs) extracted from pollen have been shown to be highly robust to chemical and physical degradation [15, 16], while also showing evidence of biocompatibility and being susceptible to degradation within the human body [17, 18]. S-SMCs find broad utility in various applications such as microparticle-stabilized Pickering emulsions, microencapsulation, and drug delivery. In addition to applications that employ native S-SMCs, there are largely untapped opportunities to explore the interfacial properties of the outer sporoderm surface and to develop facile means to modify its surface chemistry to engineer S-SMCs for an expanded range of functional possibilities.

The surface chemistry of S-SMCs is known to be amphiphilic [19] though typically more hydrophobic and therefore light-induced ultraviolet/ozone (UV-O) treatment offers an ideal means to achieve precise control over sporoderm interfacial properties [20, 21]. The outer sporoderm layer is comprised of a complex copolymer consisting of a lipidic backbone cross-linked by aromatic compounds [22]. UV-O-induced oxidative processes are known to oxidize both aliphatic [23] and aromatic structures [24] and lead to increased hydrophilicity of such compounds. Additionally, it has been recently shown that defatted pollen capsules, including sporopollenin among other components, are susceptible to UV-O treatment and exposure leads to chemical modification and increased wetting properties [25]. However, acid extraction of S-SMCs from pollen capsules is known to alter the polymer structure of the sporopollenin copolymer and hence influence sporopollenin chemical reactivity, the details of which largely remains an outstanding subject [26, 27]. Therefore, it remains unknown how acid-extracted S-SMCs will respond to UV-O treatment and whether such modification strategies would be useful for expanding the utility of S-SMCs.

When altering the surface chemistry and interfacial properties of S-SMCs, one of the most important considerations pertains to how the functionalized microparticles can be utilized for colloidal science applications as well as their behavior in more complex biological systems [28, 29]. The emulsification potential of acid-extracted S-SMCs as well as native and defatted sporomorphs has been explored in a handful of studies indicating their potential utility as stabilizing microparticles [19, 30]. Additionally, UV-O treatment has been shown to enhance defatted sporomorph dispersion properties and tune Pickering emulsion properties [25]. However, it is currently unclear whether acid-extracted S-SMCs will behave similarly and what are the advantages and limitations of UV-O treatment of S-SMCs for use in aqueous and oil/water systems? Answering such questions can guide targeted development of S-SMCs for colloidal science and microencapsulation applications. One broad microencapsulation area of interest is the loading of hydrophobic compounds into microparticles while ensuring that the consistency of a free-flowing powder remains [31, 32]. Until now, attempts to efficiently load oils and hydrophobic compounds into S-SMCs have had some success, although it has been difficult to obtain a free flowing oil-loaded powder with high loading efficiencies [17, 33, 34]. It is proposed that achieving control over the interfacial properties of S-SMCs may allow for enhanced loading potential for hydrophobic compounds, which would significantly expand the overall utility of S-SMCs in a wide range of applications.

Another key area of opportunities lie in interfacing S-SMCs with cellular materials and other biological components. Although S-SMCs are considered a biocompatible material [17, 18], there exists very limited information regarding their potential for particle/cell interactions [35, 36], and it is unclear how cells may interact with either acid-extracted S-SMCs or UV-O surface modified S-SMCs. Some evidence of cell adhesion to defatted natural pollen particles has been reported, with UV-O surface modification enhancing pollen/cell binding [25]. Extending these capabilities to the more versatile hollow core/shell microparticle structure of S-SMCs might further enable a range of particle/cell systems with broad application possibilities [37-40].

Figure 5.1 Schematic diagram showing sporopollenin sporoderm microcapsule (S-SMC) extraction with ultraviolet-ozone (UV-O) surface modification and UV-O influence on emulsification, oil loading, and particle/cell binding: (a) acid extraction of S-SMCs from pollen; (b) UV-O treatment of pollen capsules with primary chemical modifications; (c) emulsion stabilization before and after extensive UV-O treatment; (d) oil loading before and after UV-O treatment; and (e) particle/cell binding before and after UV-O treatment.

Inspired by these possibilities, the goal of this study is to explore how S-SMCs generated from *Camellia sinensis* bee-collected pollen can be modified by UV-O treatment and thereby utilized in oil/water based systems, for enhancing oil loading, and to further understand how mammalian cells interact with untreated and UV-O treated S-SMCs serving as tissue seeds (**Figure 5.1**). To this end, hollow S-SMCs were extracted from defatted *C. sinensis* pollen and extensively characterized with respect to surface morphology and other fundamental properties. The effect of UV-O treatment was observed with regards to particle morphology and wetting properties. Particle surface chemistry was explored before and after UV-O treatment *via* x-ray photoelectron spectroscopy (XPS) and Fourier-transform infrared spectroscopy (FTIR) analysis to gain insight into the fundamental chemical mechanisms driving alterations in interfacial properties. Emulsification properties were explored to elucidate the potential for S-SMCs to form Pickering emulsions and determine the limitations of UV-O treatment on tuning emulsion properties. Insights gained from studying

emulsion dynamics were used to explore and optimize S-SMC oil loading potential. Finally, particle/cell binding dynamics were explored with untreated and UV-O treated S-SMCs and Huh-7.5 liver hepatocarcinoma cells to determine whether cells can adhere to S-SMCs, and how fundamental aqueous suspension properties influence particle/cell interactions with a long view towards achieving hierarchical control over formation of tissue-like architectures.

5.2. Experimental Methods

5.2.1. Materials

Materials were used as defined in Chapter 3.2.1.

5.2.2. Defatting of Bee Pollen Granules

Defatting of bee pollen granules protocol was as defined in Chapter 3.2.2.

5.2.3. Extraction of S-SMCs

S-SMCs extraction protocol was as defined in Chapter 3.2.3.

5.2.4. Elemental Analysis of S-SMCs

Elemental analysis of S-SMCs protocol was as defined in Chapter 3.3.1.

5.2.5. Ultraviolet-Ozone (UV-O) Treatment

UV-O treatment protocol was as defined in Chapter 3.2.4.

5.2.6. Contact Angle Measurements

Contact angle measurements were performed as defined in Chapter 3.3.4.

5.2.7. X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy was performed as defined in Chapter 3.3.5.

5.2.8. Attenuated Total Reflection Fourier-Transform Infrared Spectroscopy

ATR-FTIR was performed as defined in Chapter 3.3.6.

