THE UNIVERSITY OF HULL

Sporopollenin Exines as a Novel Drug Delivery System

Being a Thesis submitted for the Degree of

Doctor of Philosophy

In the University of Hull

By

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July 2007

Acknowledgments

I would like to thank the following people who have helped me throughout the course of my research:

L. Madden, J. Halder and A. Sinclair at The University of Hull for their scientific expertise that has helped me considerably.

Mike Humphrey, Gerry James, Linda Dallman and their team at Pfizer, Sandwich, for their collaboration and hospitality during my visits to Kent.

I would like to say a huge thank you to my family and friends for their support, both financially and emotionally and also my boyfriend, Benjamin Smith, for his support, tolerance and words of wisdom.

Finally, I dedicate all the work I do to my Grandma, Ivy Blackwell, without whom I wouldn't be where I am today.

In loving memory of my Grandma,

Ivy Blackwell

Contents

Abbreviations

A. trifida*: *Ambrosia trifida

C. vulgaris*: *Chlorella vulgaris

CHN : Carbon, Hydrogen, Nitrogen Combustion Elemental Analysis

DCPIP : Dichlorophenolindophenol dye

DDS : Drug Delivery System

DMF : Dimethyl Formamide

DMSO : Dimethyl Sulfoxide

EDTA : Ethylene Diamine Tetra Acetic acid

FACS : Fluorescent Activated Cell Sorter

FAE : Follicle-Associated Epithelium

FT-IR : Fourier Transform Infra-Red

GI : Gastrointestinal

HPLC : High Performance Liquid Chromatography

IR : Infra-Red

L. clavatum*: *Lycopodium clavatum

LM : Light Microscopy

M Cells: Microfold/ Membranous Cells

4-MMNO.H_2O : 4-MethylMorpholine-*N*-Oxide Monohydrate

NMR: Nuclear Magnetic Resonance

P_2O_5: Phosphorous Pentoxide

PBS: Phosphate-Buffered Saline

PGA: Poly Glycolic Acid

PGLA: Poly Glycolic Lactic Acid

PLA: Poly Lactic Acid

RIA: Radio Immunoassay

SEM: Scanning Electron Microscopy

SGN: Simulated Gastric Fluid (Non-peptide)

UV-Vis: Ultra Violet Visible

SIF: Simulated Intestinal Fluid

SPOS: Solid Phase Organic Synthesis

TEM: Transmission Electron Microscopy

Abstract

Microcapsules are fast becoming the most successful delivery systems for the oral delivery of drugs and food additives. Since many drugs are proteins and are destroyed in acidic conditions, microcapsules offer protection against the harsh environment of the gastrointestinal (GI) tract. Although the use of microcapsules achieves controlled release of the inner material, many synthetically designed microcapsules lack consistency in their size and morphology.

The outer coat (exine) of plant pollen grains and spores is composed of the material sporopollenin. Sporopollenin exines (25μm diameter) were extracted from *Lycopodium clavatum* and were investigated as a novel drug delivery system that was inexpensive, non-toxic, from a renewable source, and exhibited a large internal cavity for loading of hydrophobic and hydrophilic substances. They showed many advantages over conventional microcapsules, including their constant chemical structure and size within a species, and their ability to offer UV and air-oxidation protection.

Previous studies have shown that particles such as pollen, spores and starch migrate into the bloodstream following ingestion by a process termed 'Persorption'[1-3]. Such findings intrigued many researchers but the phenomenon has not been unanimously accepted[4-6]. This research is a body of evidence giving unequivocal confirmation that spores of *Lycopodium clavatum* and their emptied exines were absorbed into the bloodstream of man to the same extent following oral ingestion, with a maximum of 10% (± 2%) of the dose recovered just 15-30 minutes after ingestion. These findings resolve the debate between researchers in support of persorption[3,7,8] and those

against[4-6], whom doubted the transport of particles of micron size into the bloodstream, but did not disprove such a phenomenon.

An extensive study was undertaken to investigate the effect of factors such as gender, age, quantity and the method of ingestion on the rate and extent of exine absorption into the bloodstream. In a preliminary *in vivo* experiment the successful delivery of fish oil into the bloodstream via sporopollenin exines was illustrated.

A major breakthrough has formed the foundations of this research. Although sporopollenin exhibits incredible stability to organic and inorganic solvents[9], in contrast, this research has shown that exines degrade very rapidly in blood plasma both *in vitro* and *in vivo*. *In vitro* experiments were conducted in an attempt to characterise the specific mechanism responsible for exine degradation. Progression of work has provided much evidence that the conversion of plasminogen to the enzyme plasmin is either partially or wholly responsible for the characteristic degradation of sporopollenin in the blood.

Further investigations showed that it was possible to load a sufficient quantity of substances into sporopollenin exines, such as human growth hormone (hGH), Enfurvitide (an antiretroviral agent used in the treatment of AIDS) and Cyclosporine (an antifungal agent). Their successful release from exines into different media *in vitro* and *in vivo* (carried out in Beagle dogs) was shown.

These *in vivo* experiments highlighted the need for extra protection of the drug from the GI tract and additional coatings were applied to sporopollenin exines, including a soluble form of sporopollenin. Exines with coatings were assessed to ensure they were still able to degrade in blood and release the encapsulated substance.

Current results are highly indicative that sporopollenin could become a practicable oral delivery system for molecules that are otherwise problematic to administer, such as protein drugs that degrade rapidly in acidic conditions.

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1. Introduction

1.1 Pollen, spores and sporopollenin

Non-seed bearing plants, for example ferns and mosses, produce spores as part of the process of reproduction[1]. Pollen grains are the male reproductive structure of angiosperms (flowering plants) and gymnosperms (woody plants that produce seeds not encased in an ovary, for example conifers). Spores and pollen grains can vary in size from approximately 1μm (*Bacillus subtilis*) to 250μm *(Cuburbita* pumpkin).

Both pollen and spores are made up of a bilayer (Figure 1.1.1) consisting of an intine (inner layer) and an exine (outer cell wall). The intine of the cell consists mainly of cellulose and pectin and the exine consists of sporopollenin.

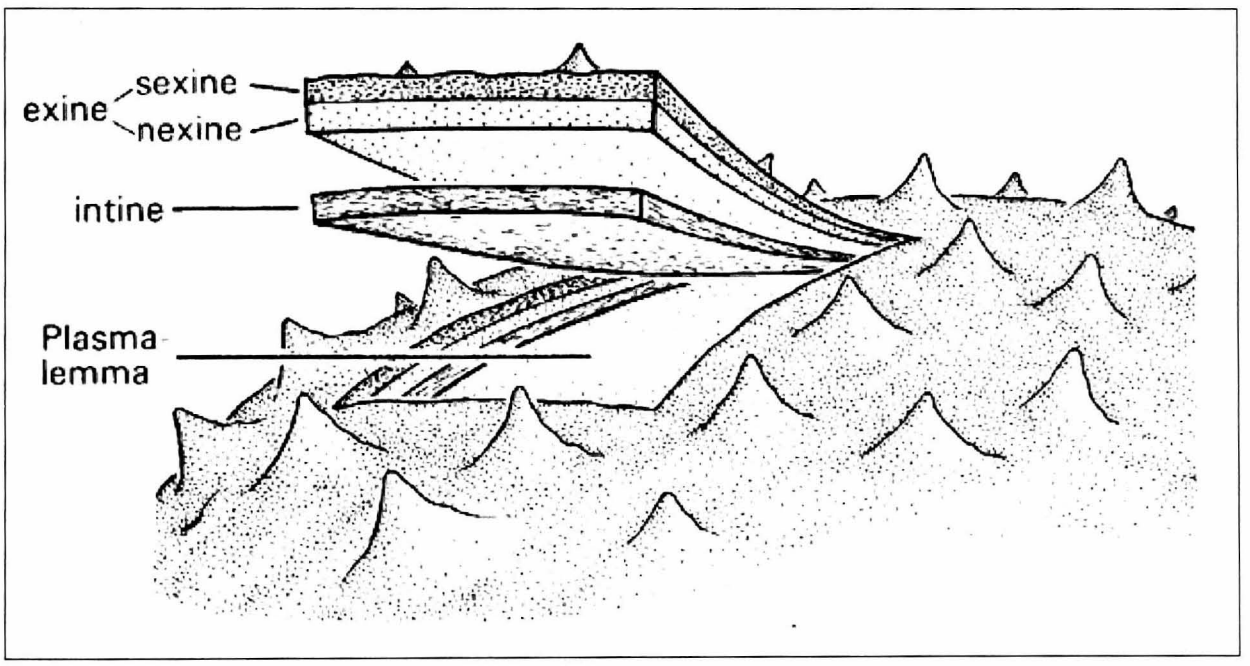


Figure 1.1.1: Terminology of the pollen grain exine[2]. The exine is composed of the nexine and sexine. The intine consists of cellulose and the exine is composed of sporopollenin.

The word 'sporopollenin' was coined from the combined efforts of researchers John (1814) and Braconnot (1829)[3], whom termed the inert exine of the pollen wall "pollenin". Early work by Zetzsche and Huggler in 1928[4] resulted in the term "sporonin" to describe the resistant material of the exine; hence the name "sporopollenin" was devised[5].

1.2 The chemical nature of sporopollenin

The exact chemical structure of sporopollenin has not yet been established due to its extreme resistance to most chemical treatments. Sporopollenin is however, believed to be a highly unsaturated oxidative polymer of carotenoids, with phenols, methyl, hydroxyl and carbonyl groups present as integral components[3]. There is little or no nitrogen present and sporopollenin is believed to have similar structural features, despite type or age and formed biochemically by an enzyme-controlled oxidative reaction involving carotenoid polymerisation[6,3].

Zetzsche et al revealed sporopollenin to be a highly unsaturated polymer containing hydroxyl groups and C-Methyl groups[4]. They presented evidence of the unsaturation of sporopollenin as it was shown to undergo bromination when reacted with bromine in carbon tetrachloride, forming bromosporopollenin with approximately 50% bromine content.

Although sporopollenin is an extremely resilient material it can be degraded into simple soluble substances by oxidative chemical attack. Sporopollenin was degraded into a mixture of simple dicarboxylic acids (C_3-C_6) and longer chain monocarboxylic acids using ozone and hydrogen peroxide[7,8].

Solid-state ^{13}C-NMR spectroscopy of sporopollenin from different varieties of pollen and spores produced many characteristic peaks, which were similar for all varieties of sporopollenin, suggesting that they have similar carbon skeletal structures[8-10].

The following table (Figure 1.1.1) summarises the peaks obtained by Hemsley et al[8-10].

^{13}C-NMR signal (ppm)	**Structural information provided**
15-20	(CH_3) and (CH_2) groups present
15-40	Cluster of peaks
100-110	Unsaturated carbons present (=CH_2)
120-130	Aromatic groups present
140	Phenolic groups present
170	(-COOH) or (–COOR) groups present

Figure 1.2.1: Table showing the solid state ^{13}C-NMR peaks obtained from sporopollenin[8-10].

Other spectroscopic methods used to investigate the chemical composition of sporopollenin include ^{1}H-NMR spectroscopy of sporopollenins dissolved in 2-aminoethanol[11] UV-Vis absorption spectroscopy[12] and IR spectroscopy[6]. Results from such studies suggest that sporopollenin consists only of carbon, hydrogen and oxygen.

A summary of the results obtained from IR spectra by Shaw et al[7] is given below:

Peak (cm^{-1})	Observations and Interpretations.
1100	A broad peak is indicative of ether groups within the molecule.
1600 – 1500	Absorption peaks in this region suggest the presence of phenolic groups.
1710 – 1640	A broad peak is evident of C=O groups. However, no absorption at $1740cm^{-1}$ indicates the absence of ester groups.
2930 – 2860	Strong, sharp peaks at $2930cm^{-1}$ and $2860cm^{-1}$ indicate the presence of CH_2 group.
3400	A strong, broad peak at $3400cm^{-1}$ suggests the presence of hydroxyl groups within sporopollenin.

Figure 1.2.2: Characteristic peaks obtained from the IR spectra of sporopollenins[7].

The empirical formula of sporopollenin derived from *Lycopodium clavatum* (club moss) in particular is based on a C_{90} unit, composed of carbon, hydrogen and oxygen; $C_{90}H_{144}O_{27}$. Sporopollenin in general is noted to have the chemical formula: $C_{90}H_{130-158}O_{24-44}$[7]. There exists a difficulty when analysing the structure of sporopollenin, since the products formed are a function of the hydrolytic method used to obtain it and may not necessarily represent natural molecular components of 'sporopollenin'.

A suggested structure of sporopollenin is given below, however it is unlikely to represent the definitive structure of sporopollenin, as the structure suggested is only based on a C_{24} unit.

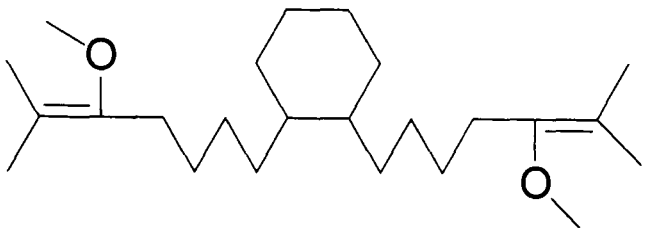


Figure 1.2.3 A suggested structure of sporopollenin[13]

L. clavatum spores have an average diameter of 28μm and a shell thickness of 1-2μm. The extracted exines have an average diameter of 25 μm. The sub-structure of *L. clavatum* spores is thought to be made up of multi-helical units[14] (Figure 1.1.4), the larger helical units appear to comprise two further helical sub-levels, suggesting that the exines of sporopollenin may be hollow once extracted from the parent spore, and subsequently, able to be filled with a substance.

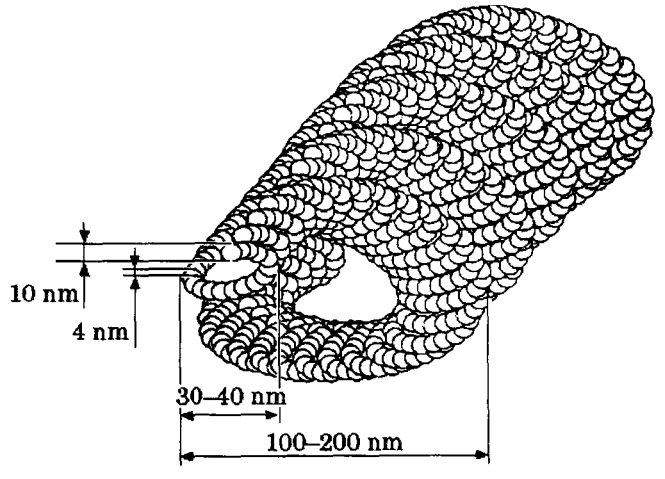


Figure 1.2.4: An interpretation of an image obtained from the exine of *L. clavatum*, showing multi-helical units[15].

1.3 The unique resistance of sporopollenin

Sporopollenin exhibits incredible resistance to certain elements and was described by Faegri and Iverson in 1964 as "...one of the most extraordinarily resistant materials known in the organic world."[16] The resistance of sporopollenin to both chemical and biological decay has been demonstrated by the discovery of individual pollen grains

and microspores in sedimentary rock over 500 million years old and also in Precambrian rocks dated up to about 3.5 x10^9 years[17,18,19]. Brooks and Shaw have also suggested that the insoluble matter found in some meteorites resembles modern sporopollenin[17,20].

Sporopollenin can withstand most chemical reagents, such as strong acid and hot alkali and can also withstand temperatures up to 250°C[3]. However, it can be degraded by prolonged exposure to oxidising agents[6] (for example ozone). Solubilisation of many different types of sporopollenin has previously been achieved using:

- 40% chromic acid[21]
- Sulphuric acid and hydrogen peroxide[5]
- Acidified sodium hypochlorite[22]
- Fused potassium hydroxide[23]
- 2-aminoethanol[24]
- 4-Methylmorpholine N-oxide monohydrate (4-MMNO.H_2O)[25-28]

Also, an insect, *Pharbitis*, was reported to contain an enzyme(s) in its intestine that is secreted into the exine after pollination and is capable of dissolving it[29].

1.4 Isolation of sporopollenin exines from pollen and spores

There are numerous methods detailed in the literature for the isolation of sporopollenin from modern pollen grains and plant spores. Some methods involve harsh treatments with strong acids and bases at high temperatures, which is believed to alter the structure of sporopollenin[30,31].

1.4.1 Treatment with alkali and phosphoric acid

This method was first reported by Zetzsche et al to remove the sporoplasm and inner intine (cellulose) of the pollen grain/spore[3,4,6]. It involves treatment of the pollen/spores with organic solvents followed by a hot alkali treatment and treatment with warm orthophosphoric acid (85%) for 6 days.

More recently, Shaw et al[32] reported that an additional treatment with H_2SO_4 (80%) was required to completely remove the cellulose.

1.4.2. Acetolysis

Erdtman first introduced Acetolysis in 1960[33]. The method involves treatment of pollen/spores with a mixture of 9:1 acetic anhydride and concentrated H_2SO_4. This method has been used and adapted by many researchers[8-10,12,34].

1.4.3. 4-Methylmorpholine-*N*-Oxide

Sporopollenin has been isolated from pollen using 4-Methylmorpholine-*N*-Oxide monohydrate (4-MMNO.H_2O). This method involves suspending the spores in molten 4-MMNO.H_2O at 75°C[27,28] or 70°C[28,35] to release the contents of the pollen/spore (known as sporoplasts)

1.4.4 Enzymes

A number of enzymes have been used to isolate sporopollenin. Examples include Mazerozym R10 and Cellulase Onozuka R10 that were used to degrade the intine of pollen wall from *Torreya California Torr.*[11] In a separate study, pine pollen was also treated with these enzymes to produce a form of sporopollenin that was free of contaminants (such as the intine)[36].

Sporopollenin was isolated from *Tulipa* and *Cucurbita* sources using other hydrolytic enzymes including protease, lipase, esterase, cellulase and amylase enzymes[37,38].

1.4.5 HF

Dominguez developed a method to isolate sporopollenin from pollen and spores by treatment with anhydrous HF in pyridine for 5 hours at 40°C[39].

1.5 Applications of pollen grains, spores and sporopollenin

L. clavatum spores have had a variety of uses for centuries. Their hydrophobic nature has allowed them to be used as dusting powders to prevent pills sticking together and external dusting powders for various skin diseases and wounds, such as stopping nose bleeds by inhalation[40,41]. *L. clavatum* spores are still used to coat tablets and have been described as 'conventional pharmaceutical excipients and additives'[42].

Pollen and spores have been noted to have therapeutic effects when used for the treatment of chronic prostasis, digestive disorders and irritability of the bladder[43]. They have also been used as explosives in fireworks and artificial lightning since they are highly flammable[44,45]. Other uses of *L. clavatum* include their addition to powders, soaps and shampoos[42].

Sporopollenin has been investigated and successfully used in peptide synthesis[46,47], as an ion and ligand exchange material and has been functionalised for use in Solid Phase Organic Synthesis (SPOS)[48].

Sporopollenin is readily available and inexpensive, since the parent pollen and spores can be purchased from many different suppliers. The table below shows the cost and availability of *L. clavatum* spores.

Supplier	Price (1Kg Quantity)
G. Baldwin & Co (UK)	£42 (63 Euro)
Tbrewala International (Nepal)	£10 (15 Euro)
Unikem (Denmark)	£42 (63 Euro)
Cedar Vale (USA)	£25 (37 Euro)
Fluka (UK catalog)	£90 (134 Euro)
Post Apple Scientific (USA)	£60 (89 Euro)

Figure 1.5.1: A table showing the commercial availability of parent pollen grains of *L. clavatum* and their prices.

Other examples of applications of pollens include *Ambrosia trifida* and Rye grass pollens. *A. trifida* pollen is harvested commercially and manufactured into pharmaceutical preparations for the treatment of allergies[49] and Rye grass seeds have been cooked and used as a cereal[50].

1.6 The Need for a Novel Drug Delivery System (DDS)

There are currently many different ways to ensure that drug molecules are administered to the target organ. These are outlined below.

- **Transdermal:** the drug diffuses through pores in the skin, e.g. Estradiol given to treat post-menopausal symptoms.
- **Respiratory:** the drug is delivered directly to the lungs, e.g. Asthma inhalers.
- **Intranasal:** the drug is absorbed through the nasal mucosa, e.g. Aerosol-based nasal spray.
- **Needleless injections:** the drug is delivered transdermally without breaking the skin.

- **Injections:** the drug in liquid form is administered directly through the skin into subcutaneous, intramuscular or intravenous compartments.
- **Peroral (tablet):** the drug is swallowed.

The peroral delivery of drugs has many advantages over other routes of administration, since it avoids the pain and discomfort associated with injections and the occurrence of infections by the use of needles. In comparison with other drug delivery methods, oral formulations are generally less expensive to produce, as they do not need to be prepared under such sterile conditions and do not require a device for delivery[51]. Trained personnel are not required to carry out oral drug delivery, ensuring a low cost of administration. Oral drug delivery also avoids the risk of introducing microbes into the patient, which can occur through injections.

The rapid growth of the biotechnology industry has resulted in the development of numerous drugs. Many of these drugs are proteins, such as hormones and vaccines, and are very sensitive to environmental conditions, for example the acidic nature of the GI tract[52]. Due to their high molecular weight they are poorly absorbed via the oral route, and the encapsulation of protein drugs is essential to protect against harsh environments that rapidly lead to their decomposition[53]. Also, many drugs have short half-lives in the blood stream and need to be administered in high doses frequently to maintain therapeutic efficacy, which can lead to side effects and varying system toxicity.

The peroral route is the most intensively investigated route of drug administration and encapsulation within a DDS has the potential to reduce the system toxicity, protect

particles from degradation in the digestive tract, provide controlled-release properties and could even mask an unpleasant taste. The use of physical encapsulation techniques is an attractive drug delivery option for a variety of drugs and nutraceuticals and is estimated to become a $74.5 billion industry in 2008[54].

1.7 DDSs Currently Available

Various DDSs are currently under development, including carriers made from soluble polymers, microparticles made of insoluble or biodegradable natural and synthetic polymers, microcapsules, cells, liposomes and micells. This research focuses on the development of microcapsules.

The term 'microcapsule' refers to particles that are between 50nm and 2mm in diameter that contain a core material different to that surrounding it.[55] The term "microsphere" refers to the empty particle.

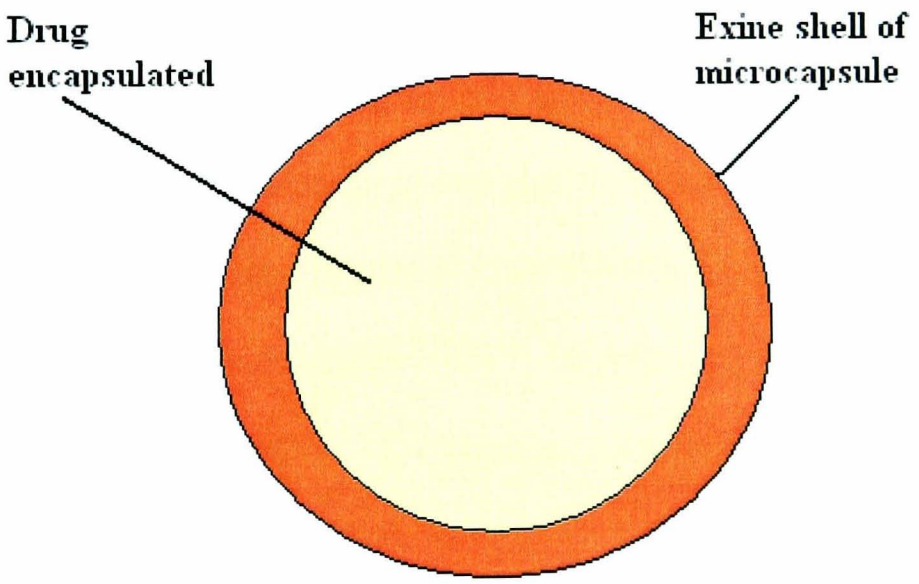


Figure 1.7: An example of a 'microcapsule'

A number of synthetic and natural polymers have been investigated for their use in drug delivery. Many synthetically designed microcapsules have been developed to

deliver drugs orally[56,57], but lack consistency in their size and morphology. Examples of biodegradable, synthetic polymers include polymers of lactic acid (PLA), glycolic acid (PGA) and their co-polymers (PLGA). Limitations with such microspheres as drug delivery vehicles are the low encapsulation efficiency (typically 61-65% for PLGA microspheres and up to 81% for alginate-chitosan-PLGA microspheres)[58]. Other disadvantages include the instability of the encapsulated drug and its incomplete release from the microsphere[59-61].

In addition there have been many attempts to manufacture microcapsules from 'natural' materials. This research focuses on naturally occurring microcapsules and some of these already under investigation are detailed below.

1.7.1 Yeast

There is current interest in the use of yeast cell walls as delivery vehicles for drugs[62]. One company in particular (MICAP) have patented microencapsulation technology that covers the use of bacteria as a capsule as well as yeast and is already licensed for flavours with Firmenich SA, and drug delivery applications with SkyePharma plc.

MICAP "melt" the yeast membrane to increase its permeability and create a space inside the cell, resulting in the formation of two barriers, an outer cell wall composed of carbohydrates and an inner fatty cell wall[63]. The resulting yeast microcapsules can store ingredients until their release on membranes such as the tongue or stomach and they offer protection of encapsulated flavours to rigorous processes, such as high temperature treatments, boiling and evaporation.

The result of this technology is that the encapsulated flavours do not break down before reaching the mouth, where they are released and gives a more intense flavour to foods such as sauces, chewing gum, cakes and soft drinks[64]. The design of this

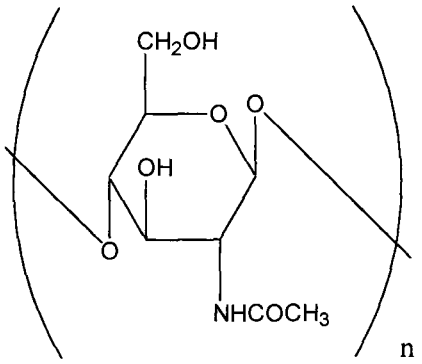
microcapsule would not offer any advantage to the delivery of drugs into the bloodstream, since the vehicle would release the drug in the mouth and would still be exposed to the harsh environment of the GI tract.

1.7.2 Liposomes

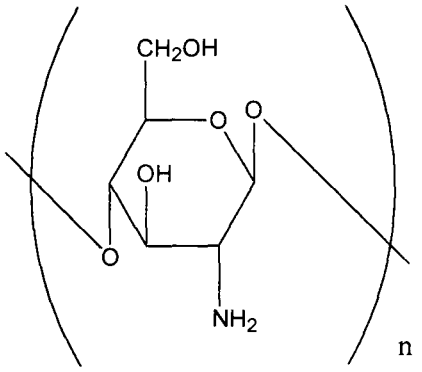
Liposomes are formed from concentric spherical phospholipid bilayers with an inner compartment[65] that has been used for encapsulating drugs[66,67]. Applications of liposomes are limited by their instability and poor permeability to polar molecules[68].

1.7.3 Chitin/Chitosan

Chitin is the second most abundant natural amino polysaccharide to cellulose[69].



Chitin, a polymer of *N*-acetyl glucosamine



Chitosan, a polymer of glucosamine

Chitosan is prepared by the deacetylation of chitin and is widely used as a drug delivery vehicle[70]. Examples of its use include the development of chitin/PLGA blend microspheres to deliver anti-cancer drugs[71] and hydrogels composed of a biodegradable chitosan backbone[72]. Chitosan is soluble in dilute acidic solutions[73] and this would be a major disadvantage for the use of chitosan as a drug delivery vehicle, since it would simply degrade when exposed to the harsh environment of the GI tract.

A study investigating the use of chitosan films for the delivery of Diazepam reported no additional advantages over the delivery of this drug when compared to commercially available Diazepam tablets[73].

1.7.4 *Chlorella*

Chlorella is a popular food supplement in Japan and is marketed as a nutritional supplement in the United States. Sporopollenin has been isolated from *C. vulgaris* and is available from RTC GmbH, marketed as 'CAELICO'[74].

The most common methods for preparing microcapsules for drug delivery are by spray-drying, spray cooling, covering in a fluid bed, extrusion, centrifugal extrusion, and co-crystallisation. The main materials currently used are dextrins, modified starch, vegetable gums, hydrocolloids and lipids and emulsifiers[75].

1.8 Particle uptake by the GI tract

It is important to consider what happens to the sporopollenin exine and encapsulated drug once it has been ingested; the movement of particles from the administration site to the systemic circulation. The particular mechanism by which molecules of different sizes are absorbed into the blood and lymph systems still remains unknown, although there have been many suggestions, such as persorption[76-78], endocytosis through enterocytes[79-81] and uptake through macrophages[82].

To better understand the process by which molecules are transported, it was necessary to explore the basic physiology associated with the human gastrointestinal (GI) tract.

1.8.1 Anatomy of the GI tract and drug absorption

Following oral administration of a drug or microcapsule, the first section of the GI tract that the drug comes into contact with is the oral cavity, but it does not remain in this area long enough for any absorption to occur, unless the drug or microcapsule was administered under the tongue (sublingual) or through the cheek (buccal)[83]. If a drug is administered sublingually absorption occurs via a different mechanism, avoiding the liver and the drug is absorbed directly into the systemic circulation[84].

The GI tract extends from the mouth to the anus and its 4 main regions are the oesophagus, stomach, small intestine and large intestine (colon). Absorption does not normally occur in the oesophagus since the oral transport mechanism is so rapid, and the drug passes into the stomach. The stomach contains hydrochloric acid, gastrin, pepsinogen and mucus. The high acidic environment (pH 1-2) of the stomach protects the body from bacteria and is also required to activate the pepsinogen. Pepsinogen is the enzyme responsible for the digestion and breakdown of proteins to peptides and is denatured above pH 5.

The next area of contact is the small intestine, the first part of which is the duodenum. The duodenum is responsible for the absorption of the majority of the drug within this 6-inch area of the GI tract. The inner lining of the absorptive part of the small intestine is greatly folded and is composed of many finger-like projections called villi, which provide a large surface area and efficient absorption of drugs. The pH of this area is around 7-8 and maximum absorption takes place.

When the drug or microcapsule reaches the duodenum it is subjected to digestive agents from the liver, pancreas and the wall of the small intestine that secrete bile, pancreatic enzymes and trypsinogen (by the action of enterokinase) respectively.

The Figure 1.8.1 below illustrates the structure of the wall of the mammalian small intestine:

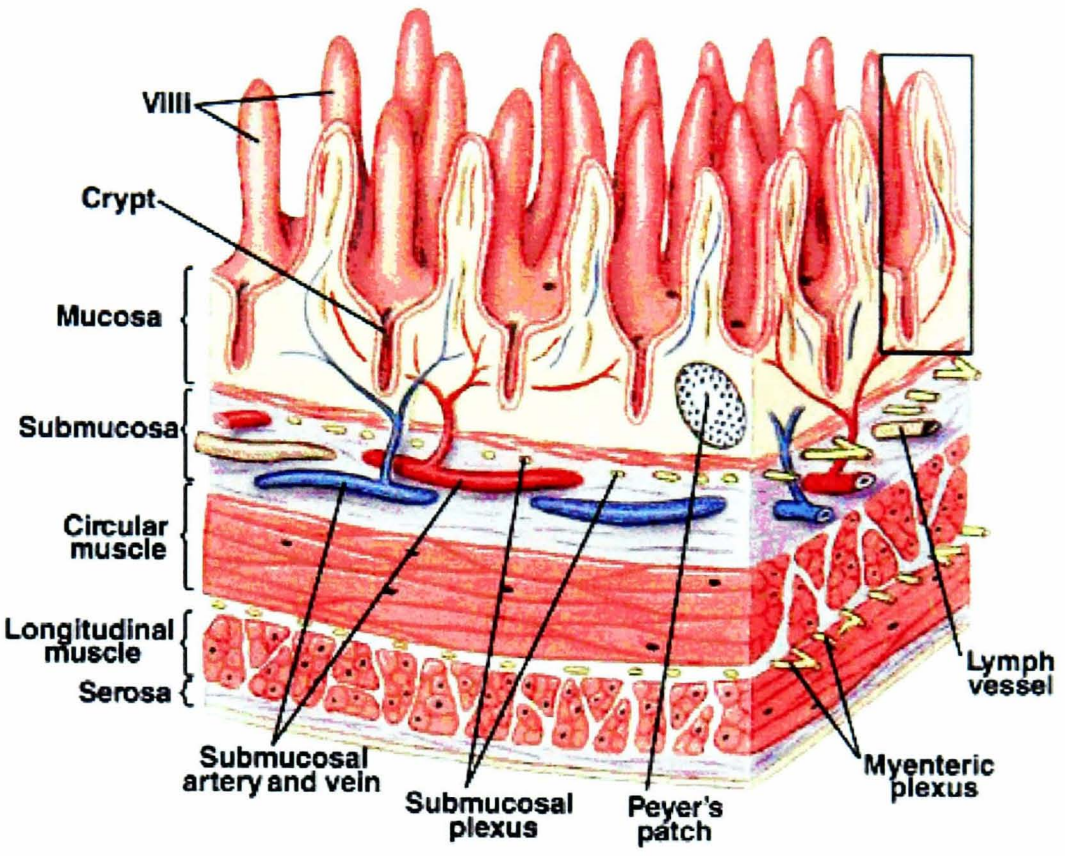


Figure 1.8.1: Diagram of the composition of the wall of the small intestine[85].

Further along the small intestine are the jejunum and ileum that lack the high surface area of the previous duodenum, so drug absorption here is minimal, although the ileum contains areas of lymphoid tissue close to the epithelial surface known as Peyer's patches, which are noted for their absorptive ability.

The final part of the digestive tract is the large intestine, which encompasses the colon and rectum. Drug absorption seldom occurs in this region.

1.8.2 Barriers to microparticles

Initially, a substance must traverse several semi-permeable cell membranes before it can reach the blood or lymph systems. These membranes act as biological barriers and

selectively inhibit the passage of certain molecules[83]. The membranes are composed of a bimolecular lipid matrix, containing mainly cholesterol and phospholipids. The lipids within the membrane provide stability and determine its permeability. Drugs and particulates must also be resistant to the chemical environment of the GI tract in order to reach the systemic circulation.

A summary of the well-documented barriers to drug absorption, which may apply to sporopollenin exines loaded with active ingredients, is given below:

- **Gastrointestinal pH**

  The pH of the duodenum is 4.9-6.4, the jejunum has a pH of 4.4-6.5 and the pH is higher in the ileum 6.5-8.0[86].

- **Luminal enzymes**

  There are pepsins and proteases in the lumen, along with lipases and amylases in the small intestine from the pancreas.

- **GI membrane**

  This lipid bilayer membrane separates the lumen of the stomach and intestines from the systemic circulation.

There are a number of possible routes of particle uptake from the small intestine. These include:

- **Transcellular active transport**, i.e. movement through the cell. For example by carrier-mediated active transport or facilitated diffusion through the cell.
- **Transcellular passive transport,** i.e. movement through the cell without aided transport.
- **Paracellular transport**, i.e. movement between adjacent cells.

The major barrier through which molecules can either be absorbed or excreted is the intestinal epithelium, of which there is approximately $200m^2$. The cells within the intestinal epithelial layer are called enterocytes and have a membrane that consists of phospholipids, as discussed above, thus restricting the movement of substances from the lumen to the bloodstream. This restriction arises because the membrane is almost impermeable to large and hydrophilic substances, such as proteins[87].

Particles are believed to be absorbed at either the level of the Peyer's patches or through the enterocyte layer and such absorption has previously been fully documented[88].

As discussed earlier, the lymphoid tissue within the gut is organised into aggregates of lymphoid follicles, known as Peyer's patches (see Figure 1.8.2 below). They induce a secretory immune response to any antigens that have been ingested. These follicles are overlaid by the follicle-associated epithelium (FAE), which comprises enterocytes and specialised phagocytic cells known as M (microfold or membranous) cells that absorb particulates into the dome-shaped structures within the Peyer's patches. M cells are known for delivering the ingested macromolecules and microorganisms to the lymphoid cells underneath[89].

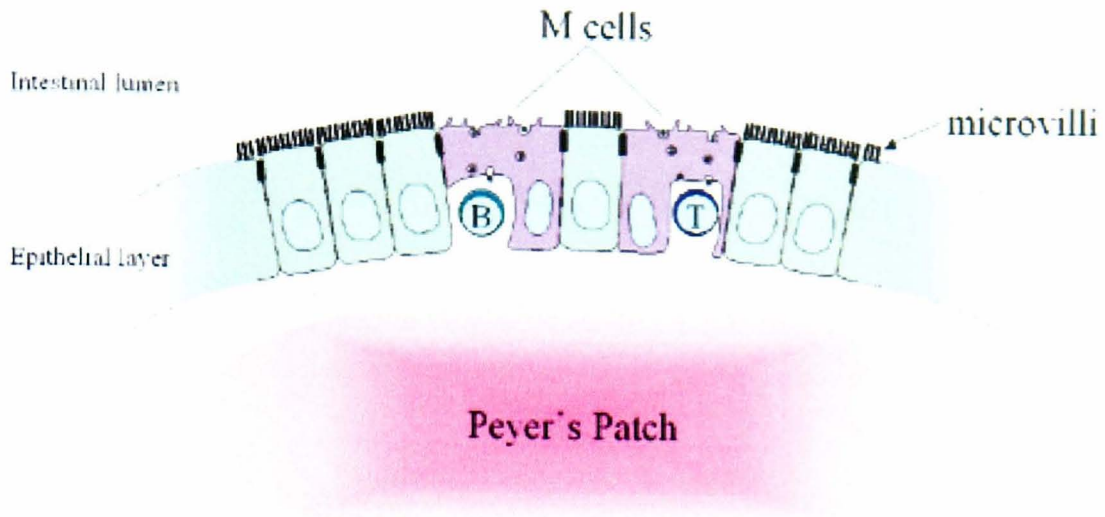


Figure 1.8.2: Diagram illustrating the location of M cells in between intestinal epithelial cells[89].

There are many factors that affect the rate and extent of uptake, including particle size, surface and intestinal target, dose of substance and administration vehicle, animal species, age and whether or not food has been taken before ingestion.

Since drugs must be transported across the epithelial cell membranes within the GI tract, differences in the luminal pH along the GI tract, surface area per luminal volume, blood perfusion, the presence of bile and mucus and the nature of the epithelial membranes all have an additional effect on the rate of uptake.

1.8.3: Previous research into the GI uptake of microparticles

1.8.3.1 Paracellular Transport (Persorption)

Paracellular transport involves the movement of substances around cells, through the gaps (tight junctions) in an epithelial cells layer[83]. Persorption refers to the process in which relatively large particles (such as 25μm spores and pollen grains) pass through the intact wall of the intestine and enter the blood or lymphatic system. Volkheimer defined this phenomenon in 1968 as "the passage of large, solid, non-deformable food

particles in the micron size range through the epithelial cell layer of the intestinal tract"[76] and reported the persorption of particles ranging from 5-150μm in diameter.

The earliest recorded study of persorption was in 1843. Gustave Herbst claimed to have discovered starch grains in the blood and lymph systems of a dog 3 hours after feeding it a suspension of starch. Persorption was thus initially termed the 'Herbst Effect'[90].

Hirsch (1906) later detected such starch grains in the blood and urine of dogs and also in human urine after the ingestion of flour[91]. Then in the 1960s Volkheimer et al[76] repeated Herbst's experiments with dogs. According to Volkheimer and his colleagues, spore-like particles were found after ingestion in arterial and venous blood, lymph, bile, breast milk, cerebrospinal fluid, urine, and were even transported across the placenta into foetal blood. He also noted that particles as large as 150μm were able to persorb and the effects of atropine, barbiturics and neostigmine on the rate of persorption are fully documented[92,93].

Whilst there is no contradicting data available with respect to the extent of the persorption of particles after repeated experiments on the same test subject, there are however conflicting theories regarding the mechanism involved in particle uptake within the literature.

Despite Volkheimer suggesting that persorption of particles occurred between intestinal epithelial cells, Weiner[94] later explained that the tight junctions between intestinal cells normally form an effective barrier to macromolecules in the 0.005μm range and stated "it is doubtful that 30μm particles gain entry via this route".

Weiner concluded his report "it is unlikely that large particles normally present in foods... would be able to penetrate the intestinal barrier by any routes mentioned due to their large size. The persorption of particles greater than 20μm...seems highly unlikely in the light of the more recent studies using more sensitive techniques"[94].

The absorption of larger, hydrophilic particles was believed to absorb by the paracellular pathway, although again this pathway had been investigated and appeared to be limited to particles below 11Å[95].

In 1973 Wilson et al[96] studied *Poa Pratensis* pollen of approximately 25μm, which had been radioactively labelled with ^{99m}Tc. Once ingested, the pollen was found to accumulate in the lower part of the stomach, being only sparingly found in the lungs and respiratory tract.

Linskens and Jorde found in 1974 that when 150 grams of *Lycopodium* or rye pollen were ingested, between six and ten thousand grains were absorbed into the bloodstream, a maximum quantity only 30 minutes after ingestion[97]. This document failed to quantify the percentage of the administered dose absorbed, but they found that the particles had been partially destroyed, a process that continued with time.

This report concluded that the experiments involving *Lycopodium* and rye pollen "confirmed the existence in man of a persorption process and a paracellular uptake of particles via the digestive tract"[97].

In another report investigating the absorption of *L. clavatum* spores and rye pollen, Linskens reported the maximum number of particles was recovered 30 minutes after ingestion[98]. The spores were described as covered with a film of serum and appeared to become smoother after 30 minutes. After approximately 90 minutes the spores

began to decay and after 120 minutes there was only debris of the particles present. It was assumed that after 120 minutes following oral ingestion, the spores had completely degraded. The following images were featured in this report:

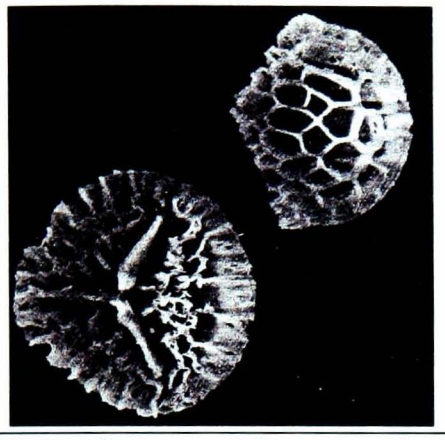
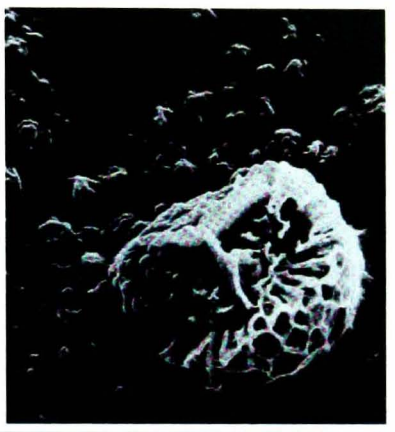
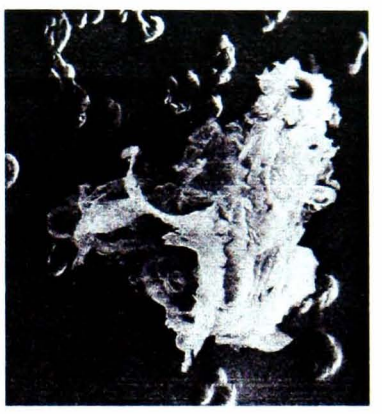
		
L. clavatum prior to oral ingestion.	30mins after oral ingestion.	90 minutes after oral ingestion.

Figure 1.8.3.1 A: Degradation of spores up to 90 minutes after oral ingestion[98].

As mentioned in the previous chapter, an experiment performed by Linskens and Jorde[98], in which pollen and spores were incubated and treated with blood *in vitro*, resulted in no decay of the spore structure. It was assumed from these results that the specific enzymes responsible for the decay of the spores were either found only within the body or were denatured when removed from the body, which is in contradiction to the results obtained within this report, as it has been shown that incubation of plasma at 37°C does indeed lead to the degradation of sporopollenin exines.

Figure 1.8.3.1 B: The persorption of large granules at the apex of the villus between enterocytes in rat jejunum[98].

G. Fabian[99] investigated the persorption of 70μm particles of cornflour and Indian ink with particles of relatively the same size, following oral ingestion. He later detected such particles in the blood only 10 minutes after administration. The transport of the particles was believed to occur subepithelially and the movement of the dye particles was traced using light microscopy[99]. These particles have also been detected in urine, bile, cerebrospinal fluid, and breast milk and are transported across the placenta into foetal blood[99].

1.8.3.2 Endocytosis through Enterocytes and Uptake through Macrophages

Weiner[94] suggested alternative routes by which macromolecules could be absorbed. These included previous accounts of particle uptake, such as endocytosis by intestinal epithelial cells (enterocytes) that eventually release their contents into the spaces between the cells. However, this route of entry was explored by Sanders and Ashworth[80] whom only found it possible for uptake of particles as large as 0.2μm.

Research investigating the absorption of intestinal ferritin in hamsters suggested that the absorption of ferritin (molecular size 0.005-0.006μm) occurred by pinocytosis (a form of endocytosis)[79]. A study by Warshaw et al[100] examining the uptake of horseradish peroxidase (molecular size 0.005μm) in the small intestine of the rat revealed that transport of horseradish peroxidase was a "pinocytosis-like process". However, since the maximum absorption occurred within the first 15 minutes of the study, passive diffusion was not ruled out as a method of transport[100].

Macrophages are cells within tissues that are formed from monocytes (leukocytes). Macrophages are phagocytes; they engulf and digest cell debris, such as pathogens, through a process known as phagocytosis (a form of endocytosis).

Uptake into the lymphoid tissue in the gut, then entry into subepithelial macrophages, was studied by LeFevre et al[101], who believe this route of entry is only effective for particles up to 7μm in diameter. In another report by the same research group the accumulation of 2μm latex particles in Peyer's patches of mice was demonstrated[102].

Wells et al hypothesised that intestinal macrophages could ingest and transport particles to mesenteric lymph nodes, as this had previously been investigated with intestinal bacteria[103]. The study revealed that segments of dog intestine were free of Peyer's patches, which indicated that the particles were not transported by this method, however, they stated that their study did not rule out the role of Peyer's patches in particle uptake. They concluded that latex particles of 1.09μm diameter were transported to the mesenteric lymph node by phagocytic cells, where they suggested "it can then enter the blood stream via the thoracic duct", but did not investigate particles in the blood[82].

The transport of horseradish peroxidase was quantified in jejunum segments with and without Peyer's patches[100] A total of 0.3% of the administered dose was recovered in jejunum tissue without patches and 2.0% was recovered in jejunum with patches, indicating that Peyer's patches play a significant role in the transport of particles.

In 1980 LeFevre et al[101] discovered that particles between 5-7μm were found in Peyer's patches, but not in the blood. Interestingly, particles of 15.8μm diameter were not found in Peyer's patches, suggesting that particles as large as this were not transported by uptake into Peyer's patches and entry into subepithelial macrophages.

A study investigating the uptake of yeast cells (*S. cerevisiae*) into the domes of Peyer's patches using *in situ* methods suggested that macrophages play only a minor role in particle transport[104]. The study involved isolation of loops from the jejunum of pigs to inject approximately $5x10^8$ yeast cells. The results showed only 1.3% of cells taken up by macrophages after 2.5h and 18.5% of the cells were detected in M cells after 1h incubation, suggesting that transport of yeast cells is "almost restricted to the M cells"[104]. After 4h 89% of the cells were found in macrophages, which were interpreted as the particles being phagocytosed and transported out of the Peyer's patch domes.

1.9 Research Aims

The difficulties in administration of drugs, such as proteins, provided the impetus for the development of a novel DDS that could deliver therapeutic levels of a drug into the body by means of a microcapsule.

1.9.1 Towards the use of sporopollenin exines as microcapsules

Oral administration of drugs, such as proteins, that are not encapsulated may undergo hepatic first pass metabolism and enzymatic degradation within the GI tract, therefore, there is a major need for the encapsulation of such drugs in order to protect them from such GI secretions and proteolytic enzymes.

Several successes have been achieved improving the bioavailability of protein drugs in different ways, such as chemical modification of the drug and co-administration of absorption enhancers and enzyme inhibitors[105,106], along with the use of polymeric delivery systems, such as swellable hydrogels[107] and enteric-coated polymers[108]. However, no single oral dosage form exists for the administration of protein drugs in general in sufficient quantity to be practicable and commercially viable.

This provided the initiative to design a delivery system that could offer the potential to deliver therapeutic levels of a drug, avoiding the damage encountered *en route* to the bloodstream, hence using a sporopollenin microcapsule.

Sporopollenin has many properties that make it suitable for use as a capsule in controlled drug delivery. It has a chemically consistent structure, despite the age or type of spore exine used. It also exhibits excellent chemical stability, as it has UV filtration properties[109] and, as mentioned earlier, has excellent physical stability due to its indestructible nature to non-oxidative chemical degradation procedures.

As previously mentioned pollen and spores are commercially available. Therefore, sporopollenin is a naturally occurring polymer that is inexpensive, easily extracted through a non-toxic route, and provides a renewable microcapsule. It is also

considered to be relatively 'elastic', since during this research the spore exines were compressed using ten tonnes of pressure into tablet form, without injury to the exine.

The non-toxicity of sporopollenin also makes it an apt material, since sporopollenin is frequently used in the confectionary industry as a filling agent and the parent pollen grains and spores can be purchased as herbal supplements from health food stores.

A. Maack[74] confirmed the non-toxicity of sporopollenin using solubilised sporopollenin derived from *C. vulgaris* in glycerol with an emulsifier. A human patch test was carried out using sporopollenin derived from *C. vulgaris.* There were no signs of toxicity in rats and ingestion of 200mg sporopollenin in rats also proved non-toxic[74].

In addition to this information, there has been no information found in the literature to suggest that ingestion of pollen and spores have any harmful or toxic effects on human or animals

The method used in this research to extract exines from the parent pollen and spores is also non-toxic, albeit aggressive, using acetone, potassium hydroxide and phosphoric acid. The extraction procedure ensures that all of the nitrogenous material within the spore or pollen grain is removed. It is this nitrogen content of pollen and spores that is believed to provoke an allergic response, for example with hay fever sufferers.

Spore and pollen particles have been shown to reach the bloodstream, following oral ingestion by a process known as 'persorption'[76]. This concept, coupled with the available space inside the emptied exines, could present a unique microcapsule technology for delivering drugs via the oral route. The microcapsules will have 0.00% nitrogen by elemental analysis, which reflects removal of allergenic proteins and so therefore will not cause an allergic reaction.

1.9.2 Limitations of previous work and application of sporopollenin exines as microcapsules

S. Kettley has focussed on the chemical attachment of drugs to sporopollenin, which has only resulted in 2mmol/g loading[48]. This would mean a very large amount of exines would need to be ingested to achieve the desired effect. With physical encapsulation, as opposed to the chemical attachment of drugs to sporopollenin, it is hypothesised that a higher mass loading will be achieved in a matter of minutes. Another beneficial aspect of encapsulation is that it is likely to mask an unpleasant taste of a substance and could help to prolong the shelf life of the encapsulated ingredient, as sporopollenin has been cited to possess UV filtration properties[109].

