Viability of plant spore exine capsules for microencapsulation

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Sporopollenin exine capsules (SECs) (outer exoskeletal wall of the spores of *Lycopodium clavatum*) were extracted and examined for their potential use as microcapsules. They were shown, by laser scanning confocal microscopy (LSCM), to be void of their inner contents. The removal of nitrogenous and other internal materials was supported by a combination of elemental and gravimetric analyses. Two different methods were investigated to encapsulate substances into SECs which were (i) mild passive migration of materials into the SECs and (ii) subjecting SECs and materials to a vacuum. A range of fluorescent dyes with different polarities were seen using LSCM to encapsulate efficiently into the SECs (up to 1 $g.g^{-1}$). Relatively unstable materials with different polarities were encapsulated into the SECs: polyunsaturated oils, which are labile to oxidation, and the enzymes streptavidin-horseradish peroxidase (sHRP) and alkaline phosphatase (ALP). Irrespective of the encapsulation techniques employed no oxidation of the oils or denaturation of the enzymes was observed following their full recovery. This study gives the first indication of the viability of SECs to microencapsulate various potentially unstable materials without causing a detrimental effect.

Introduction

The outer layer (exine) of a pollen grain is made up of sporopollenin, an organic lipid-like polymer,[1] which is extremely resilient and makes such particles, arguably, Nature's most robust microcapsules.[2] Exines have evolved to be efficient naturally occurring microcapsules as they are required to protect the fragile genetic material contained by spores, sporoplasm, from conditions such as mechanical stress, intensive UV-light exposure and aerial oxidation. Sporopollenin's physical resistance is illustrated[3] by its being the only material to remain intact in exines found in ancient sedimentary rocks, which are at least 500 million years old. Microencapsulation is a technique now widely used in cosmetic, pharmaceutical and food industries to suit oral or topical drug delivery, protection against light or air, controlled release or taste masking.[4,5] Sporopollenin, as a material used to form microcapsules, has been claimed[6–8] to exhibit advantages over other substances commonly used to form microcapsules (*e.g.* gelatine, fatty acids, chitin, cellulose, starch and silica).[4,5]

It is known that there is a natural passage of materials through the exine in a living spore,[9] which is necessary to feed the sporoplasm with nutrients.[10,11] The most evident routes identified are openings in the spore walls, whose two types are (i) apertures, large portholes (*ca.* 1–2 μm in diameter),[12] and (ii) nano-sized channels, which are much narrower in diameter(15–20 nm for *L. clavatum*).[10]

Apart from being ecological, inexpensive and readily accessible, SECs resist very harsh physical, chemical and biological conditions[2,3] and, to some extent, absorb UV light.[13] A major question in respect of using SECs is the feasibility to encapsulate and eventually recover, without damage, substances that are relatively unstable to conditions such as denaturation or oxidation. Relatively little has been published[6,14–17] to illustrate the potential of SECs in microencapsulation and to date no exercise has been reported to demonstrate the integrity of materials following encapsulation and retrieval.

In this new study we have demonstrated that a selection of compounds with differing molecular mass and polarity, some of which are vulnerable to denaturation and oxidative degradation, are capable of being encapsulated into SECs and recovered from them without significant loss of integrity. Entry of compounds into the inner cavities of SECs was observed by LSCM (laser scanning confocal microscopy), making use of the natural fluorescence of sporopollenin[18,19] and fluorescent dyes. The outer surface of the filled SECs was also examined by light microscopy and scanning electron microscopy (SEM). Further support for encapsulation was obtained from combustion elemental analysis comparing empty and filled SECs.