5.2.9. Dynamic Image Particle Analysis

DIPA was performed as defined in Chapter 3.3.2.

5.2.10. Pickering Emulsions

Pickering emulsions were prepared using protocol as defined in Chapter 3.2.5.

5.2.11. Cocoa Butter Oil Loading

Cocoa butter oil loading protocol was as defined in Chapter 3.2.6.

5.2.12. S-SMC/Cell Adhesion Study

S-SMC/ cell adhesion study was done using protocol as defined in Chapter 3.2.7.

5.2.13. CLSM Analysis

CLSM analysis was performed as defined in Chapter 3.3.8.

5.2.14. Bright Field Microscopy

Bright Field Microscopy was performed as defined in Chapter 3.3.9.

5.2.15. Surface Morphology Evaluation by Scanning Electron Microscopy

SEM was performed as defined in Chapter 3.3.3.

5.2.16. Evaluation of Pickering Emulsion by Stereomicroscopy

Stereomicroscopy was performed as defined in Chapter 3.3.7.

5.2.17. Statistical Analysis

Statistical analysis was performed as defined in Chapter 3.3.10.

5.3. Principle Outcomes

5.3.1. Extraction of Pollen Microcapsules from *C. sinensis* Bee Pollen Granules

Pollen microcapsules, S-SMCs, were extracted from bee-collected pollen by a two-step process including isolation of discrete pollen particles from bee pollen granules, followed by acid extraction of hollow S-SMCs from the discrete pollen particles. Optimization of S-SMC acid-extraction was conducted for varying acidolysis durations to determine the minimum extraction time required to achieve adequate removal of cytoplasmic contents. Elemental analysis of percent nitrogen (N%) is commonly used to identify the proportion of proteinaceous material present during S-SMC extraction, and N% multiplied by a factor of 6.25 is used to estimate percent protein [41]. Defatted pollen, and acidolysis durations of 1, 3, 5, and 10 h, resulted in protein contents of $61.5 \pm 0.3\%$, $11.7 \pm 0.1\%$, $3.9 \pm 0.0\%$, $3.4 \pm 0.3\%$, and $3.0 \pm 0.0\%$, respectively (**Figure S1, Chapter 5 Appendix**). The data indicates that 3 h treatment provides maximum protein removal based on standard acidolysis extraction methods. Numerous studies with other pollen species have indicated similar levels of proteinaceous residues even with longer duration high-temperature acidolysis [41, 42]. It has been shown by matrix-assisted laser

desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) that all proteinaceous materials above 1 kDa are effectively removed by similar S-SMC acid extraction [43, 44]. Low levels of nitrogen content commonly observed during acid extraction may be attributed to residual amino acids adhered to the S-SMC.

Micromeritic analysis of defatted pollen and S-SMCs extracted with acidolysis durations of 1, 3, 5, and 10 h, resulted in particle diameters of 36.6 ± 0.1 , 33.8 ± 0.4 , 32.8 ± 0.3 , 31.3 ± 0.3 , and 32.2 ± 0.1 μm , respectively, and minimal variation in morphological parameters such as circularity and aspect ratio (**Figure S2, Chapter 5 Appendix**). Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) analysis of defatted pollen and S-SMCs extracted with acidolysis durations of 1, 3, 5, and 10 h, indicated that all acidolysis durations appeared to remove cytoplasmic contents and produced hollow S-SMCs (**Figure 5.2; Figure S3, Chapter 5 Appendix**). Therefore, based on the results of elemental analysis, micromeritic analysis, and microscopy analysis, it was decided that 3 h acid extracted S-SMCs will be used for the remainder of the study. Of note, S-SMC extraction yields from defatted pollen were $17.3 \pm 2.1\%$, comprising 245075 ± 33032 particles mg^{-1} with a particle weight of 4.1 ± 0.5 ng. Considering a sporoderm wall thickness of 1.2 ± 0.1 μm , the sporoderm density is 1.09 ± 0.20 g cm^{-3} , which is consistent with the observation that S-SMCs sink in water.

Figure 5.2 Sporopollenin sporoderm microcapsule (S-SMC) extraction and ultraviolet-ozone (UV-O) surface modification effects on particle morphology and wetting properties: (a) photographs, optical stereomicrographs, and scanning electron microscopy (SEM) images of defatted *C. sinensis* pollen and 3 h acid-extracted hollow S-SMCs; (b) SEM images of overall particle morphology with UV-O treatment from 0-30 min; (c) SEM images of particle surface morphology with an untreated and 30 min UV-O treated S-SMC; and (d) optical microscope images of water droplets during contact angle measurements of S-SMCs with UV-O treatment from 0-30 min. Scale bars: (b) = 10 μm ; (c) = 100 nm; (d) = 0.5 mm.

5.3.2. Ultraviolet-Ozone (UV-O) Exposure Effect on Pollen Surface Chemistry

Surface Morphology and Wetting Properties

UV-O treatment of S-SMCs with UV-O treatment durations of 0, 1, 5, 15, and 30 min, resulted in no changes in overall particle morphology and no distinct changes in nano-scale surface morphology (**Figure 5.2b and 5.2c; Figure S4, Chapter 5 Appendix**). Significant increases in wetting were observed within 1 min of UV-O treatment with the particles becoming superhydrophilic within 15 min. Contact angle measurements of S-SMCs with UV-O treatment durations of 0, 1, 5, 15, and 30 min resulted in contact angles of $100.2 \pm 12.6^\circ$, $34.9 \pm 13.1^\circ$, $14.2 \pm 2.6^\circ$, $7.2 \pm 0.6^\circ$, and $5.9 \pm 2.0^\circ$ (**Figure 5.2d**), respectively. Overall, these observations suggest that enhanced particle wetting may be attributed to variations in the surface chemistry of the S-SMCs.