With chemical attachment the drug in addition to the sporopollenin would be classed as a new substance and would require vigorous tests for each different drug under investigation for the regulatory authorities of the MHRA in Europe and the FDA in America. However, with physical encapsulation of a drug, once the sporopollenin has been approved for ingestion as a vehicle, (i.e. platform technology) there would only be testing required for additional substances encapsulated.

The basis of this study was that pollen/spores are food substances and have been cited to migrate into the bloodstream and therefore could serve as a novel DDS. As mentioned previously, sporopollenin is compatible as a microcapsule. It is an inexpensive, renewable source of delivery vehicle and the raw materials can be purchased on a large scale.

1.9.3 Aims and objectives

The aim of this research was to investigate and exploit the phenomenon of the uptake of pollen, spores and their extracted sporopollenin exine particles into the bloodstream following oral ingestion for the successful delivery of drugs and nutraceuticals.

In order to determine the potential of sporopollenin for possible use in drug delivery, the following investigations were proposed:

- To determine if it was possible to load a sufficient quantity of drug into the exines and to optimise this process.
- Establishing the total number of particles in a given mass of pollen, spores or extracted exines to serve as reference values for quantification studies.
- To determine if it was possible to deliver a sufficient quantity (percentage of the dose administered) of sporopollenin exines into the circulation within a reasonable timescale. This includes optimisation of a method for quantifying the recovered particles in blood.
- To compare the rate and extent of exine absorption in human and animal models and observe any differences between individuals.
- To compare the degree of absorption of raw pollen and spores to that of the extracted exines once ingested.
- To attempt to characterise the apparent breakdown of pollen grains, spores and their extracted sporopollenin exines through *in vitro* experiments.
- To study the release of encapsulated substances into simulated gastric fluids (SGN) and blood plasma and try to achieve controlled release using additional coatings.

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2. Biological Evaluation of Sporopollenin: *In vivo* exine degradation

2.1 Introduction

Previous studies have shown that micro- and nanoparticles such as pollen, spores and starch grains are able to migrate into the bloodstream following oral ingestion[1-4]. Such findings have intrigued many researchers but the phenomenon has not been unanimously accepted[5-7] and reliable quantitative investigations into particle uptake are largely lacking.

The precise method by which microparticles are transported into the bloodstream remains ambiguous, however, several mechanisms have been suggested, such as persorption[2,8,9], endocytosis through enterocytes[6,10,11], uptake through macrophages[12] and paracellular transport[13]. The majority of research on microparticle uptake has been based on particles with diameters in the 1-2μm range[14], with the exception of Volkheimer et al[1-3,9] who reported intestinal uptake of microparticles 5-150μm in size. LeFevre et al also confirmed the possibility of 15.8μm-particle penetration of the gastrointestinal tract[7].

Throughout this report, the term 'absorption' will refer to the passage of pollen, spores and their extracted exines into the bloodstream and will not describe the site at which this process occurs or the precise method by which particles migrate, as this still remains ambiguous.

Since 'persorption' was described as a passive process[3], the extent of particle absorption was considered small and not nutritionally significant and to date there has been no effort to exploit the potential of naturally occurring spore and pollen exine particles as DDSs. However, the delivery of even low levels of potent drugs (such as

biologically active proteins and peptides that are mainly administered by injections) via this process could have important consequences. It has been reported that the extent of passive absorption may become 'appreciable' by paracellular and intracellular transport through aqueous pores and membranes, particularly via damaged mucosa[15]. The delivery of even small quantities of spore and pollen exines could have very significant impact on the level of drug administered, since there is a large space within the exine particle that could be loaded with a drug.

2.2: Results and Discussion

2.2.1 Planned *In vivo* experiments

For all of the studies Local Ethical Permission was granted from the Hull and East Yorkshire Ethics committee. The Medicine Healthcare Regulatory Authority (MHRA) considered that the pollen and the derived exines were not a medicinal product and indeed more likely to be a food.

This research was based on the transport of pollen and spore particles into the bloodstream of man following oral ingestion that had been cited in the literature[2,3,16]. It was therefore considered very likely that the extracted sporopollenin 'exine' particles, which exhibit the same structure and size as parent particles, would also be absorbed in this manner. For that reason it is anticipated that this naturally occurring polymer could have an exciting future application as a DDS and for the encapsulation of neutraceuticals for food applications.

2.2.2 Quantification of recovered particles

Several methods were explored to quantify the particles recovered in blood after ingestion. These include Fluorescent Activated Cell Sorter (FACS), Coulter counter and haemocytometery.

Several problems were encountered with the FACS method, including regular blockage of the machine, even with very dilute solutions. The method was not accurate because it was not possible to gate the detectors to a certain size limit, so many 'events' were encountered in the blank samples, making it difficult to distinguish the particles detected from blood cells. The FACS method was both time-consuming and not reproducible. Fragments were also being counted often giving an inflated result

Similar problems were also encountered using the Beckmann Coulter Counter, although it was possible to gate the detectors so that only larger particles were detected, avoiding confusion with other particles, but may have disregarded smaller fragments that were clearly of pollen/exine origin.

Haemocytometery (see Chapter 3) was the favoured counting method, since it has been described as an accurate and reliable method for quantification of particles larger than 0.5μm in a recent review[17] and has been used in previous studies[18-20]. It also enabled the particles to be positively identified due to their unique morphology, described by some as a 'natural fingerprint'[21] with 'distinguishing characteristics'[22].

The recovered particles were also positively identified by confocal microscopy due to their much-documented natural fluorescence[23], their morphological characteristics as

seen by light microscopy, accompanied by FT-IR analysis of the spores and exines before and after ingestion.

2.2.3: Transport of *Lycopodium clavatum* spores and their extracted exines into venous blood of man

A cross over study was designed to determine if both the raw spores of *L. clavatum* (25μm) and their empty exines were absorbed in man following oral ingestion and if so, to the same extent. Patients were randomised to receive the pollen or the exine by means of a labelled envelope and both the patient and the investigator were blind to the treatment given i.e. a double blind trial.

Sporopollenin exines were prepared in the manner discussed in Chapter 5 (treatment with acetone, potassium hydroxide, ethanol and phosphoric acid to leave only the exine). The parent *L. clavatum* spores (460mg ± 0.1mg) were administered orally to five human volunteers in a fasted state and the extracted exines (200mg ± 0.1mg) were administered to eight different volunteers of both sexes and ages ranging from 20 to 58 years. Both oral doses of spores and exines contained approximately 12.66×10^6 particles ($\pm 0.06 \times 10^6$ for spores and $\pm 0.07 \times 10^6$ for exines), as calculated from the calibration graph shown in Figure 2.3.1. Venous blood samples were taken at 5, 15, 30, 45 and 60 minutes after ingestion and the blood was centrifuged (3000RPM, 10 min, 4°C). The plasma (2ml) was separated from the pellet (2ml), which was lysed up to 5ml by gentle shaking with distilled water. An aliquot (10μL) of both plasma and pellet were taken for counting using a haemocytometer.

This experiment was also repeated a total of eight times on one volunteer to compare the reproducibility of the recovery of sporopollenin exines.

2.2.4 Factors affecting the rate of absorption of exines into the blood stream

Volkheimer investigated the effects of various factors on the rate of 'persorption' of starch[3]. The following table summarises these effects.

Factor investigated	Effect on the rate of 'persorption'
Age	Higher in younger subjects
Sleep	Higher during sleep
Smoking	Higher by 30% in smokers
Caffeine	Higher
Neostigmine	Higher
Metaclopramide	Higher
Papaverine	Higher
Castor oil	Higher
Polygalacturonic acid	Higher
Atropine	Lower
Barbituric acid	Lower
Tween 20 detergent added to starch	Lower

Figure 2.2.4 A brief summary of the effect of different factors on the rate of 'persorption' of starch[3].

The *in vivo* trial outlined in 2.2.3 was carried out on volunteers of different genders and ages and it was therefore possible to compare the rate of exine recovery between such individuals.

A preliminary trial was conducted to assess the effect of caffeine on the rate and extent of exine recovery in the bloodstream after ingestion. However, it was not possible to investigate the effects of sleep, smoking and the other drugs, such as Atropine, within this investigation.

2.2.5: Saturation effect with different masses of sporopollenin exines

Although much research has involved counting microparticles by tissue sectioning[24-28], researchers whom investigated particles recovered in the bloodstream after ingestion found only a small percentage of the particles. These low levels may be due to the administration of a too large dose (200g), as a 'saturation effect' has been reported during the transport of polystyrene particles (0.87μm) in rats[18] and a study administering 1-3μm latex particles to mice in smaller doses (80-95mg) resulted in a greater recovery (15%) after only 5 minutes[19]. The method by which the blood was treated prior to counting, such as leaving the blood samples at room temperature, may also have led to further 'degradation' of the particles and a lower number recovered, as explained in detail in Chapter 3.

An experiment was devised exploring the percentage of sporopollenin exines recovered in the blood after the ingestion of different doses of the same exine material. A double-blind *in vivo* study was set up with 3 volunteers who each ingested doses of 100mg, 200mg, 400mg, 600mg and 1g doses of exines (derived from *L. clavatum* 25μm) on separate occasions following fasting and the recovered particles were counted in blood using a haemocytometer.

2.2.6 Dilution effect

A study in rats revealed that the transport of polystyrene microparticles (0.87μm diameter) became dosage dependent at volumes of 0.5ml[18]. It was proposed, "The volume was sufficient to cause expansion of the GI tract lumen and so distort the lining cells"[18].

It was therefore considered that the dosage volume of sporopollenin exines (i.e. the volume of milk) could affect the percentage recovered in the bloodstream of man. As a result, an experiment was set up with three volunteers who each ingested 400mg sporopollenin exines with 15ml, 50ml, 100ml and 200ml of milk on separate occasions.

2.2.7 The effect of different dosage forms on the percentage recovery of exines in venous blood

Sporopollenin exines were administered to volunteers in different dosage forms. These included incorporation into chewy confectionary, administration via the buccal route (by swirling in the mouth without swallowing), in a tablet formulation and swallowing with water and with milk. The percentages of exines recovered in venous blood after oral ingestion via these dosage forms were evaluated to determine which method would be most suitable for maximum absorption. The dosage forms are detailed below.

2.2.7.1 Buccal absorption of exines (and chewy confectionary)

The method of transport of sporopollenin exines into the bloodstream after oral ingestion still remains unknown. It was thought that the exines might gain entry into the circulation via the mucous membranes lining the oral cavity (i.e. sublingual and buccal delivery). The buccal region has been documented to be an attractive route of administration for systemic delivery, as the mucosa has a rich blood supply and is relatively permeable[29].

Sublingual absorption involves passage of the particles through the sublingual mucosa, which is the membrane of the ventral surface of the tongue and the floor of

the mouth. Buccal absorption occurs through the buccal mucosa present in the lining of the cheek. The permeabilities of the oral mucosae decrease in the order sublingual> buccal>palatal[30].

This possible route of absorption was explored by swirling sporopollenin exines derived from *L. clavatum* (25μm) around the mouth and discarding them, taking extra care to avoid swallowing the particles. The percentage absorption of exines was evaluated by counting the number of exines recovered in venous blood over a period of one hour.

Sporopollenin exines were also prepared in chewy confectionary* (200mg per sweet). The purpose of this experiment was to determine if a higher absorption was seen with exines in confectionary compared to ingestion in milk alone. This was based on the speculation that exines might be transported into the bloodstream via buccal or sublingual routes with the particles being chewed and in contact with these areas for a longer period than when swallowing them with milk.

2.2.7.2 Tablet form of sporopollenin exines

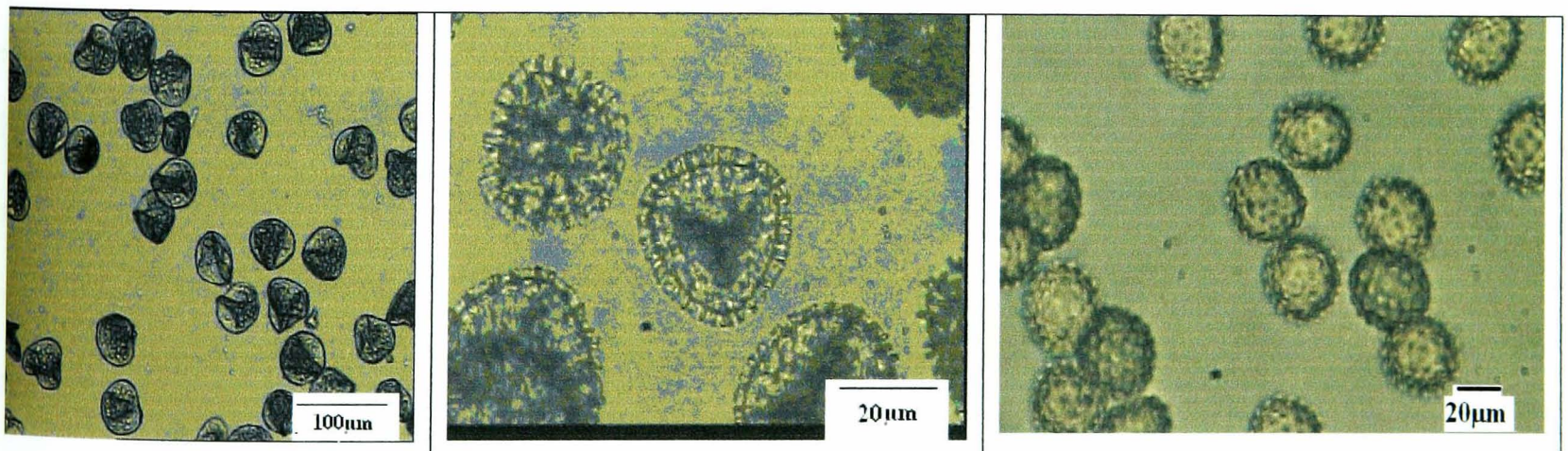
The oral route for drug delivery is convenient and usually results in high patient-compliance[31]. A popular way to administer drugs orally is using tablets. Tablets were successfully prepared from sporopollenin exines using an IR press (10 tonnes, 2min) and following *in vitro* testing to ensure the dissolution of tablets in SGN, the tablets were ingested by human volunteers. The number of exines recovered in blood was assessed over the period of one hour.

* Confectionary prepared at Nestlé PTC Laboratories, York, UK

2.2.8: Absorption of other pollens and their extracted exines

The majority of trials in this report were conducted using sporopollenin obtained from *L. clavatum* (25μm). It was considered if the size of the particle or the existence of a 'spiky' exterior as with *Lycopodium* (25μm) and *A. trifida* (15μm) influenced the rate or extent of uptake into the bloodstream. Since Volkheimer reported the 'persorption' of particles ranging in size from 5-150μm[1,3,8], the larger *Lycopodium* particles (40μm) were ingested in the same manner as the smaller particles. *A. trifida* (15μm) pollen and its extracted exines were also ingested and the percentage of particles recovered was evaluated.

Sporopollenin exines from *L. clavatum* (40μm) and *A. trifida* (15μm) were prepared using the protocols detailed in Chapter 5. Exines and pollen (400mg dose) were administered orally to one individual a total of five times for each type of exine. The percentage recovery of exines was determined by haemocytometery. The following LM images show the different exines prior to ingestion



A B C

Figure 2.2.8 LM images of 40μm *Lycopodium* exines (A) 25μm *L. clavatum* exines (B) and 15μm *A. trifida* exines (C).

2.2.9 The inter- and intra-observer error

Given that only one individual carried out the particle counting by haemocytometery, it was necessary to obtain the inter-observer error bars associated with the counting. This was achieved by taking ten separate counts from aliquots at each time point for each separate trial and each different volunteer. This revealed a typical error range within the same samples of ± 1-2% (standard deviation as determined using Microsoft Excel) and could be due to obtaining a heterogeneous blood sample and/or differences in counting technique each time.

The intra-observer error was achieved by observing the difference between haemacytometer counts on equivalent samples using identical conditions (e.g. the same equipment, centrifugation method (3000RPM, 10min, 4°C) 500mg of exines ingested in milk and timescale of 0, 5, 15, 30, 45 and 60 minutes after ingestion) by two different individuals and the maximum percentage recovery found was typically 8-10% (± 1-2%) 30 minutes after ingestion.

2.3 Results and Discussion for Chapter 2

2.3.1 Quantification

The number of particles in 1g of both spore and exine material was determined by haemocytometery. A calibration curve was plotted counting particles in different dilution solutions (1.25-100%) in aqueous ethanol using a haemocytometer.

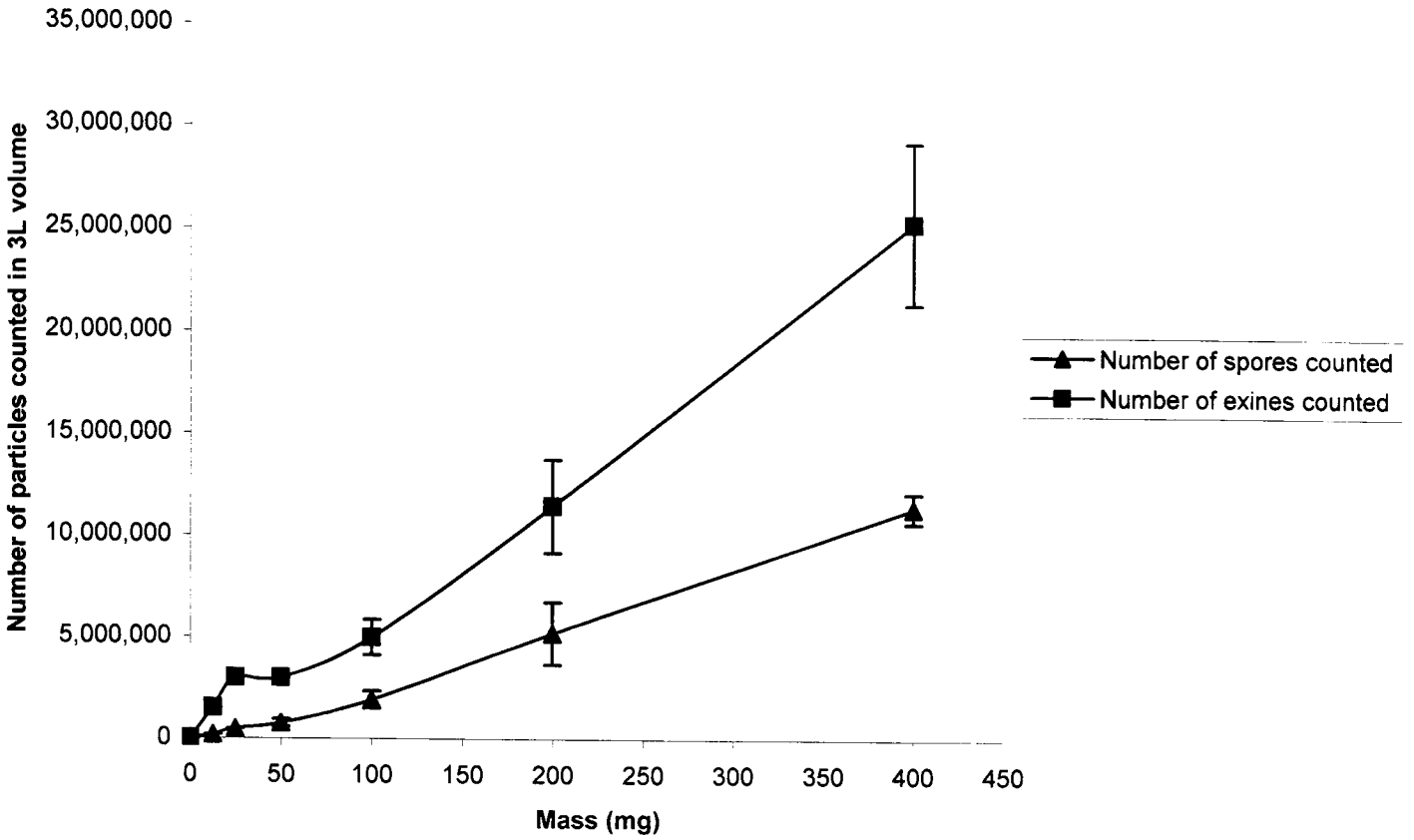


Figure 2.3.1 Calibration curve showing the number of *L. clavatum* spores and exines per gram.

The number of *L. clavatum* spores per gram was $28.65 \pm 0.72 \times 10^6$ and the number of extracted exines per gram was $63.75 \pm 2.38 \times 10^6$.

2.3.2 Transport of *L. clavatum* spores and their extracted exines into the bloodstream

A total of eight human volunteers of both sexes and ages ranging from 20 to 58 years total took part in the study. Trial volunteers ingested an oral dose suspended in milk containing approximately 12.66×10^6 particles ($\pm 0.06 \times 10^6$ for *L. clavatum* spores and $\pm 0.07 \times 10^6$ for exines) on different occasions. The particles were detected in venous blood as rapidly as 5 minutes after ingestion and the counts reached a maximum at 15-30 minutes after ingestion. The percentage recovery was based on a 3000ml circulating blood volume in man[9].

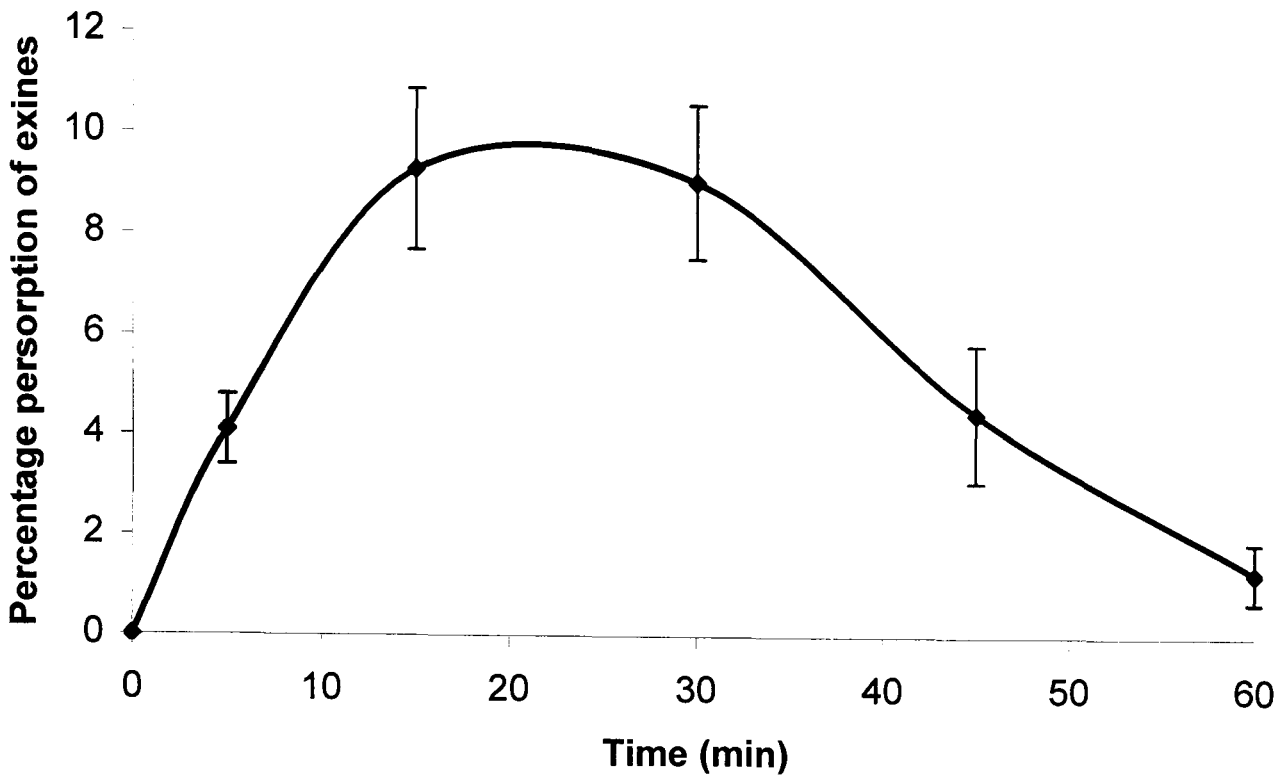


Figure 2.3.2.1 The percentage of exines recovered in blood. The data represent the mean of 8 human volunteers of both sexes ages ranging from 20-58 years.

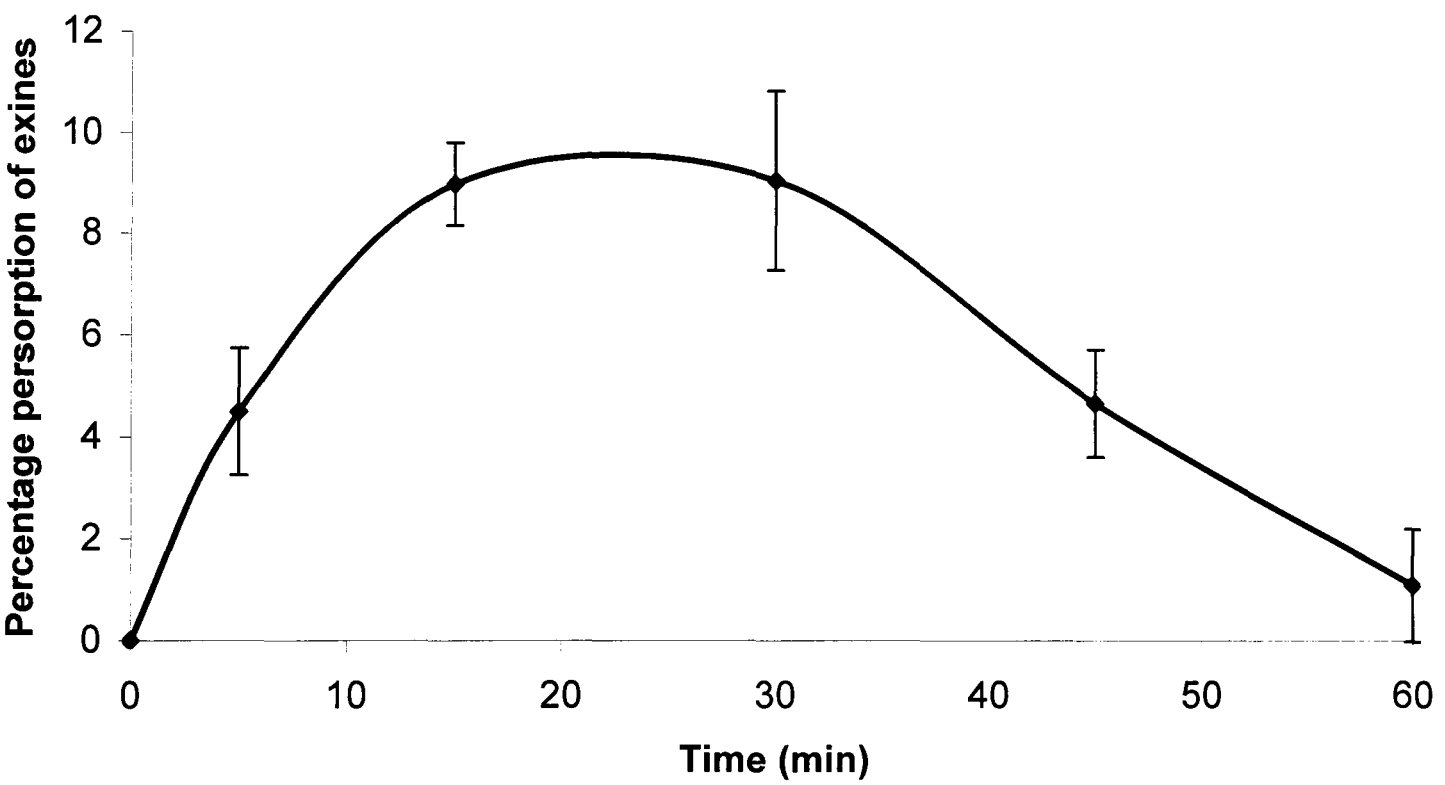


Figure 2.3.2.2 The percentage of exines recovered in blood. The data represent the mean of 8 different runs on one male volunteer aged 58 years.

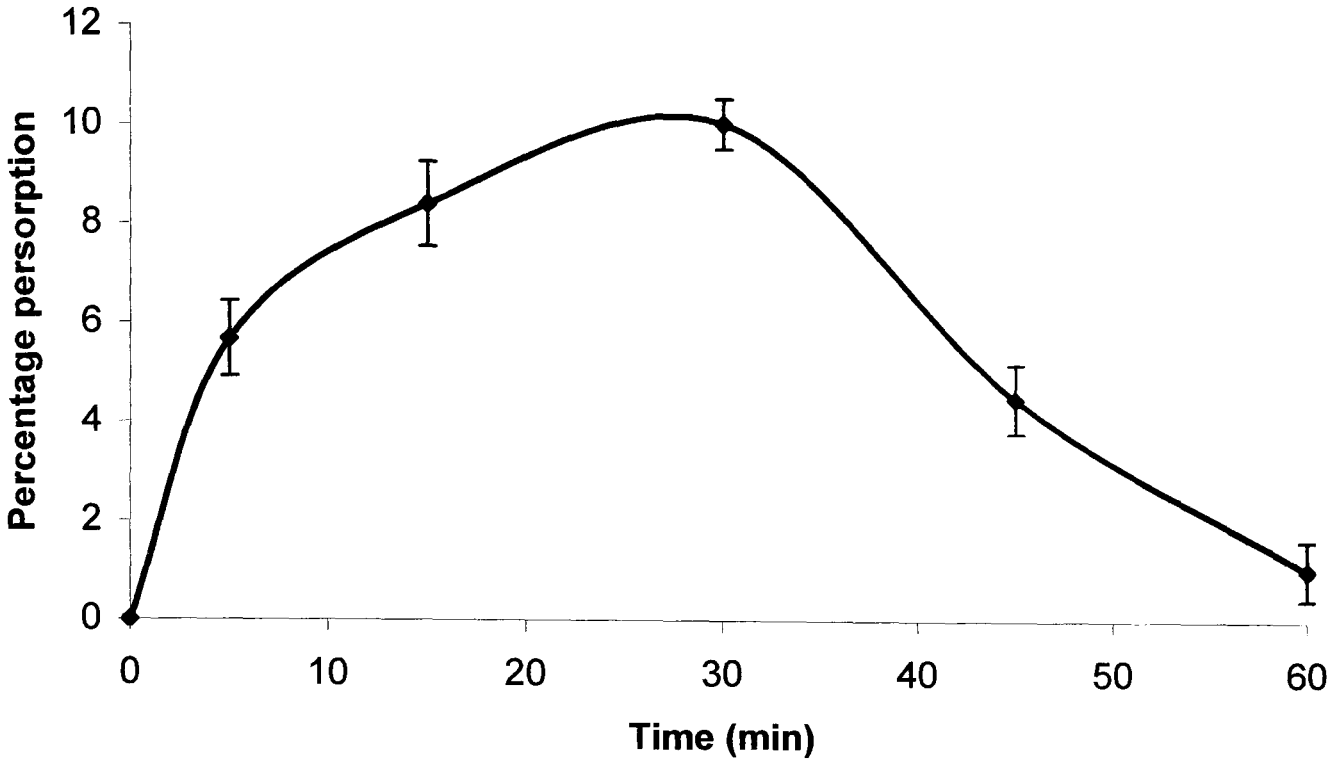


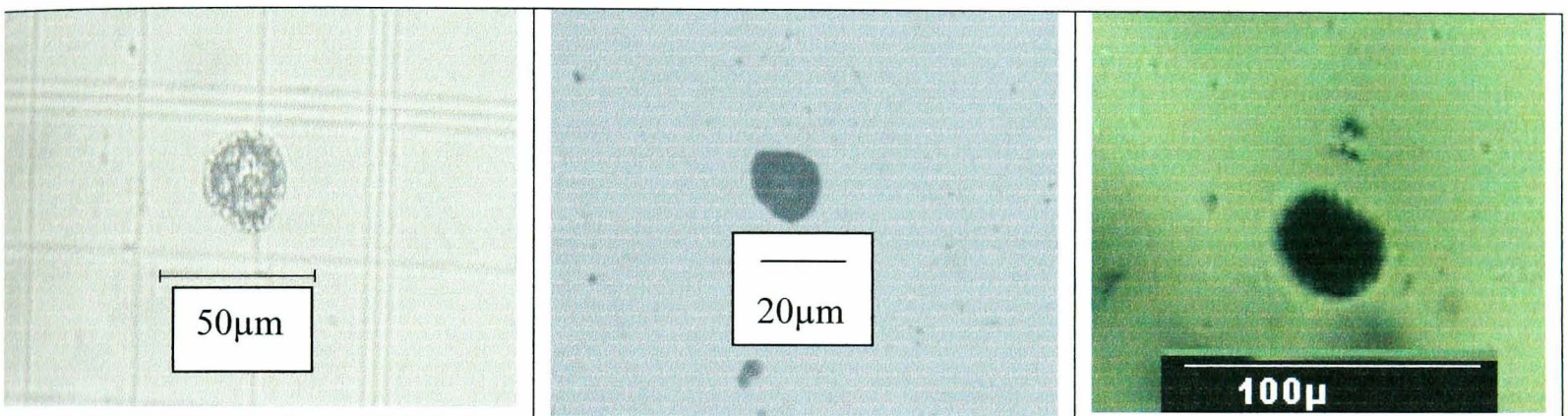
Figure 2.3.2.3 The percentage of pollen recovered in blood. The data represent the mean of 5 different runs on one male volunteer aged 58 years.

Quantification was ascertained by haemocytometery of the respective particles in venous blood taken from the arm of a volunteer at time intervals up to 1 hour following peroral administration. The recovered particles were also counted by FACS, which generally gave higher counts (typically a maximum of 20% recovery after 30 minutes) than the haemocytometer. These high counts were thought to be due to interference from blood cells, however, there were no particles counted before ingestion, which indicated that the counts could have resulted from fragments of ingested particles.

Overall it can be seen that the recovery of both pollen and their exine shells showed similar kinetic profiles. Surprisingly, both types of particles were recovered within the short time of 5 minutes following ingestion, with a maximum recovery of ca.10%

(± 2%) after 30 minutes. This maximum count after 30 minutes in the body is in agreement with results in the literature following ingestion of *L. clavatum* spores (although the quantity ingested was not stated)[32].

Relatively little difference was observed in kinetic profiles from one volunteer to another as reflected in the standard deviation bars at each time point and one volunteer being studied on five separate days.



A	B	C

Figure 2.3.2.4: An exine recovered in plasma shown on a haemocytometer (A), recovered *L. clavatum* pollen (B) and *L. clavatum* exine particles in blood plasma after 30 minutes.

Following counting, isolation of the particles was achieved by repeated centrifugation (3000RPM, 10min) and washing the resultant pellet with water to enable FTIR analysis of the resulting sediment. As shown in Figure 2.3.2.5, the sediment recovered *in vivo* produced a very similar IR spectrum to the exine particles before ingestion.

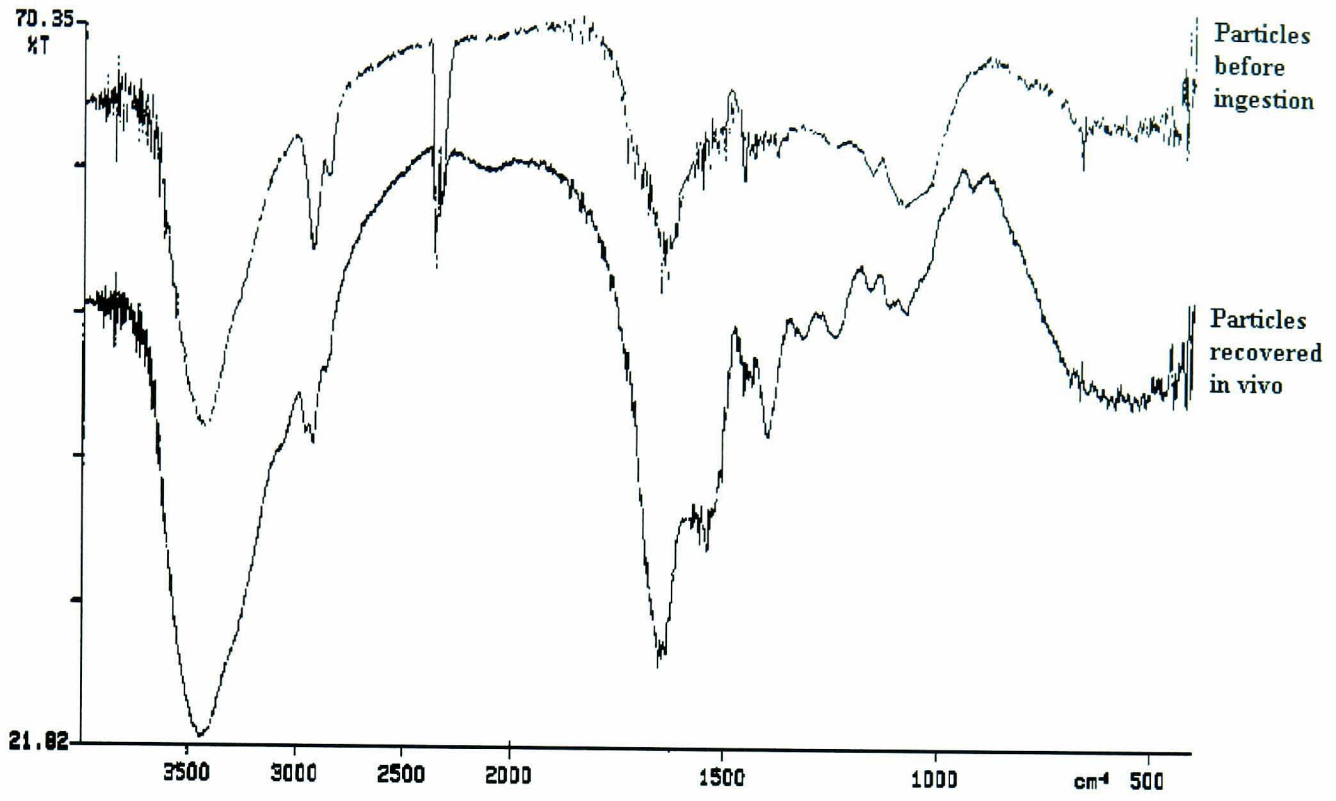


Figure 2.3.2.5: The FTIR spectra of sporopollenin exines comparable before ingestion and 15 minutes after ingestion (recovered from dried plasma).

Confocal microscopy also confirmed the presence of exine particles in the recovered blood plasma and pellet samples.

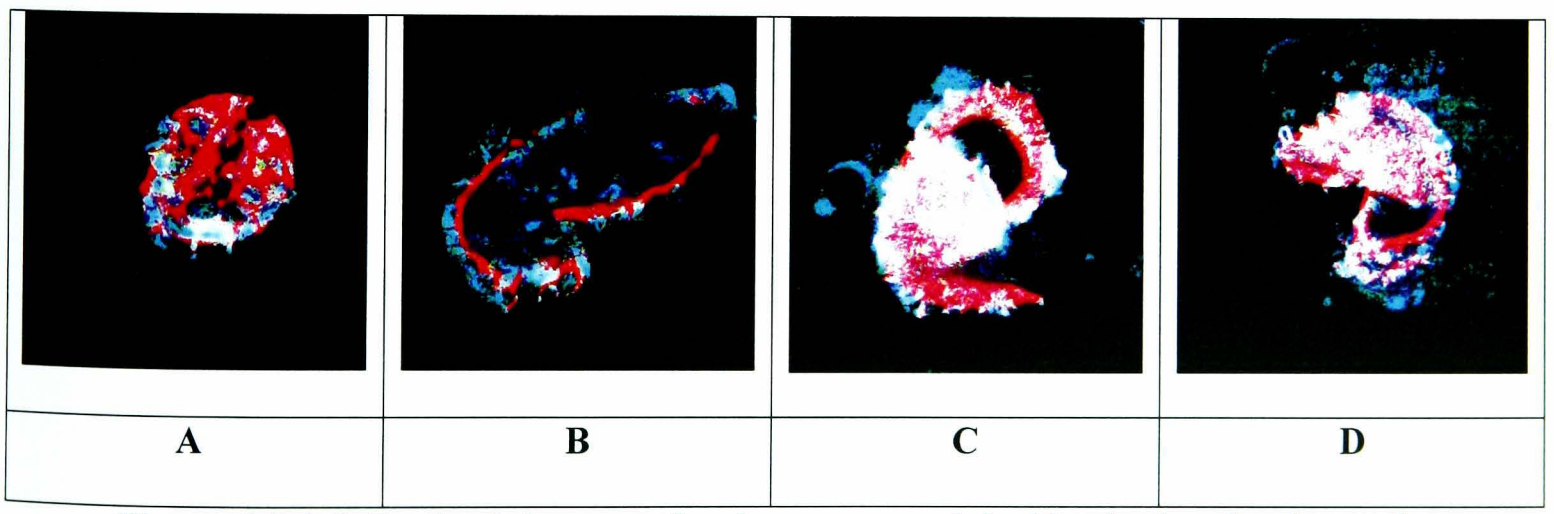


Figure 2.3.2.6 Confocal images of exines recovered in blood after 5 minutes in plasma (A), 45 minutes in plasma (B), 5 minutes in lysed blood pellet (C) and 30

minutes in lysed blood pellet (D). Confocal images were taken with the settings as described in Section 5.2.3.

Identification of exines recovered in blood was also confirmed by FACS analysis. However, the reproducibility of the FACS method was very poor considering the experiments were carried out on the same volunteer, therefore this was not pursued as a quantitative method of analysis. A typical comparison of counts obtained by FACS and haemacytometery is shown below.

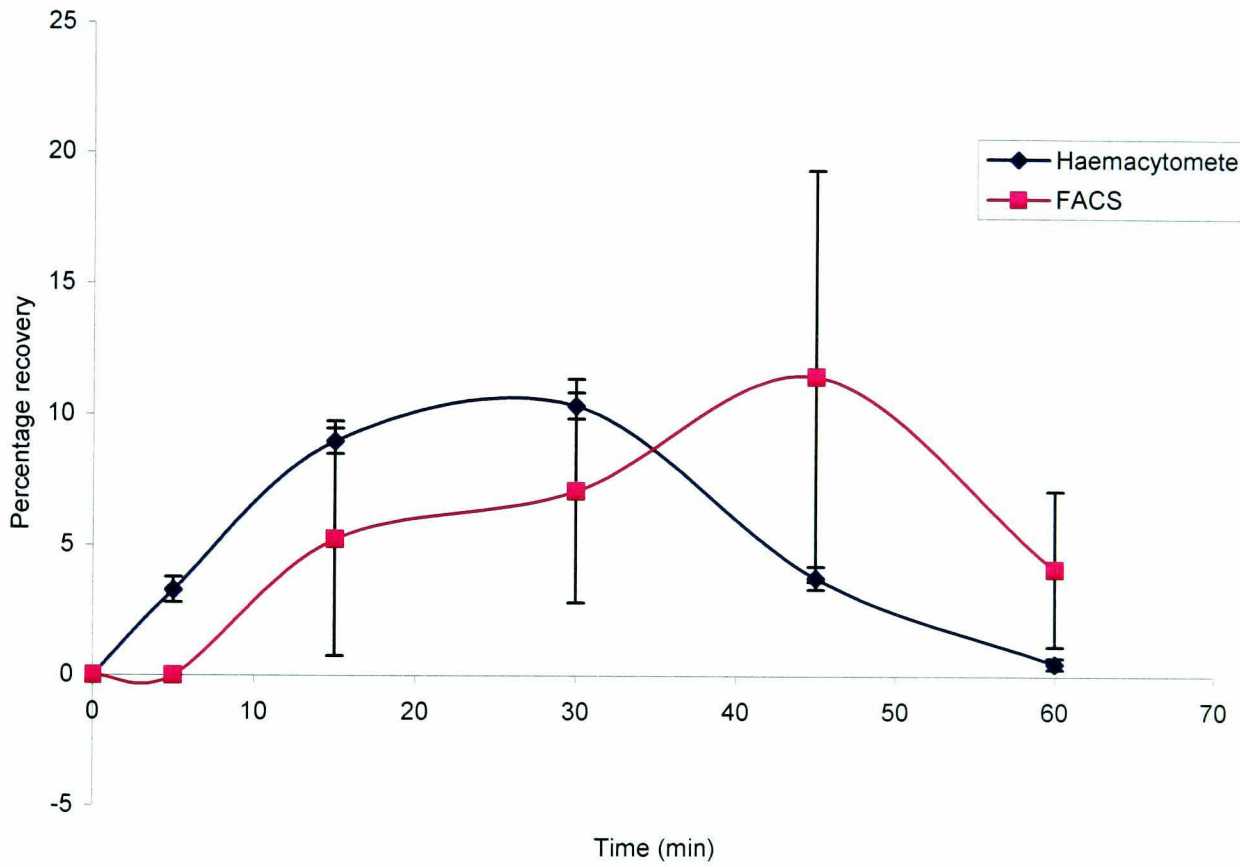


Figure 2.3.2.7 Comparison of percentage recovery of exines using haemacytometery and FACS methods.

It was promising that some particles were detected in blood by the FACS method, as none were recovered from the blood sample taken at time zero (before ingestion), which indicated that the particles detected in the other blood samples were indeed

exine particles. The FACS method provided additional evidence that exine particles were able to reach the bloodstream after oral ingestion and showed a similar profile as that demonstrated by haemacytometery, with maximum recovery after 30-45 minutes. Eyles et al[18] and Lewis et al[19] undertook two similar studies that involved administration of particles to rats and recovery of particles in the blood stream. Eyles et al investigated the transport of polystyrene latex microspheres of 0.87 ± 0.006μm diameter from the GI tract to the circulation following oral administration and Lewis et al studied the absorption of albumin microspheres of 1-3μm diameter.

The results of both research groups were in agreement with my trials using sporopollenin exines as they reported that such particles were able to penetrate the GI tract and gain entry into the circulation very rapidly; particles were seen in blood only 5 minutes after administration. The particles were also counted in the same manner as my experiments using a haemacytometer to count the recovered particles in blood. However, the model used for both studies was the rat and blood samples were taken from the tail-vein, as opposed to human trials.

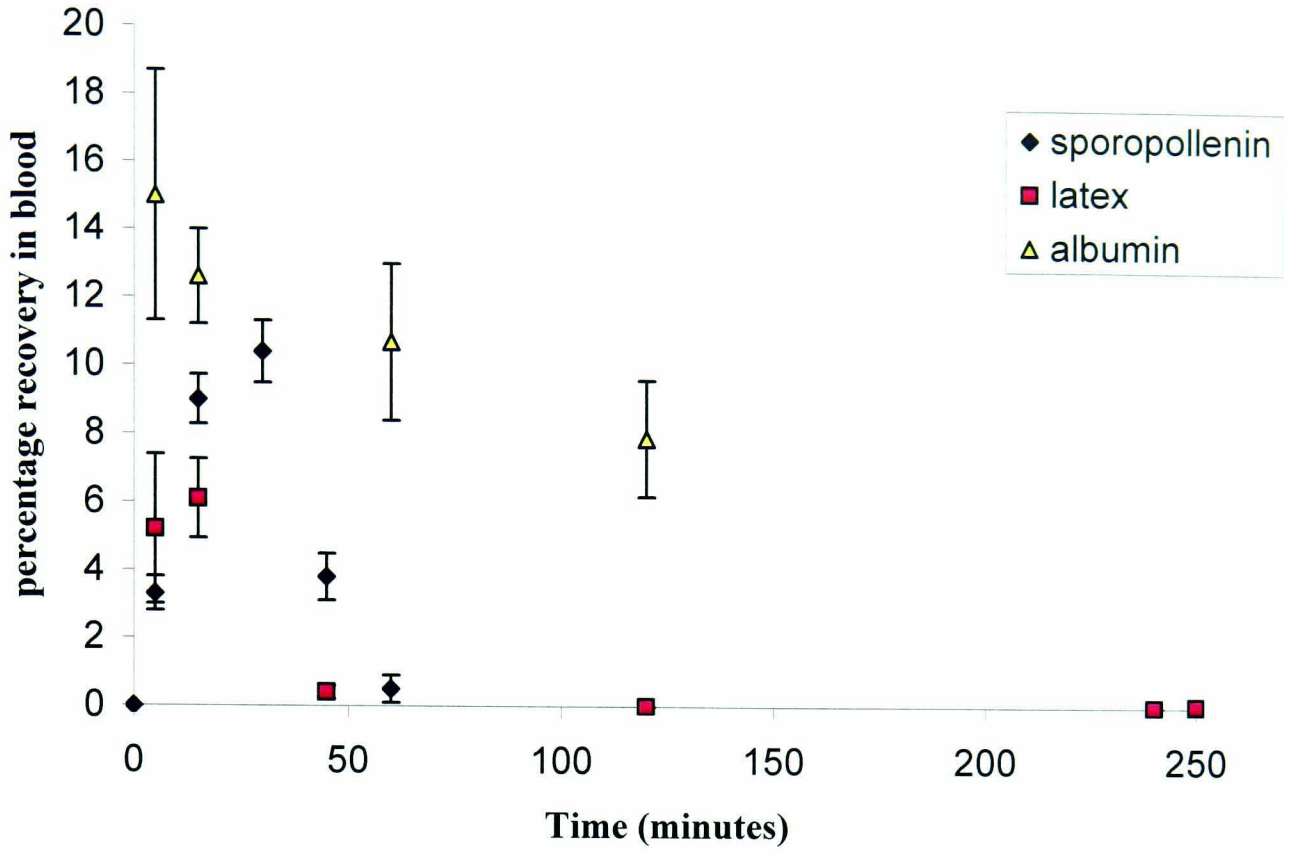


Figure 2.3.2.8 The percentage recovery of sporopollenin exines in blood in comparison to latex and albumin particles, as stated in the literature[18,19].

Although the latex and albumin particles used in both studies were much smaller than the sporopollenin particles, it would appear that there was not a significantly higher percentage recovery after the initial 5 minutes, since the 15% recovery observed for albumin microspheres could be within the error margin, which would give a similar value to that of the higher error margin encountered for sporopollenin. It is clear, however, that the profiles for the absorption of each microsphere are very different, with as much as 7.9% ± 1.7% recovery after 120 minutes for the albumin microspheres. In comparison, there were negligible sporopollenin or latex particles present in blood after this time. It should be noted that the results obtained with sporopollenin showed better reproducibility than the other two studies.

A disadvantage with the albumin microspheres is that because they are composed of protein they would be degraded rapidly in the GI tract, so 10% α-1-proteinase inhibitor must be administered to prevent such degradation.

2.4 Factors affecting the rate of absorption of particulates into the blood stream

2.4.1.1 Caffeine

There is existing literature stating that the rate of absorption of cornstarch (3-25μm size) was noticeably higher under the influence of caffeine[3]. This was confirmed in another report by Volkheimer and co-workers[33] in which caffeine was administered subcutaneously (200mg) prior to the particle to be absorbed.

Volkheimer and his colleagues found that the number of particles (starch in the literature) within venous blood was higher after administration of caffeine in comparison with an identical experiment undertaken without caffeine. This indicates an increase in the rate of persorption and it was concluded, "the motor activity of the gastro-intestinal tract is important for the process of persorption", since caffeine is a drug which stimulates the activity of the musculature of the GI tract.

He also stated in the same report that after rolled oats (200g) were ingested orally an average of 60 starch particles were detected in 10ml of venous blood after 90 minutes. This equates to an average of 18000 granules in the blood system (<1% absorption), assuming a total volume of 3000ml of blood.