Results and discussion

Extraction of SECs from *Lycopodium clavatum* spores

A variety of reagents and methods have been reported to remove the sporoplasm and other materials in and on the surface of spores to obtain SECs, as depicted in the case of *L. clavatum* in Fig. 1b and 1c. Some have involved relatively mild chemical or enzymatic treatments,[20–26] whereas others[27–36] have used aggressive and prolonged treatment with non-oxidative reagents, but in any case, intact SECs were reported. Some of these methods[33,37] are more economic and quicker to manufacture than the protocol described below; however, this method was chosen due

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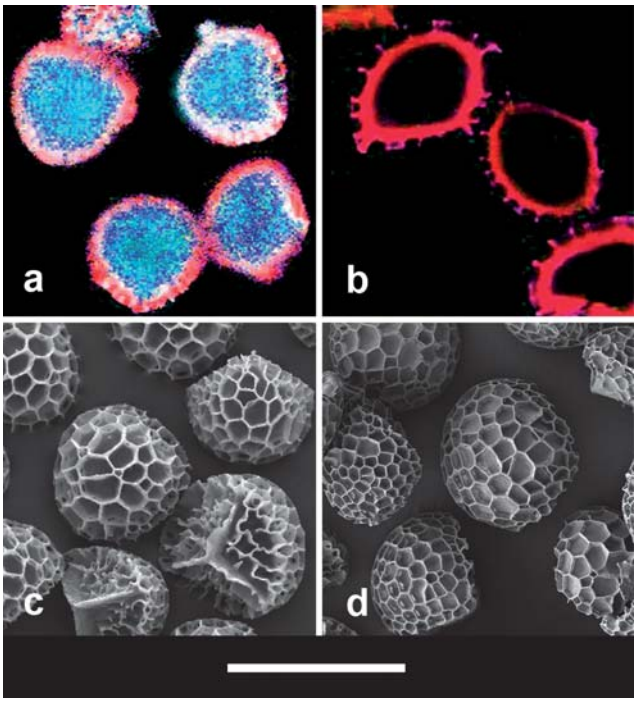

Fig. 1 LSCM images of a *L. clavatum* spore with sporoplasm and exine (a), and an empty SEC from a *L. clavatum* spore showing the exine (b). SEM images of a *L. clavatum* spore (c), and empty SEC from a *L. clavatum* spore showing a clean and intact surface (d). Scale bar = 30 μm.

to its convenient use in the laboratory and the utilisation of non-toxic and inexpensive reagents.

In this study the sporoplasm and the cellulose intine of *L. clavatum* were removed by a modified extraction protocol[31] involving successive treatments in hot acetone, potassium hydroxide and phosphoric acid accordingly. This gave an intact SEC (27 ± 2 μm in diameter), with an unbroken surface as verified by SEM (compare Fig. 1c and 1d). The inner cavity of the SEC was shown by LSCM (Fig. 1b) to be devoid of fluorescent material, in contrast to the parent spore (Fig. 1a). Also the SECs showed complete removal of nitrogenous material, at least by combustion elemental analysis (*e.g.* C, 65 ± 5; H, 7.7 ± 0.3; N, 0.0) along with a typical mass loss of 70%.

Sporopollenin as a porous material

The physical and chemical characteristics of the nano-sized channels in SECs are crucial to the viability of SECs in micro-encapsulation applications. Whilst a number of studies have been undertaken to view the nano-sized channels in different species of spores, few papers have described their physical and chemical properties. However, one exception is a detailed study by Bohne *et al.*[14] who compared the passage of different materials through the nano-sized channels of SECs extracted from the tripartite grain of *Pinus* pollen. Interestingly, they estimated the diameter of the largest channels of the external layers of sporopollenin to be around 200–300 nm, and showed that the internal layers constituted a physical barrier to non-electrolytes with a Stokes' radius >4 nm, and an electrostatic barrier to electrolytes with a Stokes' radius >1.3 nm. This study[14] indicated that restriction to the passage of products, especially macromolecules, into exines has two possible origins: (i) a physical constraint due to the size and form of the nano-sized channels, and (ii) a chemical limitation due to the presence of specific functional groups inside them. It is therefore not obvious from Bohne's work whether SECs from spores of different species can encapsulate materials with different polarities without causing damage to them in the process, especially those which are labile to oxidation or denaturation.