Pollen Surface Elemental Composition and Binding Profiles

UV-O induced surface chemistry changes are predominantly driven by increases in total oxygen content, with increased proportions of ketone ($R_2C=O$) binding and carbon-oxygen (COR) binding. Wide-scan XPS analysis indicates that total atomic oxygen increases and total atomic carbon decreases with UV-O treatment durations of 0 and 30 min resulting in oxygen concentrations of $19.4 \pm 0.6\%$ and $25.5 \pm 0.9\%$, and carbon concentrations of $75.8 \pm 1.3\%$ and $68.6 \pm 1.4\%$, respectively (**Figure 5.3a; Figure S5a, Chapter 5 Appendix**). With regards to oxygen binding distributions, narrow scan XPS analysis of both C1s and O1s peaks indicates that $R_2C=O$ binding increases by ~31%, carboxylic acid/ester ($RO-C=O$) binding decreases by ~35%, and COR binding increases by ~12% with 30 min UV-O (**Figure 5.3b; Figure S5b, Chapter 5 Appendix**). Combining both total atomic oxygen and binding distribution data it is possible to quantify overall oxygen binding distributions before and after UV-O treatment. With 30 min UV-O treatment the total proportion of $R_2C=O$ nearly doubles from $3.7 \pm 0.5\%$ to $6.2 \pm 0.8\%$, the total proportion of $RO-C=O$ decreases slightly from $6.8 \pm 0.6\%$ to $6.3 \pm 0.8\%$, and the total proportion of COR increases by half from $8.9 \pm 0.3\%$ to $13.0 \pm 0.5\%$ (**Figure 5.3c**). It is

important to note that upon more detailed analysis of the narrow scan XPS data, the increase in COR binding can be attributed to ether (COC) binding rather than COH binding (**Table 5.1**).

Figure 5.3 Surface chemistry analysis of untreated and ultraviolet-ozone (UV-O) treated sporopollenin sporoderm microcapsules (S-SMCs): (a) XPS quantification of atomic carbon and oxygen; (b) XPS quantification of proportional shifts in major oxygen binding groups; (c) XPS quantification of overall oxygen binding distributions; (d) XPS narrow scan carbon (C1s) peak of untreated and UV-O treated S-SMCs; (e) ATR-FTIR spectra and difference analysis of untreated and UV-O treated S-SMCs; and (f) normalized peak height ratio analysis of key peaks of interest from ATR-FTIR spectra.

Table 5.1 Bond-type binding energies and proportions for carbon (C1s) and oxygen (O1s) binding.

Carbon (C1s)					Oxygen (O1s)				
#	Bond type	Binding energy [eV]	0 min [%]	30 min [%]	#	Bond type	Binding energy [eV]	0 min [%]	30 min [%]
6	CC / CH	284.8	35.6 ± 4.7	30.7 ± 1.4	10	COH	532.7	23.8 ± 1.8	21.4 ± 1.1
5	COH	285.7	20.5 ± 0.8	17.5 ± 0.5	9	COC	533.0	12.3 ± 1.4	20.2 ± 1.8
4	COC	286.8	13.7 ± 1.8	22.6 ± 1.7	11	R ₂ C=O	531.6	12.9 ± 3.1	18.8 ± 1.6
3	R ₂ C=O	287.8	15.7 ± 2.4	19.7 ± 3.4	8	CO-C=O	534.1	33.7 ± 0.6	25.2 ± 0.4
2	RO-C=O	288.8	11.7 ± 2.4	6.5 ± 1.1	7	HO-C=O	535.5	17.3 ± 1.4	14.5 ± 1.7
1b	π - π	291.0		3.1 ± 0.9					
1a	π - π	291.7	2.8 ± 0.8						

UV-O treatment of S-SMCs influences π -bonding, suggesting that one mechanism of acid-extracted sporopollenin oxidation includes alterations of aromatic ring structures without causing complete ring opening. It is unknown how acid extraction of S-SMCs alters sporopollenin chemistry. However, comparing the narrow scan carbon (C1s) peak structure from S-SMCs in this study with (C1s) peak structure data from a previous study of defatted-only *C. sinensis* sporopollenin [25], acid-extraction is shown to introduce a small peak at 291.7 eV (**Figure 5.3d; Figure S5b, Chapter 5 Appendix**), which may be attributed to π -bonding from aromatic ring stacking [45]. Of note, this is the first observation of aromatic ring stacking in sporopollenin due to acid-extraction, and has important implications for material property analysis of sporopollenin, including for palynological studies of pollen fossils used in climate change analysis [46]. Further, upon S-SMC exposure to UV-O there is a clearly defined shift in the π -bonding peak energy from 291.7 eV to 291.0 eV, which suggests an alteration of aromatic ring structure without causing complete ring opening. Based on oxidation of carbon rings in graphene [47, 48], it is proposed that π -bonded rings in sporopollenin undergo a process of epoxidation leading to the insertion of atomic oxygen to replace CC bonds and form COC ether bonds, which is supported by the increase in COC bonds observed from both C1s peak and O1s peak analysis with UV-O treatment (**Table 5.1**).

Overall Trends in Surface Chemistry

ATR-FTIR analysis of UV-O treated S-SMCs supports the conclusions from XPS analysis and provides deeper insights into the overall trends in surface chemistry. FTIR spectra showed no major appearances or disappearances of peaks with UV-O treatment (**Figure 5.3e; Figure S6a, Chapter 5 Appendix**). However, a difference analysis of S-SMC FTIR spectra before and after UV-O treatment revealed that most prominent peaks observable in the raw FTIR spectra show successive reductions in peak height with increasing UV-O treatment duration (**Figure 5.3e; Figure S6b, Chapter 5 Appendix**). Additionally, with difference analysis, two regions become highlighted and show prominent increases, 1718 cm^{-1} and 1180 cm^{-1} . Peak height ratio analysis was used to quantify the trends observed from the difference analysis by comparing the height of each peak of interest to the height of the stable major peak at 1063 cm^{-1} . Previous studies regarding pollen have attributed peaks around 1063 cm^{-1} to COR bonds of cellulosic compounds present in the cellulosic inner sporoderm layer [49]. It is expected that the intine layer will be protected from UV-O treatment by the outer sporoderm layer and will provide a stable reference peak for use in peak height ratio analysis. Other FTIR peaks used for peak height ratio analysis are assigned based on existing literature as follows [50-52], A: hydroxyl (νOH , 3380 cm^{-1}), B: aliphatic (νasCH_n , 2925 cm^{-1}), C: ketone ($\nu\text{C=O}$, 1718 cm^{-1}), D: carboxylic acid/ester ($\nu\text{C=O}$, 1670 cm^{-1}), E: aromatic ($\nu\text{C=C}$, 1575 cm^{-1}), F: aromatic ($\nu\text{C=C}$, 1515 cm^{-1}), G: aromatic ($\delta\text{C-H}$, 1425 cm^{-1}), H: ether ($\nu\text{C-O}$, 1180 cm^{-1}), and I: aromatic ($\delta\text{C-H}$, 840 cm^{-1}).

