Sporopollenin micro-reactors for *in-situ* preparation, encapsulation and targeted delivery of active components

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We report a simple and robust technique for loading of sporopollenin microcapsules from *Lycopodium clavatum* with a range of inorganic and organic nanomaterials based on *in-situ* preparation of nanoparticles by a chemical reaction in the sporopollenin interior.

The outer layer (exine) of the pollen grains of mosses and ferns is composed largely of a substance known as sporopollenin[1] which has been described as "one of the most extraordinary resistant materials known in the organic world".[2] Brooks and Shaw[3] stated "sporopollenins are probably the most resistant organic materials of direct biological origin found in nature and in geological samples" as intact spores are found in ancient sedimentary rocks, which are at least 500 million years old.[1] Sporopollenin has been readily functionalised by previous workers for applications in ion exchange and solid phase peptide synthesis.[4,5] Recently, it was recognised that sporopollenin can be used to develop novel drug and nutraceutical delivery systems.[6] Sporopollenin microcapsules have the potential for oral delivery of food supplements and drugs that are usually delivered by injection or protect an active ingredient so that it can be delivered to the lower gut. The oral delivery is based upon the fact that certain plant pollens or other spores are capable of crossing the gut wall, largely intact, but are then destroyed within the blood stream, thereby releasing its contents. *Lycopodium clavatum* is a common club moss (running ground pine) that can be found in many woodland areas and rocky slopes in Europe, Central and South America, Asia and Africa. Mackenzie *et al.*[6] have developed a range of applications of sporopollenin derived from *Lycopodium clavatum* based on filling of the exine with fat (Omega 3), proteins, vitamins and enzymes. In the case of oils, a large proportion can be encapsulated but in some cases of low soluble compounds, lower loadings can be observed. The methods used to load the sporopollenin with these ingredients are based on physical adsorption on the surface of the sporopollenin and/or penetration of the active component into its interior by diffusion through the microchannels of the exine membrane[7–9] that can be can be up to 40 nm in diameter.

Here we report a simple and robust method for loading sporopollenin of *Lycopodium clavatum* with inorganic or organic nanoparticles synthesised *in situ*. The idea of this method is to use the sporopollenin microcapsules as chemical micro-reactors where a chemical reaction is used to generate a large amount of the product (*e.g.* of low solubility) inside the sporopollenin shells. Our approach allows the sporopollenin capsules to be loaded with inorganic or organic materials produced as a result of a *chemical reaction conducted selectively in the capsule interior*. As illustrated in Fig. 1, the proposed method includes the following 3 steps: (i) loading the capsules with a solution of the reagent A (or a mixture of soluble reagents); (ii) filtering and washing the capsules on the filter with a pure solvent to remove the excess of reagent A and redispersion in a solution of reagent B to conduct a chemical reaction, A + B → C, where C is a product of low solubility; (iii) filtering and washing the 'filled' sporopollenin with a pure solvent to remove the excess of reagent B, followed by redispersion

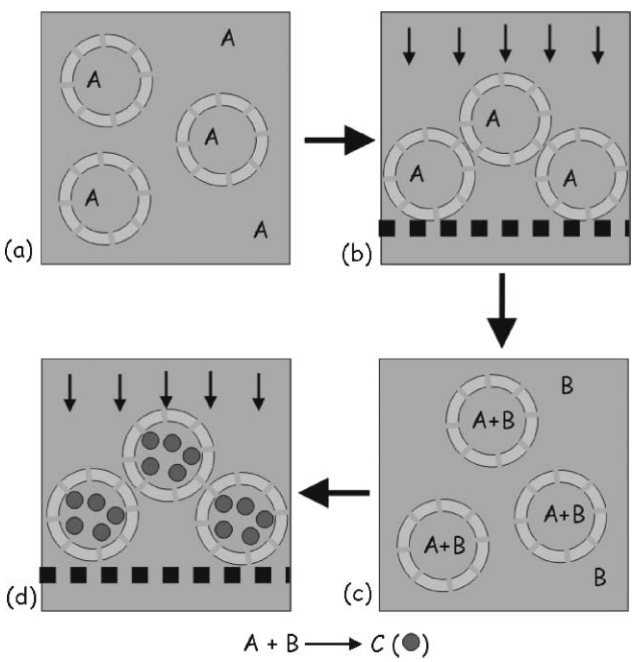

Fig. 1 Schematic representation of our method for loading sporopollenin microcapsules with nanoparticles as a result of a chemical reaction. (a) A compressed tablet of sporopollenin is dispersed in a solution of reagent A to load the capsules with this solution. (b) The sporopollenin capsules loaded with solution A are filtered and quickly washed with pure solvent to remove the excess of reagent A in the continuous phase followed by their redispersion in a solution of reagent B. (c) The chemical reaction A + B → C gives a product C of low solubility which results in the formation of nanoparticles growing inside the sporopollenin. (d) The loaded capsules are filtered and washed on the filter with a pure solvent to remove the excess of reagent B, followed by redispersion in water.