Since the structure of sporopollenin, from any plant species, has not been fully elucidated, the types of functional groups within the nano-sized channels is not known. However, those that have been found to be present in sporopollenin, as a whole, are: long aliphatic chains,[1,30] conjugated phenols,[1,24] hydroxyls,[1,30] ethers,[1,30] methyls,[1,30] and carboxylic acids.[30,31] More recently,[38] we demonstrated the accessibility of the carboxylic functional groups to reaction with ammonia to form ammonium salts and amides. However, the accessibility, proximity and orientation of the functional groups are not known on either the surfaces of the particles or within the submicron environments of the nano-sized channels. The location of such functional groups, as in enzymes, could contribute to modifying the integrity of a substance and size exclusion could be conjugated with electrostatic effects hydrophobic interactions and polarity affinities.

Encapsulation of dyes

Confirmation of materials with different polarities entering the inner cavity of SECs from *L. clavatum* spores is essential to a first proof of principle that they have general application in micro-encapsulation. An earlier study,[15] with the same derived SECs, simply used light microscopy to view the microencapsulation of aqueous inorganic solutions, but, in another study,[16] a clearer view of actual entry into the inner cavity of SECs from *L. clavatum* spores was provided by LSCM to observe micro-encapsulation of an aqueous solution of fluorescein amidite-labelled oligonucleotides.

To further explore and observe entry of materials into SECs from *L. clavatum* spores, dyes were selected systematically to explore a range of different polarities: malachite green, a cationic hydrosoluble dye; Evans blue, an anionic protein-staining dye; and Nile red, a neutral liposoluble dye. Each of them has a Stokes' radius ≤ 1.3 nm.[14] Dye solutions were made within ethanol (0.5g in 1 cm^3) and loaded, by vacuum, at a level of 1 $g.g^{-1}$, *i.e.* 2 cm^3 of dye solution in 1 g of SECs. The clear observation of each dye by LSCM (Fig. 2a, b, c and d) gave confidence of successful microencapsulation of neutral, anionic and cationic compounds. It also appeared that (i) little or no precipitated dyes remained on the surface of the SECs following their encapsulation, (ii) almost no empty SECs could be observed, and (iii) there was little debris shown surrounding the SECs.

It is interesting to note that the single chambered SECs obtained from *L. clavatum* appear to more accessible to such as Evan's blue, in contrast to the major chamber found in the tripartite sporopollenin microcapsules from pine pollen, thus indicating the possible importance of charge within the

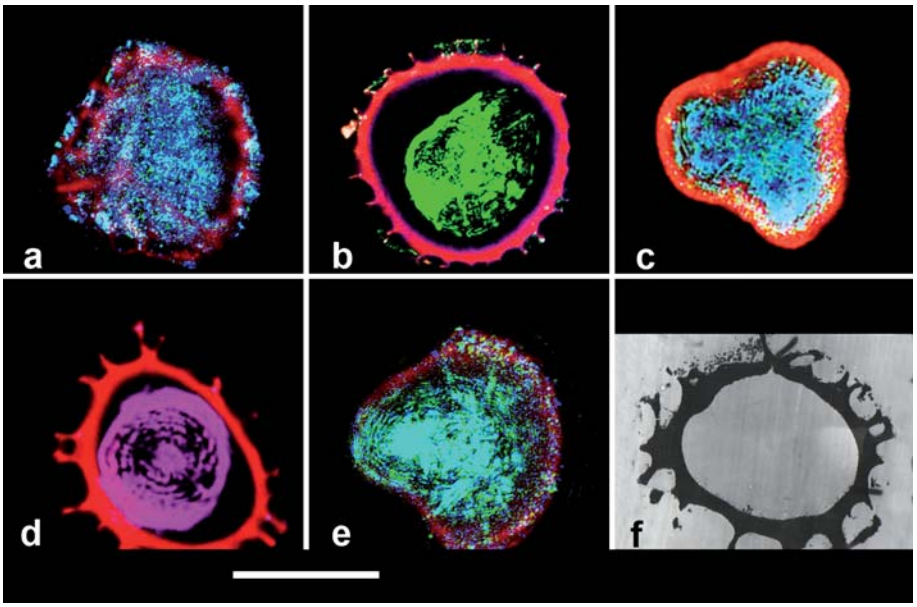

Fig. 2 LSCM images of fish oil containing lycopene (a) malachite green (b), Evans blue (c), Nile red (d) and Evans blue-stained α-amylase (e) in SECs. TEM image of a microtome section of an SEC embedded in acrylic resin (LR white) (f). Scale bar = 20 μm.