Peak height ratio analysis indicates that the major changes in polymer chemistry are increases in ketone ($\text{R}_2\text{C=O}$) binding and ether (COC) binding, with the largest proportional decreases in aromatic ring (C=C) binding (**Figure 5.3f; Figure S7, Chapter 5 Appendix**). The relative peak height ratios of all peaks of interest, after 30 min UV-O treatment, are $A = 0.92 \pm 0.01$, $B = 0.89 \pm 0.01$, $C = 1.39 \pm 0.03$, $D = 0.84 \pm 0.01$, $E = 0.94 \pm 0.1$, $F = 0.63 \pm 0.1$, $G = 0.86 \pm 0.01$, $H = 1.19 \pm 0.01$, and $I = 0.73 \pm 0.3$. The trends of increasing ketone ($\text{R}_2\text{C=O}$) binding and ether (COC) binding are consistent with the binding trends observed with XPS. Decreases in aromatic ring (C=C) binding may be

attributed to UV-O induced aromatic ring opening [25]. Importantly, the oxidative degradation of the aromatic compound *p*-coumaric acid has been shown to produce ketone ($R_2C=O$) moieties [24], and *p*-coumaric acid is known to be one of the major constituents of sporopollenin [22]. Therefore, the increase in ketone ($R_2C=O$) bonds may be directly attributable to UV-O induced aromatic ring opening within sporopollenin.

Overall, both XPS and FTIR analysis suggest that aromatic rings in acid-extracted sporopollenin present two possible paths of oxidation. Firstly, some aromatic rings may be cleaved open during UV-O exposure leading to the formation of ketone bonds ($R_2C=O$), and secondly, some π -bonded aromatic rings undergo alteration of the ring structure with the formation of ether bonds (COC). It should be noted that oxidation of the aliphatic chains present in the copolymer structure may contribute to ketone bond ($R_2C=O$) formation [23], although UV-O oxidation studies with aliphatic polymers such as polyethylene indicate increases in atomic oxygen of less than 1% after complete oxidation [53], which is much less than the ~6% atomic oxygen increase observed above.

5.3.3. Applications of UV-Ozone Surface Modified Pollen

Aqueous Suspensions and Pickering Emulsions

UV-O treatment of S-SMCs alters emulsion properties when S-SMCs are used for forming microparticle stabilized Pickering emulsions (**Figure 5.4**). Untreated S-SMCs or UV-O treatment for short duration facilitated the formation of microparticle stabilized Pickering emulsions, however, long duration UV-O treatment resulted in lower quality emulsions. Emulsions were prepared using equal parts oil and water with the addition of 0, 1, or 30 min UV-O treated S-SMCs and stabilized for 7 days. Emulsions formed with 0, 1, and 30 min treated S-SMCs resulted in systems with oil fractions (f_{oil}) of 0.65 ± 0.03 , 0.80 ± 0.01 , and 0.83 ± 0.03 , and aqueous fractions (f_{aq}) of 0.70 ± 0.03 , 0.80 ± 0.06 , and 0.74 ± 0.03 , respectively (**Figure 5.4a**).

Figure 5.4 Influence of ultraviolet-ozone (UV-O) treatment on oil/water emulsification properties of sporopollenin sporoderm microcapsules (S-SMCs): (a) visual depictions of Pickering emulsion oil/water distributions; (b) photographs of representative Pickering emulsions; (c) CLSM images highlighting oil droplet and particle settling of Pickering emulsions with oil stained by Nile Red; and (d) stereomicroscopy images of Pickering emulsions incorporating the hydrophobic dye, Nile Red. Scale bars: (c) and (d) = 100 μ m.

Emulsions formed with untreated S-SMCs appear well-structured with a relatively uniform droplet size with the majority of droplets ranging from 250-500 μ m, however, utilizing UV-O treated S-SMCs produces greater droplet size variability with an overall reduction of average droplet sizes, with the majority of droplets ranging from 50-250 μ m, and some settling of particles (**Figure 5.4b**). Observing the bottom of the emulsion layer *via* CLSM shows that for untreated particles there are relatively few discrete settled particles and most S-SMCs observed are in the proximity of an oil droplet, stained red with a hydrophobic dye (Nile Red) (**Figure 5.4c**; **Figure S8, Chapter 5 Appendix**). Incorporating 1 min UV-O treated S-SMCs results in many more settled particles and small oil droplets, suggesting a limited degree of S-SMC/oil interaction. Incorporating 30 min UV-O treated S-SMCs results in only settled particles with no oil drops visible, suggesting little S-SMC/oil interaction.

Observations of emulsion structures from above with standard optical stereomicroscopy further indicates that untreated S-SMCs produce emulsions wherein the particles surround and attach to oil droplets, with 1 min UV-O resulting in some particle/oil affinity, and 30 min UV-O resulting in minimal particle/oil affinity leading to less stable and less ideal emulsions (**Figure 5.4d; Figure S9, Chapter 5 Appendix**). It has been observed in previous studies that UV-O treatment durations of up to 120 min may be applied to hydrophobic defatted pollen particles to tune Pickering emulsion properties [25]. However, the contact angles of UV-O treated pollen plateaued at $\sim 55^\circ$, whereas, the contact angle of S-SMCs with 1 min UV-O has already reduced to $\sim 35^\circ$. Therefore, to utilize UV-O for tuning S-SMC stabilized Pickering emulsions, it may be necessary to limit the UV-O treatment duration to less than 1 min.

Oil Loading and Oil-Loaded Particle Separation

Standard emulsion preparation techniques in conjunction with UV-O treated S-SMCs provide a facile means for loading oil within S-SMCs and enables the isolation of only fully loaded S-SMCs. The above-mentioned investigations into utilizing S-SMCs in Pickering emulsions resulted in the observation that emulsion homogenization could be used to load tiny oil droplets into the hollow S-SMC internal cavity through the micron-sized apertures in the shell wall (**Figure 5.5a; Figure S10, Chapter 5 Appendix**). Upon further analysis of the loading distributions of oil-loaded S-SMCs for untreated and 30 min UV-O treated S-SMCs, it was observed that S-SMCs at the oil/water interface were only partially loaded for the untreated S-SMCs, and almost fully loaded for the 30 min UV-O treated S-SMCs. Isolation and quantification of the oil-loaded S-SMCs indicated that 0 min and 30 min UV-O treatment resulted in fully loaded particle proportions of $26.5 \pm 5.4\%$ and $93.2 \pm 3.7\%$, respectively (**Figure 5.5b; Figure S10, Chapter 5 Appendix**).