A commercially available energy drink (Red Bull®) was ingested at room temperature, which contained 80mg caffeine. The energy drink was taken 15 minutes prior to ingestion of sporopollenin exines (200mg with 15ml milk).

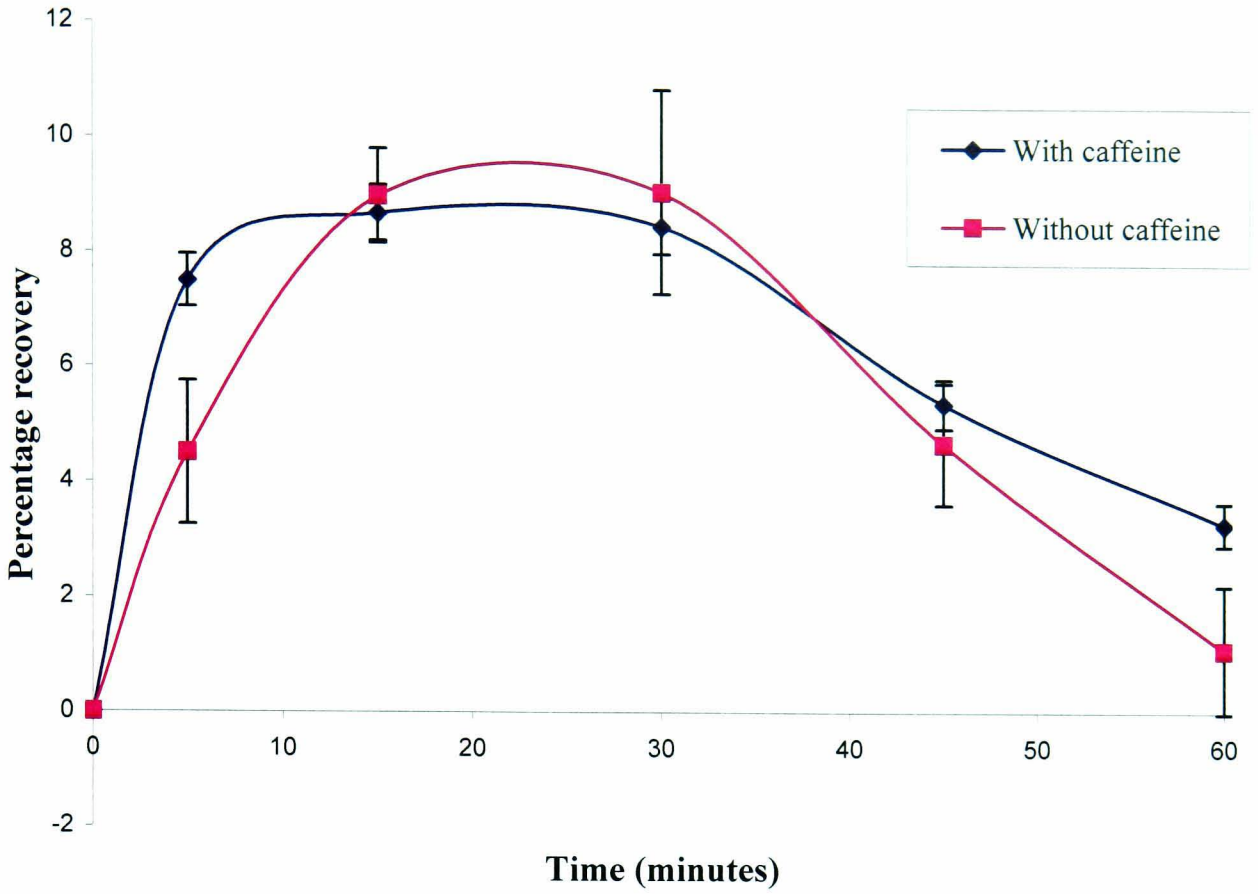


Figure 2.4.1.1: A comparison of the rate and extent of sporopollenin exine absorption into the bloodstream with and without caffeine on same volunteer

Following administration of caffeine, there was a slight increase in the initial rate of sporopollenin exine absorption by 3.0% (± 1.2%) just 5 minutes after ingestion to a maximum of 8.7% recovery. Ingestion of caffeine prior to ingestion of exines did not increase the extent of absorption into the bloodstream over the period of 1 hour, because as can be seen from Figure 2.4.1.1, the percentage recovery at each of the time points are very similar. It should also be noted that Red Bull® Energy Drink contained Taurine, which is said to enhance the ventricular functions of the body[34] and could have caused an increase in the number of particles recovered in the bloodstream.

2.4.1.2 Commercial Gaviscon®

The effect of Gaviscon® liquid on the rate of absorption of 25μm sporopollenin exines (400mg) was investigated. It was thought that Gaviscon® would increase the rate of absorption as it consists mainly of sodium alginate that coats the stomach lining to prevent heartburn. Thus, a slurry of Gaviscon® with exines was thought to increase the rate of migration into the bloodstream, as they would be exposed to the surface of the stomach and absorb to a greater extent.

In contradiction to the predicted results, administration of sporopollenin exines in a slurry of Gaviscon® caused a much slower absorption rate than taking the exines in milk alone. It was possible that the alginate acted to slow the rate by binding to the exines, making absorption more difficult.

2.4.1.3 Age

A preliminary experiment was carried out to investigate the percentage recovery of exines in the blood after oral ingestion with male volunteers of different ages. The rates of exine recovery were compared between the youngest (aged 20 years) and eldest (aged 58 years) volunteers involved with the trial, as Volkheimer stated higher 'persorption' of starch particles in younger volunteers[3].

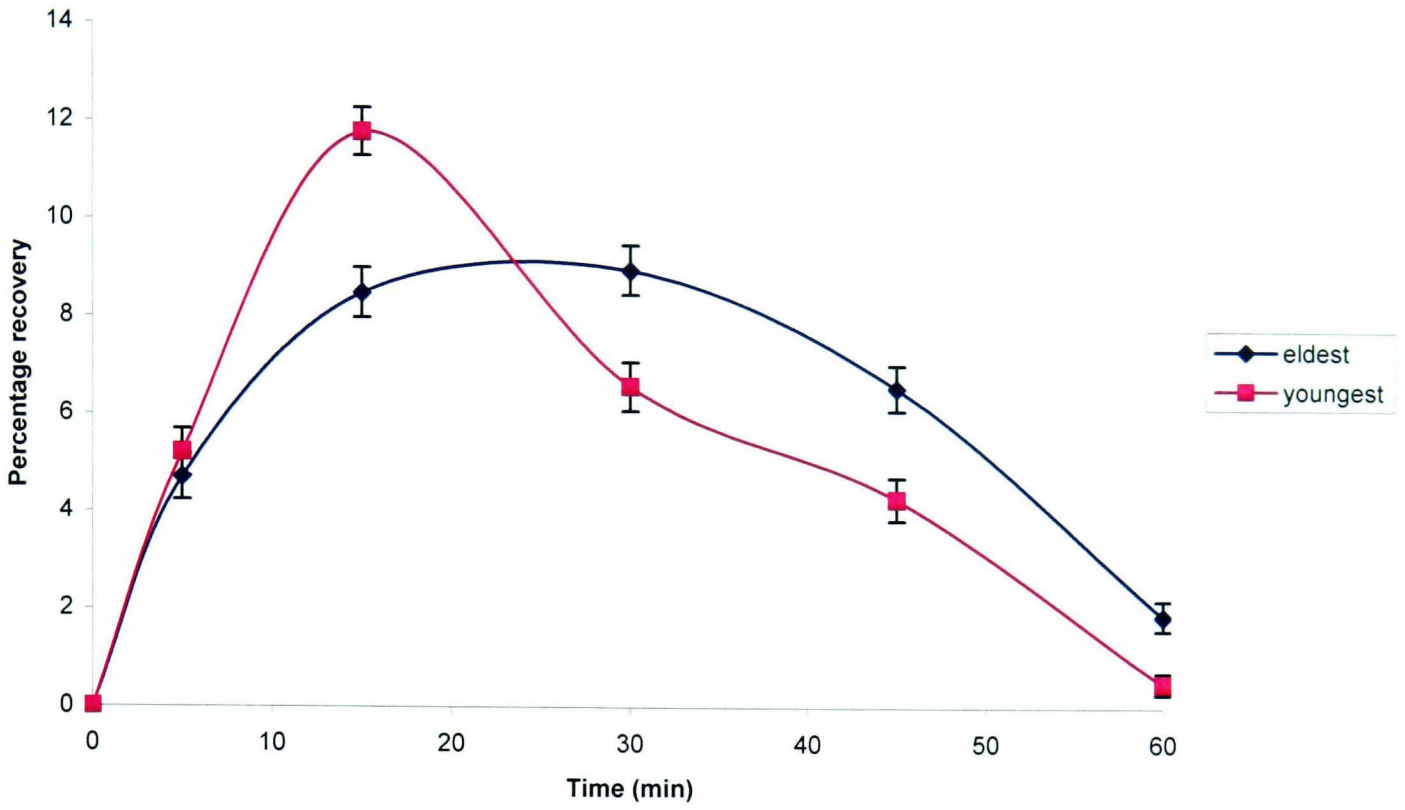


Figure 2.4.1.3 Percentage recovery of exine particles in the blood of male human volunteers of different ages.

There was a significant difference in the percentage recovery of exine particles between two volunteers of different ages, which was in agreement with the observations of Volkheimer[3] that the younger individual experienced a higher rate of particle uptake.

However, there may be other factors affecting the extent of exine transport into the bloodstream in addition to the age of the volunteers, for example individual differences in metabolism and rate of clearance, and a further study is required involving more volunteers before a certain age factor can be described to affect the rate of exine particle uptake by the GI tract.

2.4.1.4 Gender

To investigate the differences in the rate of particle absorption in male and female volunteers, from the eight volunteer trials (see 2.3.2), two male and two female volunteers were selected. The individuals were of the same approximate age (22-23 years), were all non-smokers and were fasted prior to the trial.

There were no significant differences between the percentage recovery of exine particles from male and female volunteers, given the associated error margins (mean of two male volunteers and two female volunteers).

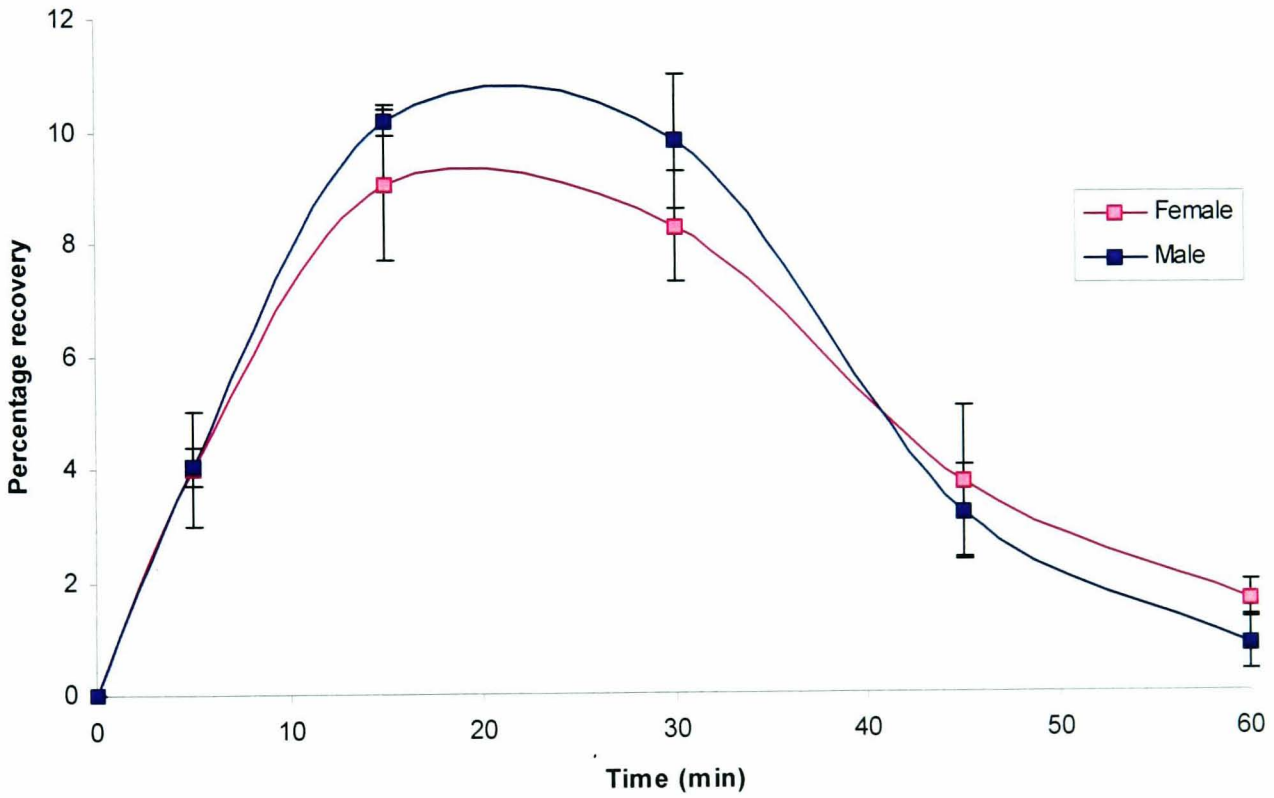


Figure 2.4.1.4 The percentage recovery of exine particles in blood following oral ingestion by male and female volunteers aged 22-23 years.

A more in-depth study with more male and female volunteers is required before any conclusions can be made with regard to differences in the rate of particle absorption between volunteers of different sexes. To date there has been no research into the effect of the gender of the volunteer on the rate of uptake of particles into the GI tract.

2.4.2 Saturation effect

Sporopollenin exines derived from *L. clavatum* (25μm) were administered orally in different doses (100mg, 200mg, 400mg, 600mg and 1g) to three human volunteers on separate days after overnight fasting.

The results were indicative of a limiting mechanism, for example a saturation effect, as a higher percentage recovery was observed with a smaller dose.

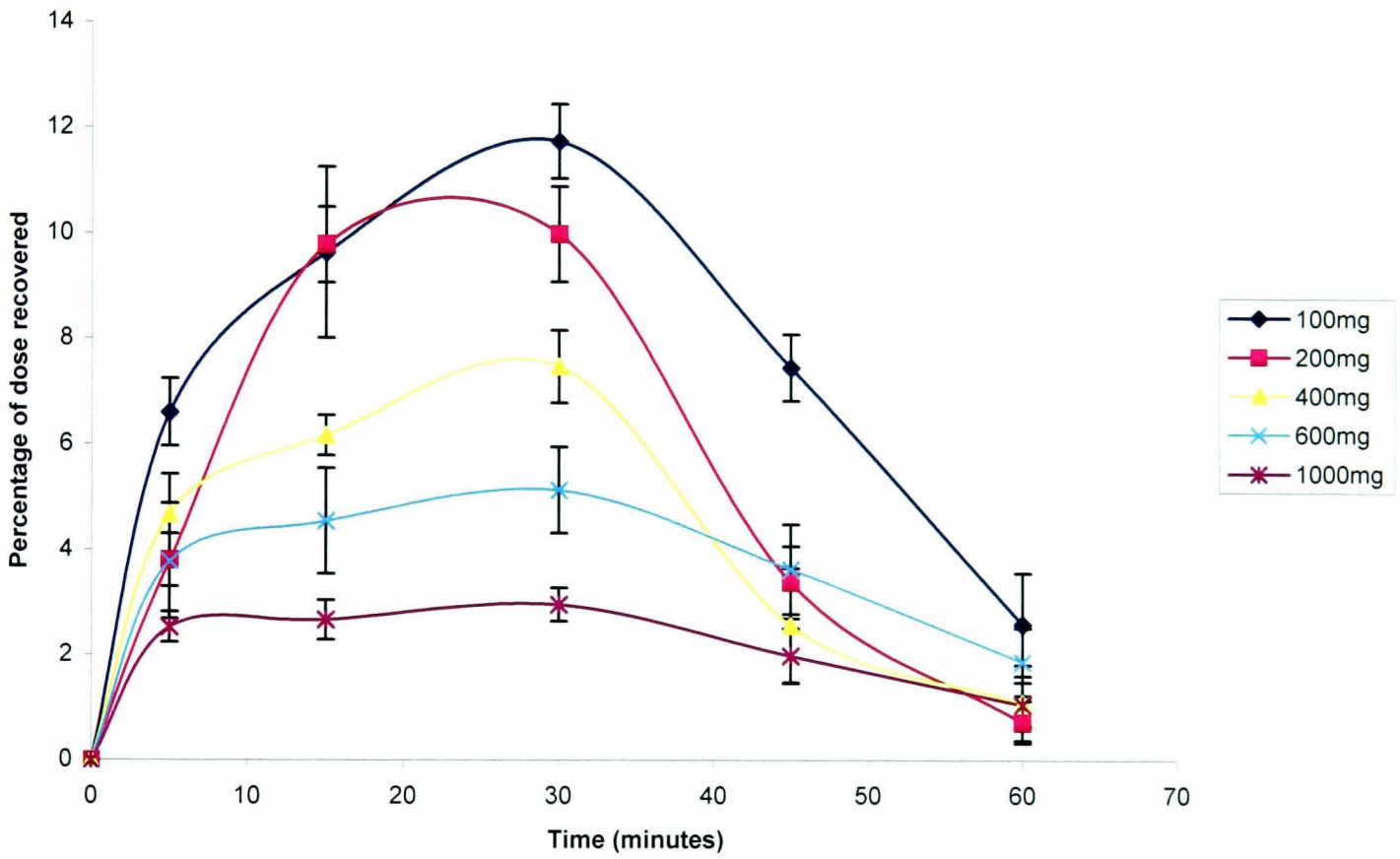


Figure 2.4.2.1 Percentage of exines recovered in blood after administration of different doses of exines (mean of three male volunteers).

A 'saturation effect' could indicate that there are only a limited number of sites through which the particles can migrate into the bloodstream an example are in Peyer's patches that were considered the main site of particulate uptake by the GI tract[17] and where many particles have been found to accumulate during biopsy experiments in animals[7,25,28]. Another explanation could be that higher doses of

sporopollenin exines have a shorter half-life, perhaps accumulate somewhere or trigger more enzyme activity.

The absolute number of particles recovered in the bloodstream was calculated (dose x % recovery) for each time point up to 1 hour, which gives an indication as to the number of particles recovered at these time points.

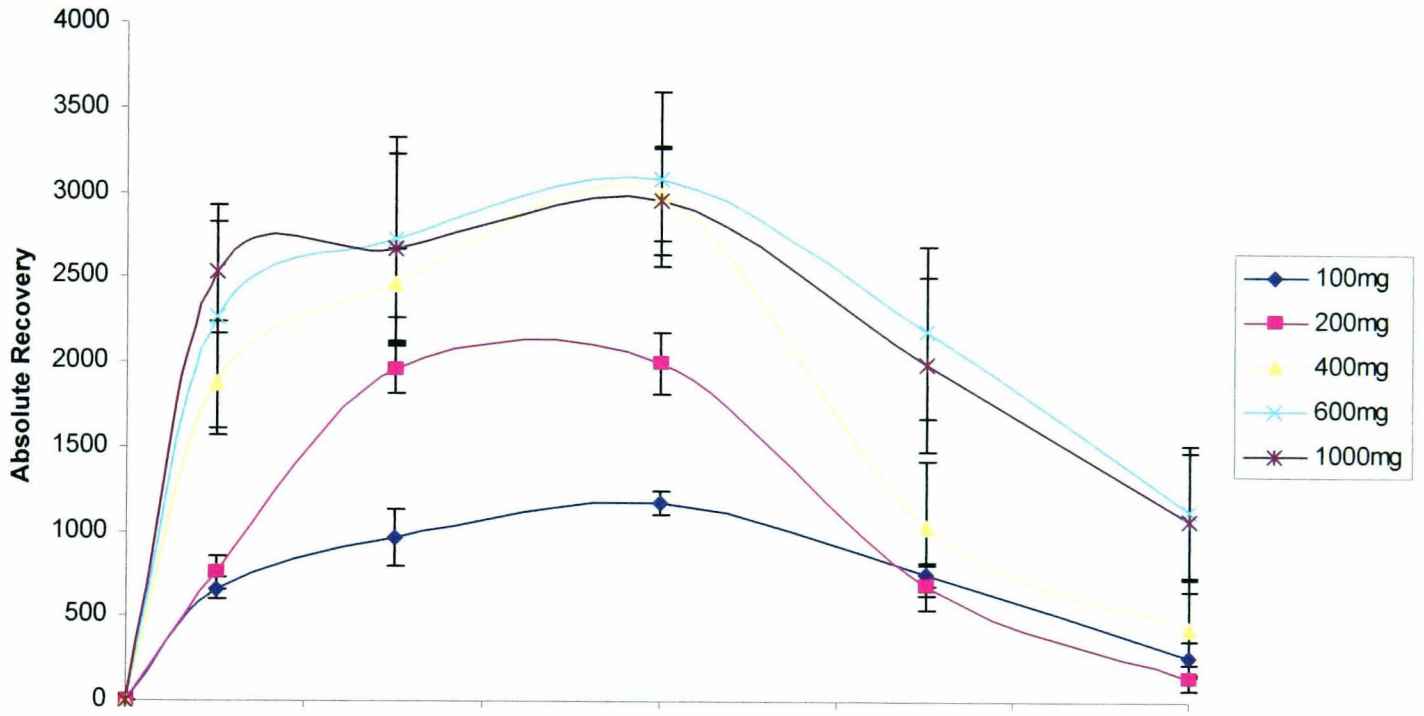


Figure 2.4.2.2: The absolute recovery of sporopollenin exines in the blood following oral ingestion of different doses.

The results show that the 200mg dose results in approximately double the particles recovered after administration of a 100mg dose, which is expected. However, the numbers of recovered particles for the 400mg, 600mg and 1000mg doses are surprisingly similar, which is important because it would indicate that it is impossible to overdose with exine particles and also suggests the saturation of the process of absorption. This finding highlights that exines are potentially a good drug delivery vehicle for drugs like insulin and psychiatric drugs or where the patient is illiterate.

The dose could be varied by encapsulating different quantities of the drug in 400mg of exine material. If a higher dose is required it is possible to administer another dose to the patient after 30 minutes.

2.4.3 Dilution effect

Sporopollenin exines (400mg) were administered to two volunteers with differing volumes of milk (15ml, 50ml, 100ml and 200ml) on separate days after overnight fasting. The percentage recovery was determined using a haemacytometer.

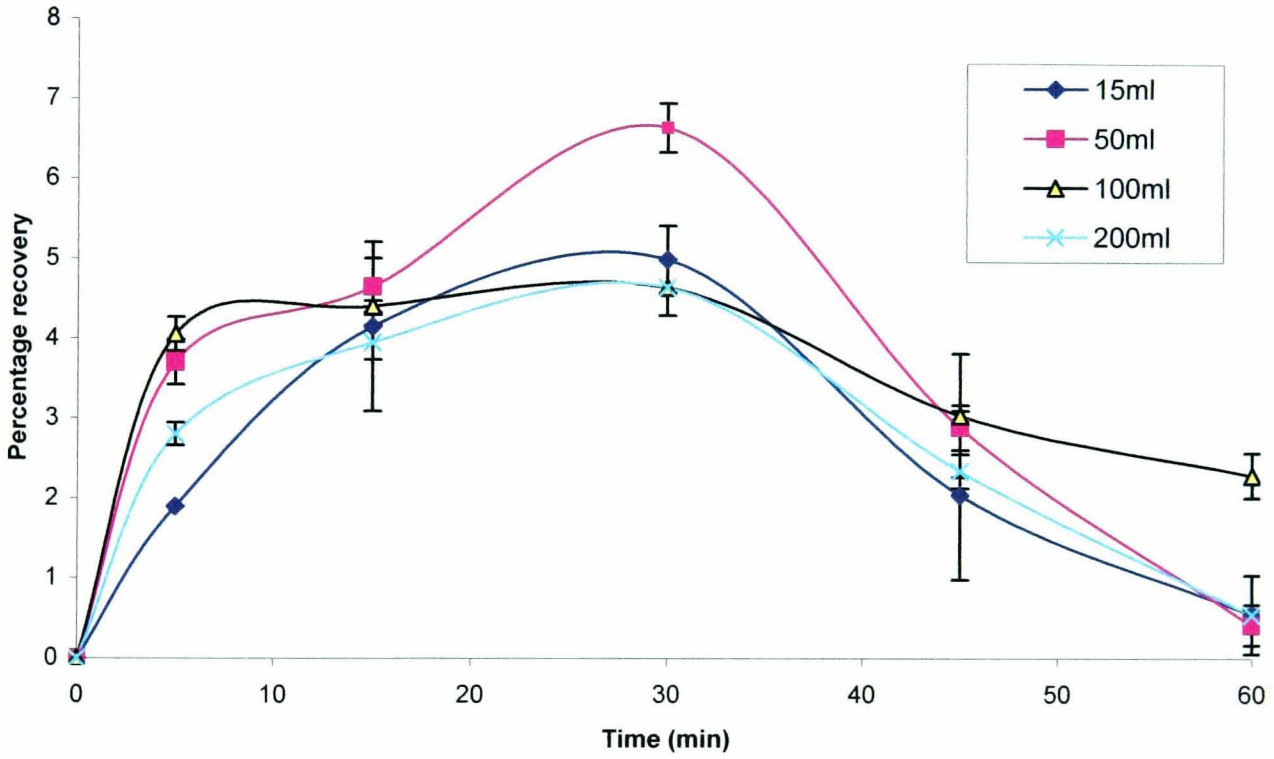


Figure 2.4.3 Percentage recovery of exines after administration of 400mg diluted with different volumes of milk.

There were no significant differences of percentage of exines recovered with 15ml, 100ml or 200ml of milk, however, after 30 minutes a higher percentage of exines were recovered when taken with 50ml milk. Therefore it can be concluded that the percentage of exines recovered in the blood after ingestion is independent of the dosage volume at the doses used.

percentage of exines recovered in the blood after ingestion is independent of the dosage volume at the doses used.

2.4.4 The effect of different dosage forms on the rate and extent of exine absorption

The following results are the mean percentage recovery of trials on 3 different volunteers following ingestion of 400mg exines in different dosage forms. All dosage forms produced similar profiles for percentage recovery in venous blood vs. time.

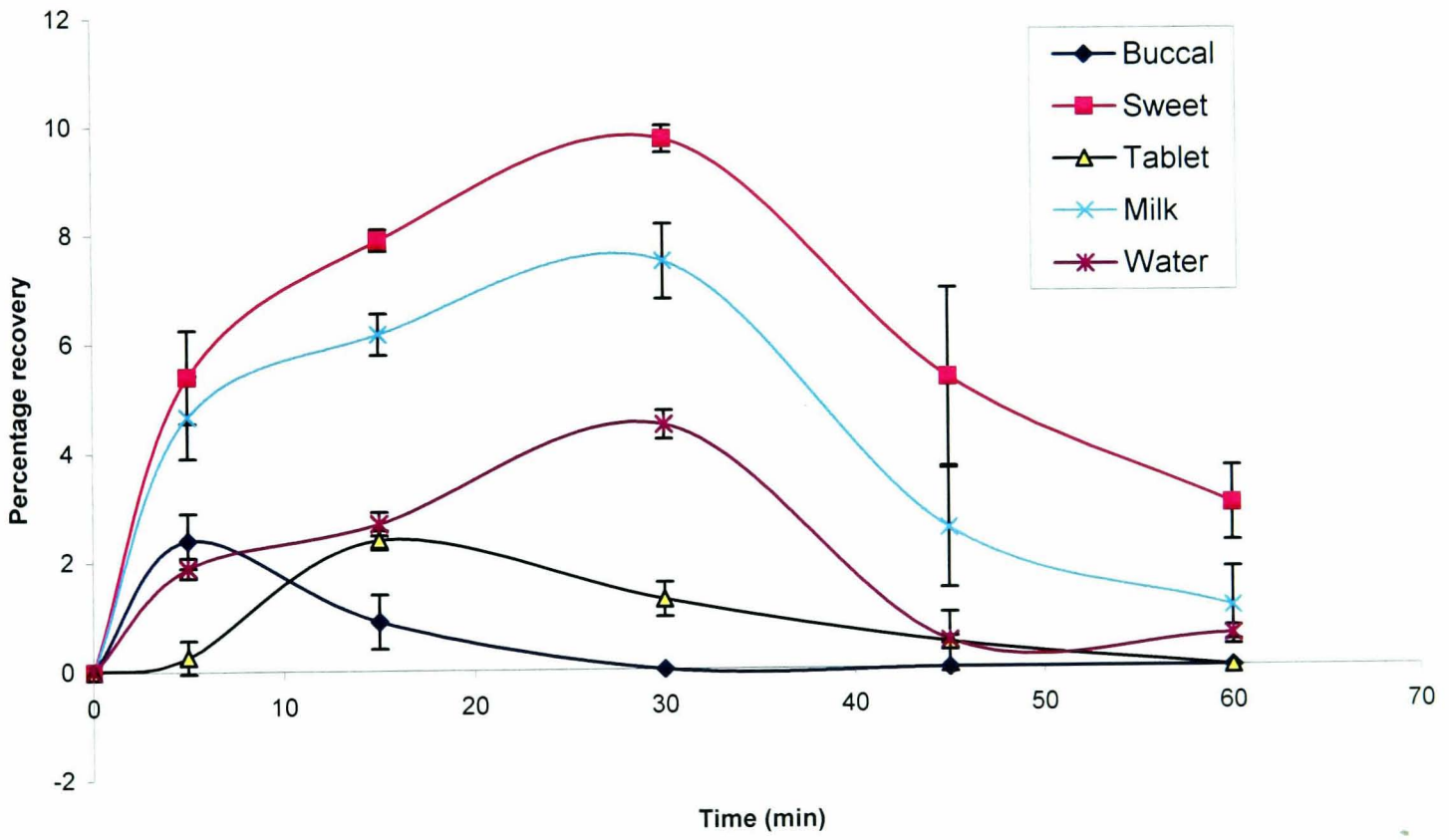


Figure 2.4.4 The effect of dosage form on the percentage recovery of sporopollenin exines (400mg) in venous blood. Results are a mean of three trials.

2.4.4.1 Buccal absorption

Following buccal administration (achieved by rinsing exines around the oral cavity for 2 minutes, then expelling them), only 2.4% (± 2%) of the particles ingested were

recovered in venous blood, which is insignificant, given the error margin. This percentage recovery may have resulted from the accidental swallowing of small quantities of exines, as it was not possible to prevent accidental swallowing of the exines. The buccal route of exine transport into the circulation was thus ruled out as the main method of absorption.

2.4.4.2 Sweet trial

A trial was undertaken with sporopollenin exines in a chewy sweet formulation (prepared by Nestle PTC Laboratories, York, UK). Three volunteers chewed two sweets (containing 400mg exines) slowly after overnight fasting. Venous blood samples were taken at 0, 5, 15, 30, 45 and 60 minutes after ingestion. Recovered particles were counted in blood plasma and lysed pellet using a haemacytometer.

The percentage recovery after ingestion was similar to that achieved after ingestion of exines in milk, with 9.8% (± 0.3%) detected in blood after 30 minutes. With a chewy sweet, the exine particles would have been in contact with the oral cavity for a longer period of time than when swallowed in milk. There was only a marginal increase (2.3%) in absorption with the sweet over the dosage with milk after 30 minutes, which indicates the buccal route of uptake did not contribute significantly, if at all, to the transport of exine particles into the bloodstream. It can be concluded that administration of sporopollenin exines in sweets resulted in the highest percentage of particles recovered in the circulation.

2.4.4.3 Tablet trial

Three volunteers ingested tablets of sporopollenin exines that contained 400mg exines, 45% lactose and 10% Bicarbonate of Soda (as prepared in Chapter 5). These tablets were previously shown to disperse into SGN, which would theoretically enable

the transport of exines into the bloodstream in the same manner as ingestion of the loose powder.

Although the tablet preparation was shown to disperse into SGN, the percentage recovery *in vivo* was low at 2.4% ± 0.17% 15 minutes after ingestion. The uptake of such particles could have been inhibited or reduced by the addition of lactose to the tablet, although this did not affect the dispersion into SGN *in vitro*. The percentage recovery reduced to zero after 60 minutes, indicating complete clearance from the circulation after this time.

During the *in vitro* trial (Section 3.10), after dispersion of the exines into SGN they were examined under the LM and appeared to be relatively intact, therefore the compression during tabletting did not affect the structure of the exines enough to be considered a reason for the low recovery.

The most obvious reason for this low percentage recovery of exines was that the tablet did not fully disperse into human gastric fluid even after 1 hour in solution, so this could have reduced the number of particles available for uptake and thus given a lower number in the bloodstream for detection.

Although this experiment proved that proof of concept that sporopollenin exines could be delivered by tablets, the percentage of exines delivered into the bloodstream via this method was lower than that achieved with ingesting the loose powder. This bioavailability needs to be improved to be commercially viable but was not explored further as this was beyond the scope of the research project.

2.4.4.4 Ingestion with milk

As seen from the dilution trial (2.4.3) in which sporopollenin exines (400mg) were ingested with milk (50ml), the percentage of recovery in the blood was significantly

higher at each time point than exines administered orally via the buccal route and those administered in tablet formulation. The percentage recovery of exines administered with milk reached a maximum of 7.5 % (± 0.7%) at 30 minutes after ingestion.

2.4.4.5 Ingestion with water

It was thought that the proteins in milk might cause a degree of coagulation in the stomach and could potentially reduce the extent or rate of exine absorption into the circulation. An experiment was set up in which one volunteer ingested sporopollenin exines (400mg) in water (approx. 50ml) instead of the usual milk. Although sporopollenin is hydrophobic, with a little shaking and stirring, the particles were successfully dispersed in drinking water.

The percentage of exines recovered in blood reached a maximum of only 4.5 % (± 0.2%) at 30 minutes after ingestion. This recovery was considerably lower than when exines were taken in sweet form and with milk. It could be possible that administration with sweets and milk enables the exine particles to become incorporated into the mixture and allow a greater extent of particle uptake into the bloodstream, perhaps by allowing greater exposure of absorption for longer within the stomach The percentage of exine particles recovered with water was greater than that achieved with tablet and buccal methods of delivery.

2.4.5 Other pollens and their extracted exines

Ambrosia trifida pollen and *L. clavatum* spores and their extracted exines (400mg) were ingested and the percentages of particles recovered in the blood stream were compared.

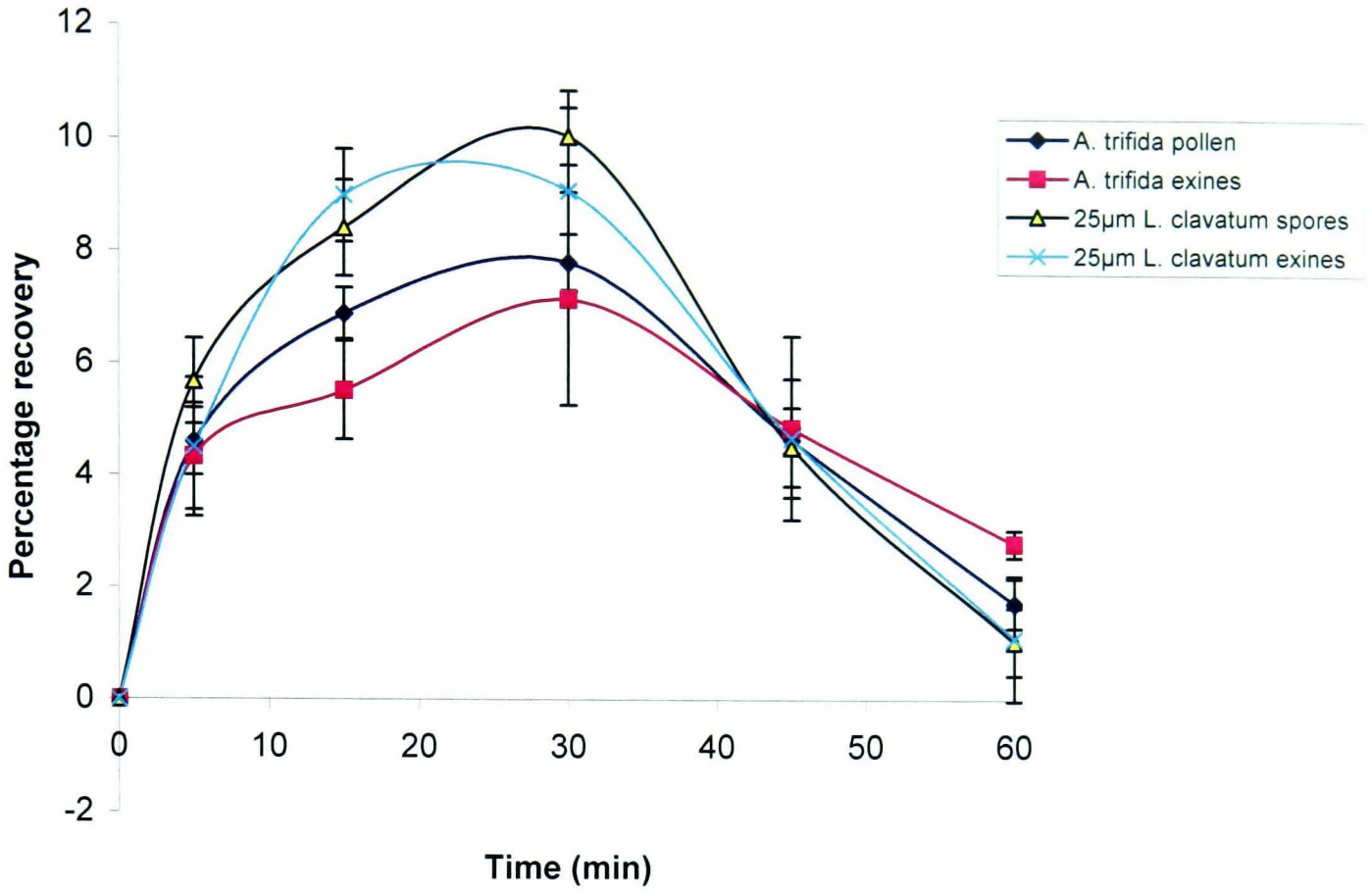


Figure 2.4.5.1 Percentage recovery of different particles in the blood after oral ingestion

In summary, the maximum proportion of particles counted in the blood after ingestion of 400mg doses of *L. clavatum* (40µm and 25µm) and *A. trifida* (15µm) were estimated by haemacytometery to be:

- Approximately 7.2% (± 1.9%) for *A. trifida* exines after 30 minutes.
- Approximately 7.8% (± 0.5%) for *A. trifida* pollen after 30 minutes
- Approximately 10.0% (± 0.5%) for 25µm *L. clavatum* spore particles after 30 minutes.
- Approximately 10.0% (± 1.7%) for 25µm *L. clavatum* exine particles after 30 minutes.
- Below 1% for 40µm *L. clavatum* raw spore and exine particles.

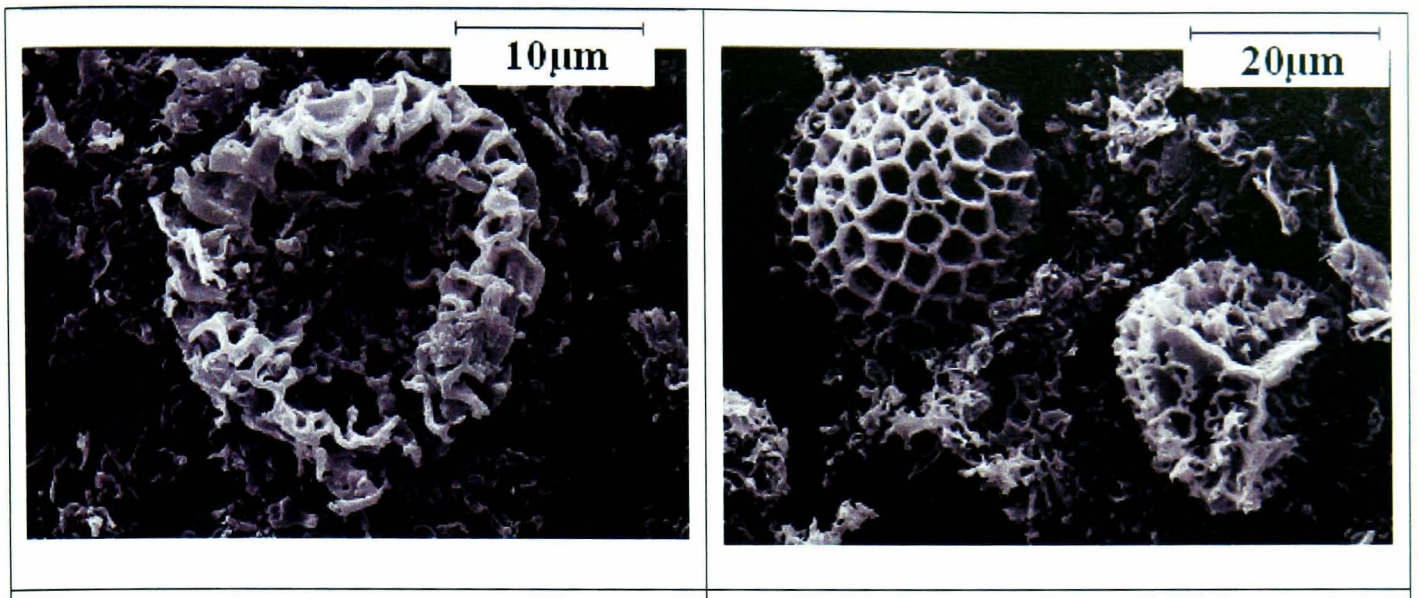


Figure 2.4.5.2: SEM images of *L. clavatum* spore particles (25µm) recovered *in vivo* 30 minutes after ingestion

The SEM images above were obtained by a concentration technique that involved centrifugation of many plasma samples 30 minutes after ingestion (when the maximum absorption occurred), the top plasma portion was then discarded, leaving a residue that was subsequently washed, centrifuged and freeze-dried.

A certain size exclusion factor exists during the transport of exine particles into the blood stream. It is not clear, however, whether this phenomenon is related to the morphology of the exines, since the larger *Lycopodium* species does not exhibit 'decoration' in the form of ridges on its surface, but both the *Ambrosia* and the *L. clavatum* exines (25µm) had decoration and both were able to reach the bloodstream. According to Volkheimer[3] the upper size limit for 'persorption' is 150µm, so theoretically, the 40µm exines could have been absorbed. This does not explain why the spores and extracted exines were not detected in the blood. It could be possible that such a spiky exterior of the *L. clavatum* (25µm) aids the passage of such particles into the blood stream, although at present this remains speculation.

Further studies are required to evaluate the persorption of different types of exines that are equivalent to or larger than 25μm in size but also exhibit a spiky exterior; for example Rye grass particles that are approximately 25μm in size but have a smooth exterior.

2.5 Dog *in vivo* trials

2.5.1 Enfurvitide-loaded sporopollenin exines

A study involving the delivery of Enfurvitide (encapsulated in sporopollenin exines) into the circulation of Beagle dogs was undertaken using the facilities at L. Hoffmann La Roche Ltd., Basel, Switzerland (as detailed in Chapter 4). PV4X was formulated and contained 273mg Enfurvitide per gram of material (determined by mass gain). A formulation of loaded Enfurvitide ('PV4X') was dispensed onto the tongue of dogs (in milk, 10ml) using a syringe. The exact doses are given in table 2.5.1.1. Blood samples were taken at 0, 0.5, 1, 1.5, 2, 4, 6, 8 and 24 hours after ingestion.

Loading of Enfurvitide PV4X (mg_{Enf}/g_{PV4X})	**Total weight sample PV4X** (g)	**Dog weight** (kg)	**Dose of loaded sp. for each (25mg/kg)** (g)
273.42	3.1600	8.4	0.768
		10	0.914
		9.1	0.832

Figure 2.5.1.1 Oral doses of Enfurvitide-loaded exines administered to dogs

The *in vivo* animal study showed that the percentage recovery of loaded exines into the bloodstream of dogs was ca. 3.4% and the three test dogs showed an equal rate of

absorption, given the accuracy of the technique. This value was a factor of three lower than the percentage recovery found in human subjects and later found in dogs (with encapsulated cyclosporine, see 2.5). This could be due to the inaccuracy of the counting method, as a 'stage micrometer' was used and involved dropping 0.1ml of sample onto a slide and counting all of the particles therein. The haemocytometer method was not in routine use at the time of the study.

Possible causes of the low counts are species differences giving different absorption rates and/or breakdown. It may be harder for the sporopollenin to gain entry into the dogs' bloodstream (horizontal *versus* vertical posture) or that the enzymes in the dogs' blood degrade the exines more rapidly, leading to recovery of fewer particles. The method of ingestion might have a small effect, but is unlikely to give the order of magnitude difference. There is also the fact that the human studies were carried out with empty spores, therefore the loaded Enfurvitide could have resulted in the lower percentage of exines recovered in blood. Images of recovered particles are shown below.

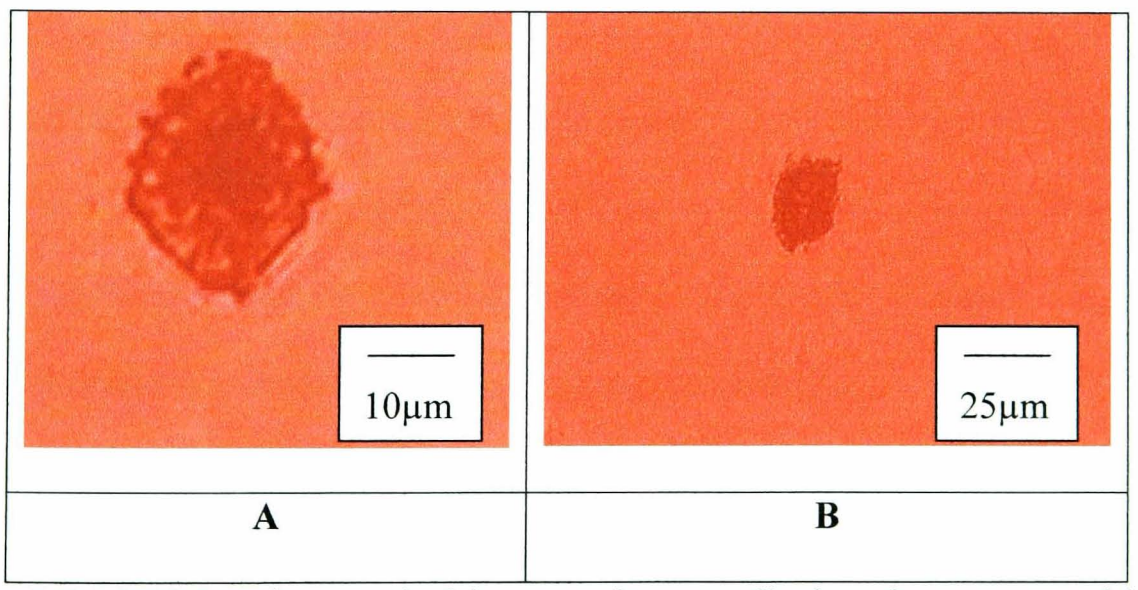


Figure 2.5.1.2 Light microscopical images of sporopollenin exines recovered in dog blood samples 30 minutes (A) and 1 hour (B) after ingestion.

It should be noted that Enfurvitide was detected in plasma samples at a low level, which confirms some absorption of exines into the bloodstream and the delivery of the loaded Enfurvitide drug into the blood. However, other *in vitro* experiments have shown that proteins are degraded rapidly in SGF (simulated gastric fluid) with pepsin, showing that some extra protection of the encapsulated drug is required (Section 4).

2.5.2 Cyclosporine-loaded exines

Cyclosporine is a cyclic peptide of 11 amino acids produced by a fungus. It is an immunosuppressant drug used routinely to prevent organ rejection after transplantation. Cyclosporine was used as a model compound as it has the required physical characteristics for inclusion into the spores and is poorly absorbed.

A feasibility study was undertaken to determine if sporopollenin exines were capable of releasing encapsulated cyclosporine into the circulation of Beagle dogs following oral administration. Full details of the study are stated in Chapter 4.

Four dogs were involved with the study, two male and two female. The results are the mean of four trials. With regards to the *in vivo* results, absorption was rapid, occurring 25 minutes after ingestion and the maximum number of exines recovered in dog blood was 30 minutes following ingestion (10.9% ± 7%). The error margins were large considering the small number of exines recovered. The number of exines recovered in the blood decreased significantly after 1.5 hours and no exines were visible in blood samples 4 hours after ingestion. This trial demonstrated the rapid transport of sporopollenin exines into the circulation and their subsequent elimination from the blood after 4 hours *in vivo*. However, the percentage recovery varied considerably between the four dogs. This could have been due to differences between the rates of

particle uptake by the GI tract of different dogs. A comprehensive study involving more subjects is required to establish a more accurate value for the rate of exine absorption in dogs.

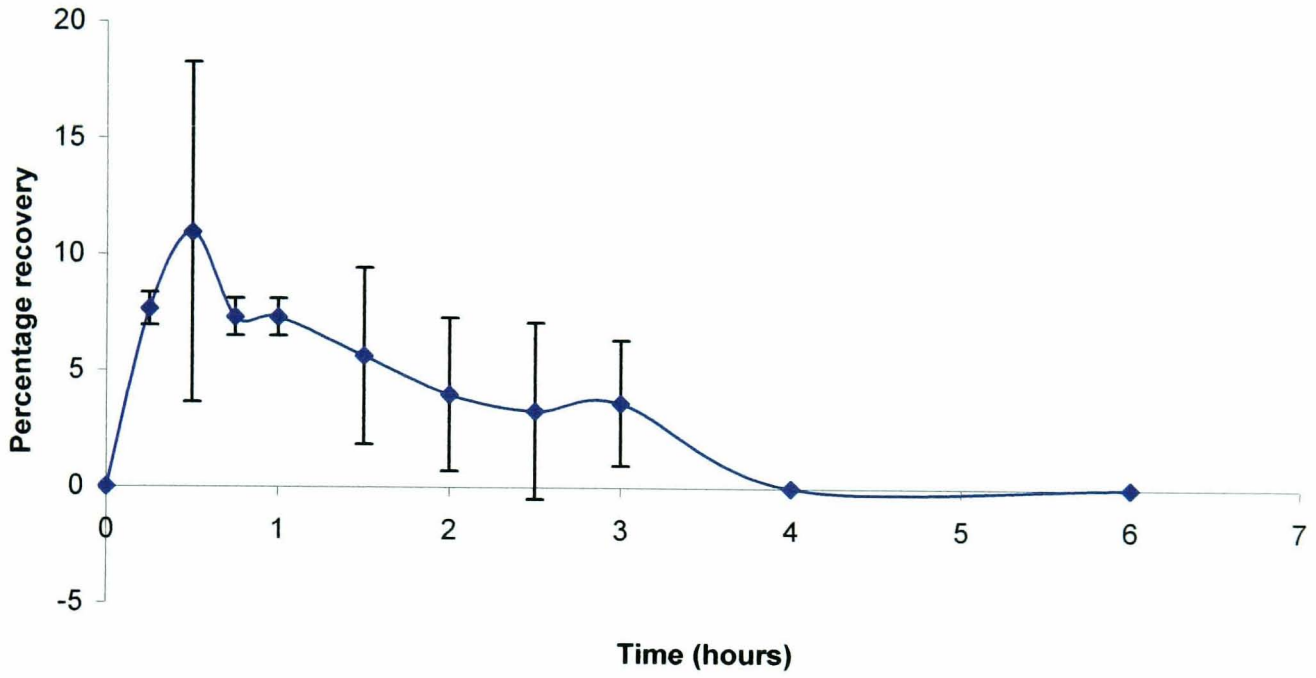


Figure 2.5.2: A graph showing the mean persorption of cyclosporine-loaded exines in 4 Beagle dogs.