nano-channels penetrating the SECs.[14] It was concluded that the effective loading of each of the three dyes in SECs was 1.0 g.g^{-1}, with a rather uniform distribution of the product inside the different microcapsules and, most importantly, no dye visible on the surface of the SECs.

Encapsulation of LR resin

Unequivocal evidence of materials being encapsulated into SECs was demonstrated by the use of LR White, which is a polar, monomer, polyhydroxylated aromatic acrylic embedding resin system.[39] The dried SECs were embedded with the LR resin and shown by TEM of a microtomed section (Fig. 2f) to have fully filled the inner cavity of SECs. No empty SECs were observed on the mounted microtome sections. Microencapsulation of LR resin was a simple proof of feasibility incidentally observed when mounting microtomed sections of embedded SECs.

Encapsulation of oils and fats

A variety of oils of different viscosities and molecular weights were examined. Initial studies were performed with *Histoclear*® since it is a common histological solvent used in palynology. The *Histoclear*® could be observed, by light microscopy, to enter the SECs quickly. As *Histoclear*® entered the SECs air bubbles could be seen exiting them. This experiment gave a simple and convenient means to view complete filling of the SECs which take place through the uniformly perforated[10,11] SECs. Such perforations allow similar passive encapsulation as observed for cod liver oil at a loading of 1 g.g^{-1}. Loadings were attempted at various levels (Fig. 3), but a loading of 1 g.g^{-1} was selected for study as this formed a good powder (Fig. 3. Confocal images of the oil dyed with Nile red showed a similar image to that in Fig. 2c, indicating encapsulation had taken place; however, when the SECs were charged with larger amounts of oil (4 g.g^{-1}), it was evident that there were initial signs of overloading of the SECs, by their becoming sticky. Distinct overloading of the SECs at 6 g.g^{-1} was observed by the mixture turning into a paste-like substance (Fig. 3c). A similar pattern of loadings was seen for the *Histoclear*® and cocoa butter. Cod liver oil, chosen since it is relatively easily oxidised, was used to compare its stability to oxidation following encapsulation and removal from the SECs. Encapsulation of melted cocoa butter at 50 °C was achieved but, at a loading of 1 g.g^{-1}, residues of the fat remained on the surface of the SECs as viewed by light microscopy. It was found that a cleaner surface could be achieved by applying a vacuum (25 hPa).

Encapsulation of proteins

Microencapsulation of proteins has potential applications, such as in drug delivery.[4] Therefore, sHRP and ALP, two well-known enzymes, were chosen for this study since their activities can be readily assayed. Successful loading of SECs was observed with four different enzymes; α-amylase (56 kDa),[40] β-galactosidase (540 kDa),[41] sHRP (100 kDa)[42] and ALP (89 kDa).[43] The α-amylase was stained with Evans blue and encapsulated in SECs in order to be observed by LSCM (see Fig. 2e). In addition, SEM and light microscopy showed no amorphous or crystalline deposits either surrounding the SECs or on their outer surfaces.

Saturated ethanolic solutions of α-amylase were encapsulated using vacuum filling. Similar solutions of β-galactosidase were also loaded into SECs using the same method. The solvent was removed by vacuum drying. sHRP was loaded into the SECs as an aqueous buffered solution, with application of vacuum as a forcing method, and subsequently lyophilised. ALP was loaded by centrifugation of a mixture of SECs with the commercial solution of ALP for 10 min under 10,000 × *g*. No amorphous or crystalline deposits either surrounding the SECs or on their outer surfaces could be viewed by SEM, showing clean exines comparable to Fig. 1d.