Figure 5.5 Influence of ultraviolet-ozone (UV-O) treatment on oil loading of sporopollenin sporoderm microcapsules (S-SMCs): (a) CLSM cross-sectional images of an oil/water system with homogenization, and floating oil-loaded S-SMCs isolated after system stabilization; (b) quantification of the proportion of fully oil-loaded floating S-SMCs; (c) schematic representation of the proposed mechanisms of oil loading and system stabilization; (d) false-color SEM images of dried oil-loaded S-SMCs highlighting clean particle surface and representative oil loading (original uncolored images can be found in **Figure S11, Chapter 5 Appendix**). Scale bars: (a) and (d) = 10 μm .

It is proposed that the untreated S-SMCs have a greater affinity to the external oil in the oil/water system, and therefore any of the fully-, partially-, or un-loaded S-SMCs may be drawn up with the oil as the oil rises during system stabilization (**Figure 5.5c**). However, 30 min UV-O treated S-SMCs become superhydrophilic and have minimal affinity to the oil in the oil/water system, and therefore only the fully-loaded particles rise due to the buoyancy of the internally encapsulated oil, whereas the partially- or un-loaded S-SMCs settle to the bottom of the oil/water system. Based on average values from S-SMC

morphological and density analysis above, and the density of cocoa butter (0.976 g cm^{-3}) used in this study, the force of buoyancy (F_b) versus the force of gravity (F_g) for volumetric oil-loading of oil-loaded S-SMCs in water indicates that at 90% loading $F_b < F_g$ (166.8 pN vs 167.0 pN), 95% loading $F_b = F_g$ (174.0 pN vs 174.0 pN), and 100% loading $F_b > F_g$ (181.3 pN vs 181.0 pN). The results indicate that at 95% volumetric loading of cocoa butter ($F_g = F_b$) there will be a transition from sinking oil-loaded S-SMCs to floating oil-loaded S-SMCs, which is in strong agreement with the experimental observations.

Upon evaporation of the water containing the oil-loaded S-SMCs it is possible to obtain a dry microparticle system with a high-degree of oil loading and minimal residual oil on the S-SMCs surface (**Figure 5.5d; Figure S11, Chapter 5 Appendix**). Importantly, it is proposed that with further optimization such a system may be automated to recycle the partially- or unloaded S-SMCs so as to achieve an industrially scalable process for obtaining an efficient and continuous supply of fully oil-loaded S-SMCs.

Pollen/Cell Spheroid and Network Formations

Particle/cell interactions are quite different between untreated and UV-O treated S-SMCs with pronounced differences in both S-SMC particle distributions and cell proliferation distributions (**Figure 5.6; Figure S12, Chapter 5 Appendix**). Overall, with 24 h incubation on a stable layer of Huh-7.5 liver hepatocytes, total S-SMC particle binding proportions were similar for both untreated and UV-O treated S-SMCs, resulting in $84.5 \pm 3.6\%$ and $75.2 \pm 5.5\%$ of particles being bound by cells, respectively. The data indicates that cells will bind well with both untreated and UV-O treated S-SMCs. However, for untreated S-SMCs, particles tend to form clusters on the scale of 100-400 μm , and the cells proliferate both throughout the S-SMC clusters and uniformly across the polystyrene dish surface. Whereas for UV-O treated S-SMCs, particles disperse more uniformly tending to form single or double layers, and cell proliferation is focused over the UV-O treated S-SMCs with notably reduced spreading across the polystyrene dish surface.

Figure 5.6 Influence of ultraviolet-ozone (UV-O) treatment on sporopollenin sporoderm microcapsule (S-SMC)/cell interactions with Huh-7.5 liver hepatocytes: (a) CLSM images of S-SMC and cell distributions and coverage areas; (b) quantification of S-SMC and cell coverage area proportions; (c) CLSM and false-color SEM images of representative S-SMC/cell spheroid and network structures (original uncolored images can be found in **Figure S16, Chapter 5 Appendix**). Scale bars: (a) 100 μ m.

Image processing of CLSM micrographs was conducted to quantify the coverage areas of S-SMCs and cells for both untreated and UV-O treated S-SMCs systems (**Figure 5.6a; Figure S13 and S14, Chapter 5 Appendix**). S-SMC particle coverage increases with UV-O treatment from $37.3 \pm 2.2\%$ to $60.7 \pm 5.5\%$, whereas cell coverage decreased from $85.4 \pm 6.0\%$ to $63.5 \pm 4.9\%$ suggesting that the cell proliferation dynamics are different in systems with untreated and UV-O treated S-SMCs (**Figure 5.6b**). The proportion of S-SMC/cell overlap also increases with UV-O treatment from $30.2 \pm 5.5\%$ to $43.9 \pm 6.2\%$, but may be attributed to the increase in total S-SMC coverage in the UV-O treated system. Most importantly, the proportion of non-S-SMC area covered by cells significantly ($p < 0.01$) decreases with UV-O treatment, with $88.0 \pm 7.7\%$ of the untreated system non-S-SMC area covered by cells, yet only

$50.2 \pm 3.9\%$ of the UV-O treated system non-S-SMC area is covered by cells. Overall, these results indicate that cells tend to proliferate more around the UV-O treated S-SMCs. UV-O treatment altering substrate/cell binding dynamics is consistent with previous observations with defatted pollen and other synthetic polymers [25, 54-56].

Observation of particle/cell spheroids and networks by CLSM and SEM provides further insights into particle/cell dynamics (**Figure 5.6c; Figure S15 and S16, Chapter 5 Appendix**). For untreated S-SMC systems, particle/cell spheroidal structures are observed along with cells proliferating throughout the 3D S-SMC system, and a thick layer of cells is visible covering the surrounding polystyrene dish. For UV-O treated S-SMC systems, particle/cell network structures are observed along with cells proliferating throughout the S-SMC network, and beyond a distance of $\sim 20\ \mu\text{m}$ from the S-SMCs there are large clear regions of polystyrene dish with no cell coverage. Close-ups of both systems indicate that cells are behaving like a mortar by binding the discrete S-SMCs together. During the washing of samples for extraction of non-bound S-SMCs, it was observed that both the particle/cell spheroid structures and single layer particle/cell network structures are robust and resistant to dissolution with agitation of the system.