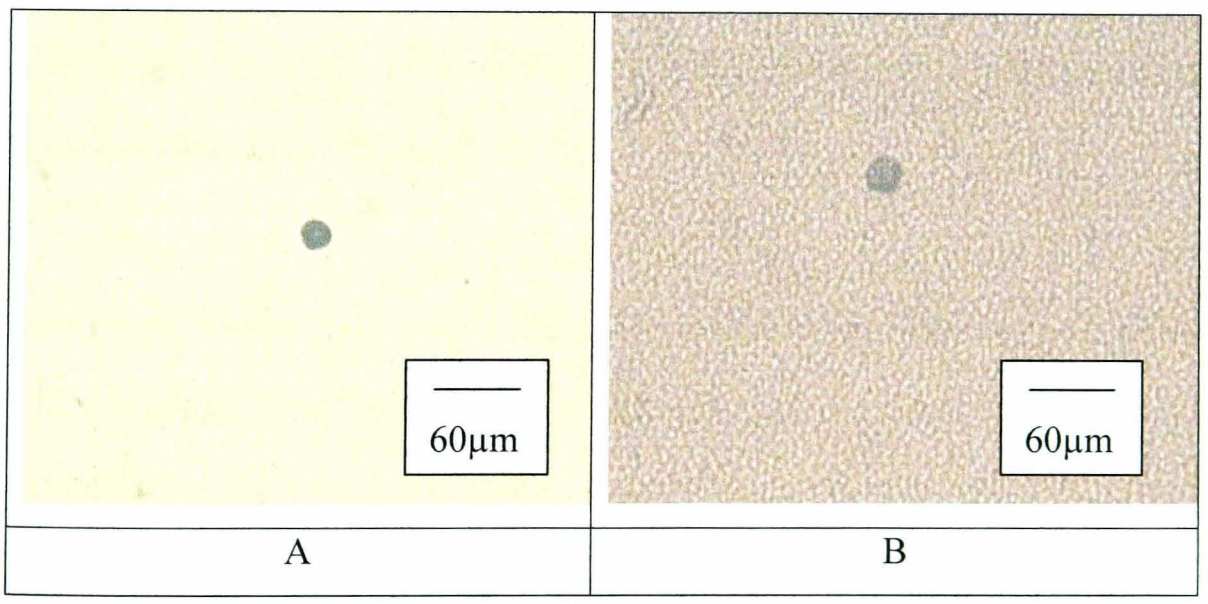


Figure 2.5.3: Exine particles recovered in dogs after 30 minutes in plasma (A) and lysed blood pellet samples (B).

2.6 Summary

It is vital that the sporopollenin-drug microcapsule must be stable to the acidic conditions of the stomach in order to allow enough time for the exine to absorb into the blood system and release the drug. Preliminary experiments detailed in this chapter have provided sufficient evidence of the passage of spore particles and sporopollenin exines into the bloodstream following oral ingestion. Administration of sporopollenin exines via the mucous membranes of the oral cavity was ruled out as a route for transport into the circulation.

The delivery of encapsulated drugs using sporopollenin exines was achieved into the bloodstream of dogs with Enfurvitide and cyclosporine, but at a lower level than that predicted based on the number of exine particles counted (assuming 100% release). It should be noted that the number of recovered exine particles that were loaded with Enfurvitide were counted using a Stage Micrometer, since the haemocytometer method was not available at the time of this study. The Stage Micrometer counting method was unfortunately not as accurate as the haemacytometer and so the results of the percentage absorption of Enfurvitide-loaded exines and cyclosporine-loaded exines were not directly comparable.

The exine particles that were recovered from venous blood following oral ingestion had been degraded and although they still retained the same fluorescence and characteristics of the particles before ingestion (Figure 2.3.2.6), it was evident that they were being degraded in the body. This aspect was investigated further in the next set of experiments.

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3. *In Vitro* Evaluation of Sporopollenin

3.1 Introduction

The biodegradability of sporopollenin in blood was first described during an *in vivo* experiment by Jorde and Linskens (1974)[1,2]. Following ingestion of 150 g of pure *Rye S. cereale* pollen, 6,000-10,000 grains were found in the blood stream, reaching a maximum after 30 minutes. A separate study by Linskens and Jorde revealed that following ingestion of *Lycopodium clavatum* spores, the particles recovered in blood were "degraded". Volkheimer has suggested that enzymes in the body are responsible for the degradation of particles such as starch grains and spores[3] and it was stated by Linskens and Jorde that "the degradation process seems proportional to the length of time the plant diaspores remain in the blood vessels"[2]. The specific enzymes and mechanism of degradation were not explained and still remains ambiguous.

Currently there is one reference that describes attempted *in vitro* experiments with sporopollenin[1]. The research involved *in vivo* and *in vitro* experiments with *L. clavatum* spores and rye pollen (*S. cereale*). Characteristic "corrosion" and "cracking" were experienced on the *L. clavatum* spores when ingested (*in vivo*) and it was believed to be due to "unknown enzymic systems"[1]. The *in vitro* experiments in the literature stated that no corrosion effects were seen after short incubation in blood outside of the body. However, this article failed to detail the sample treatment or the experimental conditions and, as explained later in the findings produced throughout this research, factors such as the timescale and temperature of the blood are very important contributors to the degradation of the sporopollenin exines.

There are previous accounts in the literature stating that spore and pollen particles are able to reach the bloodstream by a process known as 'persorption'[4]. Several of these

accounts show that the particles become degraded in the blood within the human body.

Earlier experiments cite sporopollenin to be resilient to chemical attack[5]; however it is of biological importance that they are broken down in the body because pollen grains and spores are ubiquitous and encountered in everyday activities. Since they have been shown to reach the blood, breakdown of the particles is vital to avoid potentially fatal problems due to the blockage of small blood vessels.

3.2 Results and Discussion

3.2.1 Planned *in vitro* experiments

In vitro experiments were devised to determine if spores, pollen and their extracted exines were able to degrade in blood outside of the body, and if so, to what rate and extent. Experiments were developed to investigate the effect of different fractions of blood, such as plasma, pellet and serum samples on the degradation of exines. Commercially available preparations that were either deficient in certain plasma factors or inhibited the coagulation cascade in a particular way were explored to rule out certain blood factors as the degrading mechanism.

Differences in the rate of degradation with blood plasma from differing species (human, rat and dog) were also investigated.

Sporopollenin exines were also incubated in simulated and actual stomach and small intestinal fluids to establish if they were resilient to such environments. Due to the extremely resilient nature of sporopollenin to chemical attack, as reported in the literature[6,7], it was anticipated that the exines would be stable to the acidic environment encountered in the GI tract.

3.2.2 Qualitative analysis of *in vitro* sporopollenin exine degradation

In the first instance, sporopollenin exines were simply added to human blood plasma to determine if any degradation occurred *in vitro*.

This evidence of *in vitro* degradation of exines prompted the analysis of different fractions of the blood, such as serum, plasma and lysed blood pellet for their effect on sporopollenin exine degradation. The different fractions of the blood are defined in the box below.

Plasma is defined as the aqueous phase of blood that is obtained by removing the blood cells (by centrifugation) after the addition of an anticoagulant (such as samples collected in EDTA tubes).

Serum is obtained by allowing the blood to clot, therefore the clotting cascade enzymes will have been all expended.

Lysed blood pellet refers to the fraction of blood remaining following centrifugation after removal of plasma. This is then lysed with water, which breaks up the blood cells.

Figure 3.2.2: Definitions of plasma, serum and lysed blood pellet.

3.3. Characterisation of sporopollenin degradation

3.3.1 Haemostasis and the Coagulation Cascade

Haemostasis is a well-regulated system within the body to keep the blood clot-free in normal vessels and to rapidly form a localised plug, or clot, in injured vessels[8]. Primary haemostasis is the first stage in which platelets become activated and adhere to the site of the injury, resulting in a temporary haemostatic plug. A more stable,

permanent plug is formed during secondary haemostasis, which is also known as the clotting cascade, or coagulation cascade[8].

The coagulation cascade is a sequence of serine proteases and substrates in plasma and tissues that proceeds by intrinsic and extrinsic pathways. The intrinsic pathway involves components within the blood and is activated by factor XII (Hageman Factor). The extrinsic pathway is triggered by extravascular tissue damage and becomes activated by tissue factor – a lipoprotein that is expressed at the site of vascular injury[8].

Both pathways are linked because they both lead to the activation of factor X. A list of the factors involved in the coagulation cascade and their alternative names are given below.

Factor	Alternative name	Plasma half life (h)
I	Fibrinogen	72-96
II	Prothrombin	60
III	Tissue Factor	--
IV	Calcium	--
V	Proaccelerin, Labile Factor	15
VI	Accelerin	5
VII	Proconvertin	10
VIII	Antihemophiliac Factor A, Antihemophilic Globulin (AHG)	25
IX	Christmas Factor, Antihemophiliac Factor B, Plasma Thromboplastin Component (PTC)	40
X	Stuart-Prower Factor	45-65
XI	Plasma Thromboplastin Antecedent (PTA)	60
XII	Hageman Factor	150
XIII	Fibrinoligase, Protransglutamidase, Fibrin Stabilising Factor (FSF)	72-96
-	High-Molecular Weight Kininogen	156

Figure 3.3.1.1: Alternative names and plasma half-lives of the factors involved in the coagulation cascade[8].

The coagulation pathway occurs as a sequence of positive and negative feedback loops that control the activation process. The intrinsic pathway requires the clotting factors VIII, IX, X, XI and XII. Calcium ions and phospholipids secreted from platelets are also required. Initiation of the intrinsic pathway arises when factor XI,

Hageman factor (XII), prekallikrein and high molecular weight kininogen (HMWK) come into contact with the negatively charged sub-endothelial surfaces exposed from the injury (Contact phase). The conversion of prekallikrein to kallikrein activates factor XII to factor XIIa (the "a" denotes the active form of the molecule), which in turn activates factor XI to factor XIa. In the presence of calcium, factor XIa activates the conversion of factor IX to IXa, which then goes on to activate factor Xa, with the aid of co-factor VIIIa and phospholipids expressed on platelets. Factor Xa is the point at which the two pathways merge.

The extrinsic pathway is initiated at the site of injury from tissue co-factor (III) and factor VIIa that promote the activation of factor Xa. Activated factor VIIa and tissue factor also activate factor IXa, as seen in the intrinsic pathway.

Where the two pathways combine, the conversion of prothrombin to thrombin involves factors Va, Xa and calcium. Exposure of fibrinogen to thrombin results in the formation of soluble fibrin that is subsequently converted (with calcium and factor XIIIa) to insoluble fibrin that forms a clot. The coagulation cascade is summarised below.

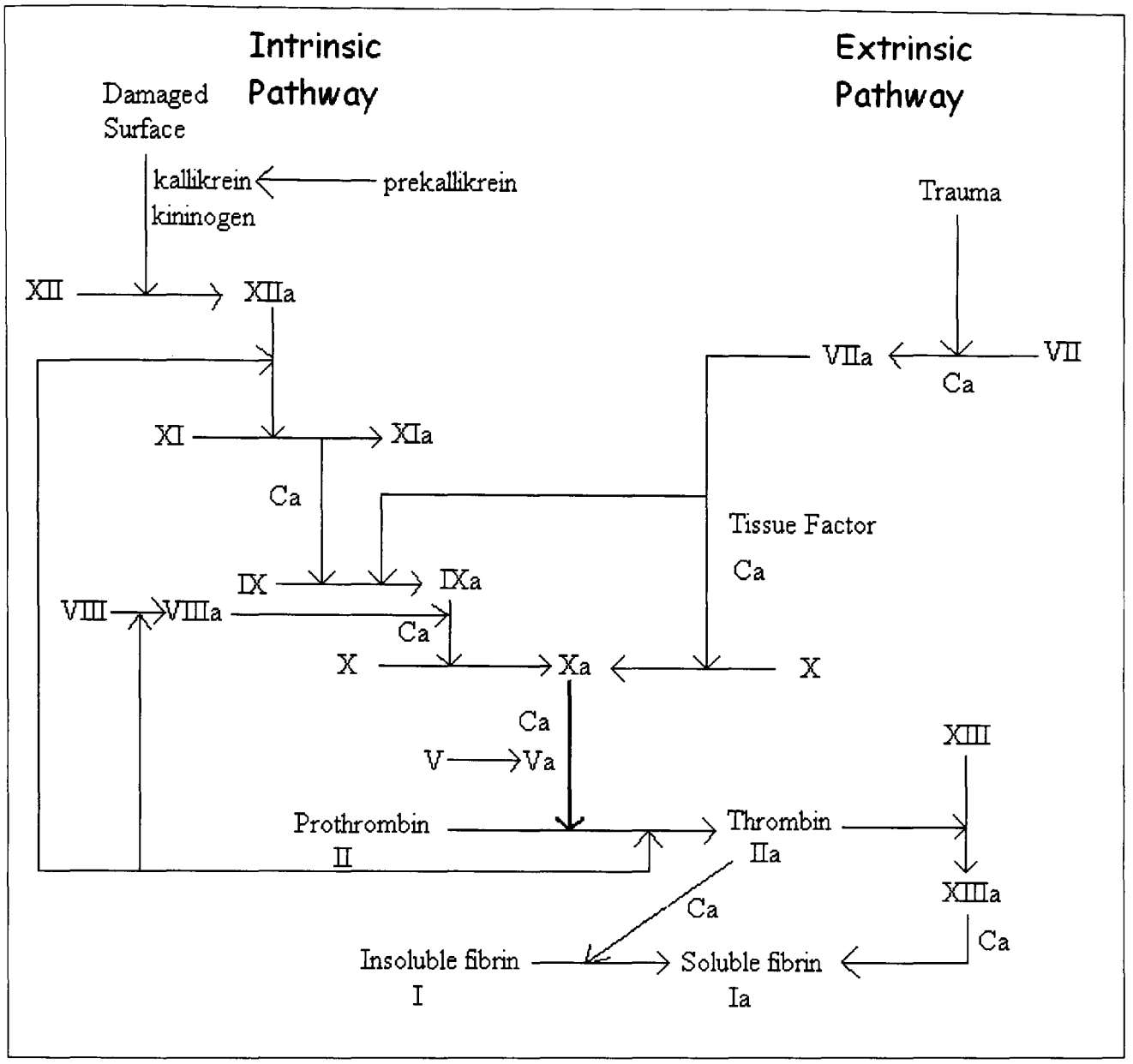


Figure 3.3.1.2: The intrinsic and extrinsic pathways of the Coagulation Cascade.

3.3.2 Experiments undertaken to characterise exine degradation

The degradation of sporopollenin exines was observed in plasma and lysed pellet, but not in serum. Different anticoagulants were explored, such as EDTA and citrate that prevent clotting by removal of calcium ions from the blood, and lithium heparin that prevents clotting by inhibiting the action of the enzyme, Thrombin, in the final stages of blood coagulation. Experiments were conducted to establish if the degradation of sporopollenin exines was caused by an enzymic method, for example observing the degradation of exines at different temperatures and assessing a possible saturation effect by adding different masses of exines into equal volumes of plasma.

Other experiments were designed in an attempt to characterise the degradation of exines, for example the use of Bovine thrombin was added to serum containing sporopollenin exines in an attempt to 'activate' serum and cause degradation of the exines. Commercial factor XII deficient plasma, prekallikrein deficient plasma and 'normal' commercial plasma were investigated for their effects on the degradation of sporopollenin.

Further experiments were then conducted to identify component of plasma responsible for exine degradation; for example using different blood collection tubes that each inhibit the coagulation pathway in a different manner.

3.4 Quantitative analysis of *in vitro* sporopollenin exine degradation

The rate of degradation was determined quantitatively using a known number of particles as a reference value for 100% recovery. Different methods, including a Beckmann-Coulter Counter, the new improved Neubauer Counting Chamber (Haemacytometer) and a Fluorescence Activated Cell Sorter (FACS) were used to assess the counts.

The degradation of the exine particles was followed by observing a decrease in the percentage recovery over a timescale.

3.4.1 Obtaining a reference value

There is no information within the literature regarding the number of sporopollenin exines per gram; however a value was noted from previous communication with a colleague[9]. *Lycopodium* tablets that were treated by acetolysis (i.e. sporoplasm of the spores was removed to leave an exine) were said to contain 160g *Lycopodium* spores

in 1,000,000 tablets. Each tablet weighed 0.00016g and there were 10679 spores per tablet. Therefore, from this reference the number of spores per gram was 66,740,000.

3.4.1.1 Flow cytometery

Flow cytometery was explored as a means of determining the number of sporopollenin exines per gram because the exines exhibit autofluorescence and so were easily identified as they passed through the laser. The flow cytometer gave a value of 1.37 x 10^9/ L, which is very different to the previous values. Accurate dilutions of the sample were required, which may have introduced sources of error and also, any debris present in the sample, such as fragments of exines would have been counted separately.

3.4.1.2 Fluorescence Activated Cell Sorter (FACS)

FACS was used to acquire a reference value. This fully automated technique was first adapted to quantify oral absorption of particles by Ebel[10] and allows analysis of a small sample volume (200μL). FACS is also able to identify single particles from aggregates[11] and can sort as many as 300,000 particles per minute .

The Becton Dickinson FACS-Calibur machine gave a value of 355,350,000 ±16,204,000 exines per gram. This value is very high in relation to the other values obtained and when using this instrument several problems became apparent. There was no actual size 'gate' and the numbers indicating particle size are all relative to each other, for example; the unit 200 will be half the size of 400 and so on.

There was also a slight delay between loading the sample onto the machine and telling the machine to "acquire" the numerical data and since the machine begins counting upon sample loading, there will undoubtedly be some particles lost. The machine was

also unable to offer a measurement of the exact volume of solution that had been taken up, but it is possible to measure this indirectly, by taking note of the volume remaining after counting if a sample of known volume is placed into the machine, however introducing its own errors.

Other problems associated with FACS are that it requires trained users to operate it and the equipment is expensive. The sensitivity is associated with the fluorescent signal generated and not on particle size and the technique involves a destructive step. For these reasons, FACS was not pursued as a method to quantify sporopollenin exine degradation.

The *in vivo* experiments that follow in this report were determined based on the ingestion of 400mg of sporopollenin or raw *L. clavatum* spore particles. Although there are approximately 5 litres of blood in an average healthy adult, it is assumed that only 3 litres are available within the immediate blood circulation[12]. Therefore, the following counts were established using serially diluted solutions containing the equivalent of 400mg exines/ 3L medium (aqueous ethanol and human serum were explored as media).

3.4.1.3 Coulter Counter

The final method used to establish the number of exines per gram was a Beckman-Coulter counter. The advantage of using a coulter counter over other automated methods is that there is a size gating that can be finely tuned to select the exact size of particle being counted. In order to optimise the size gating a sample with the equivalent of 400mg exines/3L was counted using the following gating; 25-28, 25-29, 25-30, 25-31, 25-32 and 25-33μm.

The optimum size gating was from 25μm to 30μm and was used to count the exine particles in the following diluted samples that were prepared with the equivalent of 400mg exines/ 3L. The coulter counter gave a value of 79,695,000 ± 3,650,000 exines per gram, which is comparable to the value obtained from the haemocytometery method, given the associated error margins (Figure 3.4.1.4).

3.4.1.4 Haemocytometery

A haemocytometer was subsequently used to quantify the number of sporopollenin exines per gram and involved using a light microscope to count the exines within a grid to give 63.75 ± 2.38 x10^6 particles per gram of sporopollenin.

The same tubes that were used in each of the *in vitro* and *in vivo* experiments were used for uniformity; sporopollenin exines were suspended in aqueous ethanol (4:1 water: ethanol), and separately, serum (which in earlier studies showed not to degrade the exines, but gave a medium of similar consistency)

Although this value is lower than the other counting methods, it was the most accurate with respect to the experiments undertaken, since it takes into account any loss of exines that may have occurred through:

- Not obtaining a homogenous sample
- Exines adhering to the sides of the tubes used
- Transference of exines during weighing
- The behaviour of the exines under the haemocytometer

A source of error may have been introduced because the distance between the slide and the cover slip is 100μm, which implies that counting is required on different focal planes. This involves the risk of counting the same particle more than once. In addition to this, the Brownian motion that is experienced with small particles

increases the chances of counting particles more than once. Another disadvantage of haemacytometery is that counting with a microscope is a tedious process that is only applicable to small samples.

Despite this, microscopical counting appears to be the most reliable method, especially for larger particles and smaller volumes of sample, as the exines are physically apparent and it is easy to distinguish between clusters of exines. Particle counting is considered an accurate and reliable method for the quantification of particles larger than 0.5μm[13].

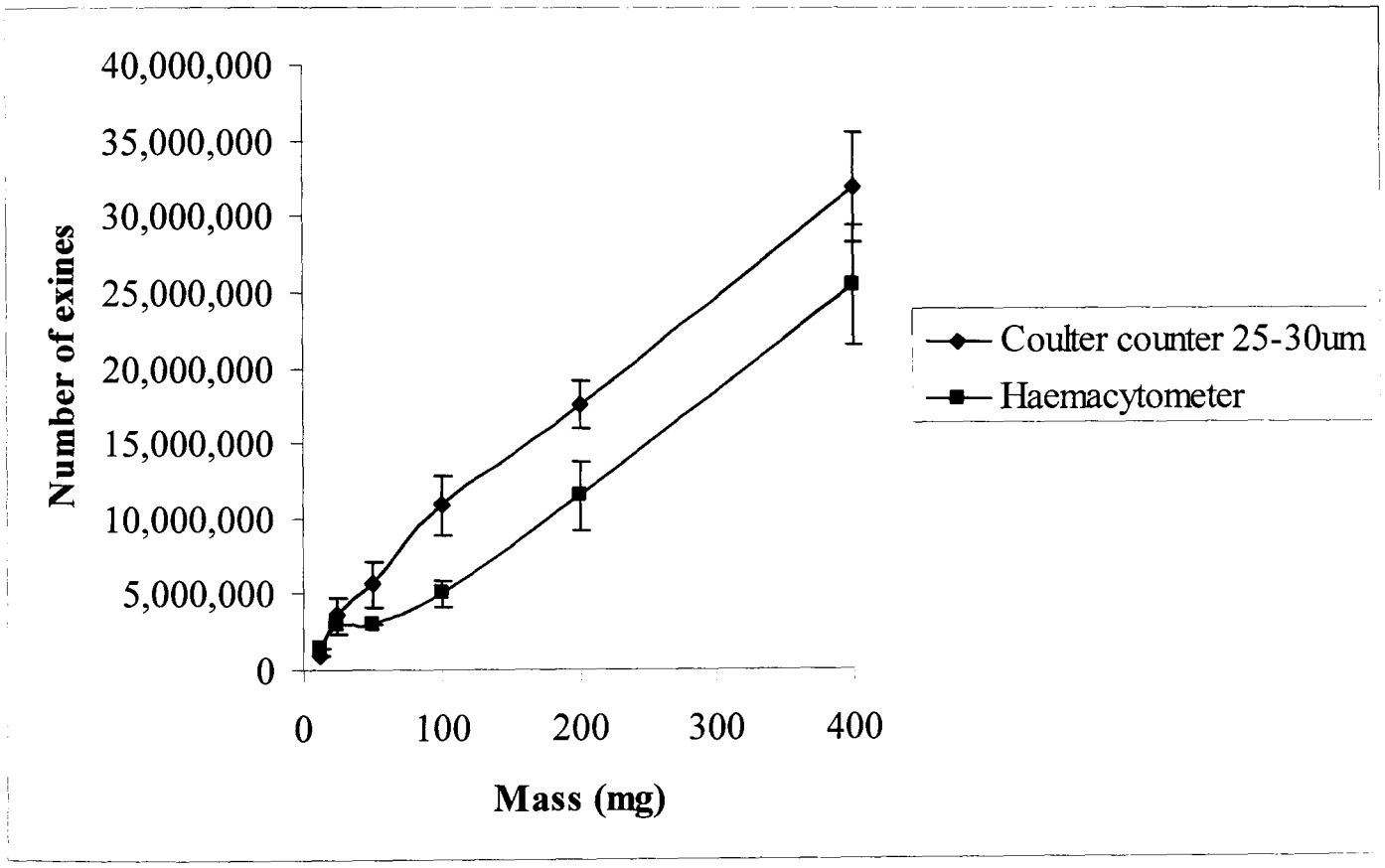


Figure 3.4.1.4: Comparison of exine counts per 3L with the haemacytometer and the Coulter Counter.

The reference values (approximate number of particles per gram of material) for other pollens, spores and their extracted exines were determined using haemacytometery and are detailed below.

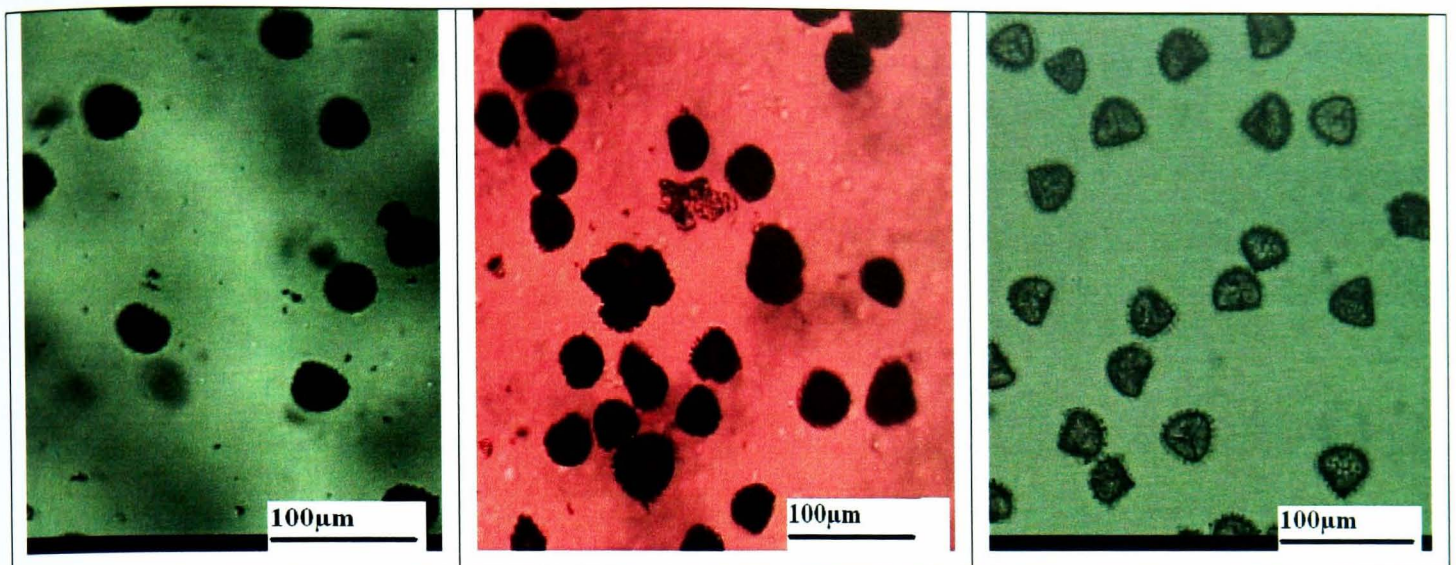
Type of particles	Approximate number of particles per gram
L. clavatum spores 25µm	28,650,000 (± 720,000)
L. clavatum exines 25µm	63,750,000 (± 2,380,000)
L. clavatum spores 40µm	17,700,000 (± 1,600,000)
L. clavatum exines 40µm	21,100,000 (± 4,470,000)
A. trifida pollen	40,900,000 (± 3,720,000)
A. trifida exines	66,000,000 (± 5,200,000)
Rye grass pollen	20,910,000 (± 1,820,000)
Rye grass exines	32,700,000 (± 1,970,000)

Figure 3.4.1.5: References values for different pollens, spores and their exines as determined by haemacytometery.

3.5. Qualitative results

3.5.1 Different fractions of the blood

The degradation of sporopollenin exines was confirmed by light microscopy by comparing exines before and after incubation in blood plasma. The images below show the sporopollenin exines upon initial contact with plasma, lysed blood and serum samples at time zero (just after addition).



Exines in plasma at time zero	Exines in lysed blood pellet at time zero	Exines in serum at time zero

Figure 3.5.1.1: Light microscopical images of sporopollenin exines remaining intact after immediate addition to plasma, lysed blood pellet and serum respectively.

The following light microscopical images detail the degradation of sporopollenin exines in plasma and lysed pellet samples, but not in serum samples over a timescale of 30 minutes.

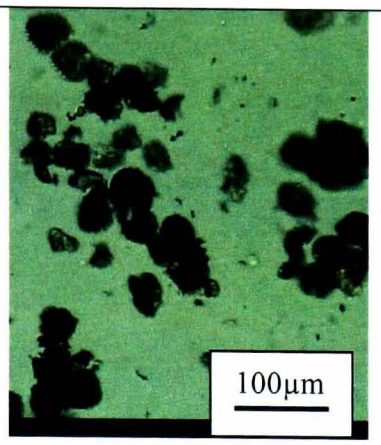
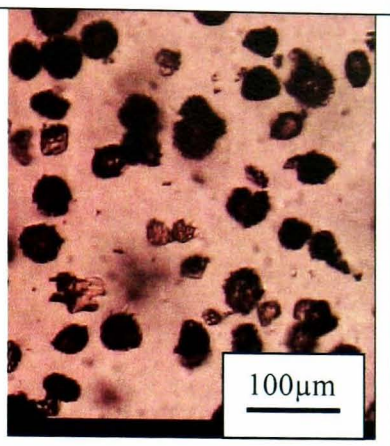
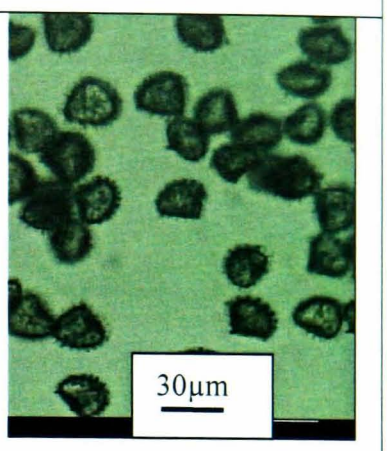
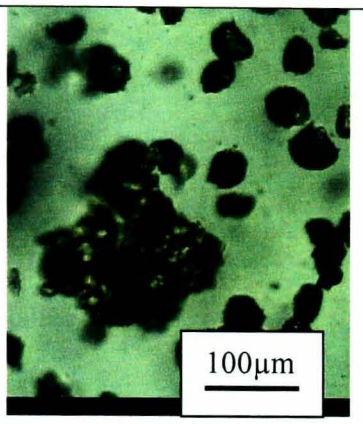
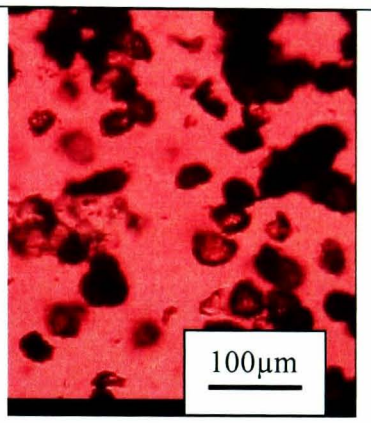
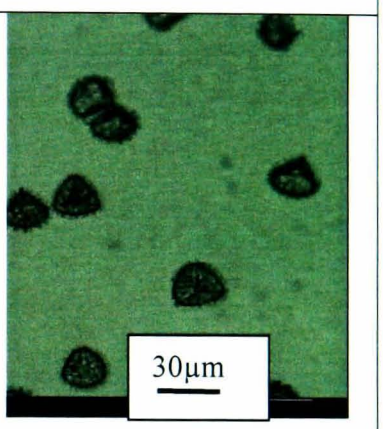
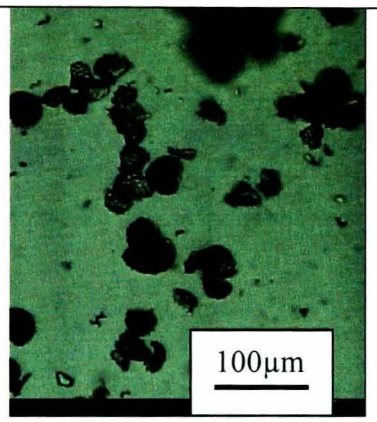
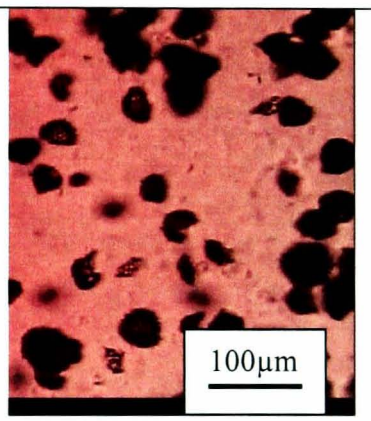
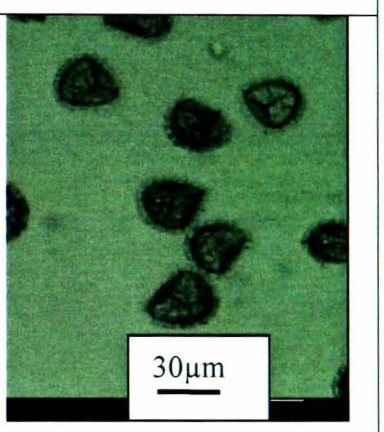
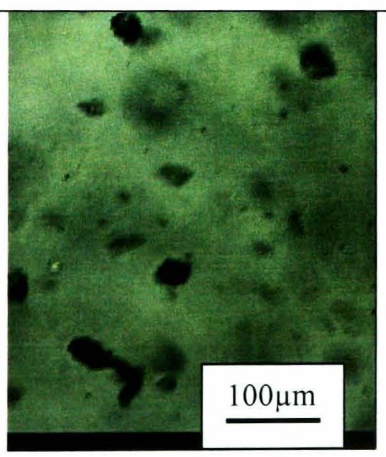
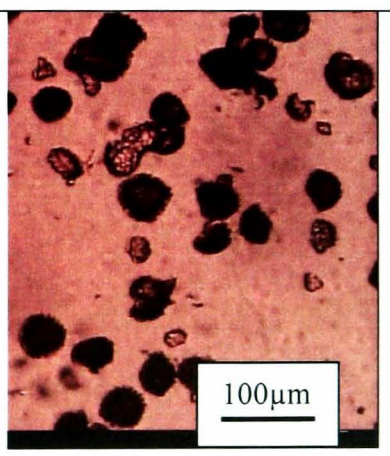
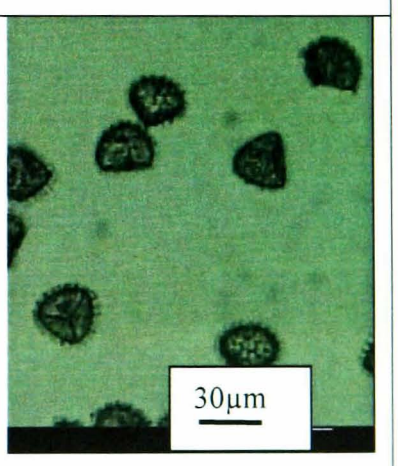
Time (Minutes)	Plasma	Lysed blood pellet	Serum
5			
15			
45			
60			

Figure 3.5.1.2 A: A table illustrating the degradation of exines along a timescale in plasma and lysed blood pellet but not in serum.

From the results it was evident that no significant degradation of the exines occurred in the serum samples, even after 45 minutes incubation at 37˚C. The exines appeared whole, whereas the plasma and blood pellet degraded the exines. This suggests that either the clotting factors, blood cells or plasmin are involved in degradation, as they are the only enzymes removed by clotting the blood before centrifugation, nearly all the other enzymes that are in plasma are present in serum.

The degradation of the exines was further verified by confocal microscopy, showing the fragments resulting after only 5 minutes incubation in human plasma and blood pellet fractions.

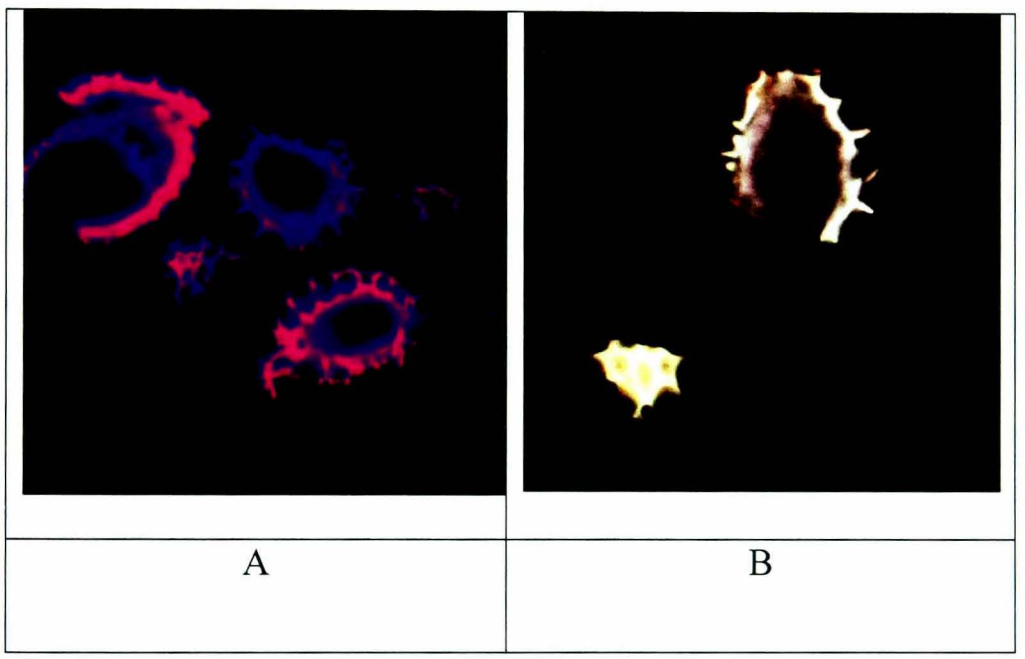


Figure: 3.5.1.2 B. Confocal microscopical images of exine fragments found in both plasma (A) and lysed blood (B) after 5mins *in vitro*.

To account for species differences, sporopollenin exines were also added to serum obtained from human, rat and dog specimens to confirm that serum did not cause degradation of the exines, as seen with the initial experiment using serum obtained from a human volunteer.

The degradation of sporopollenin exines was only evident from plasma and lysed blood pellet experiments, and not with serum, which indicated that either a clotting factor or associated enzyme was working to cause such degradation, since serum is the aqueous phase of blood that has been allowed to clot and the clotting factors were removed.

3.5.2 Different collection tubes

It can be concluded qualitatively that the sporopollenin exines were degraded in plasma obtained from citrate and EDTA tubes (that bind calcium), but not in heparin. From the literature it is known that heparin increases the activity of Antithrombin III (AT-III), which inhibits thrombin formation, factor IX and factor X by forming stable complexes with them and it also inhibits the conversion of plasminogen to plasmin[14].

Heparin is a mixture of polysaccharides that bind to AT-III and induce an allosteric change, enhancing its activity. When heparin binds with AT-III it increases the rate at which AT-III interacts with plasma serine proteases (i.e. the active site of the proteolytic enzyme contains a serine residue).

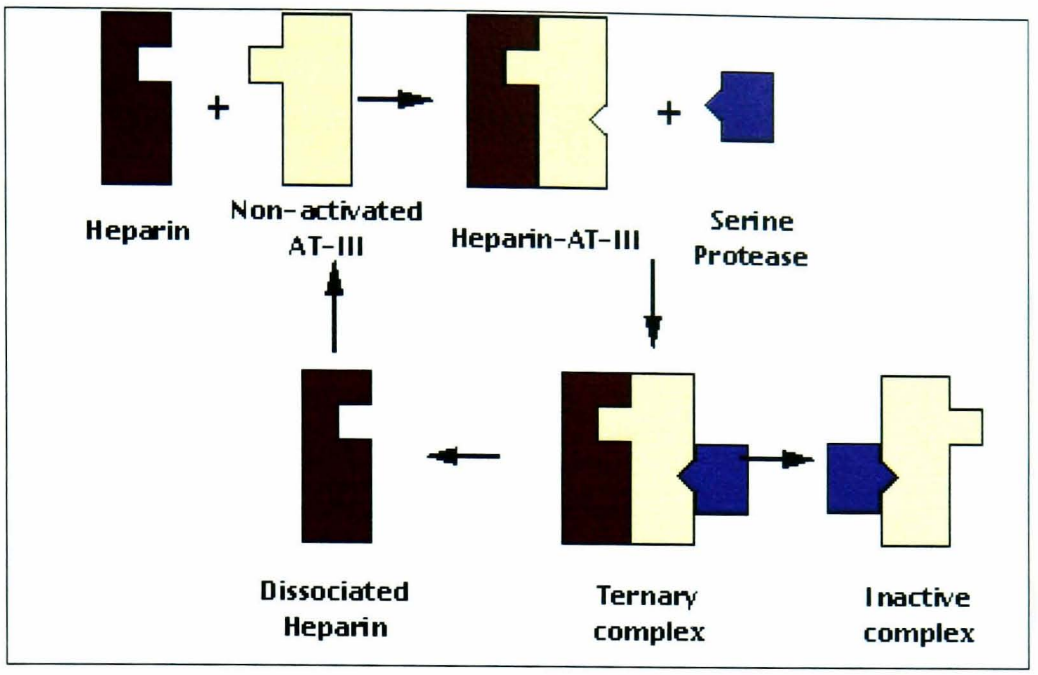


Figure 3.5.2: The binding of heparin to AT-III to produce a conformational change and thus speed up the formation of complexes[14].

These preliminary results suggest that the conversion of plasminogen to plasmin could be a possible enzymatic pathway involved with the degradation of sporopollenin exines. This preliminary information prompted a thorough quantitative *in vitro* investigation into the degradation of sporopollenin exines.

3.6 Quantitative results

Using the reference value determined by haemacytometery (63,750,000 ± 2,380,000 exines/g) it was possible to follow the degradation of sporopollenin exines in plasma, by observing a decrease in the number of exines recovered along a timescale.

3.6.1 Different collection tubes

The differences in the rate of exine degradation with plasma collected from EDTA, lithium heparin and citrate tubes were investigated. As seen from Figure 3.3.1.3, negligible degradation resulted from plasma collected in heparin tubes, and as

mentioned earlier this was predicted because heparin is a known plasmin inhibitor[15]. The number of exines recovered in EDTA and citrate tubes decreased to 44% (± 5.3%) and 60% (± 5.3%) respectively. All future experiments investigating the degradation of sporopollenin exines were performed using plasma collected from EDTA tubes because the blood collected from these tubes did not inhibit the degradation of sporopollenin exines.

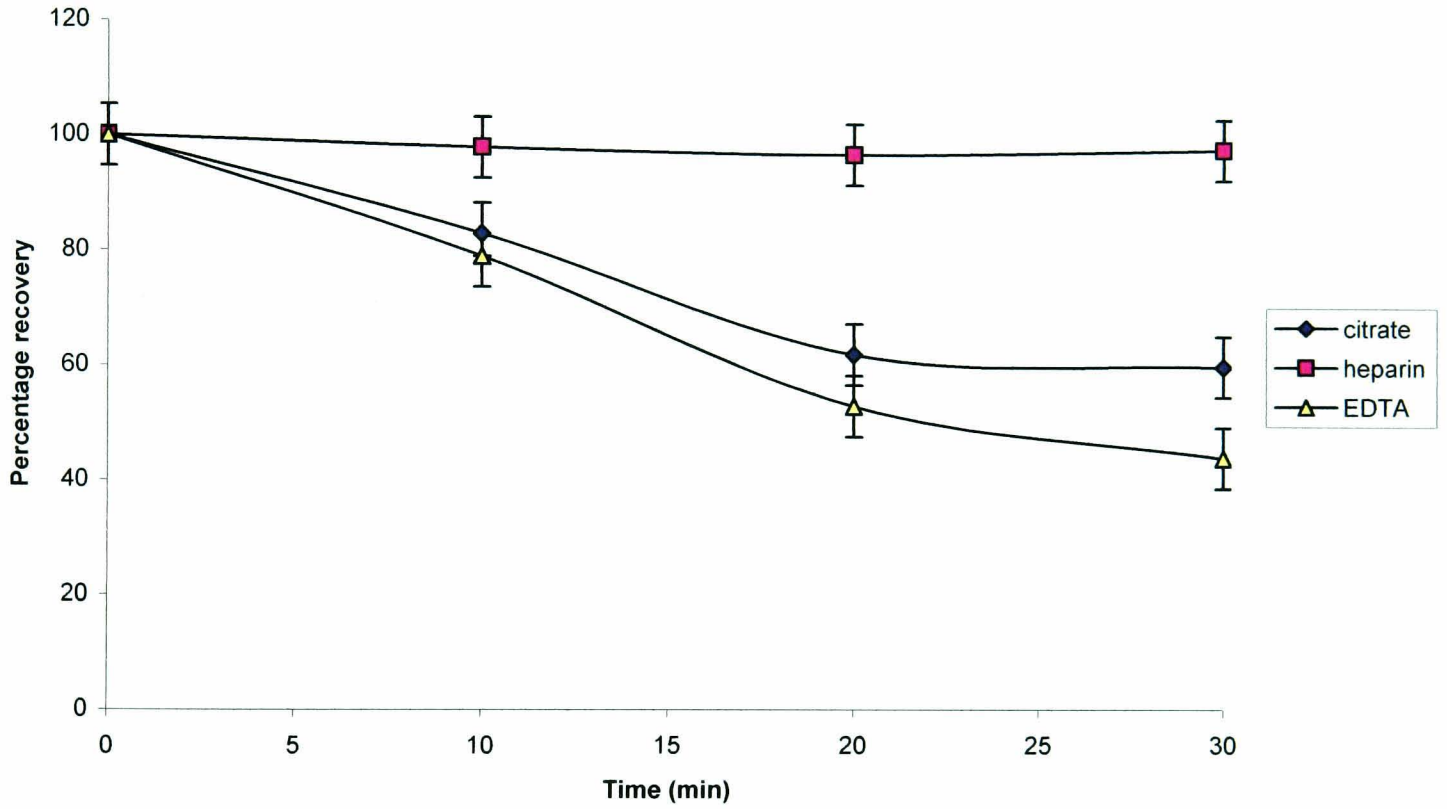


Figure 3.6.1: A graph showing the percentage recovery of exines in plasma obtained from EDTA, citrate and heparin collection tubes.

3.6.2 Effect of temperature

The effect of the temperature on the degradation of sporopollenin exines was explored. A time course study was undertaken at temperatures of 2-4˚C, 22˚C, 37˚C, and 60˚C; to determine the rate at which the sporopollenin exines were degraded and the effect that temperature had on this degradation.

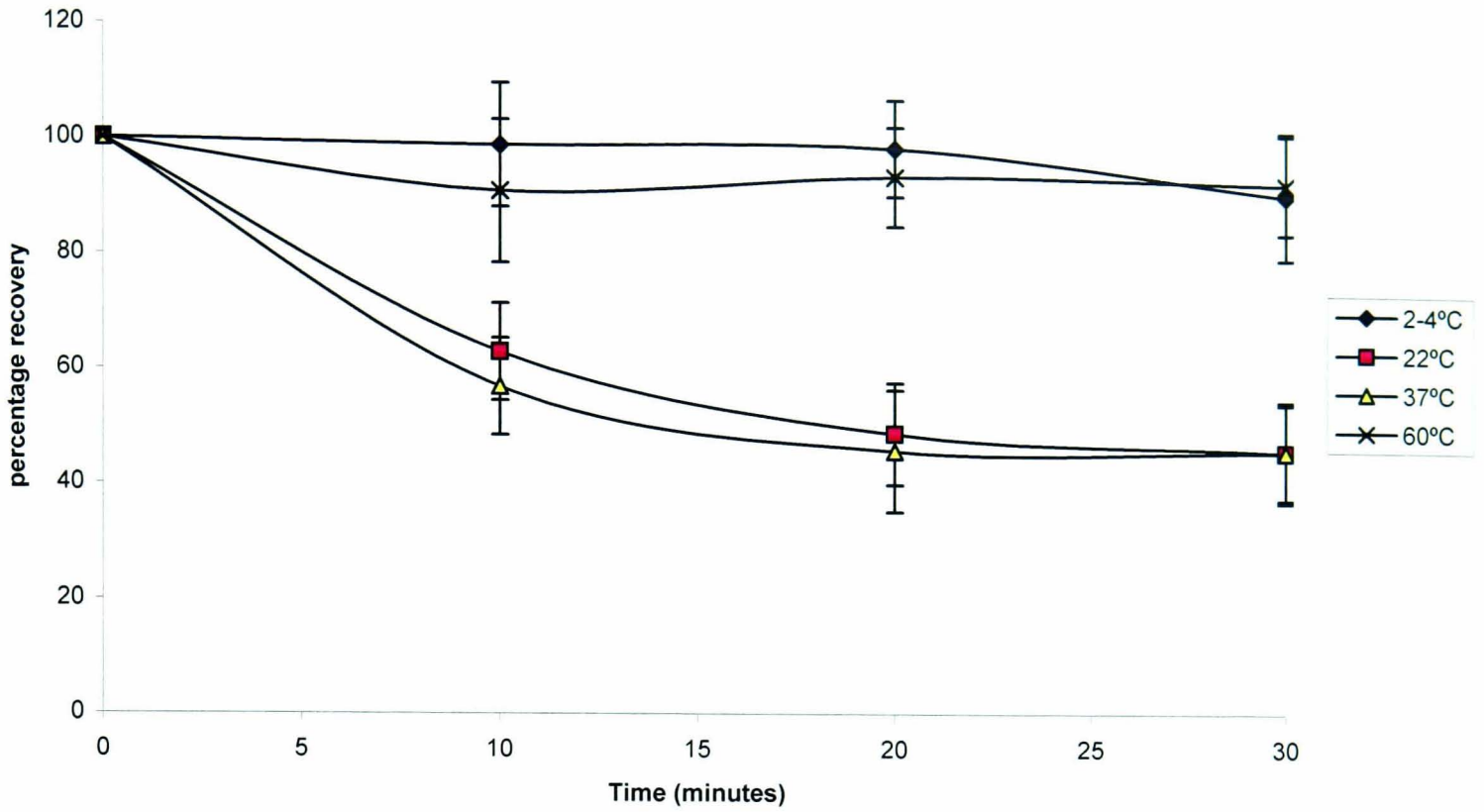


Figure 3.6.2: A graph showing the percentage recovery of sporopollenin exines at different temperatures over 30 minutes.

It is evident from the results that cooling the plasma to 2-4˚C and heating to 60˚C effectively inhibited the breakdown of exines. This was consistent with the hypothesis that the exine degradation was due to an enzyme, since heat inactivation is characteristic of enzymic activity and human enzymes start to denature (alter) quickly at temperatures above 40˚C. This is due to the protein structure becoming denatured as a result of the breakdown of the weak ionic and hydrogen bonding that stabilise the three dimensional structure of the enzyme.