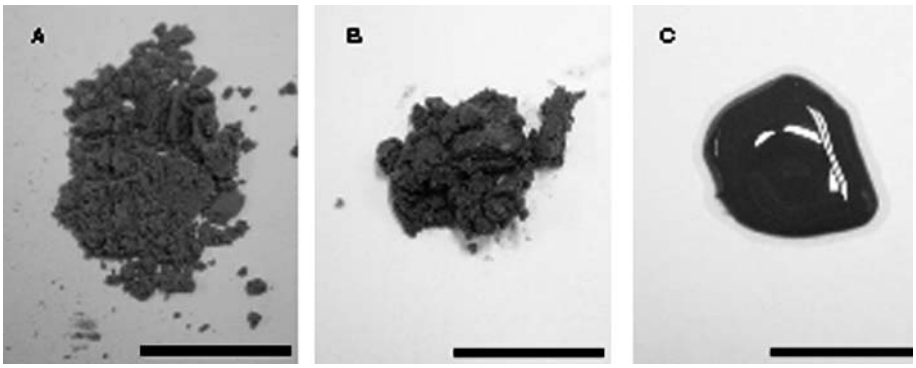

Fig. 3 Images of encapsulated cod liver oil at loadings of 1 $g.g^{-1}$ (A), 4 $g.g^{-1}$ (B) and 6 $g.g^{-1}$ (C). Scale bar = 20 mm.

Encapsulation loadings of amylase and galactosidase were 0.2 $g.g^{-1}$, whether using the compressive method or the application of vacuum. The loadings of ALP and sHRP were not evaluated since mass concentration was not known in the commercial solution, only activity concentration.

Recovery of encapsulated products from SECs

Earlier studies of encapsulation of materials into SECs have not reported evaluations of damage to such materials following their recovery. Also, if SECs can have a general application in microencapsulation, it is vital that materials can be encapsulated and retrieved without damage. Therefore in this study it was decided to examine well-known materials where microencapsulation might be beneficial due to propensity to oxidation and denaturation.

Recovery of cod liver oil. Cod liver oil has important health benefits.[44] Being rich in polyunsaturated fatty acids, it is an example of an easily oxidisable lipid and could therefore benefit from microencapsulation.[5] The state of oxidative degradation of cod liver oil, in this study, was assessed by peroxide value (PV) titration,[45,46] which is a technique widely used in industry to verify the freshness (low PV) or the rancidity (high PV; $\geq$ 20 $meq.kg^{-1}$) of fats and oils.

Cod liver oil, encapsulated by vacuum filling to a level of 1 $g.g^{-1}$, was recovered by extraction into chloroform and the SECs were removed by filtration. Chloroform gave a reproducibly high yield (96–99%) of oil removal, as verified by weight differences of dried SECs on six different samples. The PV of the oil before encapsulation (14.9 ± 1.8 $meq.kg^{-1}$), as determined on four different samples, was not significantly different from that of the oil after recovery; 14.4 ± 2.2 $meq.kg^{-1}$, when the oil had been encapsulated and then recovered. This showed that microencapsulation was not deleterious to the cod liver oil.

Recovery of proteins. Microencapsulation of various macromolecules, such as proteins, can have many advantages for application to drug delivery[4,46] and immobilisation of enzymes. Since the nature of the SEC surface and nano-channels is not known it was important to establish if such materials could be retrieved after encapsulation without loss of activity due to denaturation or adhesion to the SECs. The enzymes ALP and sHRP were chosen as candidates for this study since they are well known and their denaturation can be assessed on the basis of their activities. Solutions of each of the enzymes were encapsulated into SECs by centrifugation and application of a vacuum, respectively. They were recovered by extraction with an aqueous buffer. The amount of denaturation was verified by comparison of the activity of both enzymes before and after microencapsulation.

