Lycopodium Spores: A Naturally Manufactured, Superrobust Biomaterial for Drug Delivery

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Herein, the exploration of natural plant-based "spores" for the encapsulation of macromolecules as a drug delivery platform is reported. Benefits of encapsulation with natural "spores" include highly uniform size distribution and materials encapsulation by relatively economical and simple versatile methods. The natural spores possess unique micromeritic properties and an inner cavity for significant macromolecule loading with retention of therapeutic spore constituents. In addition, these natural spores can be used as advanced materials to encapsulate a wide variety of pharmaceutical drugs, chemicals, cosmetics, and food supplements. Here, for the first time a strategy to utilize natural spores as advanced materials is developed to encapsulate macromolecules by three different microencapsulation techniques including passive, compression, and vacuum loading. The natural spore formulations developed by these techniques are extensively characterized with respect to size uniformity, shape, encapsulation efficiency, and localization of macromolecules in the spores. In vitro release profiles of developed spore formulations in simulated gastric and intestinal fluids have also been studied, and alginate coatings to tune the release profile using vacuum-loaded spores have been explored. These results provide the basis for further exploration into the encapsulation of a wide range of therapeutic molecules in natural plant spores.

1. Introduction

Plant-based spores represent one form of natural encapsulation, and a wide range of specific plant species which produce spores are commonly found in nature.[1,2] Such natural packaging means are effective in protecting sensitive biological materials from environmental extremes in the form of prolonged desiccation, UV exposure, and predatory organisms.[3] A range of plants produce spores as a form of seed, which contains all the genetic material necessary to produce a new plant.[4,5] Such spores provide a readymade capsule scaffold with high structural uniformity and a large internal cavity which may be used to encapsulate a wide range of materials.[6,7] *Lycopodium* (*L.*) *clavatum* is one species of the genus *Lycopodium* which produces spores and which has been identified to contain a range of promising phytochemicals for therapeutic applications ranging from stomach ailments to Alzheimer's disease.[8–10] *L. clavatum* spores provide a robust capsule structure and are commercially available in large quantities across the globe.[1] *L. clavatum* spores are used in traditional herbal medicine and have been shown to exhibit a wide range of therapeutic benefits including improved osteogenesis,[11] improved cognitive function,[12] treatment of gastrointestinal disorders,[8] hepatoprotective activity,[13] and antioxidative properties.[14] Recent studies have demonstrated the use of processed *L. clavatum* shells for encapsulation,[15–19] however, the production of *L. clavatum* sporopollenin capsules requires the prolonged processing of natural spores with harsh chemical treatments at elevated temperatures, so as to isolate the sporopollenin exine shell.[20–24] In many applications, this extensive processing may be unnecessary and potential therapeutic benefits may be lost. For applications in medicine, cosmetics, and food, enhanced effects may be obtained through the encapsulation of synergistic compounds,[25] and overall, the use of natural unprocessed spores provides significant benefits in terms of processing complexity and costs for a wide range of applications.

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DOI: 10.1002/adfm.201502322

A major challenge in producing microencapsulated products is ensuring particle size monodispersity,[26,27] which can have a large effect on drug release characteristics with respect to an intended target organ.[28,29] In addition to size monodispersity, having well-defined microstructures plays an important role in exploring widespread applications.[30–32] Most conventional techniques used for encapsulation such as emulsion solvent evaporation, spray drying, and chemical conjugation fail to reliably provide either size monodispersity or well-defined microstructures.[26,27,30–32] Although several studies have reported the use of empty exine microcapsules for the encapsulation of drugs, vaccines, and magnetic resonance imaging (MRI) contrast agents, as well as for use in cosmetics and food supplements,[7,16–18,22] the use of the natural "spores" as a microencapsulation material and delivery vehicle still remains unexplored. In this regard, we have directed our efforts toward exploring systems to produce macromolecule-loaded spores using three different microencapsulation techniques. The techniques we have utilized to load natural spores are simple, cost effective, and versatile, and can be applied to the development of a range of encapsulation products so as to overcome limitations of existing encapsulants while providing well-defined micromeritic properties. The specific scientific rationalities of the present work are: (i) Encapsulation of macromolecules into natural spores and the retention of natural spores constituents. (ii) Characterization of natural spores with respect to size uniformity, shape, and structure, before and after encapsulation so as to provide valuable information on their potential usage as a pharmaceutical excipient. (iii) Encapsulating macromolecules into natural spores so as to obtain uniform sized particles with well-defined micromeritic properties as a pharmaceutical encapsulating material. (iv) Provide a feasible way to achieve a tunable release profile from macromolecule-loaded spores based on coating optimization intended to release compounds in the gastrointestinal tract. Hence, this study demonstrates the use of natural spores as a novel encapsulating material and this research provides a new dimension in the use of spores, which is strongly supported by the use of *L. clavatum* spores as a plant-based medicine[8–10] for various ailments due to the intrinsic therapeutic benefits of spore constituents. In addition, our studies demonstrate that these medicinal spores can be used to encapsulate molecules of interest for tailored applications.