The optimum temperature for human enzyme activity is 35-40˚C, since body temperature is typically 37˚C. This experiment shows that of the temperatures investigated, the temperatures that resulted in the most rapid exine degradation were 37˚C, followed by 22˚C with a decrease in percentage recovery from 0 to 30 minutes

to only 46% (± 8.3%) in both cases. The most rapid degradation of the sporopollenin exines occurred within the first 10 minutes at initially a faster rate at 37°C than 22°C.

It should also be noted that after a further 12 hours there was no additional degradation in any of the samples. This 'plateau' of degradation is also characteristic of enzymic activity. This could be because enzymes have relatively short half-lives or that due to the Lock and Key Theory[16], enzymes become saturated with substrate and so the rate at which the enzyme catalyses the reaction becomes much reduced. An enzyme becomes saturated when the active sites all of the molecules are occupied. At the saturation point, the rate of reaction will not increase when additional substrate is added, but only when additional enzyme is added.

3.6.3 Differences in the rate of degradation in plasma obtained from human, dog and rat species

The rate and extent of exine degradation was observed in plasma collected from human, dog and rat species over 30 minutes. It should be noted that the dog plasma (courtesy of Pfizer, UK) had been frozen for 3 days and defrosted prior to this experiment, whereas the plasma samples from human and rat sources were collected fresh. It was not possible to obtain fresh dog plasma at the time of this experiment.

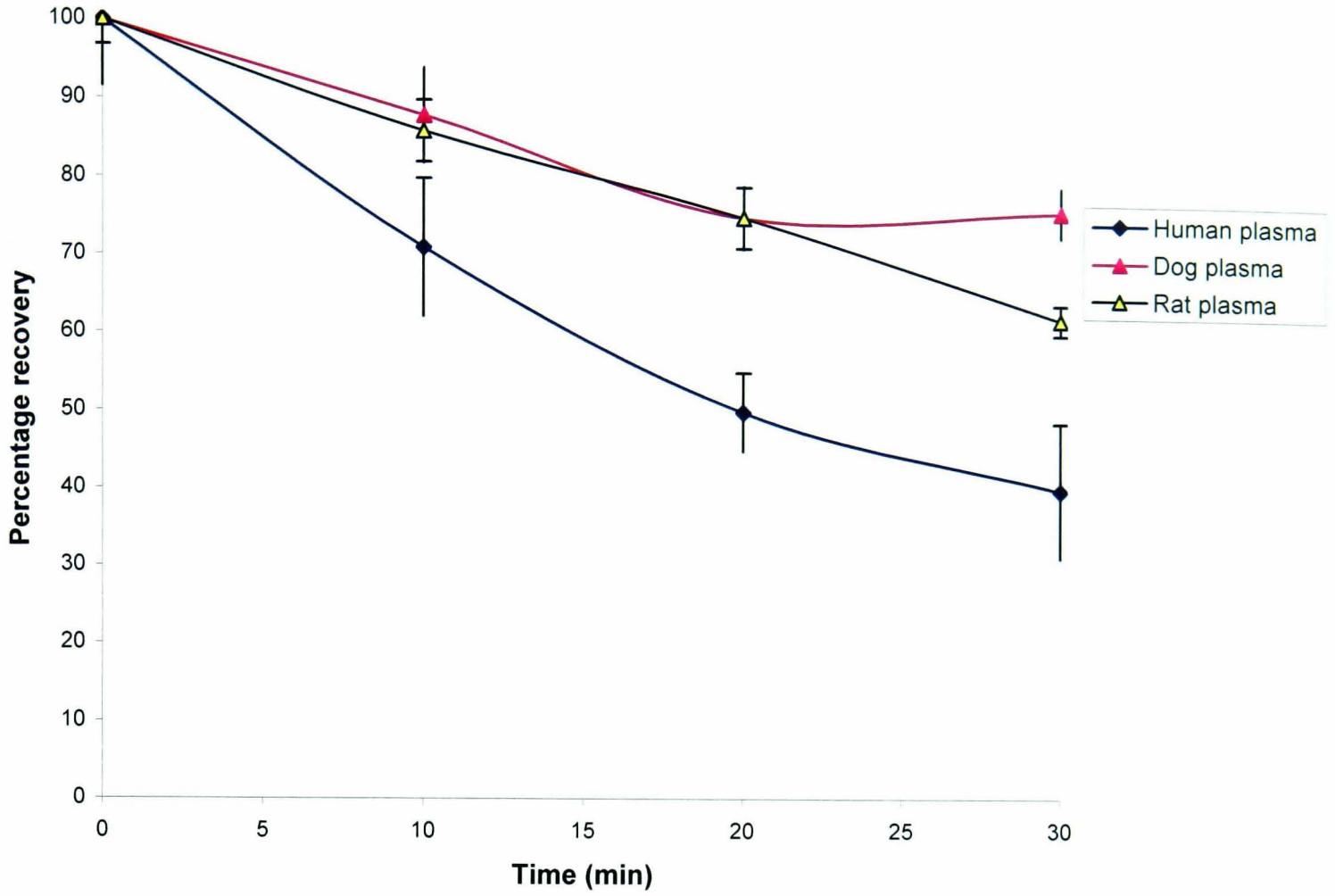


Figure 3.6.3: Rate and extent of exine degradation in plasma obtained from human, dog and rat species

There was a significantly faster initial rate of degradation with human plasma in comparison to plasma obtained from dog and rat species. Sporopollenin exines also degraded to a greater extent in human plasma than that obtained from an animal model, given the associated error margins. The rate of degradation of exines in plasma from dog and rat species was similar over 30 minutes, however, the exines in rat plasma continued to degrade by an additional 13.0% (± 0.7%) between 20 and 30 minutes, whereas the exines in dog plasma reached a plateau at this time and no further degradation was seen. This plateau of degradation of exines in dog plasma could have been due to freezing and defrosting the sample prior to this investigation. Treatment in this manner could have led to reduced activity of the factors present

within the blood that were responsible for causing such degradation of sporopollenin exines.

3.6.4 Effect of plasma storage time

It was shown that temperature had an effect on the rate of exines degradation. For this reason the storage time of plasma was investigated to examine any changes in the rate of such degradation. A study was devised to follow the breakdown of sporopollenin exines in both freshly collected plasma and plasma from the same volunteer that had been stored at -20°C for 2 weeks and defrosted before use. The rate of exine degradation was significantly reduced in the 'stored' plasma. The percentage of exine particles that had degraded over 30 minutes was 54% (± 8.3%) with fresh plasma and 19% (± 6.1%) with 'stored' plasma.

This result further supports the theory that exine degradation is due to an enzymic mechanism.

3.6.5 *In vitro* saturation effect

An experiment was developed to establish if a saturation effect is observed *in vitro*. A specific volume of plasma was incubated at 37°C and different masses of exines (4mg, 10mg and 20mg) were added, the degradation of exines was followed by haemacytometery over 30 minutes. A mass of 1mg exines was also investigated, and although the result was only 37% recovery after 30 minutes, the error in reproducibility was very high (± 37%) with such a small mass and is therefore excluded from the graph.

With an increase in mass of exines in 4ml plasma, the rate of degradation decreased. The percentage of exines recovered after 30 minutes incubation was 30.5% (± 6.3%)

for 4mg mass, 49.7% (± 4.9%) for 10mg mass and 62.8% (± 1.9%) for 20mg mass. This was indicative of a 'saturation effect', as an increase in substrate (exines) caused a decrease in degradation rate. This finding supports the speculation that an enzyme is at least partly responsible for the degradation of sporopollenin exines in blood plasma.

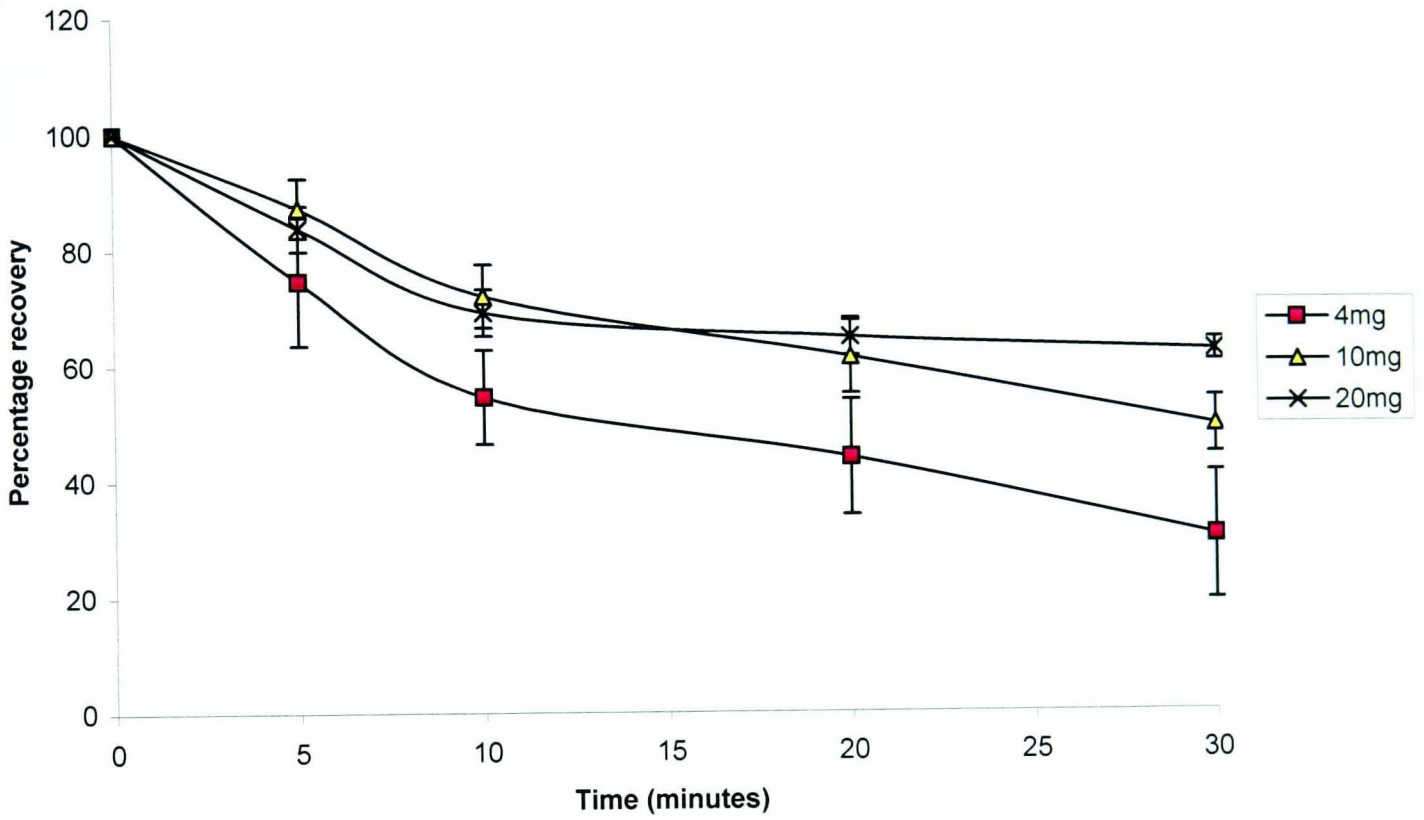


Figure 3.6.5.1: A graph displaying the percentage recovery of different masses of sporopollenin exines in plasma over 30 minutes.

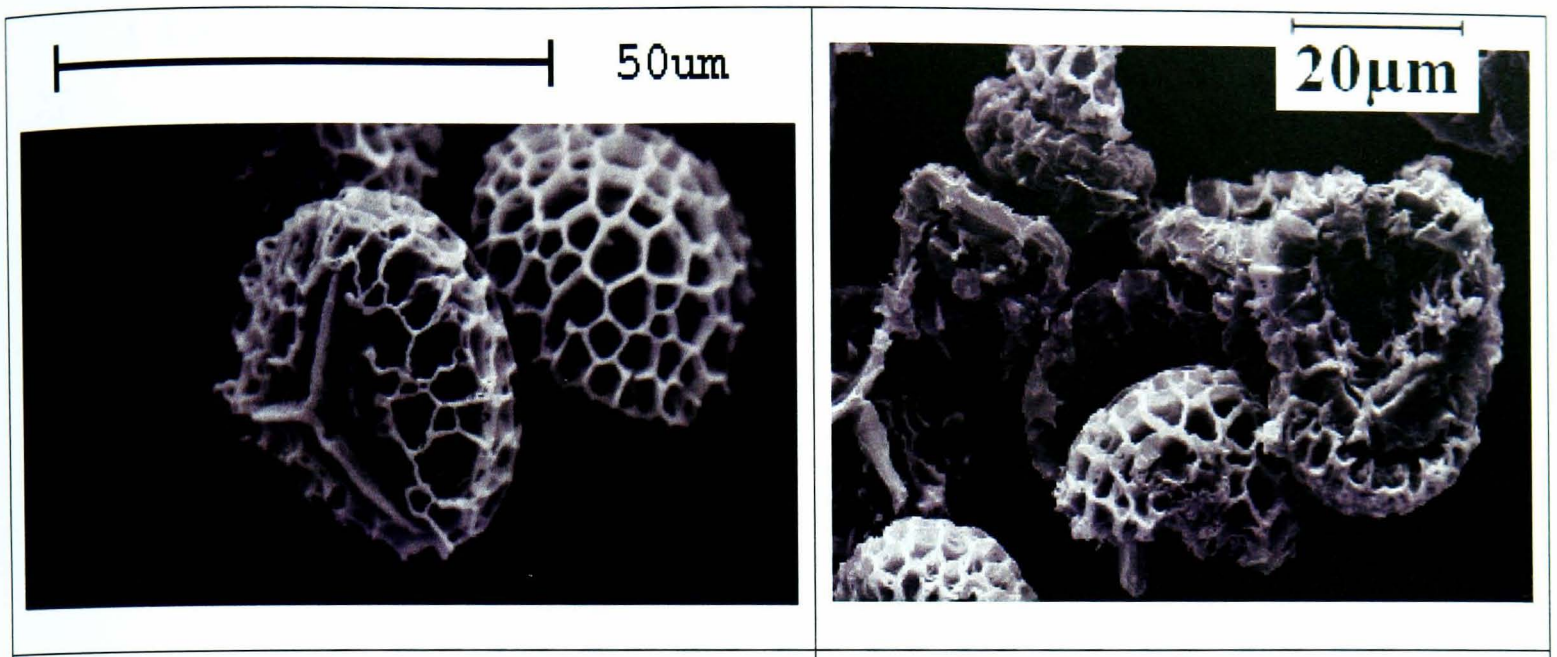


Figure 3.6.5.2: SEM images of exines untreated (A) and after incubation in plasma (B).

Assuming a linear plot of mass of sporopollenin exines degraded vs. time, an approximation to the initial velocities of degradation for each mass of exines was calculated.

	4mg exines (1mg/ml)	**10mg exines (2.5mg/ml)**	**20mg exines (5mg/ml)**
Mass exines degraded at 5min	1.01	1.26	3.22
Initial velocity (mg/min)	0.99	0.79	0.31

Figure 3.6.5.3: The initial velocities of the degradation of sporopollenin exines at different masses in 4ml of plasma.

A Lineweaver-Burk plot was produced (Figure 3.6.5.4) to evaluate whether the enzyme responsible for the degradation of sporopollenin exines followed a simple Michaelis-Menton kinetic profile or if there was a more complicated enzymic system involved with the exine degradation. The volume of plasma remained constant for each different mass of exines investigated (4ml); hence the concentration of the enzyme/s involved with the degradation of such particles would also have been constant.

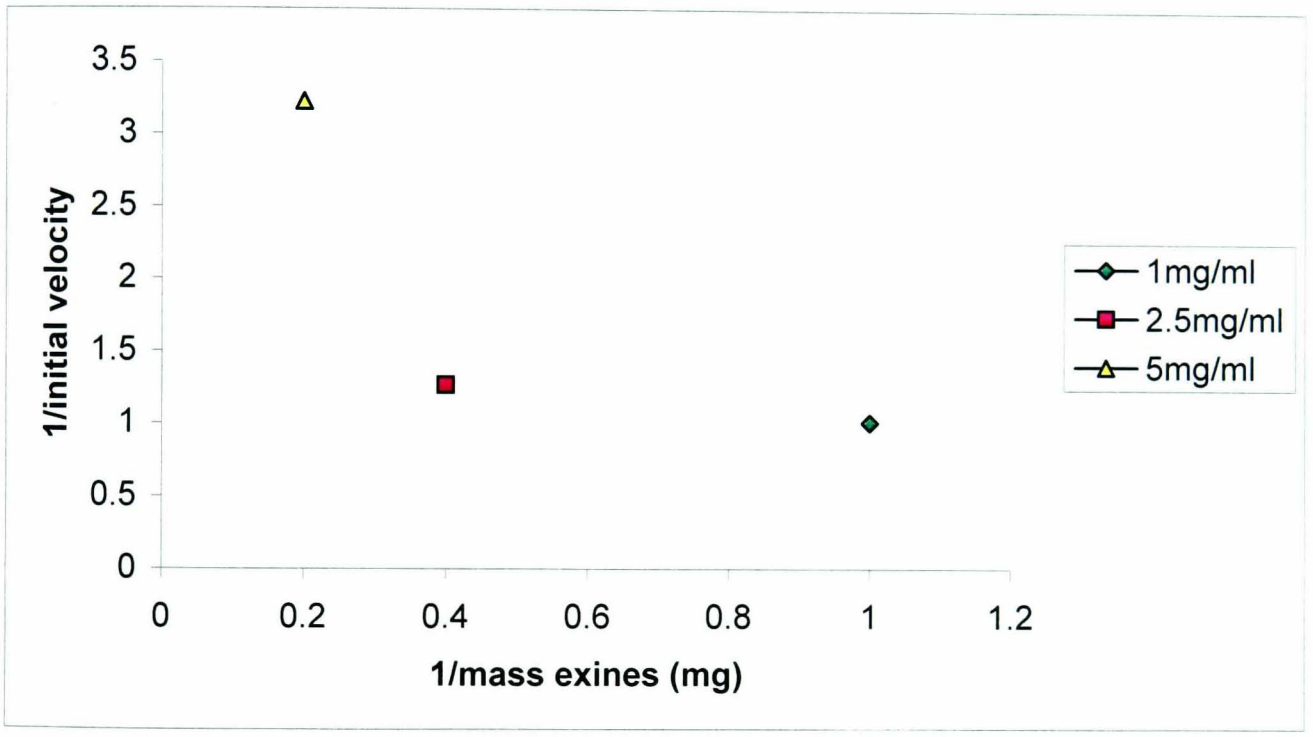


Figure 3.6.5.4: Lineweaver-Burk plot showing the degradation of sporopollenin exines in plasma does not follow simple Michaelis-Menton kinetics.

It can be concluded that the enzymic system responsible for the degradation of sporopollenin exines in plasma does not follow a linear rate and although this does not unequivocally prove that the degradation of exines is caused by a complex system of enzymes, it does indicate that a single enzyme cannot be attributed to this degradation, which would explain why the enzyme plasmin alone failed to cause

degradation of exines (Section 3.6.1.1). In order to obtain a more detailed insight into the kinetic profile of exine degradation, more data points are required.

3.6.6 Effect of Bovine thrombin on sporopollenin degradation

From initial qualitative experiments, serum did not result in any exine degradation. Therefore, bovine thrombin was added to serum to try to 'activate' any residual components to cause degradation. There was no obvious degradation occurring upon addition of thrombin and this resulted in the formation of a 'gel'. This was believed to occur because although thrombin is known to activate factors VII, VIII, V, XI, XIII it also aids the conversion of fibrinogen to fibrin monomers, and hence a clot[17]. These factors were then ruled out as the degrading factors.

3.6.7 Effect of commercial plasma preparations on sporopollenin degradation

Commercial factor XII deficient plasma, prekallikrein deficient plasma and 'normal' commercial plasma were explored for their effect on exine degradation.

There was no evidence of exine degradation using either of the commercial plasma preparations over 30 minutes at 37°C. The fact that the 'normal' commercial plasma failed to degrade sporopollenin could be due to the short half-life of an enzyme that could be responsible for the degradation and its subsequent loss during plasma preparation. Due to the fact that 'normal' plasma did not cause any degradation to the exines, the results from the factor XII and prekallikrein deficient plasmas were not conclusive.

3.6.8 Effect of Aprotinin (Trasylol) on sporopollenin degradation

An *in vitro* plasma experiment was undertaken using different volumes of Aprotinin (Trasylol). Trasylol is a natural serine proteinase inhibitor from bovine lung and is used in cardiac bypass surgery to prevent blood clotting. It is a protein consisting of 58 amino acids arranged in a single basic polypeptide with a molecular weight of 6.5kDa and is known to inhibit the activity of plasmin.

Trasylol was added to plasma (containing exines) as it was anticipated that plasmin/plasminogen could be at least partly responsible for exine degradation and so Trasylol should inhibit the degradation of exines.

It was found that the higher percentage of Trasylol added, the less degradation occurred, as more exines were recovered. The results appear to provide further evidence to support the theory that the conversion of plasminogen to plasmin degrades the exines.

Trasylol in plasma (%) v/v	Extent of inhibition (%)
0	0
5	88 (± 7%)
10	98 (± 2%)
20	100 (± 2%)

Figure 3.6.8: The extent of inhibition of the degradation of sporopollenin exines in plasma when adding different volumes of Trasylol.

3.6.9 Effect of Streptokinase on sporopollenin degradation

Streptokinase is a known plasmin activator[17]. For this reason streptokinase (20% v/v) was added to samples of serum and plasma in an attempt to 'activate' any residual plasmin that is thought to remain in serum[18] and also increase the activity of the enzyme plasmin in plasma samples to observe a faster rate of exine degradation.

The results showed that streptokinase alone does not cause exine degradation. There was a slight increase in the rate of exine degradation in plasma-spiked with streptokinase, however, this is not definitive proof that streptokinase activated the plasmin/plasminogen in plasma to allow degradation of the exines because the increase in degradation observed was just 8.7% (± 1.98%) recovery after 20 minutes incubation to 27.6% (± 1.98%) The addition of streptokinase to serum does not conclusively indicate its activation to degrade exines. If plasmin is responsible for the degradation of exines, there must be insufficient plasmin in serum to overcome the effects of inhibitors.

There was no evident degradation with serum alone, and only 3.4% (± 9.12%) degradation observed with addition of streptokinase to serum which, given the associated error margins, indicates that the addition of streptokinase to serum did not increase the rate of exine degradation. This indicates that if there was any residual plasmin/plasminogen present in serum, it was not 'activated' by addition of streptokinase.

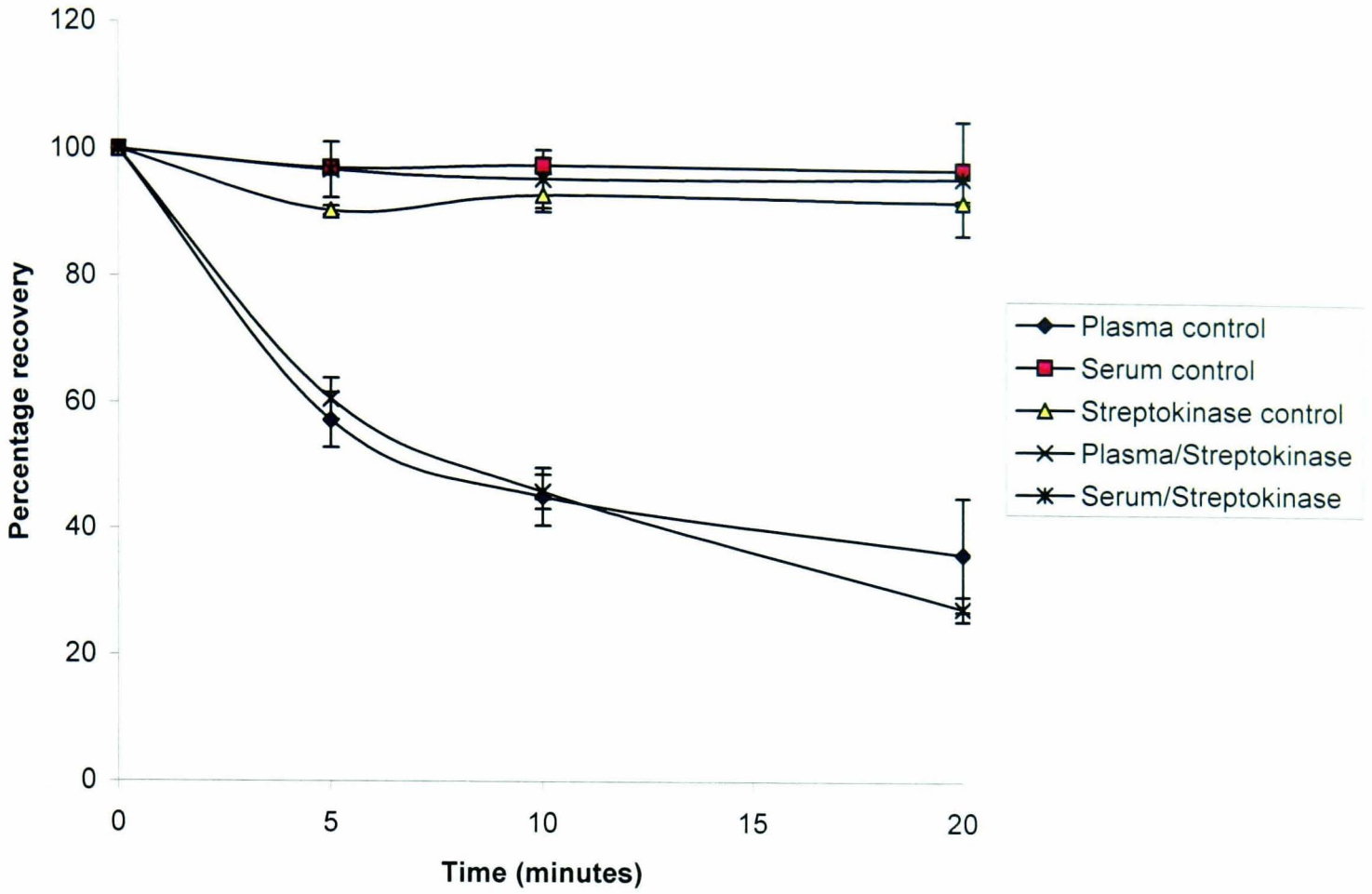


Figure 3.6.9: The effect of streptokinase on the degradation of sporopollenin exines in plasma and serum

3.6.10 Effect of Protease inhibitor cocktail on sporopollenin degradation

In a study investigating albumin microspheres in the treatment of carrageenan-induced inflammation in the rat, 10% α-1-proteinase inhibitor was used to prevent degradation of the albumin microspheres after ingestion[19].

It was thus considered that adding a protease inhibitor to plasma could potentially inhibit the degradation of sporopollenin exines. A commercial 'Protease inhibitor cocktail' was purchased which contained AEBSF, HCl, Aprotinin, Bestatin; E-64 Protease Inhibitor; Leupeptin, Hemisulfate, and Pepstatin A.

Upon addition to plasma and exines, no obvious degradation occurred after 30 minutes incubation at 37°C. This result was anticipated because Aprotinin had already been shown to inhibit the degradation of sporopollenin exines in blood plasma.

3.6.11 Effect of Plasmin on sporopollenin degradation

Since many of the above experiments suggested that plasmin could potentially be responsible for the degradation of sporopollenin exines, an experiment was conducted applying plasmin directly to the exines.

Plasmin/plasminogen has a molecular weight of 92,000 and a concentration in the circulation of 200μg/ml. It has a very short half-life in plasma and serum due to the presence of highly active inhibitors[18]. In view of this short half-life, the plasmin was made up with tris buffer (1ml).

To ensure that tris buffer did not inhibit any degradation of exines, Plasma was diluted with different volumes of buffer to make solutions of 100% plasma, 50%, 25%, 12.5%, 6.3%, 0% (control buffer). Exines were added to each solution and the exines were counted using a haemacytometer. This experiment confirmed that tris buffer did not inhibit the degradation of exines

Subsequently, plasmin (500μg) was dissolved in tris buffer and double diluted across a series of tubes, giving concentrations between 250μg/ml (similar to the physiological concentration) and 0μg/ml. There was no degradation observed with plasmin. This may be because the activity of plasmin had been compromised by addition of the buffer or because the enzyme plasmin is not the sole contributor to the degradation of sporopollenin exines. It could be that there are multiple pathways involved with the degradation and not just plasmin itself, as first thought.

3.6.12 Effect of Enoxaparin (Clexane) on sporopollenin degradation

Clexane was investigated for its effect on the degradation of sporopollenin exines in plasma. Clexane is a fragment of heparin and since heparin has shown to inhibit the degradation of sporopollenin exines it was thought that Clexane might also inhibit such degradation. Clexane alone did not cause any sporopollenin exine degradation. The control samples of Plasma from EDTA collection tubes without Clexane resulted in 68% degradation after 30 minutes, and as expected, no degradation was seen with heparin-collected plasma, given the associated error margins. Addition of Clexane to plasma in various concentrations appeared to inhibit the degradation of sporopollenin exines, although repeats of this experiment are required to confirm that this inhibition is due to Clexane.

3.7 Characterisation of the degradation mechanism

An indication that the cause of exine degradation occurs via an enzyme was evident when the breakdown was inhibited at 4°C and 60°C, yet occurred rapidly at 22°C and more so at 37°C. The 'saturation' effect shown by observing a decrease in the rate of degradation with larger masses of exines in plasma was also indicative of an enzymic mechanism. The storage time of plasma was also a factor affecting the degradation rate of exines, and longer storage led to a decrease in degradation, which was characteristic of an enzyme, since the half-life and subsequent activity of the enzyme may have been reduced over the storage period.

As a result of the degradation of sporopollenin exines only occurring in plasma, lysed blood pellet and not in serum, it was thought that an enzyme or a clotting factor was responsible because serum contains no clotting factors. Other experiments were

therefore undertaken to attempt to characterise this degradation and identify the specific enzyme responsible.

The first indication that the enzyme plasmin/plasminogen could be a contributing factor to exine breakdown was following degradation in plasma collected from EDTA and citrate tubes, but not in lithium heparin tubes. Heparin is known to inhibit the activation of plasmin and so was considered a possible attribute.

Plasmin is an important degrading serine protease enzyme that is released into the circulation as plasminogen. It degrades many plasma proteins, namely fibrin clots[15]. It is activated by tissue plasminogen activator (tPA), urokinase, streptokinase, thrombin, fibrin and factor XII. It is inhibited by anti-activators, such as alpha 2-antiplasmin, and also by aminocaproic acid.

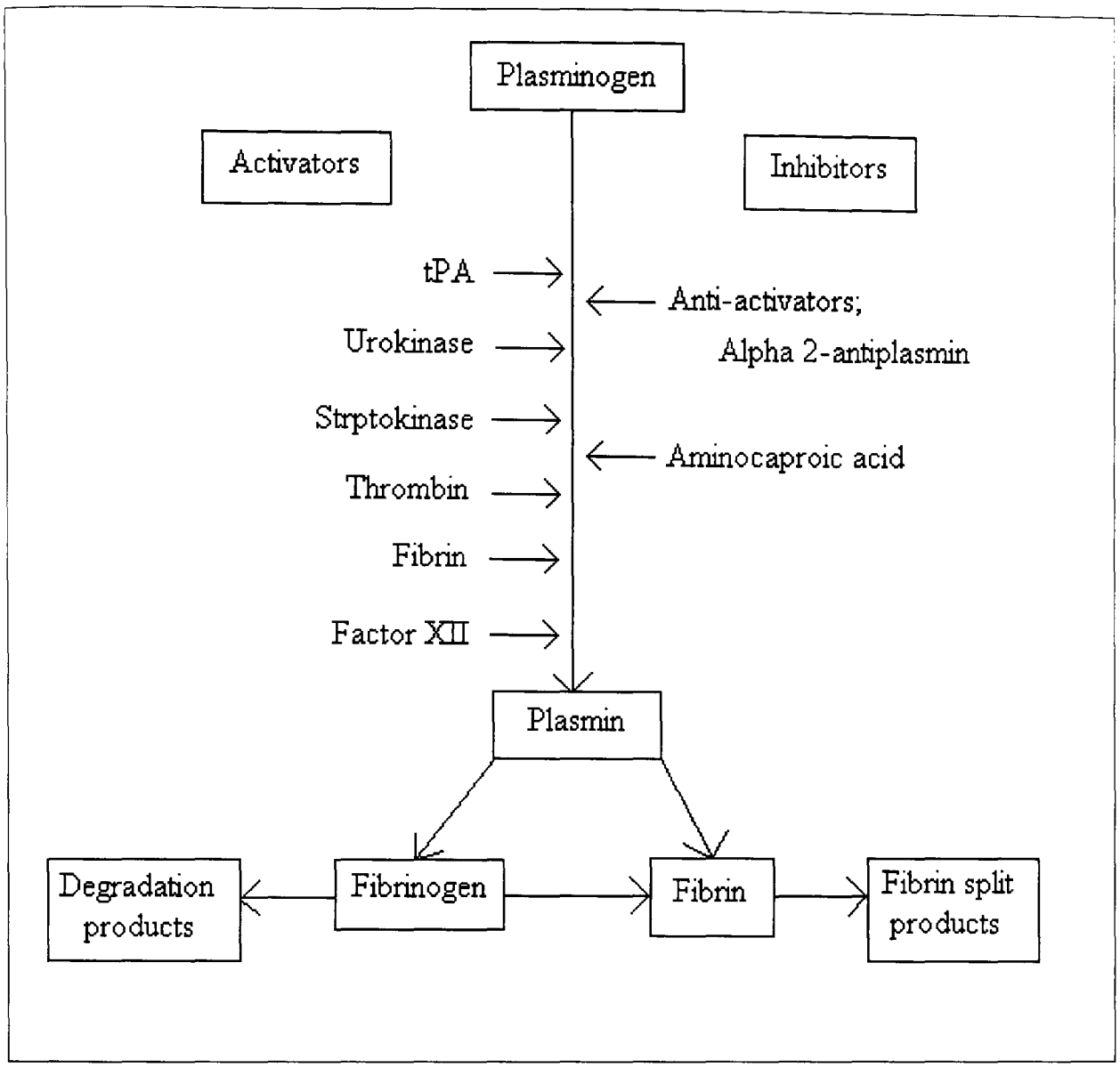


Figure 3.7.1: The factors involved with the activation and inhibition of the conversion of plasminogen to plasmin[15].

Consequently, Trasylol and a commercial protease inhibitor 'cocktail' containing Trasylol were added to plasma and successfully inhibited the degradation of exines. Plasma was spiked with streptokinase, a plasmin activator, and effectively activated degradation of exines in plasma by 8.7% (± 2%) more than in plasma alone, but no additional effect was seen with serum.

Sporopollenin exine degradation is not fully understood because plasmin is a serine protease, which causes the breakage of peptide bonds (-C(=O)-NH-) but since there is

no nitrogen present in the exines it is thought that either the plasmin is breaking other bonds, such as ester bonds, or a plasma esterase enzyme may be responsible.

Due to the exines being essentially nitrogen-free, complement was also considered as a degrading factor, because the complement system consists of a series of small proteins, which, being proteins, would also be destroyed by heating to 60°C. However, complement was disregarded as a potential degrading mechanism because all components of complement are present in fresh serum and serum did not cause any exine degradation. Similarly, antibody is also present in fresh serum and so was ruled out as a potential sporopollenin exine-degrading factor. The Lineweaver-Burk plot in Figure 3.6.5.4 showed that the degradation of sporopollenin exines did not follow a linear rate and indicated that the degradation could be caused by a more complex system of enzymes as opposed to a single enzyme.

3.8 Degradation of different pollen, spores and their extracted exines in plasma

The difference in the rate of degradation of different types of pollen, spores and their extracted exines in plasma were investigated. The degradation profiles were very similar between rye grass, *L. clavatum* (25μm and 40μm) and *A. trifida* species within the first 5 minutes. Of all the species investigated, the 40μm *L. clavatum* species appeared to degrade most rapidly over 30 minutes. The raw spores were shown to degrade more rapidly than the extracted exines.

In all other cases the extracted exines degraded more rapidly than the parent pollen or spores. In particular, after 30 minutes incubation in plasma there were significant differences between the rates of degradation of *L. clavatum* (25μm) spores (48.7% recovery ± 4.9%) in comparison to the extracted exines of 30.5% recovery (± 7.0%).

The percentage recovery of *A. trifida* pollen after 30 minutes incubation was 60.6% (± 6.1%) in comparison to the extracted exines at a more rapid 33.6 (± 6.2%) recovery. After 30 minutes the rye grass pollen degraded to give 63.5% recovery (± 4.6%) in comparison with the extracted exines that degraded to a greater extent, giving only 45.9% recovery (± 5.2%).

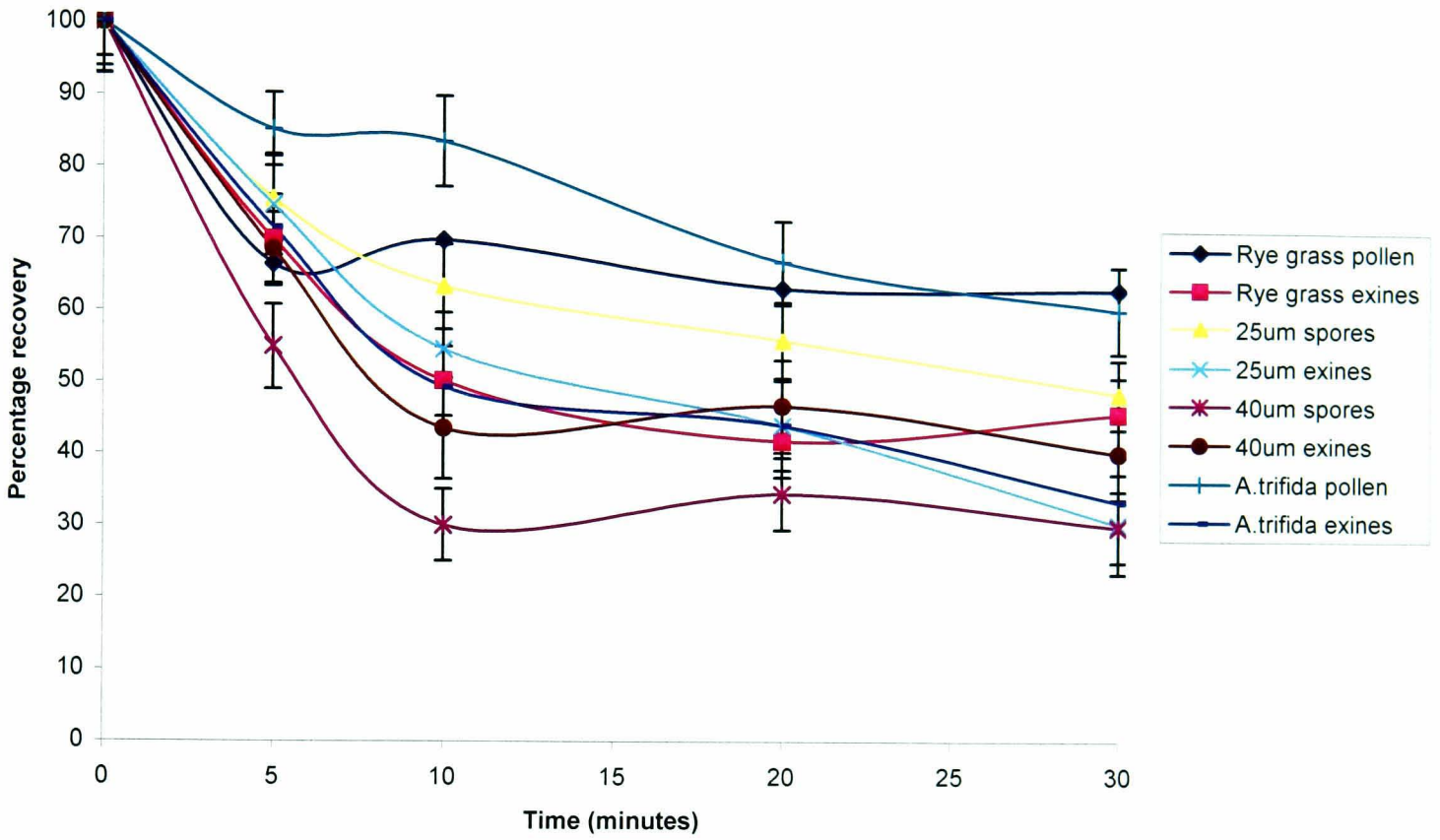


Figure 3.8.1: Degradation of different types of pollens, spores, and their extracted exines in plasma *in vitro* over 30 minutes.

Assuming the plot of percentage degradation vs. time to be linear at 5 minutes, an approximation to the initial velocities of degradation for each type of particle was calculated. The percentage degradation was converted to mass of particles consumed, since each experiment was performed on the same mass of particles (4mg in 4ml plasma).

	Rye pollen	Rye exines	25µm spores	25µm exines	40µm spores	40µm exines	*A trifida* pollen	*A trifida* exines
Mass exines degraded at 5 min (mg)	1.34	1.20	0.98	1.01	1.80	1.26	0.59	1.14
Initial velocity (mg/min)	0.75	0.83	1.02	0.99	0.55	0.79	1.69	0.88

Figure 3.8.2: The initial velocities of the degradation of different types of pollen, spores and exines in plasma.

There was little difference between the initial velocities for rye pollen and rye exines (0.75mg/min and 0.83mg/min respectively), 25µm *L. clavatum* spores and exines (1.02mg/min and 0.99mg/min respectively) and 40µm *L. clavatum* spores and exines (0.55mg/min and 0.79mg/min respectively). There was, however, a larger difference between the initial velocities of *A. trifida* pollen and extracted exines (1.69mg/min and 0.88mg/min respectively). This difference could be within the error associated with the method and does not unequivocally prove that the *A. trifida* pollen degraded at a faster rate than the extracted *A. trifida* exines. In general, given the associated error margins, the initial velocities of different types of pollen, spores and sporopollenin exines were comparable.

3.9 Resilience of exines to stomach and small intestinal fluids

An *in vitro* experiment was set up to emulate the conditions encountered in the stomach and small intestine, where persorption of pollen and spore particles has been said to take place[12,20]. The solutions were prepared as stated in Chapter 5.

Degradation in such solutions was not expected because sporopollenin is known to be stable to chemical attack[5].

No visible degradation was observed in either the simulated stomach or small intestinal fluids. It was considered that the simulated fluid may be lacking an aspect that could cause degradation *in vivo*, so actual stomach and small intestinal fluids (dog) were obtained, courtesy of Pfizer, Sandwich Laboratories.

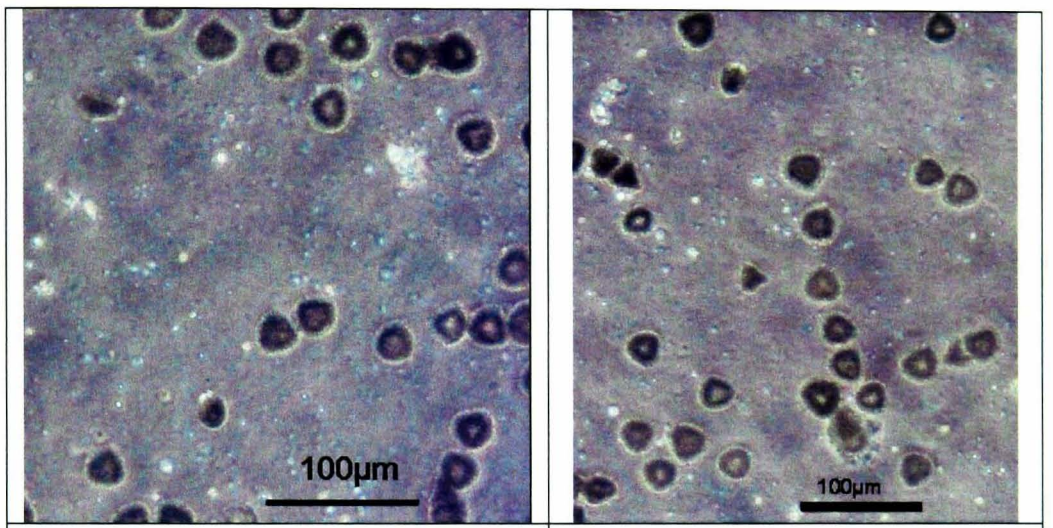


Figure 3.9: Light microscopical images of intact exines in small intestinal fluid (A) and stomach fluid (B) respectively.

Following incubation at 37°C for 1 hour there was no visible breakdown of the sporopollenin exines in either the actual stomach of small intestinal fluids (as seen above), which indicates that the particles are stable in these harsh environments. This shows the potential of exines to provide protection of encapsulated ingredients against the harsh environments of the GI tract, yet deliver them effectively into the bloodstream.

3.10 Tablet form of sporopollenin exines

Tablets were prepared from sporopollenin exines using an IR press (10 tonnes, 2 min) and their dissolution was studied in SGN (simulated gastric fluid without pepsin prepared as stated in Chapter 5). Different tablets were prepared to determine their dissolution in SGN:

A. 400mg exines
B. 400mg exines and 40mg sodium bicarbonate (10%)
C. 400mg exines, 40mg sodium bicarbonate (10%) and 180mg lactose (45%)

After 10 minutes in SGN, there was significantly more degradation of tablet C and the 10% sodium bicarbonate did not appear to aid tablet disintegration.

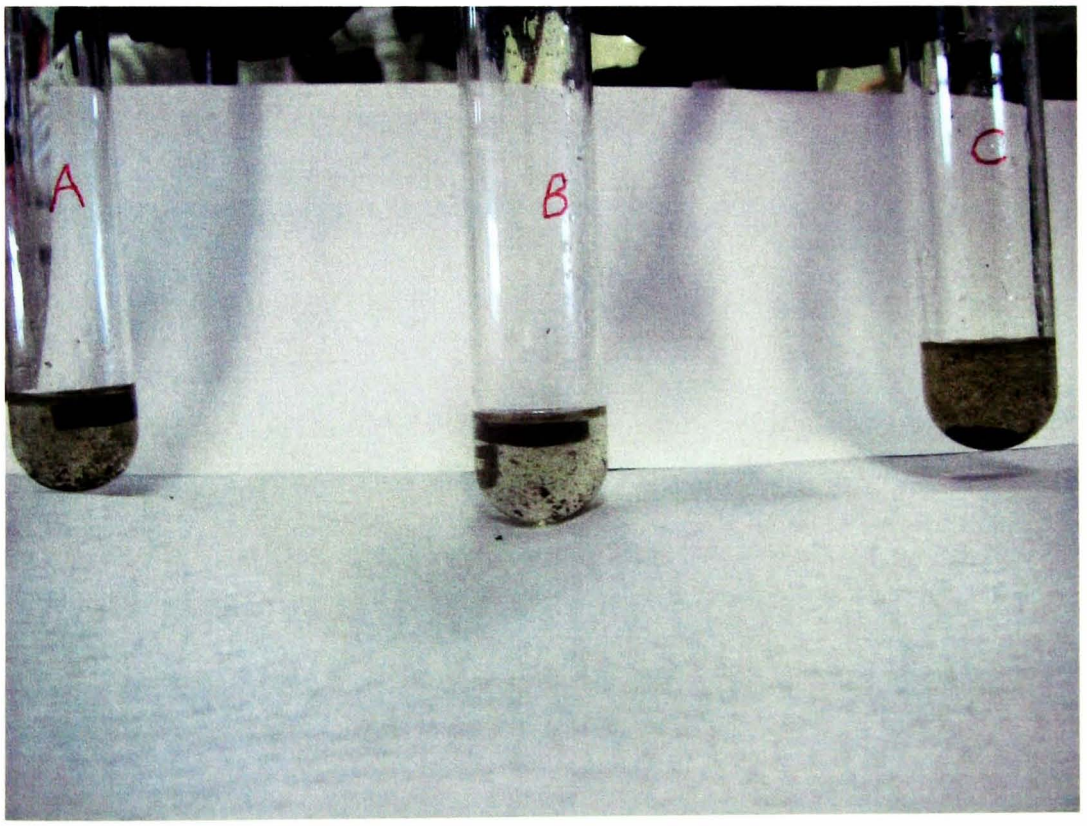
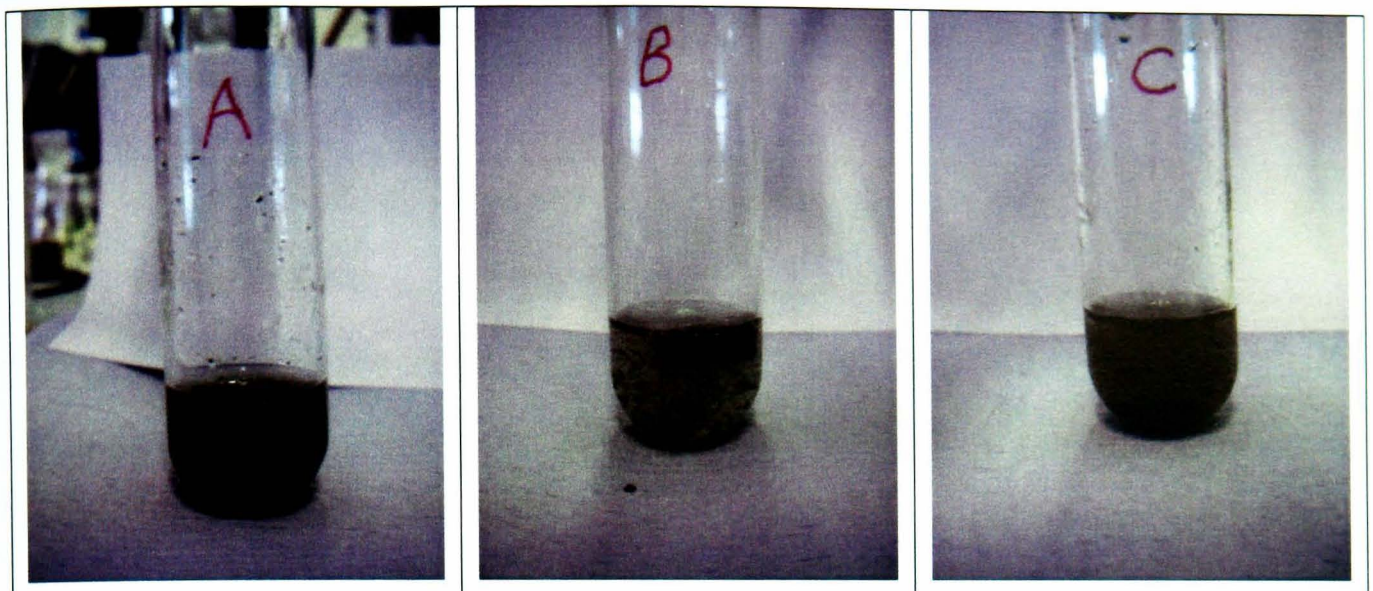


Figure 3.10.1: Tablets A, B and C after 10 minutes incubation in SGN.



Tablet A	Tablet B	Tablet C

Figure 3.10.2: Tablets A, B and C after 30 minutes in SGN.

Although tablet A broke down quite well in SGN after 30 minutes, there was still a large solid tablet at the bottom of the tube. Tablet B (with 10% sodium bicarbonate) did not appear to break down very well, even with gentle shaking. Tablet C broke down very rapidly and after 10 minutes in SGN most of the exines were in solution. For this reason, Tablet C (with 45% lactose and 10% sodium bicarbonate) was used for the *in vivo* trials on human volunteers.