The activity of sHRP was assayed using 3.3′,5.5′-tetramethylbenzidine (TMB)[47]on three occasions during the procedure of microencapsulation and recovery of sHRP. Original enzyme activity of the commercial solution was considered as the 100% reference. After the first lyophilisation and subsequent reconstitution, 76–79% of the original activity was found remaining (n = 6). After the enzymes were again lyophilised and reconstituted, with the added step of encapsulation, 49% of the original activity was assayed. The first lyophilisation led to a 21–24% decrease in the enzyme activity. We can assume the same 21–24% loss for the second lyophilisation, therefore, it can be estimated that the lyophilisations caused a 42–48% decrease in enzyme activity, leaving a 3–9% loss that can be attributed to the actual step of microencapsulation under vacuum and subsequent recovery in PBS. In summary, the overall process of encapsulation, lyophilisation and recovery was found to reduce the sHRP activity to 49% of the original activity; however, most of this loss can be attributed to the lyophilisation processes.

A second enzyme study, using ALP, was performed but without a lyophilisation step, which had already been proven to be deleterious. ALP activity was measured by its catalysing the dephosphorylation of DDAO (9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one)-phosphate) as a substrate in alkaline-buffered conditions. The rate of formation of DDAO, the product of dephosphorylation of DDAO-phosphate, was studied by UV spectrophotometry. Enzyme activity was determined, at 37 °C, for (i) fresh ALP, (ii) ALP after it had been encapsulated within SECs and (iii) ALP that was subsequently recovered, in a three-step extraction, into PBS. The loss of ALP activity due to encapsulation and recovery was 1–3%, which indicates an almost quantitative process with minimal denaturation of the enzyme.

Experimental

General experimental procedures

Optical micrographic observations were performed on an upright LMDB Leica Microscope from Leica, Germany.

Confocal images were obtained using a Bio-Rad Radiance 2100 laser scanning confocal microscope (LSCM) equipped with Ar (488 nm), Green HeNe (563 nm) and Red diode (637 nm) laser lines and connected to a Nikon TE-2000E inverted microscope from Nikon, Japan. Images were collected using Lasersharp2000 under the following conditions; laser excitation lines Ar (488nm) 15%, Red diode (637) 38%. Fluorescence from samples passed through 560 and 650nm dichroic filters and was collected in photomultiplier tubes (PMT) equipped with the following emission filters; 515/30, 590/70 and 600 long pass. The laser scan speed was set at 166 lines per sec, and the viewable area was between 20 and 200μm^2 when using a 60× oil objective. The iris was auto-set as optimal for conditions used. Gain was adjusted for optimal signal/noise ratio.

Materials

Lycopodium clavatum L. (common club moss) spores (27 μm type) were purchased from Unikem (Copenhagen, Denmark) or Tibrewala International (Kathmandu, Nepal). Cocoa butter was a gift from Nestlé Ltd. (York, UK). Cod liver oil was purchased from Seven Seas Ltd. (Hull, UK), (+)-limonene (*Histo-Clear II*®) from National Diagnostics Inc. (Atlanta, USA), LR White from London Resin Co. (London, UK), streptavidin-horseradish peroxidase (sHRP) and alkaline phosphatase (ALP) from Bio-Rad (Hercules, USA), 9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl)phosphate, diammonium salt (DDAO-phosphate) from Invitrogen (Carlsbad, USA), 3,3′,5,5′-tetramethylbenzidine from Vector Laboratories (Peterborough, UK), and α-amylase and β-galactosidase from Sigma-Aldrich (Poole, UK).

Chemicals used in peroxide value and ALP activity measurements were AR grade, other chemicals were GPR grade, and all were purchased from Sigma-Aldrich, BDH, or Fisher. The distilled water, taken from an Elgastat system, Vivendi (Buckinghamshire, UK), was of resistivity greater than 18 MΩ.cm^{-1}.

Solution preparation. Solutions were prepared using the following products.