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in water. In the case of a chemical reaction producing nanoparticles of average diameter larger than the sporopollenin pore size, the particles remain trapped in the capsules but the solvent can still access them through the sporopollenin pores. Here we present three examples of the use of this method for:

(1) Fabrication of magnetic sporopollenin capsules by *in situ* synthesis of magnetite nanoparticles (Fe_3O_4). The prepared capsules can be used for directed drug delivery by using an external magnetic field. We suggest a simple procedure to show how the magnetic sporopollenin can be loaded with an additional component (*e.g.* drug, vaccine, food supplement, *etc.*).

(2) Loading of sporopollenin (*Lycopodium clavatum*) with calcium hydrogen phosphate ($CaHPO_4$). The microcapsules prepared by this way can find application in the area of fortified foods, providing slower release and improved bioavailability of calcium and other mineral food supplements.

(3) Loading of sporopollenin (*Lycopodium clavatum*) with organic nanoparticles produced by precipitation of hydrosoluble cationic and anionic dyes. This technique allows sporopollenin microcapsules to be loaded with water-soluble organic drugs.

Details of the experimental procedure for these examples are given below. Tablets of 0.5 g *Lycopodium clavatum* sporopollenin were prepared by compressing the dry sporopollenin powder (from Polysciences, Inc.) in a mechanical press.[6] Iron(III) chloride ($FeCl_3$, anhydrous, min. 97%) was purchased from Fisher Chemicals. Iron(II) chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$, min. 98%) ammonia (33 wt%) calcium chloride (anhydrous, min. 90%) and disodium hydrogen orthophosphate (anhydrous, min. 99%) were obtained from BDH Chemicals. Ethanol (96%) and hydrochloric acid (5 M) were supplied from Sigma. Methylene blue was supplied by BDH Chemical and fluorescein sodium salt was purchased from Sigma. All solutions were prepared by using milliQ (deionised) water.

1) Preparation of "magnetic sporopollenin". The composition of solution A (see Fig. 1) was as follows: 8.11 g $FeCl_3$, 19.88 g $FeCl_2{\cdot}4H_2O$, 5 mL 5 M HCl, 40 mL milliQ water and 5 mL ethanol. These were mixed in a 100 mL flask followed by heating to 40 °C until complete dissolution of the salts. A tablet of 0.5 g compressed sporopollenin was redispersed in 15 mL of this solution and stirred for 2 hours at room temperature. The sporopollenin suspension was filtered and quickly washed with milliQ water on the filter, followed by an immediate transfer into 1 M ammonia solution in milliQ water (solution B). After 1 hour the "magnetic" sporopollenin (with cores of magnetite nanoparticles) was filtered again and washed thoroughly with milliQ water, followed by redispersion in water. A similar procedure can also be followed with sporopollenin powder (instead of a tablet). To accelerate the filling of the capsules with solution A, the suspension was kept at 250 mmHg vacuum for 15 min followed by stirring for 2 hours at room temperature.

2) Loading of sporopollenin microcapsules with calcium phosphate. The composition of solution A (see Fig. 1) was as follows: 11.10 g $CaCl_2$, 45 mL milliQ water and 5 mL ethanol. These were mixed in a 100 mL flask followed by heating to 60 °C until complete dissolution of the calcium chloride. Solution B was produced from 14.00 g Na_2HPO_4 in 100 mL milliQ water. A tablet of 0.5 g compressed sporopollenin was redispersed in 15 mL of solution A and stirred for 2 hours at room temperature. The suspension of sporopollenin was filtered and quickly washed with milliQ water on the filter, followed by an immediate transfer into the solution B (disodium hydrogen phosphate in milliQ water). After 2 hours the sporopollenin microcapsules were filtered again and washed thoroughly and redispersed with milliQ water.

3) Loading of sporopollenin microcapsules with organic salts of limited (low) solubility. In this case, the composition of solution A was as follows: 0.25 g methylene blue, 50 mL milliQ water and 5 mL ethanol. Solution B was produced from 0.36 g fluorescein sodium salt. A tablet of 0.5 g compressed sporopollenin was redispersed in 15 mL of solution A and stirred for 2 hours at room temperature. The suspension of sporopollenin was filtered and quickly washed with milliQ water on the filter, followed by an immediate transfer of the filtered capsules into the solution of fluorescein sodium salt. After 2 hours the sporopollenin was filtered again and washed thoroughly with milliQ water, followed by redispersion in water.