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4. The functional utility of sporopollenin exines as a novel drug delivery system

4.1 Introduction

Most protein-based drugs, such as vaccines and hormones, cannot be delivered orally due to problems related to degradation in the GI tract[1] and despite sophisticated new drug delivery systems, the development of an oral formulation remains a formidable challenge[2]. Microencapsulation technology (such as the use of micro- and nanoparticles) provides an exciting approach to enhance the uptake and transport of orally administered molecules.

4.2 Results and Discussion

4.2.1 Extraction of sporopollenin exines from modern pollen and spores

Several methods were explored to obtain nitrogen-free sporopollenin material from *L. clavatum* spores, as detailed in Chapter 5, including:

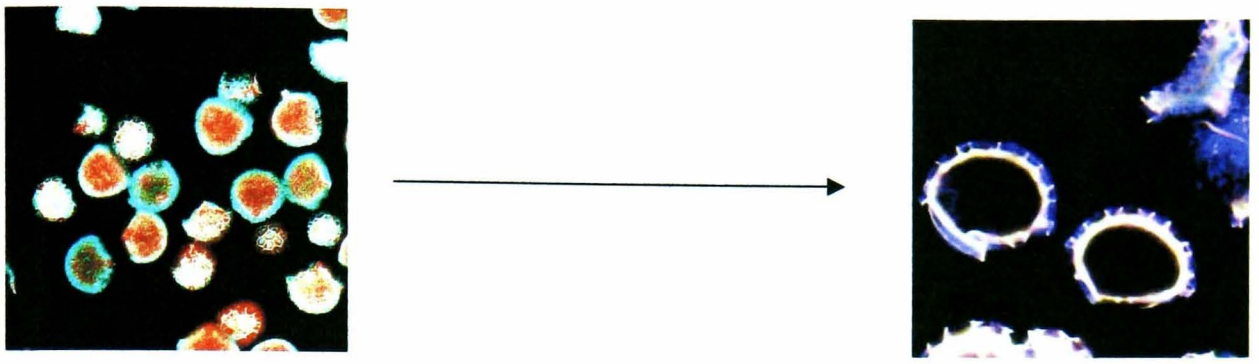
1 Treatment with potassium hydroxide and phosphoric acid adapted from the literature[3,4].

2 Acetolysis[5,6].

3 A comparison with a method used to obtain silk from silkworm moth[7].

The first extraction method was chosen over the other methods because although the latter methods were shown to be more rapid, previous elemental analysis of sporopollenin obtained this way contained high nitrogen content, which is often associated with allergens.

The confocal images below show the space created inside the exines for loading after extraction.



Raw *L. clavatum* (25µm) Extracted exines (Empty)

Figure 4.2.1: Confocal images showing the extraction of sporopollenin exines from *L. clavatum* spores.

4.2.2 Internal volume of sporopollenin exines

The exine layer was determined to be 1.5µm thick in both 40µm and 25µm *L. clavatum* sporopollenin exines by embedding particles in LR acrylic resin and cutting with a section knife (as detailed in Section 5.15.2) to observe by LM (Figure 4.2.2). Wall thickness was measured using the software *Image Pro*.

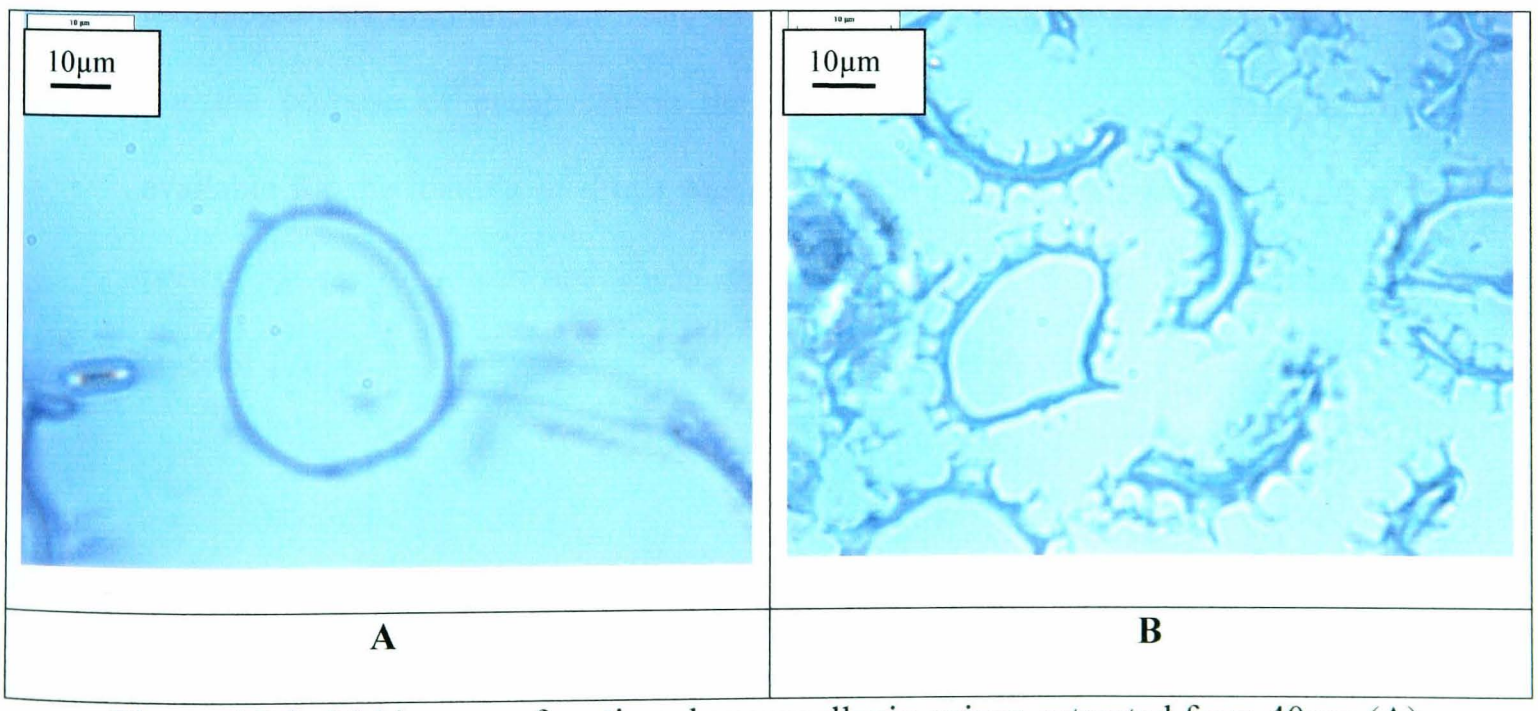


Figure 4.2.2: LM images of sectioned sporopollenin exines extracted from 40µm (A) and 25µm *L. clavatum* spores (B).

The available space for loading inside the exine was estimated based on the following geometrical data.

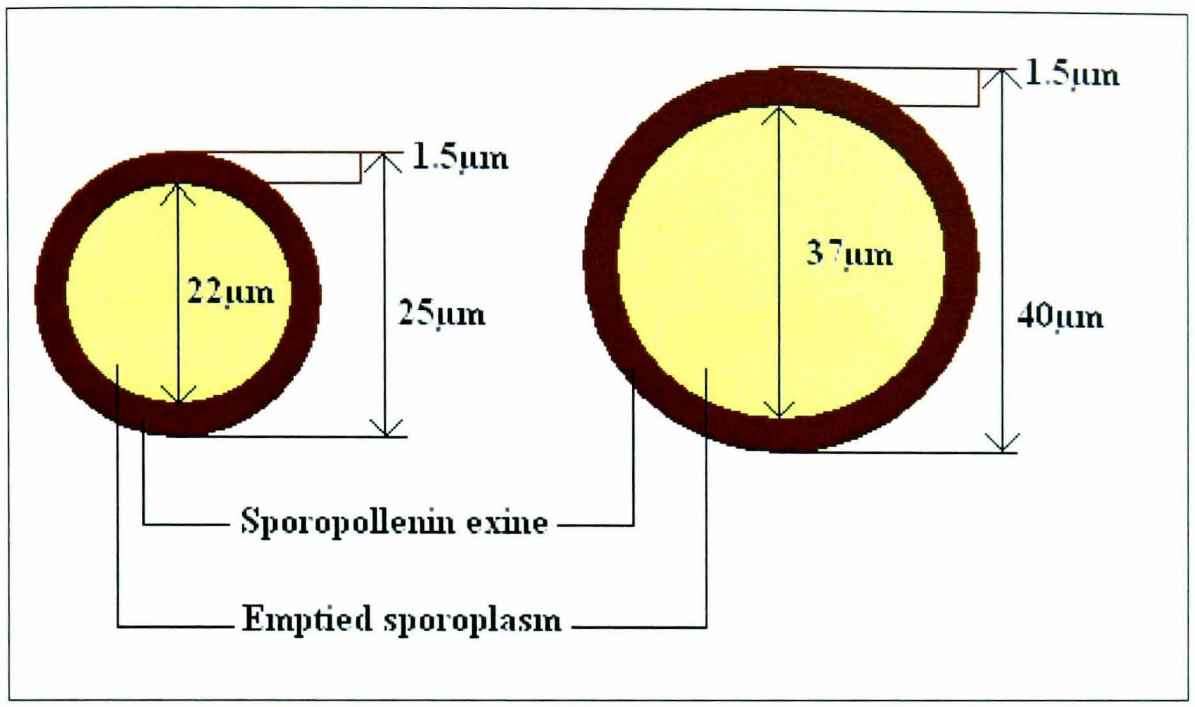


Figure 4.2.3 Measurements of sporopollenin exines assuming them to be a sphere (not to scale).

The density of 40µm and 25µm exines was determined by compressing the exines into tablets of 13mm thickness using an IR press (10 tonnes) (Figure 4.2.4).

For the purpose of encapsulation the inner volume of the exines that could be available for the loading of drugs was calculated. The particles were assumed to be perfect spheres of 40µm and 25µm diameter and TEM sections revealed an exine layer of 1.5µm.

Sporopollenin size	Tablet thickness (mm)	Tablet mass (mg)	Density (g/cm^3)
25µm	5	692	1.042
40µm	4	510	1.000

Measurement	25µm	40µm
Outer diameter (m)	0.000025	0.000040
Inner diameter (m)	0.000022	0.000037
Outer surface (m^2/exine)	1.96×10^{-09}	5.03×10^{-09}
Outer volume (m^3/exine)	8.18×10^{-15}	3.35×10^{-14}
Inner volume (m^3/exine)	5.57×10^{-15}	2.65×10^{-14}
Exine shell volume (m^3/exine)	2.61×10^{-15}	6.99×10^{-15}
Density (g/cm^3)	0.154	0.149
Statistics (exines/g)	63,750,000	21,100,000
Outer surface (m^2/g)	0.13	0.11
Outer volume (m^3/g)	5.21×10^{-07}	7.06×10^{-07}
Inner volume (m^3/g)	3.55×10^{-07}	5.59×10^{-07}
Exine shell volume (m^3/g)	1.66×10^{-07}	1.47×10^{-07}
Outer surface (dm^2/g)	13.0	11.0
Outer volume (cm^3/g)	0.52	0.71
Inner volume (cm^3/g)	0.36	0.56
Exine shell volume (cm^3/g)	0.17	0.15

Figure 4.2.4 The relative densities and measurements of sporopollenin exines

In conclusion, sporopollenin of both types could theoretically contain approximately 1ml/g of a substance, assuming an outer coating, full exines layer and full inner loading.

4.3 Loading theory

Plant spores and pollen grains are believed to possess nano-pores that run through the exine layer of the particles. These pores are prevalent in nature because they allow substances to pass readily into and out of the pollen or spore particle during their growth period. Evidence of these pores has been documented using SEM to visualise what appear to be multi-helical channels running through the exines of *L. clavatum* spores and *Fagus Sylvatica* pollen grains[8] (see below). In this respect the emptied sporopollenin exines can be regarded as permeable 'microspheres', with the potential to encapsulate 1ml/g of substance.

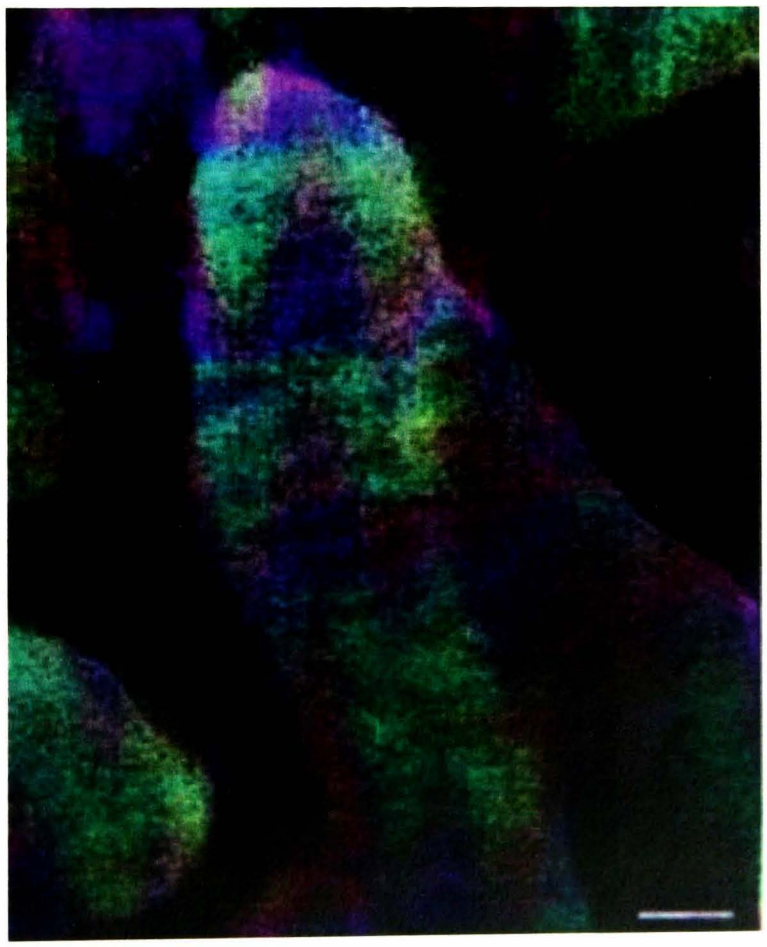


Figure 4.3.1 SEM image showing multi-helical tubules (scale bar: 100nm)[8].

4.4 Encapsulation of substances

Several loading mechanisms were explored in order to physically entrap different substances within the exines. The time taken for a substance to be absorbed into the exine cavity is strongly dependent on the presence of liquid used to aid absorption (i.e. ethanol) and also on the pressure and vacuum used during encapsulation. The presence of ethanol and vacuum increased the efficiency of filling.

1. **Passive absorption** – This involved stirring the exines at room temperature and pressure into a solution of a substance to be encapsulated. Solid substances were first dissolved in a suitable solvent that was later removed completely by evaporation, drying in an oven or under vacuum over phosphorous pentoxide. By this method, the particles were coated in the substance and did not load to their maximum efficiency.
2. **Compression** – Exines were first compressed into tablet form using an IR press (10 tonnes for 2 minutes). The tablets were then placed into a solution of the substance to be encapsulated. Although the tablets swelled up and absorbed the liquid, the exines showed slight cracking upon SEM analysis.
3. **Vacuum loading** – This method uses vacuum to remove air from inside the exine and promote loading of a solution. It was found that organic solvents (such as ethanol) load at a quicker rate than aqueous media and so typical loadings involved dissolution in water: ethanol mixtures, which were compatible with food and pharmaceutical regulations. This method was used for loading all of the substances in this report as it avoided the problems associated with exine cracking and resulted in maximum loadings.

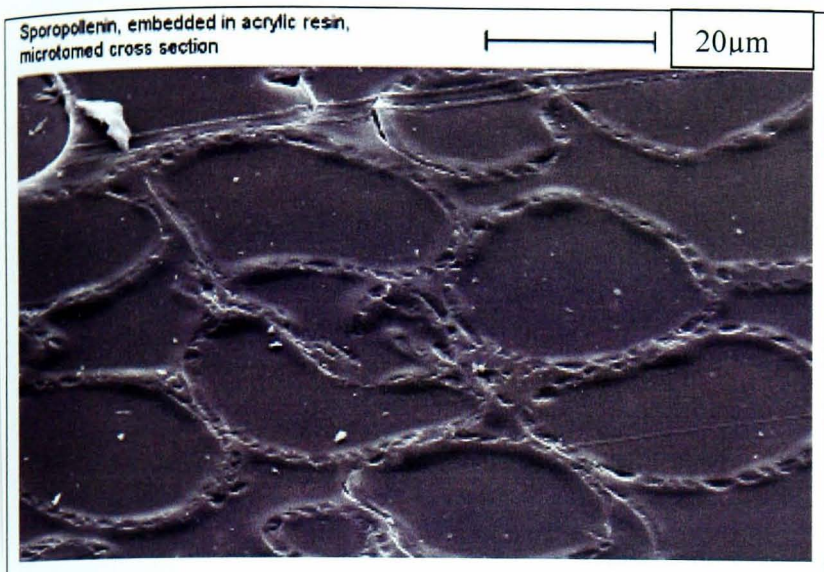
4.5 Evidence of successful loading

Sporopollenin exines possess a remarkable property. They appear to absorb and contain within them a variety of substances, including liquid, solid, inorganic, organic, hydrophobic and hydrophilic products. Often polarity is an obstacle when designing drug delivery vehicles, with hydrophobic drugs being easier to encapsulate, as they are often highly soluble in organic solvents used in many formulations[9].

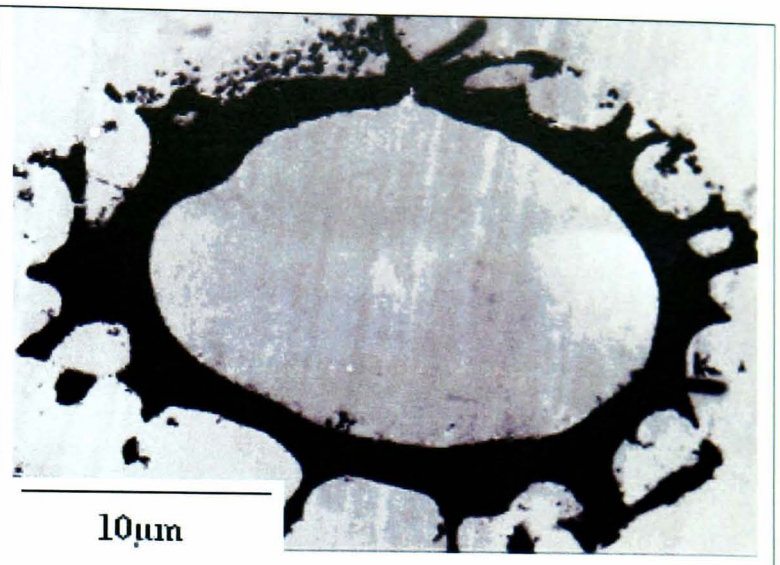
The confocal images in the following section are confirmation that different materials, including hydrophilic substances (for example ascorbic acid) and hydrophobic substances (for example oils) were successfully encapsulated into sporopollenin exines.

The use of Light microscopy also indicated the loading of products into the exines. This occurred by chance because Histoclear (D-limonene), that was used to visualise the exines under the microscope to make them transparent, appeared to soak into the particles causing air bubbles within them.

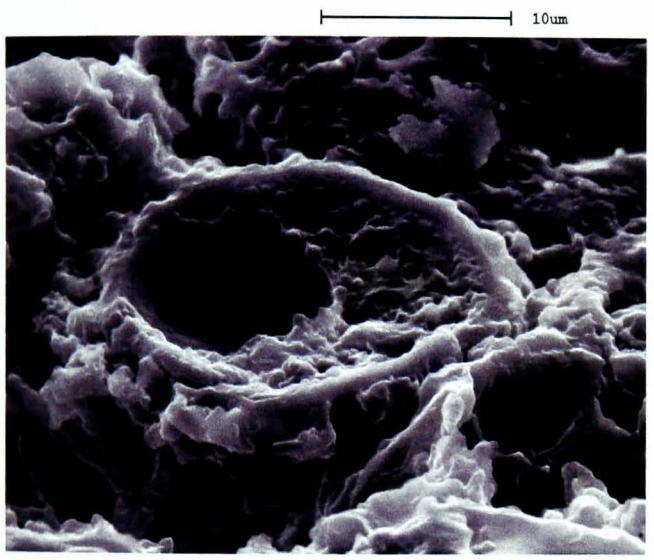
Acrylic resin was readily absorbed into sporopollenin exines during the preparation of microtome sections of exines. This is evidenced from the SEM and TEM image showing lines running through the centre of the exines that were created by the section knife (Figure 4.5.1). Other microtome sections were prepared and imaged by SEM and TEM of exines containing calcium chloride.



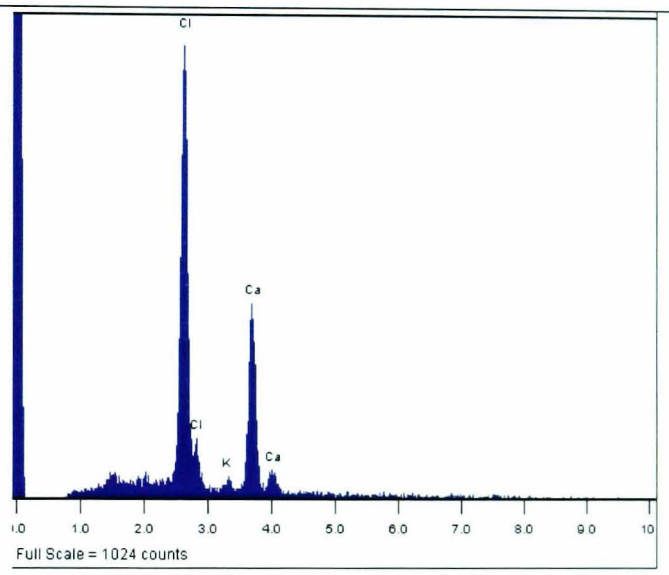
A



B



C



D

Figure 4.5.1: SEM (A) and TEM (B) evidence of sectioned sporopollenin exines filled with acrylic resin. Evidence of encapsulated calcium chloride using TEM (C, scale bar reads 10µm) and X-ray analysis (D).

The precipitation of a magnetic iron compound inside the exines resulted in attraction of the loaded exine particles to a magnet. These experiments were performed by a co-worker[10] and illustrated successful loading.

The mass gain observed during loading is also an indication of successful loading and can be confirmed by examining the diameter of the loaded exine by LM, as no change in diameter occurs and there is no obvious coating on the outer surface of the particle. This was also investigated through loading of vitamin C (ascorbic acid) and as discussed in more detail later there was no ascorbic acid detected on the surface of the loaded particles by FTIR analysis, indicating that the substance had indeed been encapsulated within the exine cavity (Figure 4.6.5.2).

4.6 Encapsulation of a range of different substances

4.6.1 Encapsulation of oils

Several oils, such as omega 3 fish oil, rapeseed oil and sunflower oil, were encapsulated with a high efficiency, with a mass loading of 4:1 w/w oil to sporopollenin exines. In comparison to a recent study investigating poly (acrylic acid) microspheres of a similar size to sporopollenin exines (15-25μm)[11], the loading of sporopollenin exines is extremely high. The highest loading of a substance achieved with the synthetic poly (acrylic acid) microspheres[11] was 4.8mg of phenolphthalein per gram of microspheres, which is very low considering that 4g of oils have been successfully loaded into 1g of sporopollenin exines in this research.

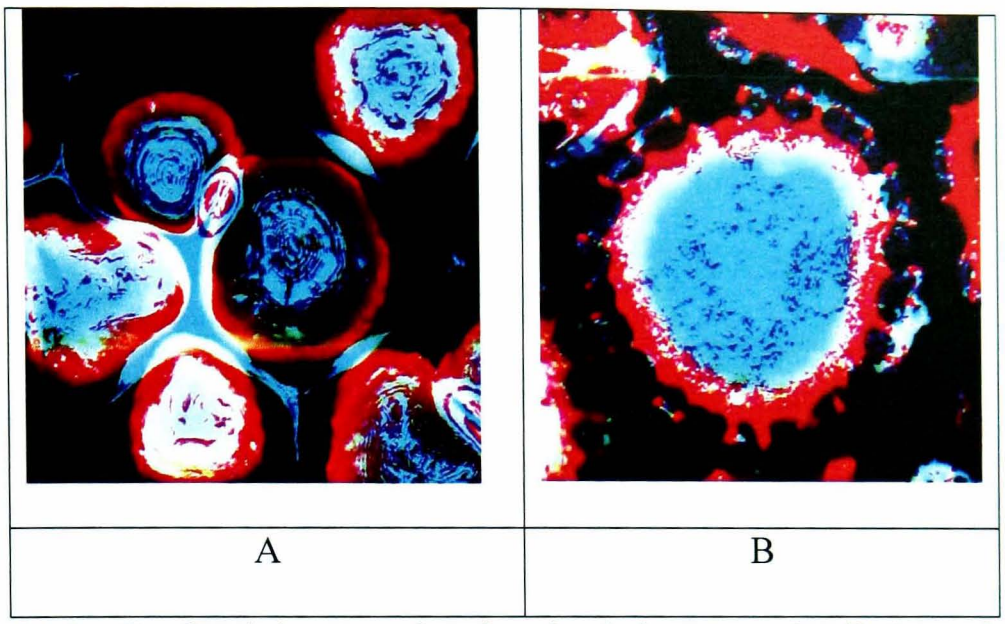


Figure 4.6.1.1: Confocal images showing loaded omega 3 oil (seen in blue) in sporopollenin exines (seen in red) that were left damp (A) and dried thoroughly (B).

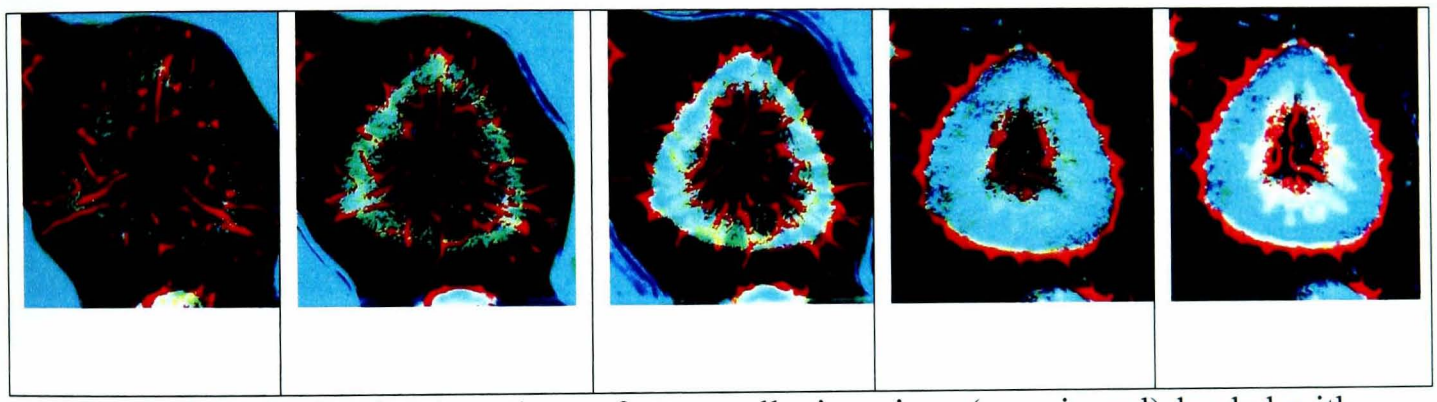


Figure 4.6.1.2: Confocal sections of sporopollenin exines (seen in red) loaded with Rapeseed oil (seen in blue).

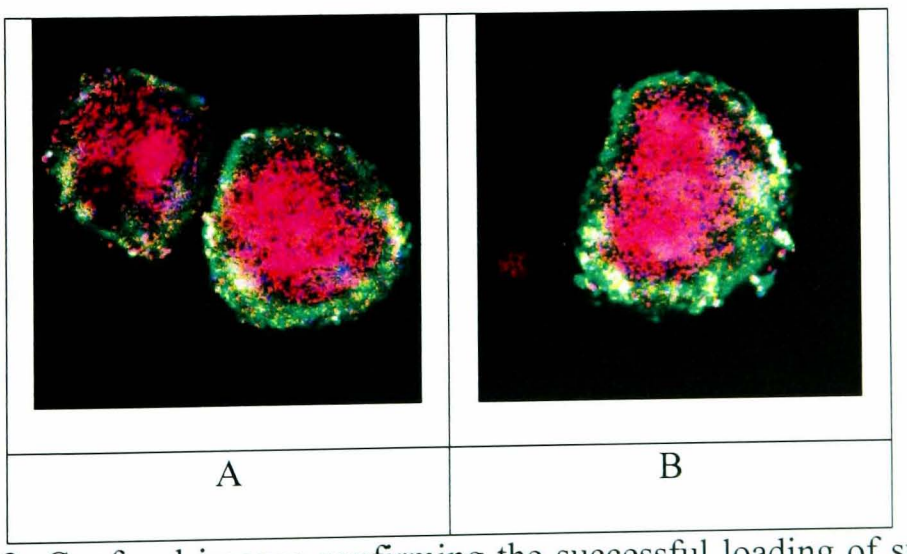
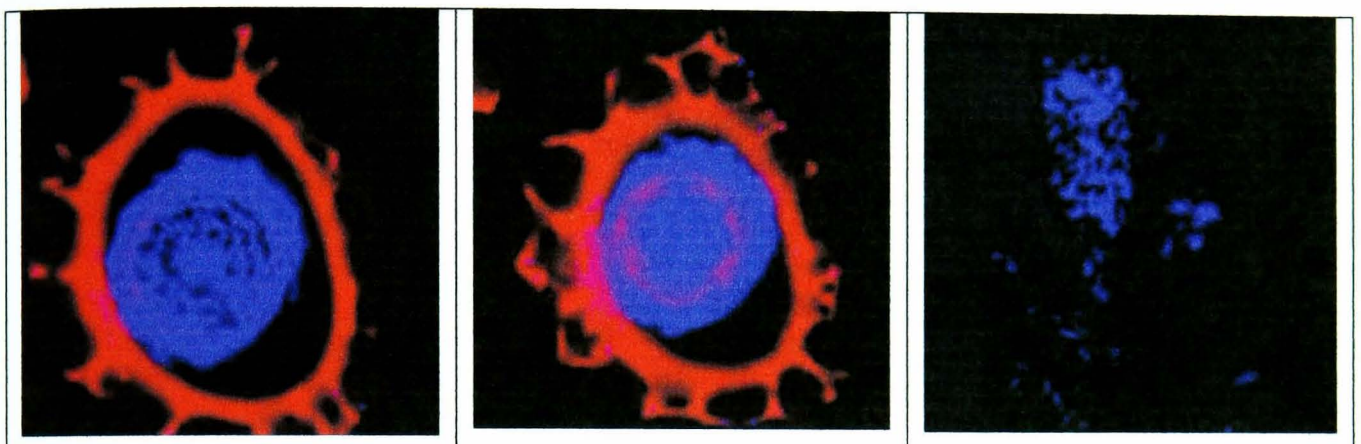


Figure 4.6.1.3: Confocal images confirming the successful loading of sunflower oil. The sunflower oil (pink) is shown within the exine (green).

4.6.2 Encapsulation of dyes

Several dyes were encapsulated into sporopollenin exines, including Nile red, Malachite green and Evan's blue.



A	B*	C

Figure 4.6.2.1 Confocal images demonstrating the loading of Nile red (shown here as blue within the red exine coating) into sporopollenin exines (A, B) and a Nile red crystal before encapsulation (C).

Malachite green (MG) is a cationic triarylmethane dye and is used as a blood-purging agent to prevent transfusion-associated transmission of a number of diseases[12].

In a recent study investigating the interaction between MG and liposome membranes, MG was shown to be absorbed into lecithin liposomes[13].

MG was successfully loaded into sporopollenin exines, as shown in the confocal images below.

* This image was selected by Yorkshire Forward to Publicise a 'Science and Art Speed Networking Evening', held at Impressions Gallery, York, June 2nd 2005

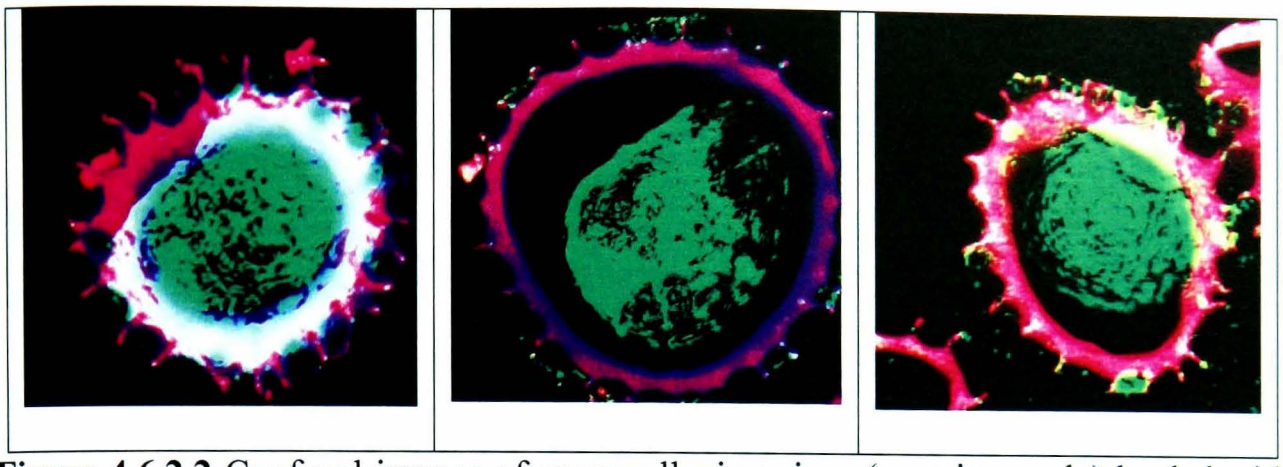
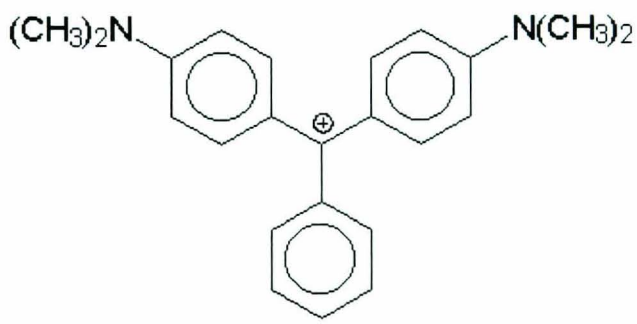


Figure 4.6.2.2 Confocal images of sporopollenin exines (seen in purple) loaded with Malachite green dye.

The loading of MG into sporopollenin exines was also confirmed by Elemental analysis because the dye molecules contain nitrogen.

	C (%)	H (%)	N (%)
Empty exines	68.62	9.18	0.00
MG-loaded exines	62.72	7.64	1.85

A



B

Figure 4.6.2.3 Typical elemental analysis of sporopollenin exines before and after loading of Malachite green dye (A) and the structure of Malachite green (B).

Encapsulation of Evan's blue was achieved (960Da molecular weight), despite it reported not to absorb into pine pollen sporopollenin exines in the literature[14]. Evan's

blue was found "not to enter the central capsule or the denatured pollen grain within 24 hours, but accumulated rapidly…when the exine was injured". The confocal image below illustrates the presence of the dye within the central cavity of the exine, which has entered passively with vacuum without causing injury to the exine.

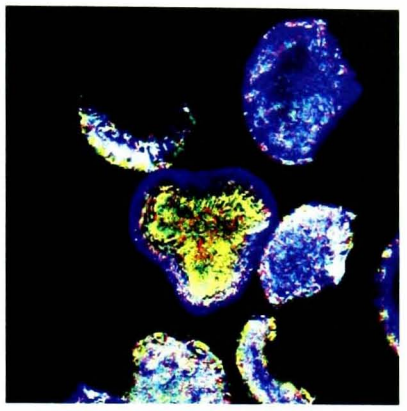


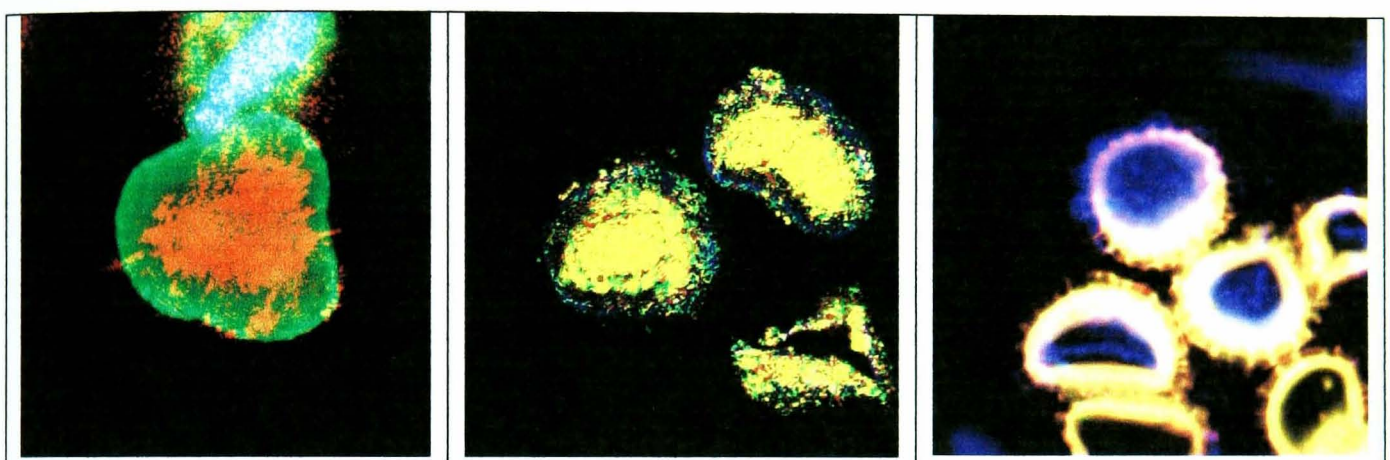
Figure 4.6.2.4 Confocal image of sporopollenin exines filled with Evans blue (shown as yellow within the exine coat shown as blue).

4.6.3 Encapsulation of proteins

Administering protein-based drugs orally is a formidable challenge due to their very short life in the gastrointestinal fluids and the oral administration of a pharmaceutical protein results in very low bioavailability[15]. There is subsequently a major need for the encapsulation of such drug molecules.

Amylase (54 kDa) and lactase (116 kDa) enzymes were successfully encapsulated into sporopollenin exines with loadings of 1:1 w/w of enzyme to sporopollenin exines (Figure 4.6.3.1). 'High' molecular weight proteins (3,340 Da) and 'low' molecular weight proteins (1,097 Da) (both supplied by Ipsen Ltd, Slough, UK) were encapsulated at maximum loadings of 40mg/g and 60mg/g respectively.

Enfurvitide (Fuzeon - 5,065 Da, Roche Ltd, Welwyn Garden City, UK) was loaded effectively into exine particles. Enfurvitide is a new class of antiretroviral drugs for the treatment of HIV in humans. It has been co-developed by Roche and Trimeris Inc. It is a peptide that usually has to be administered parenterally and was successfully encapsulated with a ratio of 273mg:726mg of Enfurvitide to sporopollenin exines.



A	B	C

Figure 4.6.3.1 Confocal images illustrating loading of amylase stained with Malachite green (shown in orange) (A), Lactase stained with Evan's blue (shown as yellow) (B) dyes into 40μm exines and Enfurvitide (shown as blue) (C) loaded in 25μm exine particles.

4.6.4 Encapsulation of Ibuprofen

Ibuprofen is an orally administered anti-inflammatory and analgesic drug. It is an appropriate drug for microencapsulation owing to its poor solubility in water, short biological half-life (which requires administration of Ibuprofen three times a day[16]), and potential hazardous broncho constriction and gastric irritation[17].

Encapsulation of Ibuprofen was achieved using sporopollenin exines at a level of 1:1w/w of Ibuprofen to sporopollenin exines.

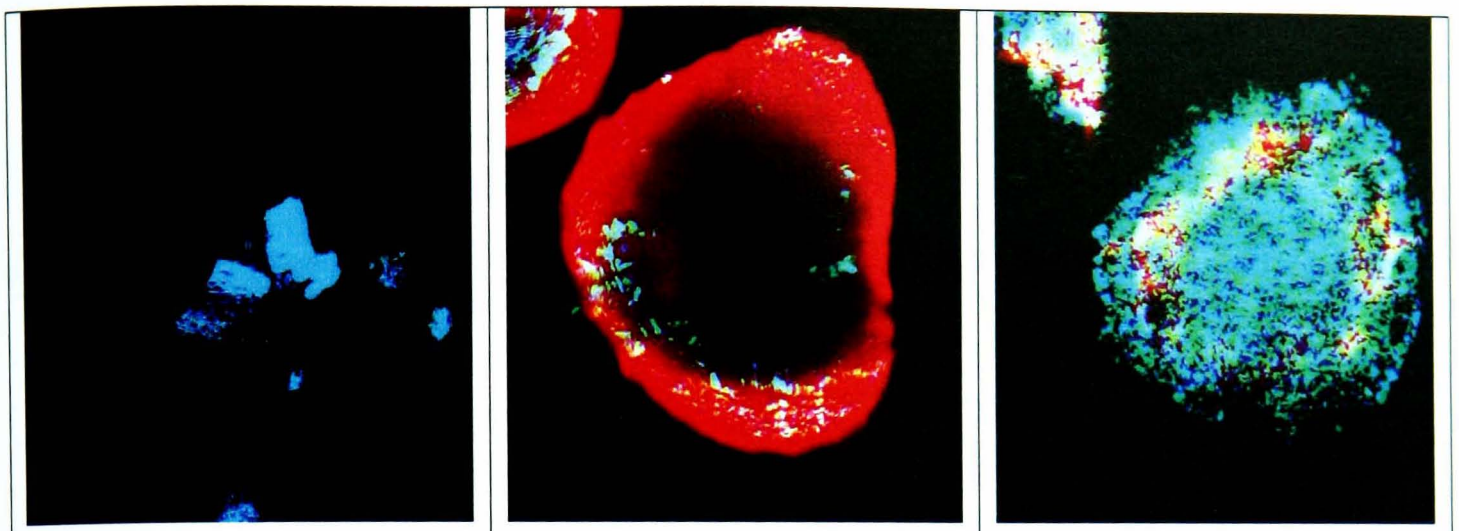


Figure 4.6.4 Confocal images of Ibuprofen (seen in blue) (A), an empty exine (B) and an exine loaded with Ibuprofen (C).

4.6.5 Encapsulation of ascorbic acid

Ascorbic acid (vitamin C) is commonly added to food products as an antioxidant to prevent premature degradation of the product. Ascorbic acid is water-soluble and its stability is affected by environmental factors such as temperature, pH value, oxygen concentration and UV radiation. Therefore, ascorbic acid was a suitable candidate for encapsulation into sporopollenin exines, which could offer protection from such degrading factors.

Ascorbic acid was encapsulated in sporopollenin exines derived from *L. clavatum* (25μm and 40μm). A maximum loading of 1:1 w/w ascorbic acid to exines was achieved (determined by the mass gain), which was considerably higher than ascorbic acid encapsulated in liposomes, described in the literature between 53-58% efficiency[18].

The high loading achieved with sporopollenin exines was verified by extracting the loaded ascorbic acid into water, simply by stirring at room temperature for 1 hour, and

subsequent titration of the filtrate with 2,6-dichlorophenolindophenol (DCPIP) dye. The *in vitro* release of ascorbic acid from sporopollenin exines is described in 4.7.1.

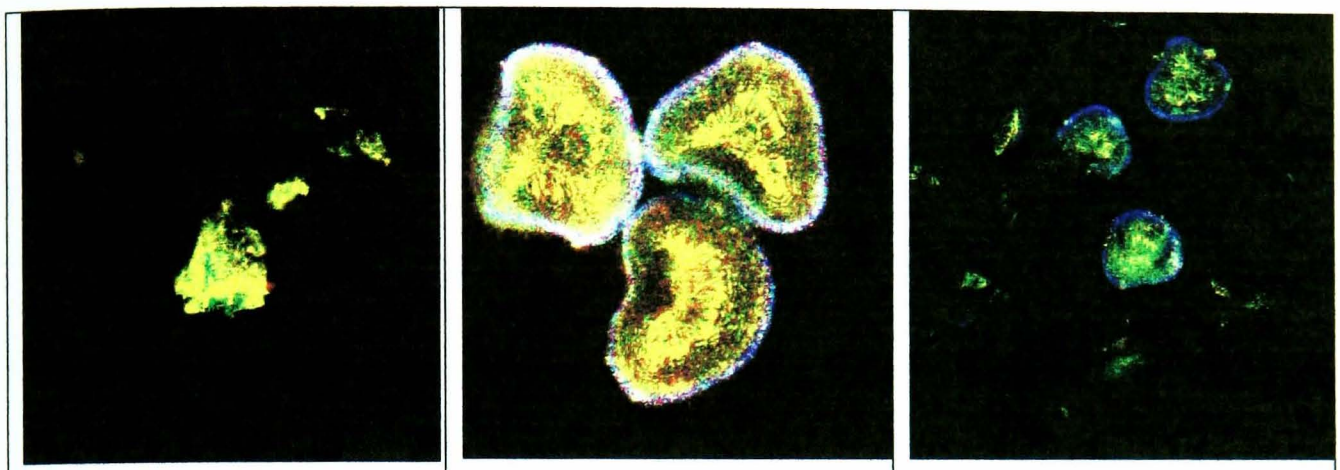


Figure 4.6.5.1 Confocal images of ascorbic acid crystals seen in yellow (A), exines loaded with ascorbic acid (B) and these loaded-exines after washing with water showing that most of the ascorbic acid has been released from the exine cavity (C).

It was important to determine whether the active ingredients were absorbed into the exine and not simply adhering to the outer surface. This prompted the analysis of ascorbic acid-loaded exines by FTIR. Three specimens were analysed:

- Unloaded sporopollenin exines
- Ascorbic acid-loaded sporopollenin exines (1:1w/w as described in Method A)
- Ascorbic acid (as a reference)

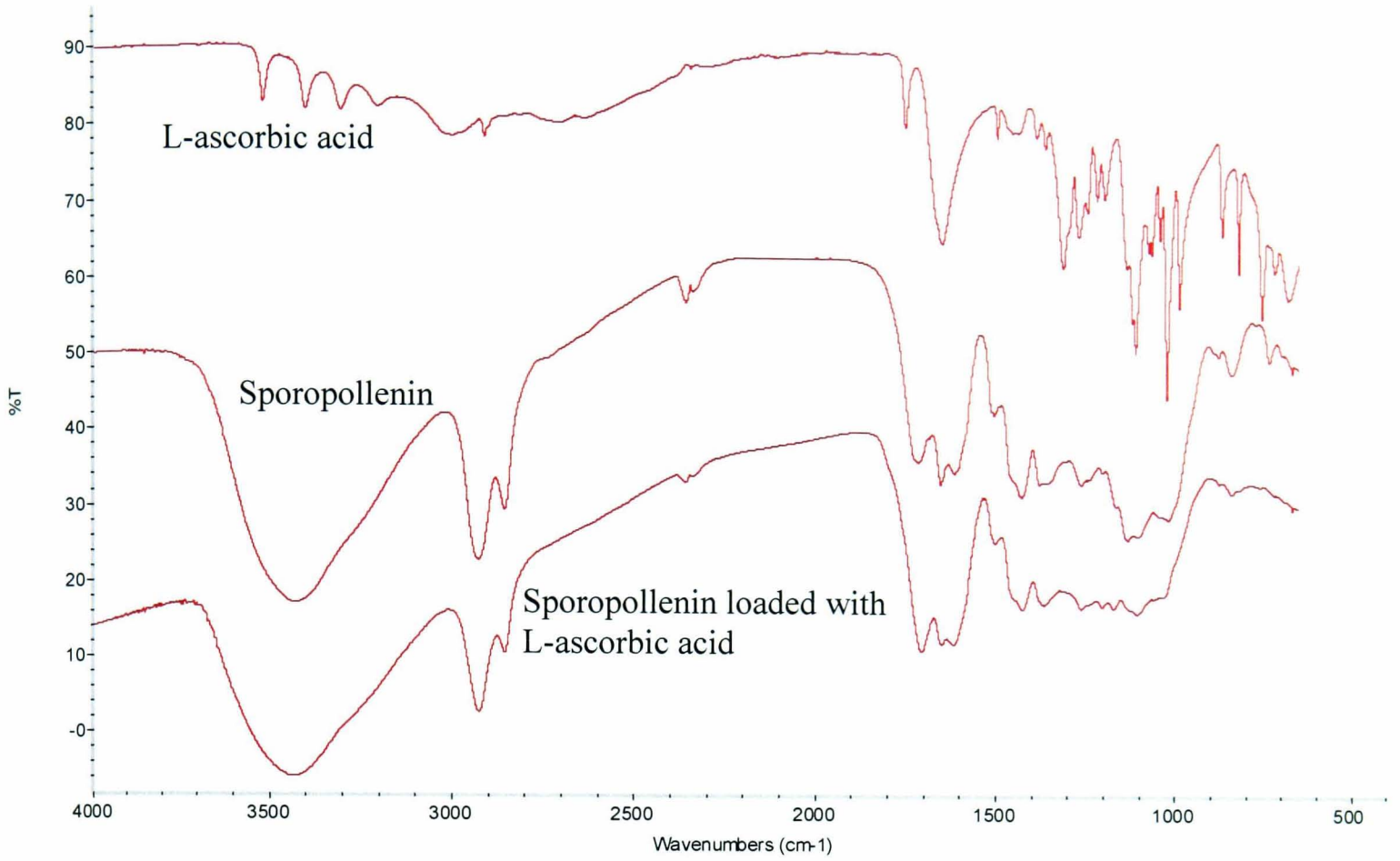


Figure 4.6.5.2 FTIR spectra showing that no ascorbic acid exists on the outer exine after loading.

There was no significant difference between the spectra obtained from the ascorbic acid-loaded sporopollenin sample and the unloaded exines. These observations were indicative of ascorbic acid absorption. The FTIR results suggested that no ascorbic acid existed on the exterior of the exine, and so indicated that the mass gain was a result of the loading of ascorbic acid into the inner cavity of the exines. Since the exines were rinsed with water after loading, it can be concluded that any remaining ascorbic acid that adhered to the surface was simply washed off.

To elaborate on this theory, Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFTS) was employed as an analytical technique. This method is

frequently used for the identification and characterisation of powdered samples, and is quite often chosen over conventional IR spectroscopy due to minimal sample preparation required. When IR radiation reaches the sample it is diffusely scattered over a wide area and it is assumed that all the light being reflected from the sample and a 100% reflecting standard can be measured. Overhead Attenuated Total Reflectance (ATR) was attempted in accordance with FTIR, with a ZnSe crystal, but was unsuccessful as an analytical technique.

4.7 *In vitro* release of loaded substances

To serve as an effective DDS, exines must allow the release of the encapsulated drug into the bloodstream. Following the successful loading of substances, it was necessary to determine if the contents of the sporopollenin exines could be effectively released into different media *in vitro*, such as SGN and plasma. This would illustrate how effective the exines would be at protecting the substance from the harsh environment in the GI tract whilst enabling the delivery of such substances into the bloodstream.