Dyes: Evans blue, malachite green and Nile red respectively (0.5g), in 4/1 water/ethanol (v/v) (1 cm^3);

Enzymes: α-amylase (1,4-α-d-glucanglucanohydrolase; EC 3.2.1.1; from *Aspergillus oryzae*; 54 kDa; 0.1g) and β-d-galactosidase (β-d-galactosidegalactohydrolase; EC 3.2.1.23; from *Aspergillus oryzae*; 116 kDa; 0.1g) in water (2 cm^3), respectively. A commercial solution of sHRP in glycerol (40 mm^3) was lyophilised and reconstituted to the original concentration with PBS (40 mm^3).

Preparation of sporopollenin exine capsules (SECs)

Raw *L. clavatum* spores (loose powder, 250 g) were suspended in acetone (750 cm^3) and stirred under reflux for 4 h. The defatted spores were filtered and dried overnight in open air. They were suspended in 6% (w/v) potassium hydroxide aqueous solution (750 cm^3) and stirred under reflux for 6 h. After filtration, this operation was repeated with a new 6% (w/v) potassium hydroxide solution (750 cm^3). The suspension was filtered and washed with hot water (3 × 300 cm^3) and hot ethanol (2 × 300 cm^3) and dried overnight in open air. It was suspended in 85% (v/v) ortho-phosphoric acid (750 cm^3) and stirred under reflux for 7 days. The solid was filtered, washed with water (5 × 250 cm^3), acetone (250 cm^3), 2M hydrochloric acid (250 cm^3), 2M sodium hydroxide (250 cm^3), water (5 × 250 cm^3), acetone (250 cm^3) and ethanol (250 cm^3) and dried in an oven at 60 °C. The SECs (75 g) obtained by the foregoing protocol were virtually nitrogen-free and almost devoid of cellulose (main component of intine). Their combustion elemental analysis was typically C, 68.9; H, 7.9; N, 0.0, as determined on a Fisons instrument Carlo Erba EA 100 C H N analyser.

Encapsulation of products

Passive encapsulation. *Histoclear*® (1 cm^3), cod liver oil (0.5 g) and molten cocoa butter (0.5 g) were added to loose SEC powder (0.5 g) at room temperature, except for cocoa butter which was melted at 50 °C prior to addition. The resulting mixture was homogenised by hand-stirring for a few seconds and immediately observed by light microscopy (Table 1).

Table 1 Encapsulation loadings of various products in SECs, as reached in the present study. Dyes used: Nile red, Evans blue, malachite green; enzymes uses: amylase and galactosidase

	Encapsulation loading (by method)	
Product	Passive	Vacuum
Histoclear®	N/A	N/A
Cod liver oil	5 g.g^{-1}	1 g.g^{-1}
Melted cocoa butter	5 g.g^{-1}	1 g.g^{-1}
Dyes	N/A	1 g.g^{-1}
Enzymes	N/A	0.2 g.g^{-1}
Ascorbic acid	N/A	0.5 g.g^{-1}

Encapsulation by vacuum

General process of vacuum encapsulation. The liquid or solution to encapsulate was mixed with loose SECs, gently stirred by hand for a few seconds and subjected to a vacuum (25 hPa) for 1 h. The products encapsulated were: molten cocoa butter (1 g.g^{-1}), cod liver oil (1 g.g^{-1}), dye solutions (2 cm^3.g^{-1}), sHRP solution in PBS (1 cm^3.g^{-1}) and amylase and galactosidase solutions (4 cm^3.g^{-1}). Vacuum encapsulation was carried out at room temperature. However, prior to addition to SECs, cocoa butter was melted at 50 °C.

SECs loaded with dye, amylase, galactosidase and sHRP solutions were freeze-dried after the application of the vacuum (Table 1).

Vacuum encapsulation of LR White resin. SECs were treated with 2.5% glutaraldehyde using 0.1% cacodylale buffer, post fixed in 1% osmium tetroxide. Dehydration was undertaken stepwise over 5 days, starting with water/ethanol 7/3 (v/v) and finishing with absolute ethanol. The SECs were embedded in an acrylic resin (LR White) under vacuum, polymerized at 80 °C for 8days and finally stained in toluidine blue. Embedded particles were sectioned using a microtome.