We demonstrated the viability of our technique by loading sporopollenin derived from *Lycopodium clavatum* spores (Fig. 2A) with magnetite nanoparticles. The reaction in this case involves precipitation of a stoichiometric mixture of $FeCl_2$ and $FeCl_3$ with ammonia solution. Fig. 2B shows an optical image of the obtained magnetic sporopollenin capsules containing magnetite nanoparticles, some of which have aggregated in larger clusters, visible in transmitted light in the capsule interiors. The SEM images of cracked-open sporopollenin capsules loaded with magnetite reveal that the magnetite nanoparticles are predominantly localised on the inside of the capsule (Fig. 2C). The magnetite nanoparticles are organised in a thick layer as can be seen from the high magnification SEM image of the internal wall of the sporopollenin capsule (Fig. 2D). To prove that the sporopollenin capsules really contain a high load of magnetite particles we tested their response in an external magnetic field, created by a permanent magnet. Fig. 2E–F illustrate the behaviour of the magnetic sporopollenin capsules (settled due to gravity) when brought into contact with the magnet. Note that the sporopollenin capsules redisperse and are attracted to the magnet which proves that they have a significant load of aggregated magnetic nanoparticles. A similar result was obtained by initial redispersion of the magnetic sporopollenin in the aqueous phase followed by exposure to the external magnetic field (Fig. 2G–H). The result clearly shows that the dispersion of magnetic sporopollenin behaves like a ferrofluid (if the volume fraction of magnetic sporopollenin is high enough). Such magnetic capsules can find application for targeted drug delivery aided by using an external magnetic field. We anticipate that the capsules can be additionally loaded with other materials (the magnetite will remain in the capsules). This can be accomplished by drying the magnetic sporopollenin followed by its compression into a tablet and its redispersion in the solution of the active component which is to be loaded into the magnetic sporopollenin. This would ensure that both the magnetite and the other active component (*e.g.* drug, vaccine, food supplement, *etc.*) are loaded in the capsules. To check whether the proposed loading technique works with other materials, we also loaded the sporopollenin from *Lycopodium clavatum* with calcium hydrogen phosphate which was produced as a result of the following ionic reaction: $Ca^{2+} + HPO_4^{2-} \rightarrow CaHPO_4$. Fig. 3A shows an image of the produced sporopollenin filled with precipitated $CaHPO_4$. As seen in the optical image, aggregates of $CaHPO_4$ particles are visible inside the sporopollenin microcapsules. Finally, we performed an additional experiment to demonstrate that the same