4.7.1 Release of ascorbic acid

Ascorbic acid was loaded (as described in 4.6.5) at a ratio of 1:1 w/w ascorbic acid to sporopollenin exines was released into PBS pH 7.2 for 5, 15, 30 and 60 minutes and the resulting solutions were titrated against Dichlorophenolindophenol (DCPIP) dye. This method was based on the reduction of DCPIP with ascorbic acid and has been documented as an official method for the determination of ascorbic acid[19]. This confirmed the release of 16.4% of the encapsulated ascorbic acid after 5 minutes and 23% after 60 minutes in PBS.

Ascorbic acid (loaded at 1:2 ascorbic acid to sporopollenin exines as stated in Chapter 5) was also released into PBS and the concentration of recovered ascorbic acid was determined using a voltammetric method because of the reported high sensitivity and selectivity associated with this method[20]. A co-worker[21] carried out this work and showed the percentage of ascorbic acid recovered intact from exines was 94% of the loaded material. The recovered ascorbic acid was in its reduced form before loading and after release, indicating that the loading procedure did not affect the functionality of the loaded ascorbic acid.

4.7.2 Release of Ibuprofen

Sporopollenin exines containing Ibuprofen (1mg per mg sporopollenin exines as prepared in Methodology) were stirred into plasma and SGN samples over the period of 45 minutes.

Release of Ibuprofen from exines was very rapid, with 91% ibuprofen released into plasma after just 5 minutes and after 45 minutes incubation 95% of the Ibuprofen was released into plasma (Figure 4.7.2). Ibuprofen appeared to be effectively protected from SGF, since after 45 minutes incubation in SGF, only 17% was released. This release rate of contents is extremely rapid in comparison with similar-sized synthetic microcapsules[11], in which only 75% of the contents were released after 30 minutes. The release of Ibuprofen from sporopollenin exines was very rapid in comparison with ethylcellulose microspheres[17] and recent inorganic porous micron-sized particles made from silica, calcium carbonate or calcium phosphate[22].

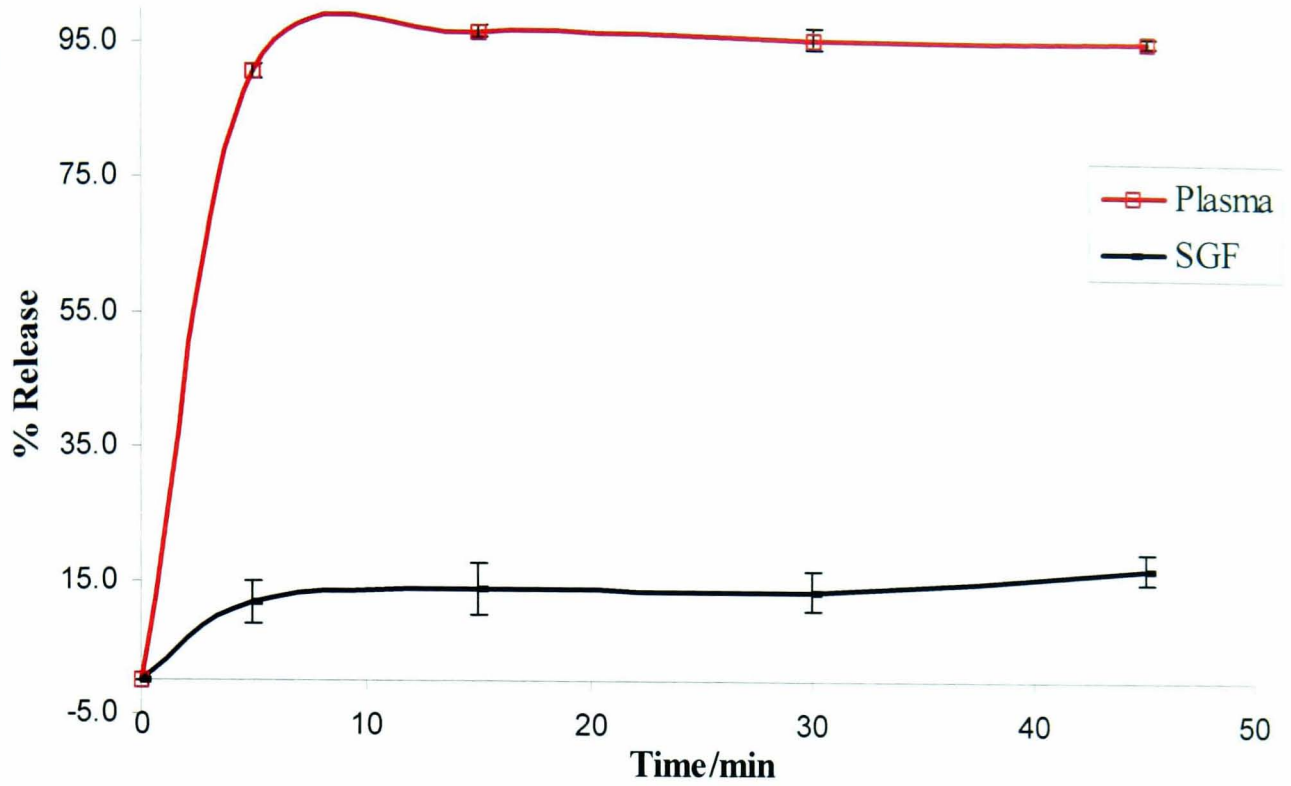


Figure 4.7.2: The release of Ibuprofen into SGN and plasma over 45 minutes.

4.7.3 Release of Human Growth Hormone (hGH)

Sporopollenin exines loaded with hGH were stirred into PBS and SGN containing pepsin (pH 2). The hGH was readily released from the sporopollenin exines within 10 minutes into both solutions and the hGH was destroyed by the enzyme pepsin. However, there was still discernable activity (0.6mU/L after 60 minutes), which may suggest a small degree of protection from the exines. Additional coatings were also applied to sporopollenin exines loaded with hGH and the release from these preparations is discussed in Section 4.8.3.2.

4.7.4 Release of Amphotericin

Amphotericin B is an antifungal agent that traditionally has been given parenterally. It is insoluble in water and has a number of toxicities that are reduced when incorporated into a carrier[23], so it was therefore an ideal drug to encapsulate.

Amphotericin was released into DMSO at Pfizer R & D, Sandwich, Kent, from liposomal formulations and bile salt preparations of loaded sporopollenin exines.

An average of 27% of amphotericin was released from the bile acid loaded preparation, whilst 79% was released from the liposomal loaded form of amphotericin after 24 hours (determined by HPLC-UV assay).

	Weight Spores (mg)	Theoretical Amount Amphotericin in Spores (ug)	Inj 1 (ug)	Inj 2 (ug)	Mean (ug)	Recovery (%)	Average Recovery %
Liposomal preparation	0.9	10.6	10.6	10.6	10.6	100	79
	2.4	28.3	16.1	16.0	16.1	57	
	1.2	14.2	11.3	11.3	11.3	80	
Bile salt preparation	1.3	75.5	21.6	21.5	21.6	28.6	27.0
	2.3	133.6	27.4	27.4	27.4	20.5	
	0.9	52.3	16.7	16.7	16.7	31.0	

Figure 4.7.4: Release of amphotericin from sporopollenin exines into DMSO and detected by HPLC UV assay.

4.8 External coatings and their stability *in vitro*

To offer extra protection of the loaded ingredients from the harsh conditions of the GI tract, the exines were coated with different substances (see Methodology). Unloaded

exines were coated to assess if these coatings hindered their degradation in SGN and plasma. The first coating investigated was soluble sporopollenin.

4.8.1 Soluble sporopollenin

Despite the documented resilient nature of sporopollenin to biological and non-oxidative chemical attack, there have been numerous literature references stating the solubilisation of sporopollenin in substances such as ozone, and in particular, 2aminoethanol.

Complete solubilsation of sporopollenin was first demonstrated using sporopollenin exines from the pollen *Typha angustifolia* L[24]. The reaggregated material was analysed by confocal microscopy and showed auto-fluorescence similar to that of the exines. FTIR revealed similar spectra for both products and they were stained with toluidine blue dye, which indicated that they were large molecules with many anionic sites[25]. They also stated that treatment of the pollen with acetolysis and phosphoric acid altered the exine and lead to subsequent insolubility in 2-aminoethanol. In contradiction to this work, another group of researchers had previously found that "treatment with phosphoric acid to remove the polysaccharide intine did not alter subsequent reactions with 2-aminoethanol"[26]. This protocol involved acetolysis of the pollen in order to remove the intine and the cytoplasm, as the literature suggests structures that resist acetolysis are related to lignin and sporopollenin[27].

2-aminoethanol was chosen since it is an organic solvent that has previously been used to study pollen exines and intines and is known to dissolve only the outer exine layer of many gymnosperms and angiosperms.

Due to the successful transport of sporopollenin exine particles into the blood stream during the *in vivo* trials, it was thought that the mechanism by which sporopollenin exines are absorbed is by persorption. Also, the protection offered by sporopollenin exines to an encapsulated ingredient in the GI tract had been demonstrated using *in vitro* experiments. Therefore it was proposed that a soluble form of sporopollenin would be created to act as a coating for exines that have an encapsulated ingredient (hGH for example) to prevent any of the loaded ingredients leaching out into gastric fluids.

L. clavatum sporopollenin (25μm and 40μm) and *Chlorella vulgaris* spores were successfully dissolved in 2-aminoethanol. A reaggregate was not achieved with either 25μm or 40μm *L. clavatum* sporopollenin, however, cold ethanol was used to obtain a reaggregate of *Chlorella vulgaris* and an FTIR spectrum of this reaggregate was achieved (Appendix). The failure to achieve a reaggregation with *L. clavatum* sporopollenin may be due to the greater stability of this sporopollenin (possibly due to cross-linking occurring during phosphoric acid treatment), which is in accordance with the literature, with the exception of this sporopollenin did dissolve in 2-aminoethanol. Solubilisation and reaggregation of the parent *L. clavatum* pollen was not attempted due to the results in Figure 4.8.3.1, showing that sporopollenin did not offer extra protection. The reaggregated solid was analysed by confocal microscopy and fluoresced at the same wavelengths as the *L. clavatum* sporopollenin exines. Under the microscope it did not have a structure since there is no step during the procedure that could give a structure to the reaggregated material. This made it difficult to observe degradation in plasma, although it was assumed that the material would degrade in the same manner as the sporopollenin exines in blood *in vivo*.

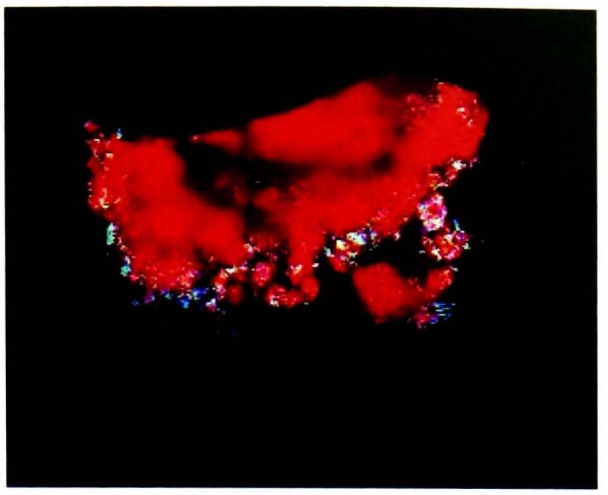


Figure 4.8.1: Confocal image of the reaggregated material

4.8.2 Other coatings

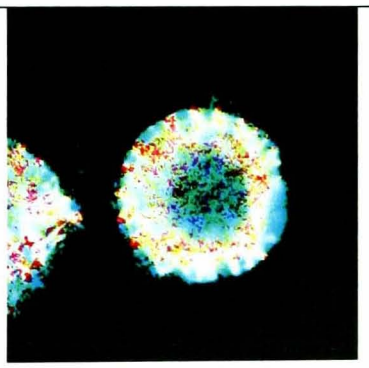
The following coatings were used to coat unloaded sporopollenin exine particles, as described in Chapter 5. They were chosen due to their routine use in microencapsulation and the food industry[28,29], and therefore their non-toxicity:

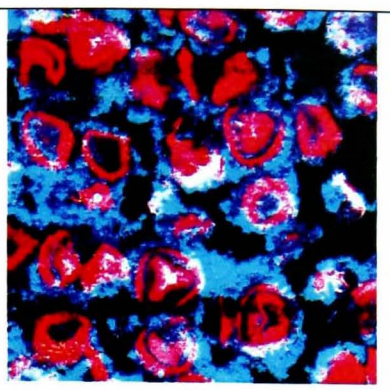
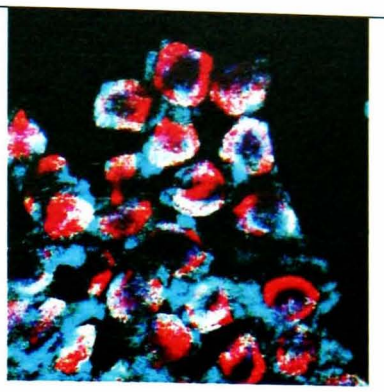
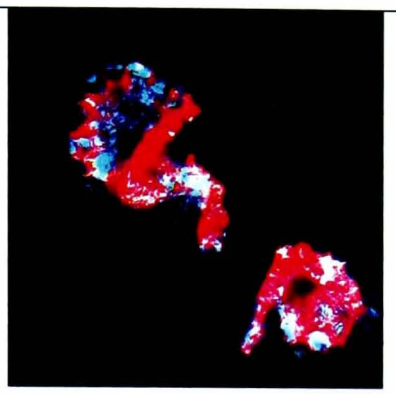
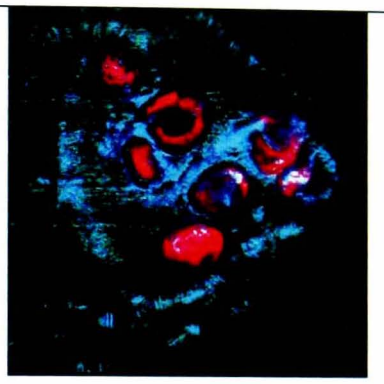
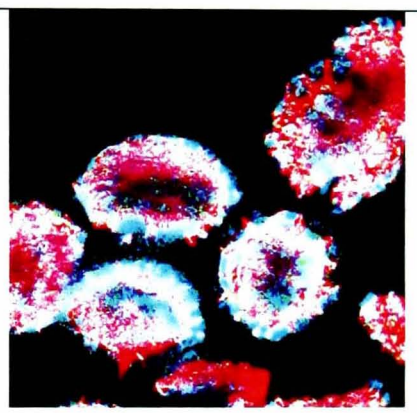
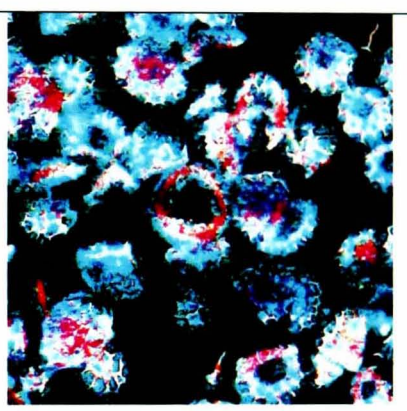
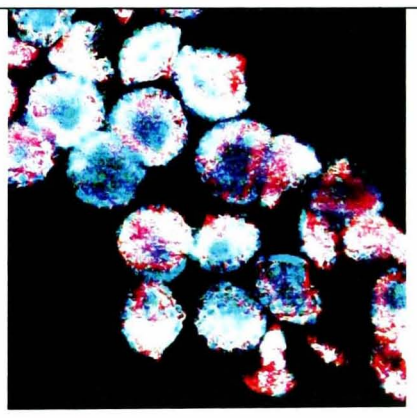
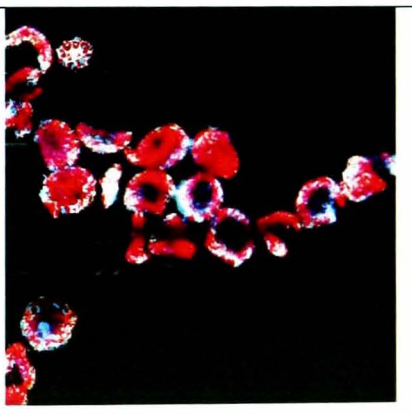
- Starch
- Gum arabic
- Cocoa butter
- Beeswax
- Shellac
- Cod liver oil

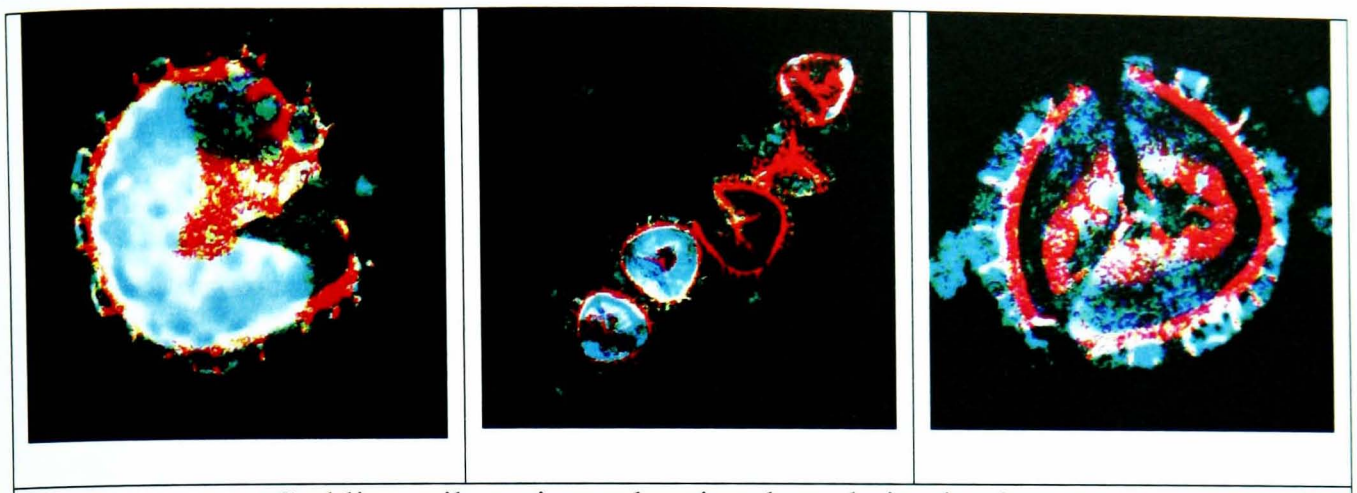
The coated-exine particles were then incubated in SGN and plasma to ensure they were able to withstand the SGN to offer protection of potential-loaded ingredients, but did not impede the degradation of exine particles in plasma.

Confocal microscopy revealed that starch and gum arabic-coated exines were still intact in SGN and although gum arabic-coated exines seemed to remain intact in SGN, gum arabic itself degraded in acid and so would not offer protection in the GI tract. However, it should be noted that gum arabic is classed as a food fibre and so is not absorbed by the body. This means that although it may be affected by SGN, it may also retain its structure and could still help to delay the release of the hGH compared to sporopollenin exines without a coating. The other coatings showed little or no degradation after 30-minute incubation with SGN.

Gum arabic and cod liver oil coatings showed the most degradation in plasma after 30 minutes incubation. The least degraded exines were those coated with shellac, cocoa butter and beeswax. The starch exine coating may be problematic in the body since none of the exines were shown to break down in human plasma.

Sample coating	**Confocal images**	
	Stability in SGF	**Instability in plasma**
Gum arabic		

Cocoa butter		
Shellac		
Beeswax with ethanol		
Starch		



Cod liver oil coatings: showing degradation in plasma.

Figures 4.8.2: Confocal images showing the stability of coated exines in SGN but their instability in plasma (excluding the coatings mentioned above).

4.8.3 The delayed release of substances

Human growth hormone (hGH) was selected as an ideal candidate to examine the effect of different sporopollenin exine coatings on the rate of release because it was routinely assayed at the Hull Royal Infirmary Pathology Laboratory and was readily available in the laboratory.

4.8.3.1 Soluble sporopollenin

The soluble sporopollenin obtained from *C. vulgaris* was used to coat exine particles loaded with hGH. The coating was achieved by simple stirring. The exines coated with soluble sporopollenin degraded at 37°C in human plasma, however, as seen in the confocal images (Figure 4.8.3.1.1 (B)), the exine is seen in red and the centre is also seen in red, indicating that the contents of the exine are indeed sporopollenin and not hGH, which is usually seen as blue/green. A potential problem with coating the exines with soluble sporopollenin is that the encapsulated hGH may have leached out during coating. Due to the degradation of exines in plasma, but not in serum (Chapter 3), the coated exines containing hGH were incubated in serum for 30 minutes at 37°C

to avoid interference of exine degradation products when detecting hGH released into solution. As seen in Figure 4.8.3.1.1 (C) the surface hGH had leached into serum.

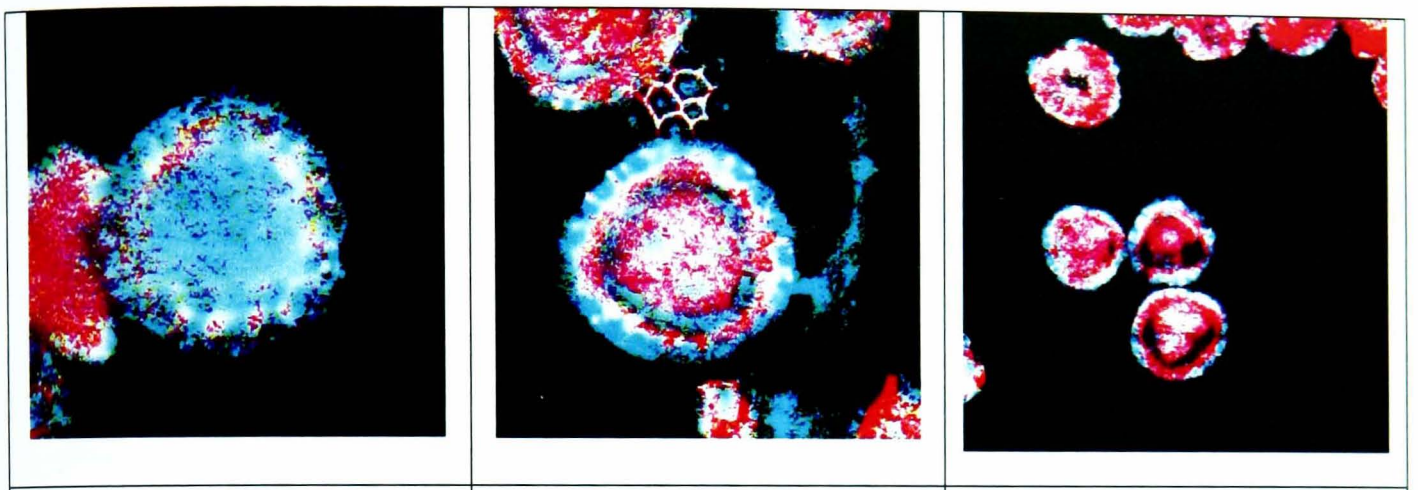


Figure 4.8.3.1.1 An exine particle loaded with hGH (A), exines loaded with hGH and coated with soluble sporopollenin before (B) and after 10 minutes in serum at 37˚C (C). The exine and soluble sporopollenin are shown in red and the hGH is shown in blue.

Time (min)	hGH release in serum (mg/l)	hGH release in plasma (mg/l)	hGH with soluble sporopollenin coating release in serum (mg/l)	hGH with soluble sporopollenin coating release in plasma (mg/l)
0	0.49	0.37	0.39	0.26
10	0.42	0.46	0.46	0.59
30	0.73	0.85	0.69	0.77
60	0.50	-	0.6	-

Figure 4.8.3.1.2: Release of hGH from sporopollenin exines into serum and plasma samples before and after coating of the exine particles with a soluble form of sporopollenin.

The results show that hGH leaches out of the sporopollenin exines into both serum and plasma at a steady rate that increases with time. The rate of release is marginally faster in plasma than serum and appears to be unhindered by the soluble sporopollenin coating.

Upon confocal microscopy it was evident that the soluble form of sporopollenin had become encapsulated during the coating procedure, which may compromise the amount or alter the activity of the encapsulated hGH. Also, the reaggregate was later found to be soluble in acid (0.2M HCl), which would mean that it would not offer protection of the exine from the acids present within the GI tract. For these reasons the soluble form of sporopollenin was not pursued as a coating material for the exines and other coatings were explored.

4.8.3.2 Other coatings

The effect of different coatings on the retention of hGH was explored. The following coatings were applied to hGH-loaded sporopollenin exines:

- Carnauba wax
- Cod liver oil
- Starch and hGH as an emulsion
- Starch and cod liver oil
- Starch and echium oil
- Liposomal hGH and starch
- Gelatine and Eudragit

Release of hGH from these samples into SGN and serum yielded the following results (determined by RIA)

Coating	Assay result mU/L	
	SGN	Serum
Blank	>520	>104
Control (no coating)	>520	>104
Soluble sporopollenin	>520	>104
Carnauba wax	322	>104
Cod liver oil	>520	>104
Starch emulsion	>520	>104
Starch and cod liver oil	0.21	-
Starch and Echium oil	-	-
Liposomal hGH with starch	54.6	>104

4.8.3.3 Release of hGH into SGN and serum *in vitro*

Encapsulated hGH was readily released from the uncoated exines into both SGN and serum. Coating with soluble sporopollenin, encapsulation of an emulsion of hGH with cod liver oil or starch did not alter the rate at which hGH was released from the exines into SGN or serum. The carnauba wax coating appeared to delay the release of hGH into SGN, but did not alter the release into serum, thus could offer protection against the GI tract but release into the bloodstream. It should be noted that the exines coated with carnauba wax were not shown to degrade in plasma, so could be potentially problematic. Coating with starch and cod liver oil together and the liposomal form of hGH loaded together with starch led to a lower concentration detected in SGN, this could be due to the coatings offering extra protection or it could be due to hGH leaching out during the coating procedure. Further experiments are required to

confirm these findings and future samples were prepared loading a lower concentration of hGH into the exines, since the upper limits of the RIA were 0.4ng/ml.

Sporopollenin exines loaded with hGH were coated with gelatine and Eudragit and stirred into SGN containing pepsin. The samples were then taken out of SGN and incubated in plasma for 30 minutes.

Time (min)	**HGH detected in SGN (mU/L)**	**HGH detected in plasma (mU/L)**
0	3.5	1.6
20	0.2	1.5
40	0.2	0.8

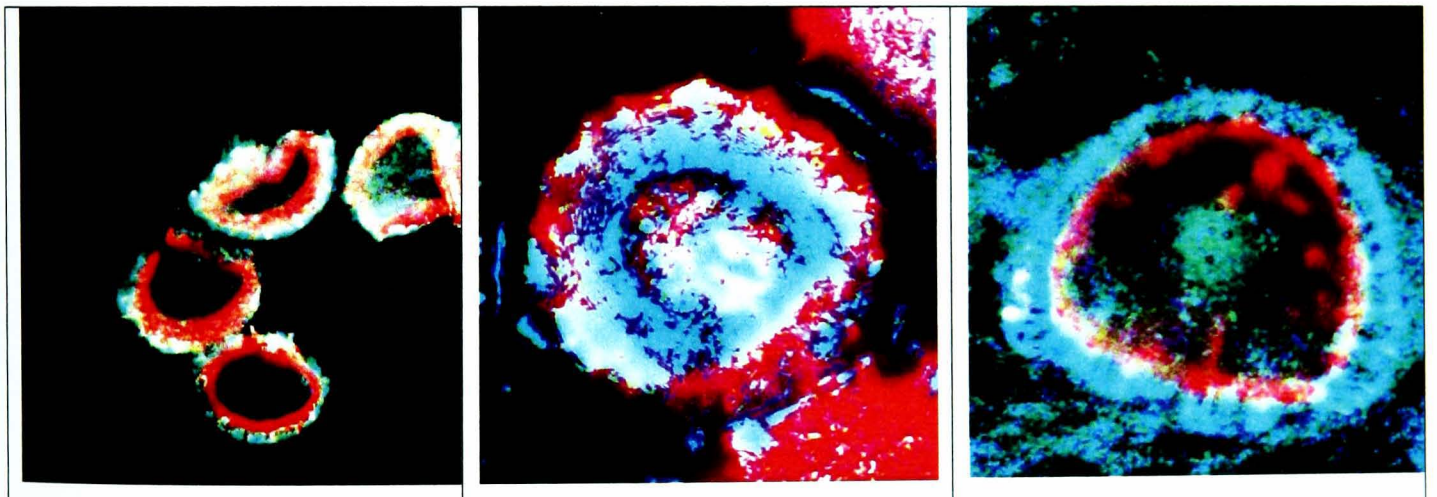
Figure 4.8.3.4 Release of hGH into SGN and plasma

The results show that after 20 minutes incubation in SGN, the loaded exines released some hGH, but the value was at the lower limit of detection, indicating that either little hGH was actually released from the exines or that released was rapidly destroyed (presumably by pepsin) before detection. It should be noted however, that once this sample was then removed from SGN and placed into plasma, most of the hGH detected in the baseline sample was released and detected, indicating that there was nearly complete protection of hGH from the previous SGN exposure. After 40 minutes, the concentration of hGH detected in plasma (after previous SGN incubation decreased by 50% to 0.8mU/L, but still indicated protection of 50% of the hGH that was released without SGN contact.

Other results performed by a co-worker show that with additional drying steps involved in the coating of hGH-loaded exines (with gelatine and Eudragit) there was as high as a 69% release of loaded hGH into plasma[30].

4.9 Loaded exines: *in vivo* delivery

The oral delivery of exines loaded with rapeseed oil (1:3 w/w of sporopollenin to rapeseed oil) was achieved. A full taste-trial with encapsulated oils was carried out by a co-worker and showed the exines to effectively mask the taste of the oils[31]. Ten minutes after ingestion exine particles were found in the lysed pellet portion of the blood. The particles were first identified by LM, then confirmed to be exines by confocal microscopy.



A	B	C

Figure 4.9.1: Confocal images of empty exines before loading with oil (A), the loaded exines before ingestion (B) and recovered in blood (C), showing what appears to be oil (shown in blue) leaching out of the exine.

The outer layer of fluorescence surrounding the loaded exine was initially thought to be oil 'leaching' out from the exine into the blood, but as seen above, the empty

exines that display this fluorescence, although not as intense. It may be due to attraction of components within the plasma surrounding the exine upon contact. It should be noted that the oil did not impede the degradation of the sporopollenin exines as they were still seen to break down in the blood (Figure 4.9.2).

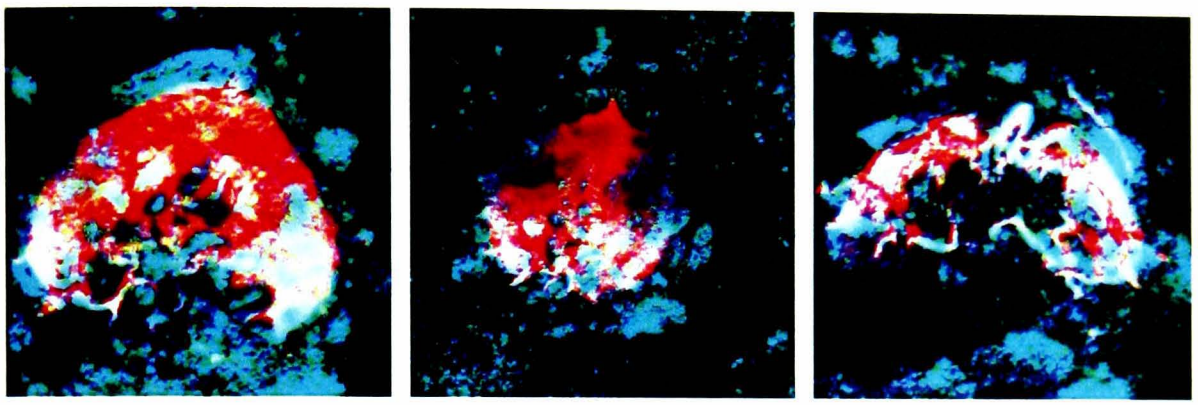


Figure 4.9.2: Confocal images showing degradation of the oil-loaded exines that were recovered from the blood.

4.10 Sporopollenin exines to deliver Enfurvitide (Fuzeon)

Enfurvitide was encapsulated into sporopollenin exines and animal trials were undertaken using the facilities at F. Hoffmann-La Roche Ltd, Basel, Switzerland.

Enfurvitide was loaded into sporopollenin exines with 50% w/w efficacy (product called 'PV4X') and was used for *in vivo* trials with Beagle dog subjects (as described in Chapter 2).

With respect to the functional utility of sporopollenin exines as a drug delivery system, the assay showed that some of the encapsulated Enfurvitide had been delivered into the bloodstream of Beagle dogs following oral ingestion. However the blood levels of detected Enfurvitide were only 29 ng/ml after 30 minutes, which was significantly lower (only 1-2%) than expected based on the exine counts at that time

point. Also, there was not a direct correlation between sporopollenin exines counts and Enfurvitide levels over that time period studied.

Both exines and Enfurvitide were found over a long period (4 hours and >8 hours respectively), showing that there was further absorption after the initial counts.

It was possible that PV4X released the drug very quickly, since the maximum drug level was detected at 30 minutes; hence there was the possibility that a significant amount of PV4X (and hence Enfurvitide) may have been absorbed into the bloodstream before this period. Alternatively, the majority of the Enfurvitide could have been destroyed *en route*, presumably in the relatively short transit time in the stomach where it could have come into contact with acid and proteases such as pepsin. Further work is required to develop sporopollenin exines as a drug delivery vehicle and coatings (as explored earlier) may increase the concentration of drugs recovered in the bloodstream after oral dosing. It should be noted that the coating work had not been investigated at the time of the Enfurvitide dog trials.

4.11 Sporopollenin exines to deliver cyclosporine

An experiment was designed to investigate the potential of sporopollenin exines as a novel technique to orally dose drugs that are poorly absorbed from the GI tract. Cyclosporine was chosen as a model drug because it has the required characteristics for inclusion into the exines and it is poorly absorbed.

Cyclosporine was loaded into sporopollenin exines with 50% w/w efficacy and the encapsulation was verified using confocal microscopy and also SEM to ensure that the drug was not adhering to the outer surface of the exine (Figure 4.11.1).

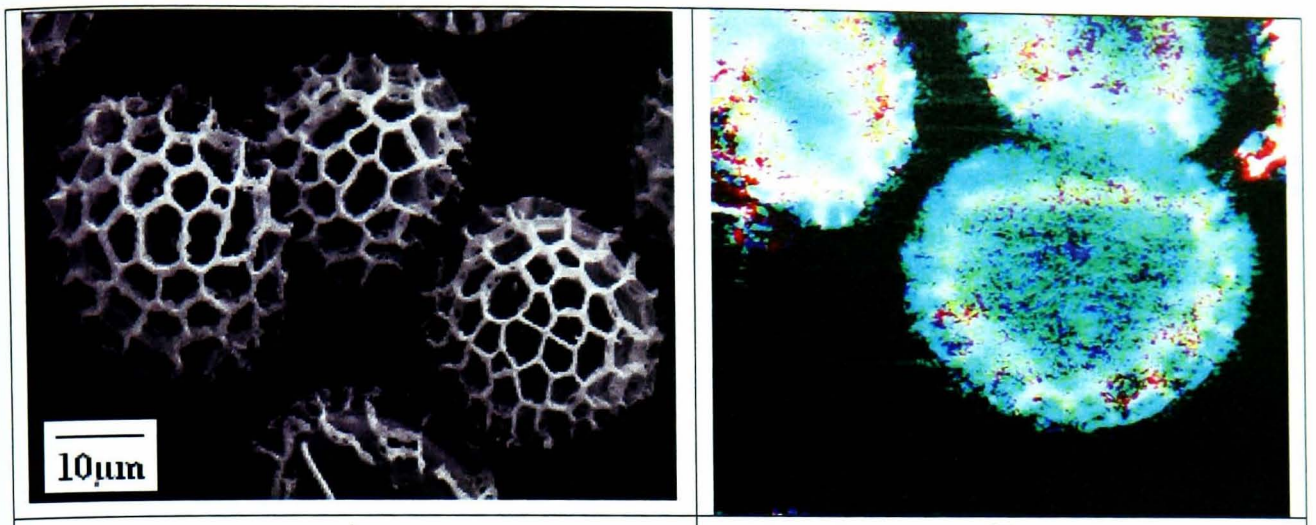


Figure 4.11.1: SEM (A) and confocal images (B) of cyclosporine loaded into sporopollenin exines

Animal trials were undertaken using the facilities at Pfizer R & D, Sandwich, Kent. Four Beagle dogs were fasted prior to the study. Two dogs were dosed with cyclosporine in methylcellulose suspension as a control and two were dosed with the exines loaded with cyclosporine (45mg), also in a methylcellulose suspension. The assay detected cyclosporine recovered in the blood at levels that increased with the exine count, but these data are confidential and so are not included in this report.

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5. Experimental Methods and Materials

5.1 Materials

Chemicals were purchased from the following companies, Sigma, Aldrich, Fluka and Fischer Scientific. *Lycopodium clavatum* spores (40μm) were purchased from Post Apple Scientific and *Lycopodium clavatum* spores (25μm) were purchased from G. Baldwin and Co. and Fluka on separate occasions. *Ambrosia trifida* pollen was purchased from Fluka. Aprotinin (Trasylol), Clexane, Streptokinase and the Commercial plasma preparations were a kind gift from the Hull Royal Infirmary Chemical Pathology Department. Enfurvitide (Fuzeon) and cyclosporine were kind gifts from F. Hoffmann-La Roche Ltd, Basel, Switzerland and Pfizer, Sandwich Laboratories, Kent, UK respectively.

5.2 Methods used to analyse sporopollenin exines

The following methods and techniques were used throughout this investigation to analyse spores, pollen grains and their extracted sporopollenin exines.

5.2.1 CHN Elemental Analysis

Fisons instrument Carlo Erba EA 100 C H N analyser. Analyses were carried out in duplicate for each sample and the values shown are the mean values of the analysis. Determinations were carried out at the University of Hull by the analysis service, Department of Chemistry.

5.2.2 FT-IR Spectroscopy

Perkin-Elmer Paragon 1000 Fourier Transform Infra Red Spectrometer. Samples were ground with anhydrous KBr (Analytical grade) to produce disks. Infrared spectra were a result of 4 scans.

5.2.3 Microscopy

Confocal microscopy images were obtained using a Bio-Rad Radiance 2100 laser scanning microscope equipped with Ar (488nm), Green HeNe (563nm) and Red diode (637nm) laser lines connected to a Nikon TE-2000E inverted microscope from Nikon, Japan. A Nikon Upright Transmission Microscope was used to obtain light microscope images. Scanning electron micrographs were obtained using a Leica Cambridge Stereoscan 360 Scanning Electron Microscope (SEM). Tony Sinclair, Department of Biology, University of Hull, performed the SEM images.

5.2.4 Beckmann Coulter Counter

A Beckman Coulter Counter Model ZM was used for particle counting. The optimum gating for the detection of *L. clavatum* particles was between 20-28μm.

5.3 Extraction of sporopollenin from modern *L. clavatum* spores

5.3.1 Method A (KOH/ ortho-phosphoric acid treatment)

The following method was adapted from the literature[1-3] to produce empty sporopollenin exines. This particular method was used to remove the inner contents (sporoplasm) of the spores and leave a product that was completely free of nitrogen, which is usually associated with allergens.

The *L. clavatum* spores (250g) were treated with acetone (750ml for 4 hours at 60°C) and hot aqueous potassium hydroxide twice (6%, 850ml for 6 hours at 80°C) in order to remove the sporoplasm, along with some allergenic proteins present within the spores, leaving just the exine for further investigation. The acetone used in this procedure removed the surface coating and a small amount of fat. Refluxing with aqueous Potassium hydroxide was undertaken to release the sporoplasm, including any remaining fats, proteins and waxes. Solvent and aqueous washes followed to remove the resulting breakdown products. Treatment with 85% *o*-phosphoric acid over 5 days was undertaken and then repeated again to remove the intine layer (cellulose) of the spore and other existing polysaccharides, leaving a brown coloured powder, the homogenous exine. The sporopollenin derived by this method was dried *in vacuo* over P_2O_5 until constant weight.

It was evident from the extraction procedure that treating in the manner described above did not cause any changes to the exine morphology and similar results were observed with different batches using the same extraction process. However, it has been noted "variations in the time and temperature of each extractive step provide a somewhat different final residue"[4], so the extraction process was kept constant for each batch.

It should be noted that although the spores did not exhibit the same external 'decoration' as those described in the literature and appeared to be larger in size (approx. 40μm) these smooth-surfaced spores were also purchased from other companies, and were thought to be a different strain of *Lycopodium*. A yield of 52% was achieved from 250g using this method.

5.3.2 Method B (Acetolysis)

Acetolysis has previously been used to obtain sporopollenin[5,6] and to prepare pollen grains for microscopical examination because it is known to dissolve cellulose, hemicellulose and chitin[5].

A method was adapted from the literature[5] to isolate sporopollenin from modern pollen and spores. The pollen or spore material was prepared by first washing with glacial acetic acid to remove water because the acetolysis solution reacts violently with water.

The acetolysis mixture (110ml) was prepared by slowly mixing a 9:1 (v/v) solution of acetic anhydride (99ml) with conc. sulphuric acid (11ml) (CARE: EXOTHERMIC REACTION). This was added drop-wise to *L. clavatum* (25µm, 50g) and was heated (60°C) with stirring for 4 hours. The spores were allowed to cool to room temperature and were filtered (porosity grade 4), rinsed with glacial acetic acid (50ml), distilled water (50ml) and ethanol (3 x 30ml). The resulting sporopollenin was dried over P_2O_5 *in vacuo*. A yield of 43% was achieved from 50g using this method, which is only slightly lower than the yield obtained with Method 1 (52%) but the exines contained more nitrogen than those extracted with Method 1.

5.3.3 Method C (Silkworm moth patent[7])

To produce exines that were nitrogen-free in a high yield, another sporopollenin extraction method was explored, based on a Japanese silkworm moth patent (U.S 6156330)[7]. The patent claims that the microsporidian spores produced by their method consist only of glucans, chitin and chitosan.

A 'hydrolysis' cycle was followed. A solution of NaOH (1M, 100ml) was added to *L. clavatum* (25µm, 50g) with stirring for 12 hours at room temperature. The spores

were allowed to cool to room temperature and were filtered (porosity grade 4). and added to a solution of HCl (1M, 100ml) with stirring for 12 hours at room temperature.

This hydrolysis cycle was repeated a total of 3 times, the spores were filtered (porosity grade 4) and the sporopollenin was washed with distilled water (2 x 100ml) and refluxed in ethanol (95%, 2h) to 'sterilise and dehydrate' the particles. The resulting sporopollenin was dried over P_2O_5 *in vacuo* until constant weight. A yield of 26% was achieved from 50g using this method, which was considerably lower than that obtained in Method A and the exines also contained higher % nitrogen.

Combustion elemental analysis of the sporopollenin produced the following results:

Extraction method	**Elemental analysis (%)**			**% Yield**
	C	**H**	**N**	
Raw spores (25μm)	65.35	10.14	0.67	-
A	66.74	7.07	0.00	52
B	63.90	10.00	0.23	43
C	46.71	6.40	1.07	26

Figure 5.3.3: Elemental combustion analysis comparing sporopollenin exines prepared by different extraction protocols.

5.3.4 Extraction of *Ambrosia trifida* sporopollenin exines

This method was adapted from the extraction of *L. clavatum* (Method A).

Raw *Ambrosia trifida* (2g) was refluxed with stirring in acetone (30ml, 1h). The solid was filtered (porosity grade 5) and refluxed in NaOH (5%, 60ml, 1h) with gentle

stirring. The solid was filtered (porosity grade 5) and refluxed with stirring in ethanol (60ml, 1h) the solid was filtered (porosity grade 5) and finally refluxed in orthophosphoric acid (60ml, 1h). The resulting sporopollenin was filtered (porosity grade 5) and washed with distilled water (5 x 50ml), and ethanol (5 x 50ml). The sporopollenin was dried over P_2O_5 *in vacuo*. A yield of 36% was achieved from 2g using this method and although some nitrogen remained, a more aggressive treatment (refluxing at each step for 2h) that did remove all of the nitrogen resulted in damaged particles.

	C (%)	H (%)	N (%)
Raw pollen	55.66	10.22	3.71
Exines	57.51	10.34	0.65

Figure 5.3.4: Combustion elemental analysis results comparing raw *A .trifida* pollen with extracted exines.

5.4 Quantification

A Becton Dickinson FACS-Calibur machine, a Beckmann Coulter Counter and an improved Neubauer counting chamber (haemacytometer) were used to quantify the number of particles in 1g of *L. clavatum* spores (both 25μm and 40μm sizes), *Ambrosia trifida* (15μm) and their extracted exine material. Of these methods the haemacytometer gave the most reproducible results and was considered an accurate and reliable method for the quantification of particles larger than 0.5μm[8]. Therefore the haemacytometer was the favoured method. It should be noted that before the haemacytometer method was selected as the standard counting method, a stage micrometer was used to count particles which involved suspending 0.1ml of sample

onto a glass microscope slide and counting the particles therein using a light microscope. This method was time-consuming and not as accurate as the haemacytometer. The stage micrometer was only used for the Enfurvitide experiment (Section 2.5.1).

With the haemacytometer, stock solutions were made up of 400mg/3L of exine or pollen/spore material in water: ethanol (1:4). Different dilution solutions (100%, 50%, 25%, 12.5%, 6.25% and 3.13%) were made up to 10ml diluted with distilled water. A calibration curve was deduced and the number of particles per gram determined. Magnification was x 20 lens.

Type of particles	**Approximate number of particles per gram**
L. clavatum spores 25µm	28,650,000 (± 720,000)
L. clavatum exines 25µm	63,750,000 (± 2,380,000)
L. clavatum spores 40µm	17,700,000 (± 1,600,000)
L. clavatum exines 40µm	21,100,000 (± 4,470,000)
A. trifida pollen	40,900,000 (± 3,720,000)
A. trifida exines	66,000,000 (± 5,200,000)
Rye grass pollen	20,910,000 (± 1,820,000)
Rye grass exines	32,700,000 (± 1,970,000)

Figure 5.4: The number of spore/pollen particles and their extracted exines per gram (determined by haemacytometer).

5.5 Biological Evaluation of Sporopollenin: *In vivo* exine degradation

5.5.1 Transport of *L. clavatum* spores and exines into the bloodstream

Eight human volunteers of both sexes and ages ranging from 20 to 58 years were each given an oral dose containing approximately 12,660,000 ± 287 particles (200mg exines or 640mg raw *L. clavatum* spores) in milk (ca. 15ml) after fasting overnight. This dose was chosen because it gave the highest percentage recovery (see Saturation trial). Venous blood samples (4ml) were taken at time 0, 5, 15, 30, 45, 60 minutes following ingestion.

The sporopollenin exines (200mg) were taken by eight volunteers, one volunteer of which repeated the trial a total of eight times on separate days. The raw *L. clavatum* spores (640mg) were taken by one volunteer a total of five times on different study days. The results were a mean of 20 counts per time point.

5.5.2 Treatment of blood samples

In all cases, venous blood samples were collected into EDTA tubes at time 0, 5, 15, 30, 45 and 60 minutes after ingestion and centrifuged immediately (3000RPM, 10min, 4°C). The plasma aliquot (2ml) was removed for analysis and the remaining pellet (blood cells) was lysed with distilled water (up to 5ml) and was then analysed for particle content using a calibrated haemacytometer. Magnification was x 20 lens.

5.5.3 Concentration technique for SEM analysis

Lycopodium clavatum spores (25μm, 400mg) were ingested with milk (ca. 15ml) after overnight fasting. It should be noted that the particles were well wetted with milk by agitation using a plastic spatula. Venous blood samples (8 x 4ml) were

collected into EDTA collection tubes 30 minutes after ingestion and were centrifuged (3000RPM, 4°C, 10 min). The plasma portions of the blood (4ml) were combined and centrifuged again in the same manner as before. The sediment was recovered and water (10ml) added to wash the sediment, which was centrifuged again in the same manner as before. The resulting sediment containing recovered spore particles was freeze-dried overnight and SEM performed.

5.5.4 FT-IR of sporopollenin exines recovered *in vivo*

Plasma samples taken form a volunteer after ingestion of sporopollenin exines (200mg) were centrifuged, the top fraction of plasma was discarded and the remaining sediment was dried *in vacuo* over P_2O_5. This sample was ground using a pestle and mortar and a disc was produced using an IR press (10 tonnes, 2min). An IR spectrum was produced and compared to that of sporopollenin exines before ingestion.

5.5.5 FACS method

Two human volunteers ingested sporopollenin exines (200mg) derived from *L. clavatum* (25μm) in milk (ca. 15ml) after overnight fasting. Venous blood samples were taken at 0 (baseline), 5, 15, 30, 45 and 60 minutes after ingestion. The blood samples were diluted with PBS (see table below) and recovered particles were measured with the FACS machine. Different voltages for sideward scattering (155 and 269) and different thresholds (500 and 550) were investigated. Separation of sporopollenin exines and blood cells within the samples was achieved when a lower threshold was used.

5.5.6 The inter- and intra-observer error

The difference in the number of particles recovered in the bloodstream after oral ingestion of sporopollenin exines (i.e. the inter-observer error) was investigated. Ten separate counts were taken from aliquots at each time point for each separate trial and for each volunteer using a haemacytometer.

The difference between two researchers counting the number of particles recovered in the bloodstream after oral ingestion of sporopollenin exines (i.e. the intra-observer error) was investigated. A human volunteer ingested sporopollenin exines (400mg) in milk (ca. 15ml) and venous blood samples were collected in EDTA tubes in duplicate. Both samples were processed separately using the same conditions (the same centrifugation method, 3000RPM, 4°C, 10min and lysing the blood pellet up to 5ml with distilled water) by two different researchers. The number of exine particles recovered in the blood plasma and lysed pellet samples were counted in each case using a calibrated haemacytometer.

5.6 Factors affecting the rate of absorption of particles into the blood stream

5.6.1 Caffeine

A commercially available energy drink ('Red Bull' containing 80mg caffeine per 250ml) was taken 15 minutes prior to ingestion of sporopollenin exines (200mg) derived from *L. clavatum* (25μm) with milk (ca. 15ml). Blood samples were taken and treated in the manner described in 5.5.2.

5.6.2 Commercial Gaviscon®

Sporopollenin (400mg) derived from *L. clavatum* (25μm) was suspended in Gaviscon® liquid (10ml) and swallowed. Venous blood samples were taken and treated as in 5.5.2 and the recovered particles were counted using a stage micrometer because haemacytometery was not available at the time of this study. The results were a mean of 20 counts.

5.6.3 Age

Sporopollenin exines (200mg) derived from *L. clavatum* (25μm) were ingested by two male volunteers aged 20 and 58 after overnight fasting. Venous blood samples were taken and treated as in 5.5.2 and the results were a mean of 20 counts.

5.6.4 Gender

Sporopollenin exines (200mg) derived from *L. clavatum* (25μm) were ingested by two male and two female volunteers aged 23 after overnight fasting. Venous blood samples were taken and treated as in 5.5.2 and the results were a mean of 20 counts.