Based on these observations, both untreated S-SMCs and UV-O treated S-SMCs provide compelling materials for use in cellular adhesion and tissue engineering applications. Through protocol variations and longer duration studies it may be possible to provide important contributions for developing novel spheroidal or network-type cell culture platforms [57]. Additionally, both untreated and UV-O treated S-SMCs exhibit the potential for cell compatibility and may be used for developing novel applied systems for oral, intravenous, or topical drug delivery.

5.4. Conclusion

UV-O treatment enhances the utility of acid-extracted S-SMC hollow microcapsules for colloidal, microencapsulation, and tissue engineering applications. Highly versatile S-SMCs may be extracted from bee-collected pollens and surface functionalized with short duration exposure to UV-O resulting in wetting properties ranging from hydrophobic to superhydrophilic. The lipidic/aromatic copolymer exhibits oxidative alterations in surface chemistry by the chemisorption of atomic oxygen through the formation of ketone ($R_2C=O$) and ether (COC) bonds in conjunction with the ring opening and alteration of aromatic moieties within the copolymer structure. Both ketone and ether functionalities are considered hydrophilic and may be directly attributed to the observed enhancement in wetting and hydrophilic properties of the co-polymer structure. Modification of the surface chemistry of the acid-extracted S-SMCs provides an important means to tune the interfacial properties of the material for achieving desirable behaviors in various fundamental systems and applications.

Understanding the influence of surface modification on the behavior of S-SMCs in various applications provides insights into how the UV-O treatment of S-SMCs may be utilized to control systems of interest. In oil/water systems, untreated and short duration (1 min) UV-O treated S-SMCs are shown to form microparticle stabilized Pickering emulsions. However, beyond 1 min UV-O treatment, superhydrophilic S-SMCs fail to form a stable emulsion, yet lead to a means to obtain ideal fully oil-loaded microparticles. Both untreated and UV-O treated S-SMCs are shown to exhibit favorable particle/cell interaction dynamics, with untreated S-SMCs offering a means to obtain a novel particle/cell spheroid system, and UV-O treated S-SMCs resulting a more disperse particle/cell network system with increased particle/cell interaction.

Overall, S-SMCs and UV-O surface functionalization are shown to provide a highly versatile technology for utilization in a wide range of potential applications and there is much potential for the further exploration of the systems highlighted in this study. The behavior of UV-O treatment on S-SMC

stabilized Pickering emulsion properties with various oil phase compounds, a detailed analysis of oil-loading of S-SMCs for various microencapsulation applications, and the optimization of particle/cell spheroids or 3D network structures for various tissue engineering applications all warrant further investigation.

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Chapter 6

Implications and Recommendations for Future Work

The objectives of this Master of Engineering project are to surface modify microparticles using UV-O, to characterize the microparticles for changes due to treatment, and to explore microparticles as functional microbeads by understanding the emulsification characteristics and particle/cell adhesion properties. The microparticles utilized in this project are pollen particles and S-SMCs. Previous chapters have provided justification, detailed summary of the materials and methods, and the result and discussion for the exploration of pollen particles and S-SMCs as functional microbeads. Overall, this chapter a summary of the contributions, implications and the recommendations for future work.

6.1. Summary of Contributions and Implications

In this thesis, the author has done studies that further increase the understanding of sporopollenin and help facilitate further exploration or utilization of sporopollenin microcapsules for various applications. The author has shown that it was possible to extract loose pollen particles from bee pollen granules that yield pollen of high purity (monospecies) and that it was possible to extract S-SMCs from *C. sinensis* pollen. UV–O surface modification *C. sinensis* pollen and S-SMCs for developing Pickering emulsion-based formulations and enhancing pollen/ cell adhesion properties was possible. UV–O treatment results in enhanced surface wetting. The resulting degradation leads to altered pollen and S-SMCs surface chemistry characterized via XPS and ATR-FTIR.

The ability to tune emulsion properties through the simple process of UV–O treatment expands the potential applications for utilizing pollen in natural consumer products. UV–O treatment is shown to enhance the binding affinity of pollen and liver hepatocarcinoma cells. Pollen particles are known to be biocompatible; however, this is the first study to highlight and enhance pollen/cell binding affinities, and an awareness of pollen/cell binding has potential implications for a wide range of fields, from biology and pollination, to natural product development and drug delivery. Looking forward, pollen particles offer an attractive material for developing natural oil/water based products for natural foods, cosmetics, and herbal therapeutics.

This thesis provides an understanding of the influence of surface modification on the behavior of S-SMCs in various applications provides insights into how the UV-O treatment of S-SMCs may be utilized to control systems of interest. In oil/water systems, untreated and short duration (1 min) UV-O treated S-SMCs are shown to form microparticle stabilized Pickering emulsions. However, beyond 1 min UV-O treatment, superhydrophilic S-SMCs fail to form a stable emulsion, yet lead to a means to obtain ideal fully oil-loaded microparticles. Both untreated and UV-O treated S- SMCs are shown to exhibit favorable particle/cell interaction dynamics, with untreated S-SMCs offering a means to obtain a novel particle/cell spheroid system, and UV-O treated S-

SMCs resulting a more disperse particle/cell network system with increased particle/cell interaction.

This study also provides an opportunity to compare various properties of UV-O treated pollen in comparison to UV-O treated S-SMCs. In terms of hydrophilicity, although both pollen and S-SMCs exhibited improvement in wetting, S-SMCs showed the largest and fastest increase in hydrophilicity. In terms of surface chemistry, both exhibited increases in ketone moieties. S-SMCs also exhibited increases in ether moieties which can be attributed to ring opening. In terms of Pickering emulsions, UV-O treatment can aid in the tuning of Pickering emulsion properties and the formation of stable emulsions for both pollen and S-SMCs. However, care must be taken with S-SMCs as excessive UV-O exposure results in particles which are too hydrophilic. In terms of particle/cell adhesion properties, UV-O treatment of pollen particles enhances cell adhesion while UV-O treatment of S-SMCs allow for control of cell growth structures. Additionally, UV-O treatment of S-SMCs has the added advantage of facilitating optimal oil loading.

Overall, pollen, S-SMCs and UV-O surface functionalization are shown to provide a highly versatile technology for utilization in a wide range of potential applications and there is much potential for the further exploration of the systems highlighted in this study.