Encapsulation by centrifugation. A commercial solution of ALP in glycerol (2 mm^3) was mixed with SECs (0.01 g) and the resulting mixture was subjected to centrifugation (10,000 × *g*) at 4 °C.

Recovery of the products from SECs

Cod liver oil-loaded SECs (1 $g.g^{-1}$, 2 g) were suspended in chloroform (10 cm^3) and stirred at room temperature for 1 h in a stoppered flask. The oil solution was recovered by vacuum filtration on a 16–40 μm-mesh glass sinter and used for PV titration, considering the chloroform previously added as part of the titration protocol.

SECs filled with ALP (0.01 mg) were suspended in PBS (1 cm^3). The suspension was shaken for 30 s and filtered through a 0.4 μm-pored syringe-tip filter for evaluation of the enzyme activity of the filtrate. Particles and filter were flushed again several times with phosphate buffered saline (PBS) (1 cm^3) and enzyme activity of each filtrate was determined.

sHRP was studied using the following protocol: lyophilisation of the commercial solution of sHRP and reconstitution to the same concentration with PBS solution; mixture of the reconstituted solution of sHRP in PBS with SECs and application of a vacuum; lyophilisation of the resulting product; and suspension of the freeze-dried product in PBS, reconstituting the original volume. sHRP encapsulated in SECs (38 mg) was suspended in PBS (1 cm^3) and assayed for enzyme activity. SECs were removed by filtration through a 0.4 μm-pored syringe-tip filter and enzyme activity was evaluated in the filtrate.

Enzyme activity measurement

The activities of ALP and sHRP were assessed using DDAO-phosphate and 3,3′,5,5′-tetramethylbenzidine as respective substrates.

Peroxide value measurement[45,46]

Acetic acid (15 cm^3) was added to a solution of fat (1 g) in chloroform (10 cm^3), followed by saturated aqueous potassium iodide (1 cm^3). The mixture was shaken for 1 min and set aside away from light for exactly 5 min at room temperature. The reaction was quenched by dilution with water (75 cm^3). The liberated iodine was titrated against a 0.01 M standardised sodium thiosulfate solution (volume *V*), using a starch indicator. A blank test was carried out simultaneously where the volume V_0 of titrating solution did not exceed 0.05 cm^3. PV, expressed in milliequivalents of active oxygen per mass unit of fat ($mmeq.kg^{-1}$), was given by the formula:

$$PV = \frac{V - V_0 \times T}{m} \times 1000$$

V and V_0 are the volumes of titrating solution (cm^3) for the sample and for the blank respectively, T the concentration of titrating solution ($mol.dm^{-3}$) and m the mass of fat in the test portion (g).

Conclusion

SECs extracted from *L. clavatum* are able to microencapsulate and subsequently permit relatively unstable materials including enzymes (ALP and sHRP) and cod liver oil to be recovered without significant deleterious effect to them. No barriers were also found to three dyes with differing polarities. It would appear that the chemical and physical features of the nano-sized channels present in SECs extracted from *L. clavatum* are able to permit passage of a variety of polar and non-polar materials with sizes up to a Stokes' radius of 4 nm and probably up to 20–30nm, in keeping with the dimensions reported in the literature.[10] Although all products could be encapsulated using passive filling over a short period, on occasion this produced a product that was found to show debris on the outer surface. Vacuum filling, which would possibly incur more turbulence through a nano-channel and hence be more deleterious to enzymes, was found not to damage the products being encapsulated and, after just 1 h, gave loaded SECs with little or no evidence of debris. We would therefore recommend filling by vacuum. Almost full recovery of encapsulated oil or enzymes was possible by suspension and/or mixing within a solvent. The lipids suffered little oxidation and horseradish peroxidase and alkaline phosphatase were not significantly denatured.

These preliminary results give an indication that SECs, from *L. clavatum* spores, and perhaps other plant spores, can form useful templates or shells for microencapsulating a variety of substances and in particular where there is a need for uniform particle size to be employed, such as in food texture.

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