5.6.5 Saturation effect

A double-blind study was conducted with two volunteers, who were each given oral doses of 200mg, 400mg, 600mg and 1000mg sporopollenin exines derived from *L. clavatum* (25μm) with milk (ca. 15ml) on separate occasions after fasting overnight. Venous blood samples were taken and treated as in 5.5.2 and the results were a mean of 20 counts.

5.6.6 Dilution effect

Human volunteers ingested sporopollenin exines (400mg) derived from *L. clavatum* (25μm) with different volumes of milk (15ml, 50ml, 100ml and 200ml) on separate occasions after fasting overnight. Venous blood samples were taken and treated as in 5.5.2 and the results were a mean of 20 counts.

5.7 The effect of different dosage forms on the rate and extent of exine absorption

5.7.1 Buccal absorption

Sporopollenin exines (400mg) derived from *L. clavatum* (25μm) were rinsed around the oral cavity for 2 minutes with extra caution taken to avoid swallowing. The exines were then discarded. Venous blood samples were taken and treated as in 5.5.2 and the results were a mean of 20 counts. This experiment was repeated twice on one volunteer.

5.7.2 Sweet trial

Sporopollenin exines (200mg) extracted from *L. clavatum* (25μm) were encapsulated in chewy confectionary using the facilities at Nestle PTC, York. Each sweet contained 200mg sporopollenin and two were chewed by three volunteers to give a 400mg dose. Venous blood samples were taken and treated as in 5.5.2 and the results were a mean of 20 counts.

5.7.3 Tablet trial

Tablets of sporopollenin exines derived from *L. clavatum* (25μm) using method A (400mg) were prepared by compression using an IR press (10 Tonnes, 2min). They contained 10% sodium bicarbonate, along with 45% lactose to aid tablet dispersion in the stomach. The total mass of each tablet was 620mg and they measured 3mm thickness and 13mm diameter. Three human volunteers ingested one tablet with water after overnight fasting. Venous blood samples were taken and treated as in 5.5.2 and the results were a mean of 20 counts.

5.7.4 Ingestion with milk

Sporopollenin exines (400mg) derived from *L. clavatum* were ingested by three volunteers orally with milk (ca. 15ml) after overnight fasting. Venous blood samples were taken and treated as in 5.5.2 and the results were a mean of 20 counts.

5.7.5 Ingestion with water

Sporopollenin exines (400mg) derived from *L. clavatum* were ingested by two volunteers with drinking water (ca. 15ml) after overnight fasting. Venous blood samples were taken and treated as in 5.5.2 and the results were a mean of 20 counts.

5.8 Other pollens and their extracted exines

Lycopodium spores (40μm, as prepared in 5.3.1), *A. trifida* pollen particles (15μm, as prepared in 5.3.4) and their extracted exines were ingested by two human volunteers at a dose of 400mg in milk (ca. 15ml) on separate occasions after overnight fasting. Venous blood samples were taken from the volunteers and treated as in 5.5.2. The results were a mean of 20 counts.

5.9.1 Encapsulation of Enfurvitide

The encapsulation was carried out on exines derived from *L. clavatum* (25μm) using Method A. A solution of Enfurvitide (2g) in ethanol: water (1:4) was mixed with sporopollenin exines (4g). The mixture was subjected to a vacuum (ca. 20mmHg) for 2 hours at room temperature, quickly rinsed with water, filtered and dried under vacuum over P_2O_5 for 12 hours at 5°C. Successful encapsulation of the Enfurvitide resulted in the formation of product 'PV4X' and successful loading was verified by combustion elemental analysis and microscopy.

5.9.2 Dog *in vivo* trials

The sample 'PV4X' was dispensed onto the tongue of the dog from a wide bore plastic syringe (in milk (ca. 10 ml); a further amount of milk (20ml) was used to rinse the remaining product from the tube into the dogs mouth. Most of the product was swallowed in each case. Samples of blood (5ml) were collected into EDTA tubes at the following time intervals following ingestion: 0 (baseline), 0.5, 1, 1.5, 2, 4, 6, 8 and 24 hours. The sample was centrifuged and the separated plasma was decanted into a second tube. Of this a sample (ca. 1ml) was reserved for immediate microscopy and another (ca. 4ml) for an Enfurvitide assay undertaken in Berlin.

The blood samples were centrifuged (3000 RPM, 8min). The pellet (2.4 ml) and plasma (2.6 ml) were separated into two tubes and a third tube was used for an aliquot (1ml) of the plasma for Enfurvitide assay in Berlin. The blood cells (2.4 ml of pellet) were lysed with distilled water (5ml); therefore total volume of 7.4ml. Because the haemacytometer was not in routine use at the time of this experiment, an aliquot (0.1ml) was dispensed onto a stage micrometer and viewed under a light microscope (x10 magnification) and all remaining particles (exines and fragments thereof) were

counted within the said volume. This measurement was repeated for each blood sample taken.

5.9.3 Encapsulation of cyclosporine

Cyclosporine was loaded into sporopollenin exines derived from *L. clavatum* (25μm). Cyclosporine (300mg) was weighed into a filter (sinter porosity 2). Ethanol (10ml) was added to form a solution, to which sporopollenin exines (25μm, 600mg) were added. The excess ethanol was removed by vacuum filtration. The sample was dried *in vacuo* until constant weight. Cyclosporine was loaded into sporopollenin exines with 50% w/w efficacy.

5.9.4 Dog *in vivo* trails

Four dogs were fasted prior to the study. Two dogs were dosed with cyclosporine in methylcellulose suspension and two were dosed with the exines (45mg) that were loaded with cyclosporine (1:2 w/w Cyclosporine to exines), also in a methylcellulose suspension. Blood samples were taken in duplicate at the time points: 0 (pre-dose), 15min, 30min, 45min, 1h, 1.5h, 2h, 2.5h, 3h, 4h, 6h, 10h, 24h. One sample was assayed by HPLC-UV to determine the concentration of Cyclosporine released into the circulation and one sample was taken to count the number of exines that had reached the bloodstream using a calibrated haemacytometer.

5.10.1 Encapsulation of Amphotericin

As amphotericin is not soluble in either water or ethanol, several solvents were explored (diethyl ether, chloroform, DMF) however; amphotericin could not be precipitated into the exines. Four samples were prepared:

- Amphotericin (30mg) was dissolved in DMSO (1ml) as a control sample.
- Amphotericin (30mg) was dissolved in DMSO (1ml) and exines were added (0.5g)
- Amphotericin (60mg) was dissolved in DMSO (1ml) as a control sample.
- Amphotericin (60mg) was dissolved in DMSO (1ml) and exines were added (0.5g)

The samples were placed under vacuum for 1 hour. Ethanol (4ml) was added to each sample and was centrifuged (3000 RPM, 4 min). The ethanol washings were removed and ethanol (4ml) was added again and the step was repeated a total of 5 times. A solid yellow/orange precipitate formed in the control samples. A portion of each sample was added to 4ml water to see if the residual DMSO could be washed from the surface of the exines to produce a dry sample. The loading of amphotericin was determined on a mass gain basis. For the 30mg sample: 9mg/0.5g sporopollenin was loaded (30%) and for the 60mg sample: 40.2mg/0.5g sporopollenin was loaded (67%). Successful loading was confirmed by mass gain and confocal microscopy

5.10.2 Release of Amphotericin

Sporopollenin exines loaded with amphotericin (0.9mg, 2.3mg, and 1.3mg for 3 different runs) were placed into clean culture tube and DMSO added (10ml). The tubes were gently mixed for 24 hours at room temperature then centrifuged. An aliquot of the supernatant was diluted 1:2 with HPLC mobile phase (2mM ammonium acetate in 95% methanol, 5% water.) and 10µl injected onto the column for analysis and quantification against reference standards.

5.11 *In vitro* experiments

5.11.1 Different fractions of the blood

Blood from a human volunteer was collected into EDTA tubes to produce plasma and serum tubes to produce serum. The blood was centrifuged (3000RPM, 10min, 4°C) and the plasma and serum portions of the blood (2ml from each tube) were removed into separate tubes, leaving the pellet portion of the blood that contained the blood cells. This pellet was then lysed with distilled water (5ml).

Sporopollenin exines (4mg) were added to plasma, serum and lysed blood samples (4ml) and were incubated at 37°C with gentle shaking for 30 minutes. After this time, an aliquot (10µl) was viewed under the light microscope (x20) and images were taken.

Quantitative experiments were performed investigating plasma collected from EDTA, lithium heparin and citrate tubes. Four different tubes were set up, each containing plasma (4ml) from each of the tubes (i.e. 4 x EDTA, 4x lithium heparin and 4 x citrate plasma). Sporopollenin exines (4mg) were added to each tube and an aliquot (10µl) was taken after immediate addition as time zero. The other three samples were incubated at 37°C for 30 minutes and additional aliquots (10µl) were taken at 10, 20, and 30 minutes after addition. Particles were counted using haemacytometery and the mean of three runs was given.

5.11.2 Different collection tubes

The following 3 samples were prepared:

- 4 x 4ml plasma collected into citrate tubes
- 4 x 4ml plasma collected into heparin tubes

- 4 x 4ml plasma collected into EDTA tubes

Exines (4mg) were placed into a series of four tubes containing plasma (4ml) collected from either heparin/citrate/EDTA, which had been incubated at 37°C. An aliquot (10µl) was taken immediately from one tube after addition of the exines. This was analysed by haemacytometer and the count at time zero constituted a reference value of 100% recovery* (i.e. no time for the exines to degrade). The other 3 samples of each type were incubated at 37°C and aliquots (10µl) were taken at 5, 10, and 20 minutes. This method was repeated for each of the 3 samples above. The counts were a mean of 15 repeats.

5.11.3 Effect of temperature

Human plasma (4ml) was heated to 2-4°C, 22°C, 37°C and 60°C using a heating block or ice bath. Sporopollenin exines (4mg) were placed into 4 separate tubes of plasma for each time point and shaken. An aliquot (10µl) was taken immediately from tube 1 (at zero time point). The samples were incubated at the desired temperature for 10,20 and 30 minutes. The haemacytometer count at time zero acts as a reference* for the other counts and is given a 100% recovery value. The mean of 15 counts was recorded using a haemacytometer.

5.11.4 Differences in the rate of degradation in plasma obtained from human, dog and rat species

The rate of exine degradation in plasma from human, dog and rat origin was observed over 30 minutes *in vitro*. Sporopollenin exines (2mg) were added to 4 separate tubes

containing plasma (2ml) at 37°C. The rate of degradation was observed over 30 minutes (time points 0, 10, 20 and 30 min) using a haemacytometer.

5.11.5 Effect of plasma storage time

Sporopollenin exines (4 x 4mg) were added to human plasma samples (2 x 4ml) incubated at 37°C, two of which were freshly collected into EDTA tubes and two had been stored at –20°C for 2 weeks and had been thawed out. An aliquot (10µl) was taken after immediate addition of the exines (i.e. at time zero), and after 30 minutes. Sporopollenin exines were counted using haemacytometery and the results were a mean of five counts.

5.11.6 *In vitro* saturation effect

Plasma collected from EDTA tubes (5 x 4ml in 5 separate tubes for each of the 3 masses of exines) was incubated at 37°C. Different masses of sporopollenin exines (4mg, 10mg and 20mg) were added and the degradation of these exines was followed by haemacytometery at time zero (removal of 10µl after immediate addition), 5, 10, 20 and 30 minutes after addition of the exines.

5.11.7 Bovine thrombin

Bovine thrombin (200U) was diluted with distilled water (0.5ml) and different volumes of thrombin (50µl, 100µl, and 300µl) were added to fresh human serum samples (2ml). Sporopollenin exines (5mg) were added to each tube and incubated at 37°C for 30 minutes. It was difficult to count any recovered particles in the samples because the thrombin had formed a gel.

5.11.8 Commercial plasma preparations

Commercially available factor-deficient plasma samples (Commercial factor XII deficient plasma, prekallikrein deficient plasma and 'normal' commercial plasma) were incubated at 37°C for 10 minutes. Sporopollenin exines (5mg) were added and the degradation was observed up to 30 minutes after addition using a haemacytometer. Results were a mean of 10 counts.

5.11.9 Aprotinin (Trasylol)

Trasylol was added to fresh human plasma (12 x 2ml) in the following dilutions: 0.1ml (5%), 0.2ml (10%) and 0.4ml (20%). Four separate tubes containing plasma (2ml) were set up for each dilution. Sporopollenin exines (4mg) were added to each sample and incubated at 37°C for 30 minutes. An aliquot (10µl) was taken for counting at time zero (immediately after addition), 10, 20 and 30 minutes after addition of exines. Particles were counted using a haemacytometer. Counts were a mean of 3 repeats.

5.11.10 Streptokinase experiment

The following 5 samples were prepared and incubated at 37°C:

A. Serum control samples x 4 (4ml)
B. Serum with streptokinase x 4 (4ml, 20% v/v streptokinase)
C. Plasma control sample x 4 (4ml)
D. Plasma with streptokinase x 4 (4ml, 20% v/v streptokinase)
E. Streptokinase control x 4 (4ml)

Exines (4mg) were added to all 4 tubes of samples A-E and shaken immediately. The timer was started. An aliquot (10µl) was taken immediately from one tube after addition of the exines. This was analysed by haemacytometer and the count at time zero constituted a reference value of 100% recovery (i.e. no time for the exines to degrade). The other 3 samples of A-E were incubated at 37°C and aliquots (10µl) were taken at 5, 10, and 20 minutes. This method was repeated for each of the 5 samples above. The counts were a mean of 15 repeats.

5.11.11 Protease inhibitor cocktail

Protease inhibitor cocktail was purchased from EMD Biosciences (Calbiochem) and consists of AEBSF, HCl, Aprotinin, Bovine Lung; Bestatin; E-64 Protease Inhibitor; Leupeptin, Hemisulfate, and Pepstatin A. The inhibitor was defrosted to room temperature and an aliquot (20µL) was added to plasma (4ml). Sporopollenin exines (4mg) were added to plasma (4ml) – containing the inhibitor, in a series of 4 tubes (for time 0, 5, 10 and 20 minutes). Particles were counted using a haemacytometer. Results were a mean of 10 counts.

5.11.12 Plasmin experiment

To ensure that tris buffer did not inhibit any degradation of exines, fresh human plasma was collected and diluted with buffer. Tris buffer was prepared as follows:

Tris (6.1g), NaCl (0.7) and distilled water (800ml) were mixed and the pH was adjusted to pH 7.4 using HCl (1M) and a calibrated pH meter. The solution was made up to 1L.

Plasma was diluted with different volumes of buffer to make solutions of 100% plasma, 50%, 25%, 12.5%, 6.3%, 0% (control buffer). Sporopollenin exines (4mg)

were added to each solution and the exines remaining after 0, 5, 15 and 30 minutes were counted using a haemacytometer.

Next, plasmin (500μg) was dissolved in tris buffer (1ml) and double diluted across a series of tubes, giving final concentrations of 250μg/ml, 125μg/ml, 63μg/ml, 31μg/ml, and 0μg/ml (buffer control). The total volume made up was 1ml. sporopollenin exines (1mg/ml) were added to each tube and sampled over 30 minutes at 37°C. Particles were counted using a haemacytometer. Results were a mean of 10 counts.

5.11.13 Enoxaparin (Clexane) experiment

The following solutions were prepared in duplicate:

- Plasma from EDTA (4ml)
- Plasma from Heparin (4ml)
- 50 Units (5μL) Clexane made up to 4ml with Plasma (EDTA)
- 100 Units (10μL) Clexane made up to 4ml with Plasma (EDTA)
- 150 Units (15μL) Clexane made up to 4ml with Plasma (EDTA)
- 200 Units (20μL) Clexane made up to 4ml with Plasma (EDTA)
- Clexane control (no plasma) 1ml contained 10,000 Units – only 2mg exines added

Solutions were incubated at 37°C for 10 minutes, and sporopollenin exines were added (4mg to each tube, except 2mg into Clexane control tubes). At time zero (i.e. upon immediate addition) an aliquot (10 μL) was removed and exines were counted from the first tube of each sample using a haemacytometer. After 30 minutes incubation an aliquot (10 μL) was removed from tube 2 for each sample. The results were a mean of four counts.

5.11.14 Degradation of different pollen, spores and their extracted exines in plasma

Pollen or exines derived from *L. clavatum* (25μm and 40μm) and *Ambrosia trifida* (4mg) were placed into a series of five tubes containing plasma (4ml) at 37°C. An aliquot (10μl) was taken immediately from one tube after addition of the pollen or exines. This was analysed by haemacytometer and the count at time zero constituted a reference value of 100% recovery* (i.e. no time for the particles to degrade). The other four samples for both pollen and exines were incubated at 37°C and aliquots (10μl) were taken at 5, 10, 20 and 30 minutes. The counts were a mean of 15 repeats.

5.11.15 Resilience of exines to stomach and small intestinal fluids

The following solutions were prepared using methods from Pfizer Pharmaceutical R & D, Sandwich Laboratories (methods stated in USP).

Simulated stomach fluid (Non peptide)

Sodium chloride (2g) was dissolved in 500ml water. Hydrochloric acid (conc. 7ml) was added and this was made up to 1L with water. The pH was 1.2 and the solution was incubated at 37°C.

Simulated intestinal fluid

Potassium dihydrogen orthophosphate (6.80g) was added to sodium hydroxide (1.57g) and sodium chloride (2g). Water was added (30ml) and the pH was adjusted to 7.50 ± 0.10 with sodium hydroxide solution (0.1M). The solution was made up to 1L with water.

Actual small intestinal fluid and stomach fluid were obtained from Beagle dogs courtesy of Pfizer Pharmaceutical R & D, Sandwich Laboratories, UK.

5.11.16 Preparation of Simulated Gastric Fluid (SGN)

Simulated gastric fluid was prepared according to the literature reference[9] by dissolving NaCl (2mg/L) in water and adjusting the pH to 1.5 with HCl (1M). If Pepsin was added to this preparation pepsin was dissolved into the SGN solution (3.2g/L).

5.11.17 Tablet form of sporopollenin exines

Sporopollenin exines (400mg) were made into tablets using the IR press (10 tonnes, 2 min). Tablets measured 3mm thickness and 13mm diameter. Three tablets were prepared to determine their dissolution in SGN:

A. 400mg exines
B. 400mg exines and 40mg sodium bicarbonate (10%)
C. 400mg exines, 40mg sodium bicarbonate (10%) and 180mg lactose (45%)

Each tablet was added to SGN and their dispersion into solution was observed.

5.12 Functional Utility of Sporopollenin Exines

Several loading mechanisms were explored for the encapsulation of substances into the sporopollenin exines.

5.12.1 Passive protocol

Sporopollenin exines (1g) were stirred at room temperature and pressure into a solution of a substance to be encapsulated. Solid substances were first dissolved in a suitable solvent that was later removed completely by evaporation, drying in an oven or under vacuum over phosphorous pentoxide.

5.12.2 Compression protocol

Sporopollenin exines (500mg) were compressed into tablet form using an IR press (20mmHg pressure for 2 minutes). The tablets were then placed into a solution of the substance to be encapsulated and left for 1 hour. The mixture was then dried under vacuum over phosphorous pentoxide for 2 days.

5.12.3 Vacuum protocol

Sporopollenin exines (1g) were placed into a solution of the substance to be encapsulated, stirred and placed under vacuum (20mmHg) for 60 minutes. The mixture was then dried under vacuum over phosphorous pentoxide for 2 days. Liquids encapsulated in this manner are listed below:

- Histoclear® (D-limonene)
- Omega 3 fish oil
- Vegetable oils (rapeseed, sunflower, echium, soybean)
- Molten cocoa butter

- Lycopene (as Lyc-o-mato commercial preparation)
- Molten beeswax

Solids encapsulated in this manner are detailed below:

- Ascorbic acid (vitamin C)
- Various dyes (malachite green, Evan's blue, Nile red, solvent blue, solvent yellow, solvent red)
- Insulin – 6 kDa
- α amylase – 54 kDa
- β galactosidase – 116 kDa
- Human growth hormone (Humatrope – 22,125 kDa)
- Other proteins (Ipsen)

  Light 1,097 Da loaded max 60mg/g

  Heavy 3,340 Da max loading 40mg/g
- Enfurvitide (Fuzeon – 5,065 Da)

5.13 Loading of substances into sporopollenin exines

5.13.1 Oils

A stirred mixture (2ml) of the oil under investigation (omega 3, sunflower and rapeseed oils) in ethanol (1:1) was added to sporopollenin powder (0.5g) with stirring and placed under vacuum (20mmHg) for 30 minutes. The product was dried to constant weight over phosphorous pentoxide and examined by microscopy. A maximum mass gain of 4:1 w/w oils to sporopollenin exines were achieved in this manner.

5.13.2 Dyes

A range of dyes (Nile Red, Malachite Green and Evan's Blue) was loaded into sporopollenin exines in a series of experiments. A solution of the dye in water/ethanol (5ml, 4:1 v/v) was added to sporopollenin exines (typically 500mg) and placed under vacuum (20mmHg) for 1 hour. The samples were then dried *in vacuo* over P_2O_5 until constant weight. Loadings of 1:1 w/w were achieved based on mass gain calculations and successful loadings were verified using confocal microscopy.

5.13.3 Enzymes (amylase and lactase)

Solutions of lactase and amylase (200mg) in water/ethanol (6ml, 4:1 v/v) were added to sporopollenin exines (typically 400mg) in a series of experiments. The mixtures were placed under vacuum (20mmHg) for 1 hour and dried *in vacuo* over P_2O_5 until constant weight. A loading of 1:2 w/w enzymes to sporopollenin exines was achieved and verified by mass gain and confocal microscopy.

5.13.4 Enfurvitide

A solution of Enfurvitide (approx. 500mg) in water/ethanol (10ml, 4:1 v/v) was added to sporopollenin exines (3.5g). The mixture was placed under vacuum (20mmHg) for 2 hours at room temperature, quickly rinsed with water (5ml), filtered and dried *in vacuo* over P_2O_5 for 12 hours at 5°C. The loaded exines were then freeze-dried. Successful encapsulation was verified by combustion elemental analysis, mass gain and confocal microscopy. A product (PV4X) with a ratio of 273:726 of Enfurvitide to sporopollenin exines was achieved.

5.13.5 Ibuprofen

A homogenous mixture of an ethanolic solution of Ibuprofen (800mg Ibuprofen in 4ml ethanol) was mixed with sporopollenin exines (700mg) and subjected to a vacuum (20mmHg) for 1 day and dried *in vacuo* over P_2O_5. A loading of 1:1 was achieved, determined by mass gain.

5.13.6 Human Growth Hormone (hGH)

A solution of hGH (1.5mg) in water (0.5ml) and of EtOH (0.5ml) was added to sporopollenin exines (25μm, 1g) with stirring and was placed under vacuum for 1h. The sample was freeze-dried until constant weight. The loading of hGH was 1.2 mg per gram of sporopollenin. Higher loadings were possible (highest loading is currently 7.1mg hGH/g exines) but this lower loading was ideal because assuming 100% release, the hGH was within the detection limits for the RIA.

5.13.7 Amphotericin

Bile acid form of amphotericin: Amphotericin (60mg) was dissolved in DMSO (1ml) and sporopollenin exines were added (0.5g). The samples were placed under vacuum (20mmHg) for 1 hour. The loading of amphotericin was 80.4mg per gram of sporopollenin (i.e. 67% efficacy) determined by mass gain.

Liposomal form of Amphotericin: A total phospholipid mixture of 56 mg (soy lecithin/cholesterol in the weight ratios of 4:1) in Et_2O (2.5 mL) and a solution of amphotericin (12 mg) in PBS (0.75 mL) (pH 7.4) were mixed and stirred for 30 min at room temperature, and emulsified with a bath-type ultrasonifier for 30 min. The emulsion was formed and ethanol was removed by rotary evaporator in 20°C water

bath under reduced pressure. After this, the sporopollenin was poured into the solution (203mg) and was let under vacuum for 1h. A loading of 50.9μg of preparation was achieved (which contained 45% amphotericin and 35% sodium deoxycholate in a bile acid formulation) which equated to 10.6 μg of Amphotericin B per gram of exines.

5.13.8 Cyclosporine

Cyclosporine (301mg) was weighed into a pre-weighed round bottom flask. Ethanol (10ml) was added to form a solution, to which sporopollenin exines (25μm, 600mg) were added. The solution was stirred for 10 minutes and the excess ethanol was removed by rotary evaporation. The ratio of cyclosporine to sporopollenin in this formulation was 1:2 w/w.

5.13.9 Ascorbic acid

Method A: Loading with ethanol

Ascorbic acid (500mg/5ml) was added to sporopollenin exines derived from *L. clavatum* (25μm, 500mg) with a few drops of ethanol to aid the loading. The mixture was stirred and placed under vacuum at room temperature for 2 hours and filtered on a sinter (porosity 3). The loaded sample was rinsed with water and dried over P_2O_5 until constant weight. A loading of 1:1 w/w was achieved, which was determined by the mass gained.

Method B: Liposomal ascorbic acid Encapsulation

A total phospholipid mixture of 1g (soy lecithin/cholesterol in the weight ratios of 4:1) in Et_2O (9.8ml) and a solution of ascorbic acid (336mg) in a buffer solution of PBS (2.95ml) (pH 7.4) were mixed and stirred for 30 min at room temperature, and

emulsified with a bath-type ultrasonifier for 30 min. After this, the emulsion was formed and the Et_2O was evaporated using rotary evaporator in 20°C water bath under reduced pressure. The resulting liposomal dispersion was stirred for 15 min to form the liposomal suspensions. After this, the sporopollenin exines were poured into the liposomal suspensions with gentle stirring and were placed under vacuum for 1h. A loading of 251mg per gram of sporopollenin was acheived (w/w).

Method C: Encapsulation of ascorbic acid as an emulsion in oil

A mixture of ascorbic acid (330mg) in water (2.6ml) and fish oil (5ml) was mixed and stirred for 30 min at room temperature, to produce an emulsion. After this, the sporopollenin exines were added with gentle stirring and were placed under vacuum for 1h. A loading of 210mg per gram of sporopollenin was achieved.

Method D: Loading of ascorbic acid at 50% efficacy

Ascorbic acid (100mg) in distilled water (0.6ml) was added to sporopollenin exines (200mg) with stirring to produce a homogeneous mixture. This was placed under vacuum (20mm Hg) for 1.5h. The sample was freeze-dried until constant weight. Ascorbic acid was loaded at 50% efficacy (1:2 w/w ascorbic acid to sporopollenin exines).

5.14 Evidence of loading

5.14.1 Confocal images

Particles to be examined were mounted onto a glass slide using clear varnish to secure in place. Confocal images were obtained using a Bio-Rad Radiance 2100 Laser Scanning Microscope as described in 5.2.3. The method was Blue/Green/red using

lasers: Argon (514 4.1%, 488 34.6%, 476 OFF, 457 5.6%), Green HeNe (543 57.6%), Red diode (OFF), and Blue diode (405 4.9%)

5.14.2 Loading of acrylic resin

2% potassium permanganate was used to preserve the specimen; there was no vacuum for 45 minutes. It was washed four times with distilled water to remove potassium fix. The sample was then dehydrated in ethanol of increasing strength (i.e. 50, 75, 90, and 100%) in order to avoid shrinkage. LR White Resin® [80% polyhydroxy substituted bisphenol A dimethacrylate resin (MWt 452), 19.6% C12 methacrylate ester, 0.4% dimethyl para toluene] was absorbed into the spores after pre-treatment with ethanol. The sample was then embedded in gelatine capsules and placed into an oven at 60°C for 12 hours to polymerise and harden the resin.

The section was cut with a diamond knife and the resulting 'Reichert OMU2 Ultra Microtome sections' were placed onto formvar coated copper grids. The samples were then stained with 2% uranyl acetate for 30 minutes and 2% lead citrate for 5 minutes to be viewed by SEM. The sporopollenin exines were also examined by TEM performed by J. Halder[1].

5.14.3 Loading with calcium chloride

A highly concentrated solution (10ml) of calcium chloride (114g in 100ml ethanol: water, 9:1) was added to sporopollenin (0.5284g) and stirred for 8 hours. The exines were dried in an oven, fixed in LR acrylic resin and sectioned. The particles were viewed with SEM and the presence of calcium chloride was confirmed by X-ray analysis.

5.14.4 Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFTS)

DRIFTS of the sporopollenin loaded with ascorbic acid prepared by Method A (Section 5.14.9) were attempted following an Overhead Attenuated Total Reflectance (ATR) method with a ZnSe crystal. The sporopollenin samples were diluted with KBr by mixing thoroughly. A micro-cup was used and the mirrors realigned after each run. A KBr blank sample was also run as an energy throughput to disregard any background readings.

5.14.5 Fourier Transform Infra-Red Spectroscopy (FT-IR)

KBr discs were prepared using an IR press of samples of sporopollenin exines before loading, encapsulated ascorbic acid (prepared by Method A, Section 5.14.9), and ascorbic acid as a reference. The samples were diluted with KBr by mixing thoroughly (ratio of approx. 1:4 sporopollenin to KBr). Spectra were a result of 4 scans.

5.15 Release of substances from sporopollenin exines

5.15.1 Ascorbic acid

Titrimetric analysis

Exines loaded with ascorbic acid (20mg prepared by Method A, Section 5.14.9) were stirred in PBS pH 7.2, SGN pH2 or plasma (10ml). This was then filtered and 1ml of the filtrate was diluted up to 10ml with distilled water. An aliquot of this sample (5ml) was taken for the assay, added to acetic acid (2ml, 5%) and the solution was titrated against DCPIP dye. This was repeated for the following time points: 5, 15, 30, 60 minutes. The concentration of the solution was determined assuming a 1:1 reaction stoichiometry between DCPIP and ascorbic acid (RMM 176.12g/mol.).

Number of moles of titrant = Volume DCPIP used (L) X moles of Titrant ($6.89x10^{-4}$)

Voltammetric analysis (carried out by a co-worker)[10]

A calibration plot for ascorbic acid in PBS (pH 7.2) was determined using a voltammetric method. Sporopollenin exines loaded with ascorbic acid, as prepared by Method D (20mg) were immersed in PBS (pH 7.2, 10ml) and subsequent filtration (porosity 3). This was repeated twice, the filtrate was diluted up to 50ml and the amount of ascorbic acid was estimated using the above calibration curve, giving a concentration of 0.79mmol/L.

% Vitamin C= [AA detected]/[AA initial loading]

5.15.2 Ibuprofen

Release profiles of Ibuprofen in SGN and plasma were monitored by UV-Vis spectroscopy (performed by a co-worker[11]). A calibration plot for Ibuprofen was constructed at 263.61nm. The initial loading of Ibuprofen was determined by immersing loaded exines (40mg) into ethanol (25ml) for 30 minutes. This was filtered and the concentration determined using the calibration curve, giving a value of 1mg Ibuprofen/mg exines.

The loaded exines (40mg) were immersed in SGN or plasma collected in EDTA tubes (150ml). The suspension was filtered at 5, 15, 30 and 45 minutes and subsequently immersed in ethanol (25ml) for 30 minutes. These ethanolic solutions were taken for determination of Ibuprofen by UV-Vis spectroscopy.

5.15.3 Human Growth Hormone (hGH)

Samples of encapsulated hGH (5mg) were placed into a PBS control solution (4ml) and SGN with pepsin (4ml) and stirred. Aliquots (1ml) were taken at time 0. and 10 minutes after addition of the loaded-sporopollenin and assayed for hGH using the RIA facilities at Hull Royal Infirmary Chemical Pathology Laboratory.

5.15.4 Amphotericin

Sporopollenin exines loaded with liposomal and bile salt preparations of amphotericin (as described in Section 5.14.7) were sent to Pfizer R & D, Kent for analysis. They were placed into clean culture tubes (approx. 1-2mg, actual masses given in Section 4.4.4) and DMSO was added (10ml). The tubes were mixed gently for 24 hours at room temperature and centrifuged. An aliquot of the supernatant was diluted 1:2 with HPLC mobile phase (2mM ammonium acetate in 95% methanol, 5% water). 10μL was injected onto the column for analysis and quantification of amphotericin against reference standards.

5.16 External coatings and their stability *in vitro*

5.16.1 Preparation of soluble sporopollenin

L. clavatum sporopollenin (25μm and 40μm) and *Chlorella vulgaris* spores were dissolved in 2-aminoethanol and cold ethanol was used to obtain a reaggregate, since it was not possible to achieve an aggregate using distilled water. Although the *L. clavatum* sporopollenin did dissolve in 2-aminoethanol, it was not possible to obtain a reaggregate from any *L. clavatum* sample. The 2-aminoethanol was removed from the samples by heating under vacuum. FT-IR was performed on the reaggregate from *C. vulgaris*.

5.16.2 Coatings

Sporopollenin exines of 25μm (200mg, unloaded) were mixed for 30 minutes at room temperature with the following coatings, and dried over P_2O_5 without vacuum to avoid filling of the 'empty' exine particles:

- Starch solution (4ml of a 50g/L solution)
- Gum arabic (4ml of a 1g/10ml solution)
- Cocoa butter (500mg, molten)
- Beeswax (1g, molten)
- Shellac (2ml of a solution in 4:1 water/ethanol)
- Cod liver oil (approx. 2ml)

5.16.3 Degradation of coated-exines

An aliquot (2mg) of the different coated-exines were added to human plasma (4ml). Separate tubes were set up for each time point. The degradation of the exines was observed by microscopy (light and confocal) after 30 minutes incubation.

5.17 Coating of hGH-loaded sporopollenin exines

Sporopollenin exines that were loaded with hGH (at a level of 1.2mg per gram of sporopollenin) (100mg in each case) were mixed for 20 minutes at room temperature with the following coatings, and dried over P_2O_5 without vacuum to avoid filling of the loaded particles:

- Carnauba wax (approx 1g, molten)
- Cod liver oil (approx. 1ml)
- Starch and cod liver oil (2ml of a 50g/L starch solution mixed with 1ml cod liver oil)

- Starch and echium oil (2ml of a 50g/L starch solution mixed with 1ml echium oil)
- An emulsion of hGH in starch: A mixture of hGH (2.6mg) in water (0.5ml) and starch (1ml of 50g/L starch solution) to provide an emulsion. Sporopollenin exines (1g) were added to this mixture and let under vacuum (20mmHg) for 1h. After this, the mixture was dried over P_2O_5 until constant weight and yielded a loading of 1.3 mg hGH per gram of sporopollenin by mass gain.
- Liposomal hGH with starch: A total phospholipid mixture of 45mg (soy lecithin/cholesterol in the weight ratios of 4:1) in ethanol (2ml) and a solution of hGH (3.9mg) in water (0.6ml) were mixed and stirred for 30 minutes at room temperature, and emulsified with a bath-type Ultrasonifier for 30 minutes. An emulsion was formed and the ethanol was removed using a rotary evaporator in 20°C water bath under reduced pressure. The resulting liposomal dispersion was stirred for 15 minutes to form the liposomal suspension. Sporopollenin exines were poured into the suspension and this was let under vacuum (20mmHg) for 30 minutes. Starch (1ml of 50g/L starch solution) was added to this loaded formulation with stirring to give an additional coating. The sample was then freeze-dried until constant weight. A loading of 1.9mg hGH per gram of sporpollenin exines was achieved.
- Gelatine and Eudragit coatings: A solution of hGH (1.3mg) in water (0.5mL) and ethanol (0.5mL) was added to sporopollenin exines (1.12g) and was placed under vacuum (20mmHg) for 1h. The sample freeze-dried until constant weight. A solution of Eudragit L-100 (293.1mg) and lauric acid (279.4mg) in ethanol (2mL) was poured onto the loaded-exines with mixing

and was placed under vacuum for 1h. The sample was dried until constant weight. The operation was repeated with a solution of same concentration of Eudragit L-100 and lauric acid in ethanol (2mL) and the sample was dried until constant weight. The loading of hGH was 1.2 mg/g of sporopollenin.

5.18 The effect of coatings on the release of hGH from sporopollenin exines

Human plasma samples (2ml) were collected from EDTA tubes and were incubated (37°C, 10 min) to allow optimal enzyme activity. An aliquot (2mg) of the following coated exines were added to the plasma and a 10µl sample taken at 0, 10 and 30 minutes respectively. An aliquot (approx. 1ml) of each sample was submitted for growth hormone assay.

The release of hGH from sporopollenin exines coated with gelatine and Eudragit was achieved by adding the coated exines (4mg in each case) to SGN containing pepsin and then removing the SGN solution. Plasma was then added to the remaining exines. The exines were incubated at 37°C in SGN for 0, 20 and 40 minutes, with plasma added for an additional 30 minutes at 37°C after removal of the SGN. Separate tubes were set up for each of the three time points.

5.19. Loading of rapeseed oil and *in vivo* experiment

Sporopollenin exines (25µm, 300mg) were left overnight in rapeseed oil (15ml). The loading was 1:3 w/w of sporopollenin to rapeseed oil. The samples were filtered and rinsed with water, before being dried over P_2O_5.

A sample of exines loaded with rapeseed oil (250mg, 3:1 oil to exines) was administered orally in with milk (ca. 15ml). Blood samples were taken and treated in the manner described in 5.5.2. Particles recovered in blood samples were mounted onto glass slides and confocal microscopy was performed.

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6. General Conclusions and Further work

6.1 Objectives

Previous studies have shown the uptake of particles of micron size, such as pollen and spores, into the bloodstream following oral ingestion to be a controversial topic[1-6], thus the aim of this research was to evaluate whether absorption of such particles occurred and to what rate and extent. It was planned to assess the suitability of sporopollenin exines as a novel DDS. In order to achieve this, it was proposed to load the exines with a variety of drugs, such as hGH, Enfurvitide and Cyclosporine, to achieve protection from the GI tract but also obtain maximum release into the bloodstream. It was also proposed to characterise the degradation of the sporopollenin particles using both *in vitro* and *in vivo* methods.

6.2 Major New Finds

6.2.1 *In vivo*

This research provided unequivocal proof that pollen grains (*A. trifida*), plant spores (*L. clavatum*) and their extracted exines were able to reach the bloodstream of man following oral administration. Particles were detected as rapidly as 5 minutes after administration with a maximum of 10% (± 2%) of the recovered dose accounted for 15-30 minutes after initial ingestion. No further uptake was observed after 1 hour. The results of an extensive *in vivo* trial with human volunteers showed *L. clavatum* spores (25μm) and their extracted exines migrated into the bloodstream following oral ingestion to the same rate and extent over 1 hour. Intra- and inter-observer errors were low (± 2% typical error). A method was developed to count the recovered particles and involved the use of a cold centrifuge (4°C) to prevent uncontrolled degradation of

the particles and a haemacytometer to count the particles. This new method allowed for quantitative analysis, unlike the literature by Jorde and Linskens, which only identified recovered particles qualitatively[3,7].

These findings resolve the debate between researchers in support of the uptake of particles into the bloodstream[1-3] and those against, whom doubted the transport of particles of micron size into the bloodstream, but did not disprove such a phenomenon[4-6].

The rate of sporopollenin exine uptake into the bloodstream was increased by 3.0% (± 1.2%) with administration of caffeine prior to ingestion of exines and appeared to be independent of dosage volume (i.e. the volume of milk taken with the dose) and the age of the volunteers. There was little difference in the percentage of recovered exines with increased doses, with similar numbers of exines recovered after ingestion of 400mg, 600mg and 1000mg doses, suggesting a saturation effect of the absorption process. This is important because it could mean that a sporopollenin exine DDS would prevent an overdose of an encapsulated drug because only a certain number of exines can reach the bloodstream at any given time. It was assumed that the exines that did not reach the bloodstream were simply excreted from the body, since the elimination of ingested particles, such as starch, was investigated in studies by Volkheimer[1].

The method of administration was an important factor in determining the percentage of exines recovered in the bloodstream. The highest percentage recovery was achieved when exines were incorporated into chewy confectionary and also when

taken with milk. Fewer exines were detected in venous blood when ingested with water, possibly because the hydrophobic nature of the exines did not allow for thorough dispersion of the particles. Buccal delivery of exines into the circulation was ruled out as a main method of absorption and exine tablets that dispersed well into SGN *in vitro* did not result in a high percentage recovery.

A. trifida pollen grains (15μm), *L. clavatum* spores (25μm) and their extracted exines were able to reach the bloodstream following oral ingestion. These particles exhibited 'decoration' to their exterior, whereas *Lycopodium clavatum* (40μm) spores and exines were found at levels of only <1%. There exists either a size-exclusion mechanism that does not allow 40μm particles to enter the bloodstream or the existence of exterior 'decoration' on the particles could have aided their migration into the bloodstream.

Enfurvitide and cyclosporine were successfully delivered into the bloodstream of dogs after oral ingestion, but at low levels (with Enfurvitide only 1-2% of the expected concentration was detected in blood based on the exine counts), indicating that extra protection of the encapsulated drug would be required to achieve controlled-release at a desirable level. This prompted research into different additional coatings in an attempt to offer additional protection of the encapsulated drug against the GI tract. These coated exines were then investigated to ensure they were still able to degrade in blood and release their loaded drug.

6.2.2 *In vitro*

Despite the documented resilience of sporopollenin to chemical attack[8] both *L. clavatum* spores and their extracted sporopollenin exines were shown to degrade very

rapidly in the blood through *in vitro* and *in vivo* experiments (the initial velocities *in vitro* were 1.02mg/min for spores and 0.99mg/min for exines). A reproducible, reliable method was developed for counting the exine particles recovered *in vitro* and *in vivo* to assess the rate and extent of degradation. The new method involved the use of a cold centrifuge (4°C) to prevent any further degradation of the particles and a haemacytometer to count the particles.

The degradation of sporopollenin exines was only evident from plasma and lysed blood pellet experiments, and not in serum, which indicated that either a clotting factor or associated enzyme was causing such degradation, since serum is the aqueous phase of blood that has been allowed to clot and all the clotting factors were removed.

The mechanism of exine degradation was considered to be due to an enzyme because breakdown was inhibited at 4˚C and 60˚C. In addition, when using different volumes of plasma to degrade the same mass of exines, the rate of degradation reached a plateau after 30 minutes, which is in accord with the saturation of an enzymatic process. The enzyme plasmin was considered, because treatment of plasma with Trasylol (a known plasmin inhibitor) resulted in a reduction in the rate of exine degradation, with 20% v/v Trasylol inhibiting 100% of the degradation. However, when plasma and serum samples were spiked with streptokinase (a known plasmin activator) there was no obvious increase in the rate of degradation and plasmin itself did not cause degradation of sporopollenin exines. This could indicate that a combination of factors is responsible for sporopollenin degradation and not plasmin alone.

In contrast to the exines degrading rapidly in blood plasma, the morphology of the exines was not altered during incubation in SGN or SIF and remained intact after

incubation in actual dog stomach and intestinal fluids, which emphasizes the suitability of such particles to offer protection against the GI tract.

6.3 New Work

For the eventual clinical exploitation of sporopollenin exines as a novel DDS, more needs to be determined about the mechanism by which the particles reach the bloodstream and the related kinetics in order to achieve a level of control over the delivery of the encapsulated drug. Future work should involve radioactive labelling of the sporopollenin exines prior to ingestion to determine the precise route by which they enter the bloodstream. Such labelling of pollen and spore material has been carried out in the literature using ^{99m}Tc[2].

Further studies are required to evaluate the absorption of different types of exines that are equivalent to or larger than 25μm in size but have a smooth exterior (such as Rye grass, 25μm) to determine whether there is a size-exclusion factor of if the presence of exterior 'decoration' aids the absorption of such particles into the bloodstream following oral ingestion. Future work involves gaining a better understanding of the process of particle uptake into the bloodstream following oral ingestion and to develop a controlled-release drug delivery system. Sporopollenin exines provide a novel DDS with an exciting future to deliver drugs that are problematic to administer and other substances (have the potential to protect and deliver probiotic microorganisms that aid mammal's digestive and immune systems).

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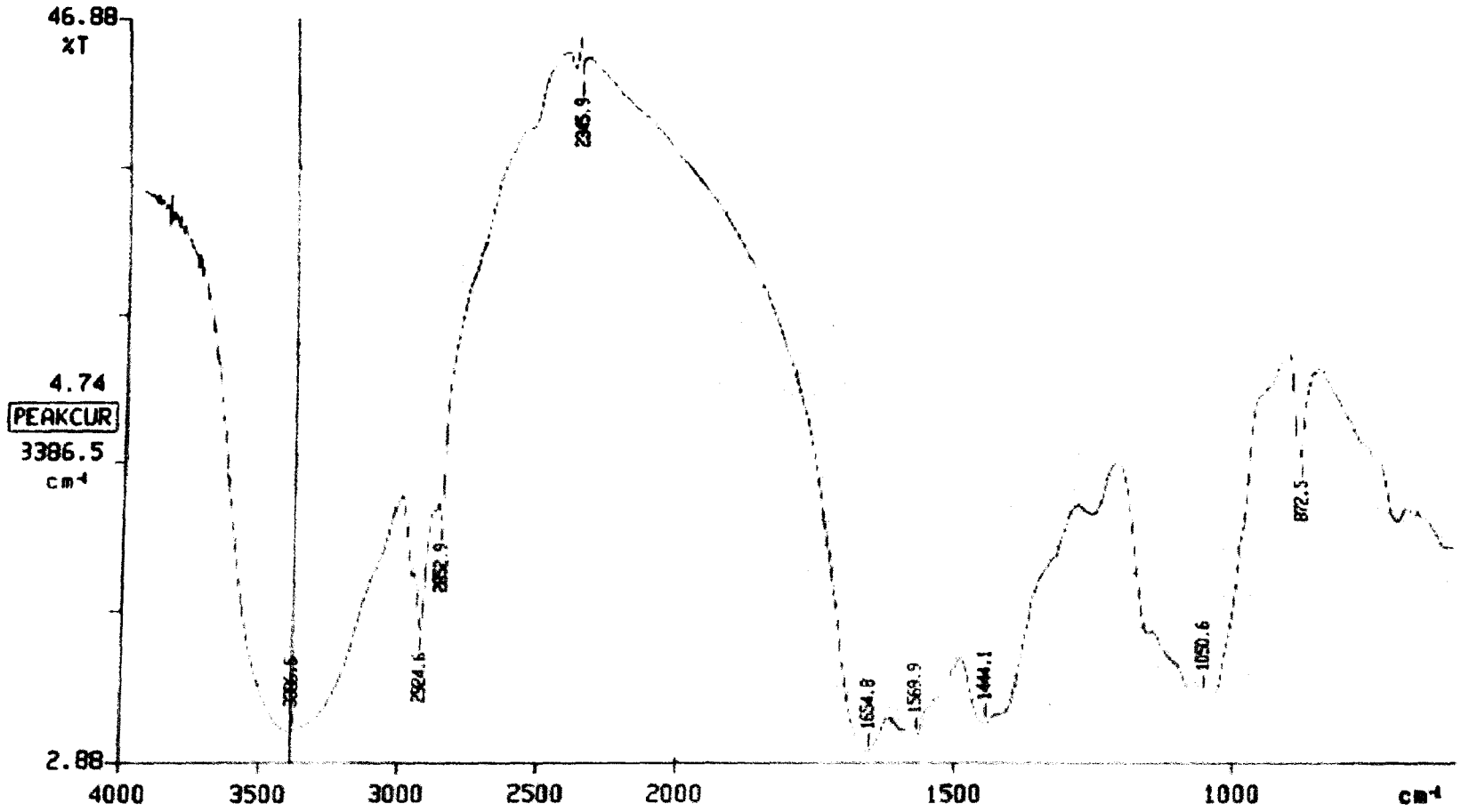
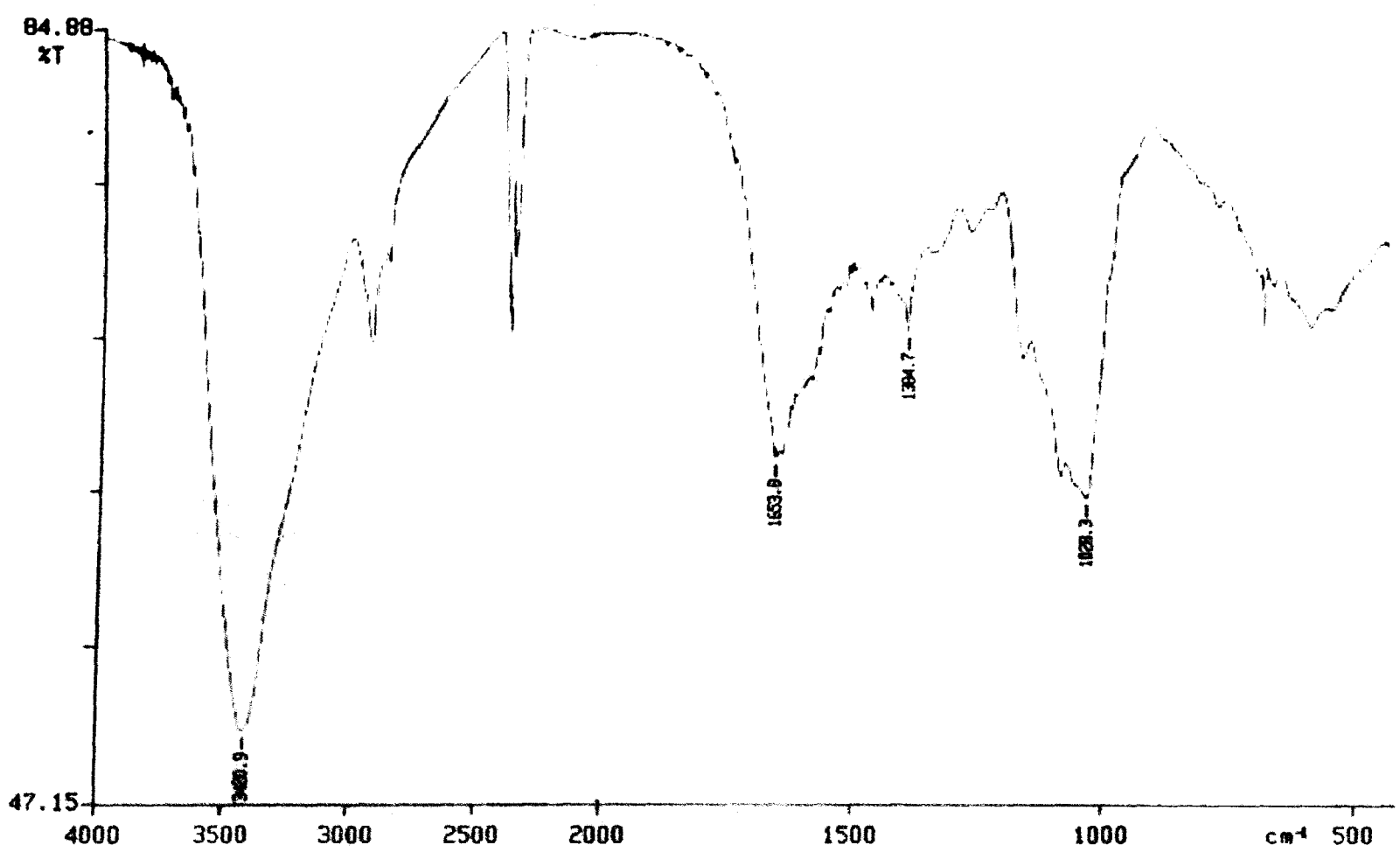
Appendix



Appendix 1: FT-IR of *Chlorella vulgaris* before treatment



06/04/06 09:47
X: 4 scans, 4.0cm-1

Appendix 2: *Chlorella vulgaris* reaggregate after solubilsation with 2 aminoethanol.