6.2. Recommendations for Future Work

6.2.1. Defatting of Bee Pollen Granules to Obtain Pollen Particles

This study has shown that it is possible to obtain pollen particles of high purity and monodispersity from *C. sinensis bee pollen granules*. In the future, there could be a series of studies investigating the general applicability of the defatting protocol used. This is because pollen from different sources might have different lipid or wax coating and might require different solvents. Solvents that are more polar or less polar. One could also compare the efficiencies defatting for various defatting solvents.

6.2.2. Pickering Emulsions

This thesis has only presented on Pickering emulsions made with IPM, however there are many other oil phases available in the market. Studies can be done investigating if the behaviours of the system are exhibited for systems using oils like soy bean oil, sunflower oil or other non-polar hydrocarbons. Comprehensive stability tests can also be done in the future on how other parameters affects the Pickering emulsions made with pollen particles and S-SMCs. Other parameters that can be varied are temperature, shear forces, addition of another compound, flow, etc. This is because in real world application in food science industry or personal care require would have more than three components and the production or the manufacturing process may be done at varying temperatures or shear forces.

6.2.3. Cocoa Butter Oil Loading into S-SMCs

This project has shown that super hydrophilic S-SMCs is a mean to obtain ideal fully oil loaded microparticles. In the future, one can explore the possibility of recycling or reusing the microparticles that are not filled. One can also investigate the release of such systems as well as the mechanism of release. Exploration of the encapsulation of non-polar compounds with cocoa butter as a carrier phase or solvent can also be done. Stability of the encapsulated hard oil over various storage conditions or durations can also be investigated.

6.2.4. Cell Adhesion Study

This thesis has shown that UV-O enhance the binding affinity of pollen and liver hepatocarcinoma cells. This study also showed that both untreated and UV-O treated S- SMCs are shown to exhibit favorable particle/cell interaction dynamics, with untreated S-SMCs offering a means to obtain a novel particle/cell spheroid system, and UV-O treated S-SMCs resulting a more disperse particle/cell network system with increased particle/cell interaction. In the future, studies could be done to investigate if observations in this study is applicable towards other cell types such as muscle cells, skin cells or stem cells.

Comprehensive studies into the functionality of the cells incubated with (untreated and treated) pollen particles and S-SMCs can also be done.

There are a range of specific examples which would make for promising future studies depending on applications. Compound loaded S-SMCs or pollen particles could be used as a form of topical cream for cosmetics purposes or as a natural wound healing cream or gel, therefore it would be appropriate to study cell lines such as HEKn (Human Epithelial Keratinocytes) and HSkMC (Human Skeletal Muscle Cells). Compound loaded S-SMCs or pollen particles could be used for oral drug delivery or nutraceutical applications, therefore it would be appropriate to study cell lines such as KMU-CS12 (Human Gastric Cells). Another possible cell type would be skin cancer cells because the studies conducted were done using hepatocarcinoma cells and suggested potential benefits to developing topical formulations for treating cancers.

APPENDIX

Chapter 4 Appendix

Figure S1. Pictorial diagram of defatting of *Camellia sinensis* bee pollen: (a) Pre-defat, sonication, and mesh filtration, (a.i.) *C. sinensis* bee pollen granules, (a.ii.) defat in acetone, (a.iii.) vacuum filtration, (a.iv.) bath-sonication of pollen in water, (a.v.) nylon mesh filtration to remove large contaminants; (b) Vacuum filtration and washing, (b.i.) vacuum filtration, (b.ii.) transfer pollen mass to beaker for washing, (b.iii.) rinsing of vacuum filter, (b.iv.) pollen washing, (b.v.) transfer to round-bottom flasks with acetone; and (c) Defatting and drying, (c.i.) defat separately in acetone and diethyl ether, (c.ii.) vacuum filtration, (c.iii.) partial drying during vacuum filtration, (c.iv.) disperse pollen clumps in drying dishes, (c.v.) prepare for drying.

Figure S2. Images of bee-collected *Camellia sinensis* pollen defatting process: (a) Stereo optical microscope images and scanning electron microscope images of natural bee-collected pollen granules; and (b) Stereo optical microscope images of defatted pollen particles.

Figure S3. Micromeritic properties of defatted pollen particles: particle distributions of diameter, circularity, and aspect ratio, and representative optical microscope images of discrete pollen particles.

Figure S4. Scanning electron microscope (SEM) images of particle distributions, single particles, and particle surface morphology for untreated (0 min) pollen and ultraviolet-ozone (UV-O) treated (15, 30, 60, 120 min) pollen particles.

Figure S5. Wide scan XPS-spectra of untreated (0 min) and ultraviolet-ozone (UV-O) treated (15, 30, 60, 120 min) pollen particles.

Figure S6. Peak fitting of narrow scan XPS data for chemical binding distributions of carbon (C1s) and oxygen (O1s) peaks for untreated (0 min) and ultraviolet-ozone (UV-O) treated (120 min) pollen particles.

a.

b.

Figure S7. ATR-FTIR analysis for untreated (0 min) and ultraviolet-ozone (UV-O) treated (15, 30, 60, 120 min) pollen particles: (a) Sets of six ATR-FTIR spectra for each of untreated and UV-O treated pollen particles, highlighting key peaks of interest; (b) Peak-height ratio shifts of untreated and UV-O treated pollen for major peaks of interest.

Figure S8. Histogram data of particle cluster diameter for untreated (0 min) and ultraviolet-ozone (UV-O) treated (120 min) pollen particles: (a) Full set of histogram data; and (b) Reduced normalized frequency range to highlight trends for 90-150 μm and 150+ μm particle cluster diameter ranges.

Figure S9. Optical microscope images of representative particle clusters for each particle cluster size distribution range of 30-50, 50-90, 90-150, and 150+ μm , with particle cluster examples in increasing cluster size for each size distribution range.

Figure S10. Images of pollen particle stabilized Pickering emulsions with untreated (0 min) and ultraviolet-ozone (UV-O) treated (15, 120 min) pollen particles.

Figure S11. Optical microscope images of pollen particle stabilized Pickering emulsions with untreated (0 min) and ultraviolet-ozone (UV-O) treated (15, 120 min) pollen particles: (a) Pickering emulsions at day 0 incorporating a hydrophobic dye, Nile red, indicating an oil in water emulsion system; and (b) Pickering emulsions at day 7, without hydrophobic dye.