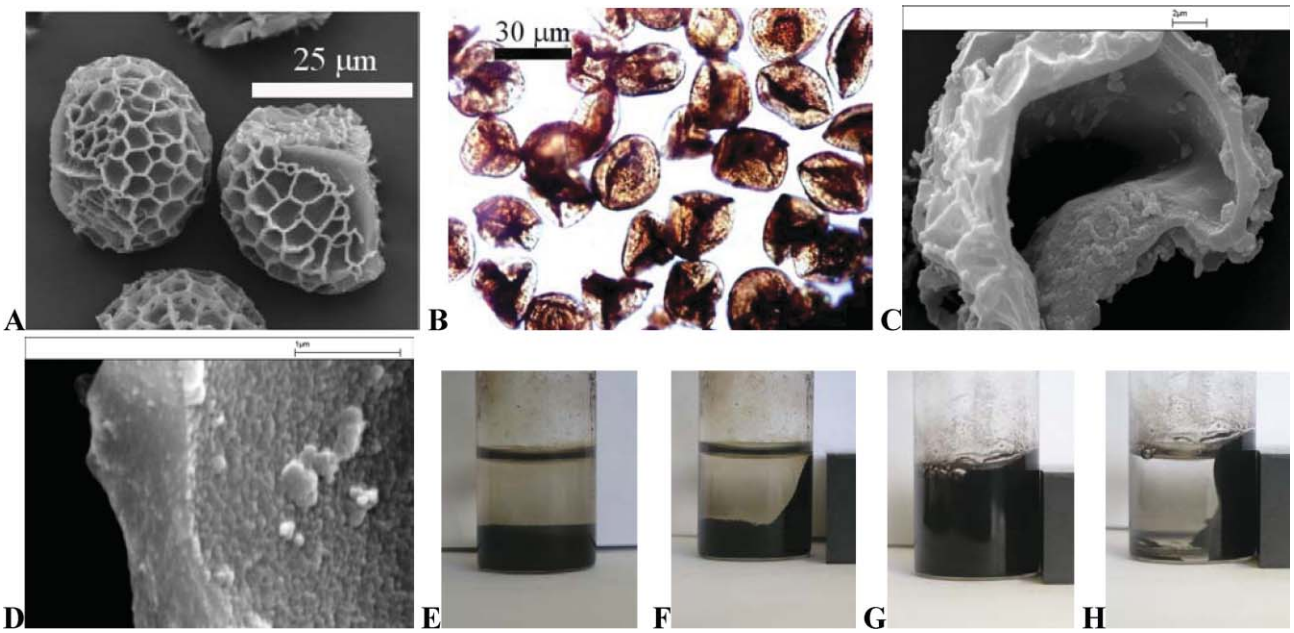

Fig. 2 (A) SEM image of empty sporopollenin exines extracted from *Lycopodium clavatum*. The average diameter of the sporopollenin capsules is 28 μm. (B) An optical microscope image of the same sporopollenin loaded with magnetite (Fe_3O_4) nanoparticles. (C) and (D) SEM images of cracked-open sporopollenin capsules indicate a deposit of magnetite nanoparticles on the inner wall of the sporopollenin capsules. (E) The suspension of the magnetic sporopollenin has settled due to gravity. (F) A permanent magnet positioned at the right-hand side of the tube attracts the magnetic sporopollenin. (G) A suspension of magnetic sporopollenin redispersed in water. (H) A permanent magnet settles the microcapsules from the dispersion within a few seconds.

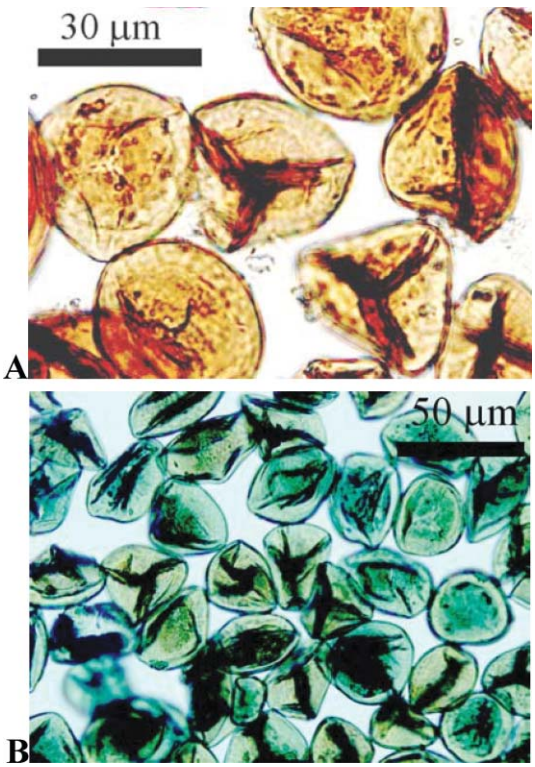

Fig. 3 Optical microscope images of sporopollenin (*Lycopodium clavatum*) loaded with precipitated salts: (A) calcium hydrogen phosphate ($CaHPO_4$) and (B) a complex of methylene blue and fluorescein (green colour, low solubility in water).

technique works not only with inorganic nanoparticles, but also with organic nanoparticles. For this purpose we produced sporopollenin capsules loaded with particles obtained as a result of the ionic reaction of methylene blue (cationic dye) with fluorescein sodium salt (anionic dye). The product is a complex of low solubility and green colour which can be clearly seen in the optical microscope image in Fig. 3B. These results suggest another possible use of sporopollenin microcapsules for slow release of drugs by following a similar strategy. A number of water soluble drugs have ionic character which allows their precipitation with a hydrophobic counter-ion inside the sporopollenin capsules where they would form hydrophobic nanocrystals of low solubility that would dissolve slowly from the capsules to the continuous phase thus supporting a constant concentration of the active component (*e.g.* drugs, insulin, vaccine, minerals, *etc.*). Such a study is under way.

In summary, we have developed a simple method for loading of sporopollenin microcapsules with nanoparticles and insoluble salts by using a chemical reaction or a precipitation process that generates the encapsulated compounds inside the sporopollenin shell. We demonstrate the method by producing magnetic sporopollenin (loaded with magnetite nanoparticles), and sporopollenin filled with calcium phosphate and organic salts of low solubility.

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