Figure S12. Confocal laser scanning microscopy (CLSM) z-stack images of pollen particle stabilized Pickering emulsions incorporating a hydrophobic dye, Nile red, with untreated (0 min) and ultraviolet-ozone (UV-O) treated (15, 120 min) pollen particles. Scale bars: 100 μm .

Figure S13. Confocal laser scanning microscopy (CLSM) 3D z-stack images of untreated (0 min) and ultraviolet-ozone (UV-O) treated (120 min) pollen particles adhered to Huh 7.5, hepatocarcinoma cells, with additional cell-only images. Scale bars: 100 μm .

Figure S14. Confocal laser scanning microscopy (CLSM) z-stack images of untreated (0 min) and ultraviolet-ozone (UV-O) treated (120 min) pollen particles adhered to Huh 7.5, hepatocarcinoma cells. Scale bars: 100 μm .

Figure S15. Scanning electron microscopy (SEM) images of ultraviolet-ozone (UV-O) treated (120 min) pollen particles adhered to Huh 7.5, hepatocarcinoma cells: (a) Top view images of pollen/cell adhesion; (b) Side-view images of pollen/cell adhesion; and (c) Top view images of pollen/cell adhesion.

Chapter 5 Appendix

	Carbon (%)	Hydrogen (%)	Nitrogen (%)	Protein Content (%)
Defatted Pollen	50.29 ± 0.14	7.87 ± 0.03	9.84 ± 0.04	61.48 ± 0.26
1 h S-SMCs	58.70 ± 0.15	8.10 ± 0.04	1.87 ± 0.02	11.70 ± 0.13
3 h S-SMCs	60.75 ± 0.20	8.17 ± 0.04	0.63 ± 0.00	3.94 ± 0.03
5 h S-SMCs	62.32 ± 0.09	8.34 ± 0.02	0.54 ± 0.05	3.37 ± 0.32
10 h S-SMCs	62.54 ± 0.10	6.93 ± 0.03	0.47 ± 0.01	2.96 ± 0.03

Figure S1. Protein content based on elemental analysis of *C. sinensis* pollen before and after acid extraction of sporopollenin sporoderm microcapsules (S-SMCs)

Figure S2. Micromeritic properties of *C. sinensis* pollen before and after acid extraction of sporopollenin sporoderm microcapsules (S-SMCs): particle distributions of diameter, circularity, and aspect ratio, and representative optical microscope images of discrete particles.

Figure S3. Morphological observations of *C. sinensis* pollen before and after acid extraction of sporopollenin sporoderm microcapsules (S-SMCs): (a) SEM images of pollen and S-SMCs with cross-sections highlighting effective removal of cytoplasmic contents with acid extraction; and (b) CLSM images of pollen and 3 h extracted S-SMCs with cross-sections highlighting effective removal of cytoplasmic contents with acid extraction.

Figure S4. Scanning electron microscope (SEM) images of particle distributions, single particles, and particle surface morphology for untreated (0 min) pollen and ultraviolet-ozone (UV-O) treated (1, 5, 15, 30 min) sporopollenin sporoderm microcapsules (S-SMCs).

Figure S5. XPS surface chemistry analysis of untreated (0 min) and ultraviolet-ozone (UV-O) treated (30 min) sporopollenin sporoderm microcapsules (S-SMCs): (a) wide scan XPS spectra; and (b) peak fitting of narrow scan XPS data for chemical binding distributions of carbon (C1s) and oxygen (O1s) peaks for untreated (0 min) and UV-O treated (30 min) S-SMCs.

Figure S6. ATR-FTIR surface chemistry analysis of untreated (0 min) and ultraviolet-ozone (UV-O) treated (1, 5, 15, 30 min) sporopollenin sporoderm microcapsules (S-SMCs): (a) ATR-FTIR spectra; and (b) difference analysis of ATR-FTIR spectra for UV-O treated S-SMCs relative to untreated S-SMCs.

Figure S7. ATR-FTIR analysis for untreated (0 min) and ultraviolet-ozone (UV-O) treated (15, 30, 60, 120 min) pollen particles: (a) Sets of six ATR-FTIR spectra for each of untreated and UV-O treated pollen particles, highlighting key peaks of interest; (b) Peak-height ratio shifts of untreated and UV-O treated pollen for major peaks of interest.

Figure S8. CLSM z-stack images of sporopollenin sporoderm microcapsule (S-SMC) stabilized Pickering emulsions incorporating a hydrophobic dye, Nile red, with untreated (0 min) and ultraviolet-ozone (UV-O) treated (1, 30 min) S-SMCs.

Figure S9. Stereomicroscope images of sporopollenin sporoderm microcapsule (S-SMC) particle stabilized Pickering emulsions with untreated (0 min) and ultraviolet-ozone (UV-O) treated (1, 30 min) S-SMCs.

Figure S10. CLSM images of homogenized oil/water system with sporopollenin sporoderm microcapsules (S-SMCs) and oil-loaded floating S-SMCs from untreated (0 min) and ultraviolet-ozone (UV-O) treated (30 min) oil/water systems.

Figure S11. SEM images of dried oil-loaded sporopollenin sporoderm microcapsules (S-SMCs) highlighting clean particle surface and representative oil loading for untreated (0 min) and ultraviolet-ozone (UV-O) treated (30 min) S-SMCs.

Figure S12. CLSM DIC images of sporopollenin sporoderm microcapsules (S-SMCs) and cells for untreated (0 min) and ultraviolet-ozone (UV-O) treated (30 min) S-SMC systems.

Figure S13. CLSM z-stack images of sporopollenin sporoderm microcapsules (S-SMCs) and cells for untreated (0 min) and ultraviolet-ozone (UV-O) treated (30 min) S-SMC systems. Scale bars: 100 μm .

Figure S14. CLSM images of sporopollenin sporoderm microcapsule (S-SMC) and cell distributions and coverage areas for untreated (0 min) and ultraviolet-ozone (UV-O) treated (30 min) S-SMC systems.

Figure S15. CLSM images of representative sporopollenin sporoderm microcapsule (S-SMC) / cell spheroid and network structures for untreated (0 min) and ultraviolet-ozone (UV-O) treated (30 min) S-SMC systems.

Figure S16. SEM images of representative sporopollenin sporoderm microcapsule (S-SMC) / cell spheroid and network structures for untreated (0 min) and ultraviolet-ozone (UV-O) treated (30 min) S-SMC systems.