Here, we have explored for the first time natural spores as advanced materials to encapsulate macromolecules by three different simple encapsulation techniques including passive, compression, and vacuum loading. The natural spore formulations developed by these techniques were extensively characterized with respect to size uniformity, shape, encapsulation efficiency, and localization of macromolecules in the spores. We have also studied in vitro release profiles of developed spore formulations in simulated gastric and intestinal fluids. Further, vacuum-loaded formulations were chosen to achieve tunable release profiles by the use of alginate, a natural biomaterial, as a secondary coating material. Our encapsulation techniques involve the use of bovine serum albumin (BSA) as a model hydrophilic macromolecule[26,29,30] to load into natural *L. clavatum* spores. The selection of BSA as a model protein is based on its use as a well-studied hydrophilic macromolecule for diverse encapsulation strategies involving single and double emulsion solvent evaporation techniques.[26,30]

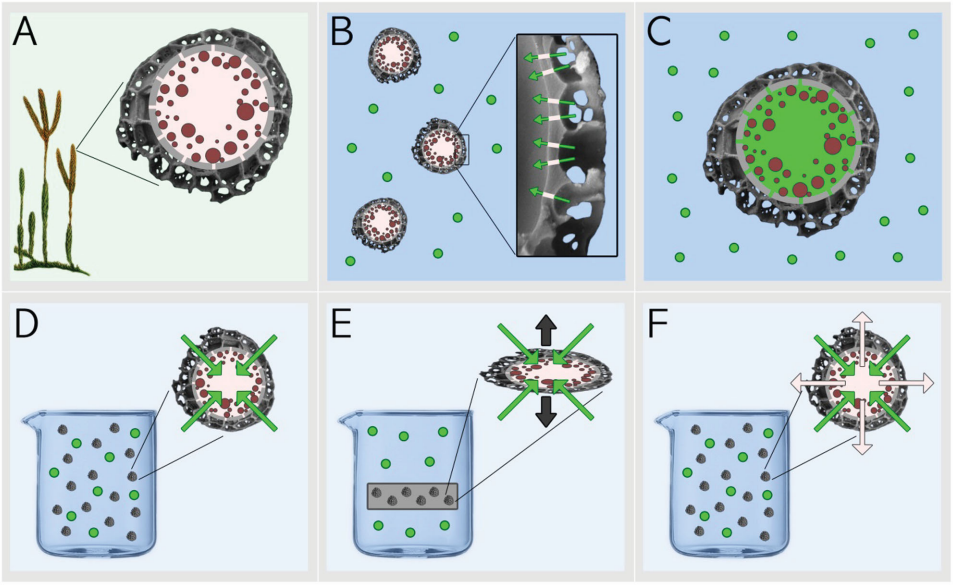
2. Results and Discussion

2.1. Macromolecular Encapsulation and Characterization of Natural Spores

Scheme 1 presents a schematic of the different encapsulation techniques used to utilize natural spores as an advanced encapsulating material. Attractive features of these techniques include both versatility and simplicity with the potential to allow for further application to a variety of small or large biomolecules under ambient processing conditions. Image (A) shows the origin of natural spores from the vascular plant *L. clavatum*, wherein these spores exhibit both uniformity of size and microstructures. When these spores are suspended in a macromolecule-containing solution (B) the macromolecules enter the internal spore cavities through natural nanochannels in the spore wall of ≈15–20 nm size,[20] and image (C) depicts a macromolecule-loaded spore along with the spore's natural cytoplasmic constituents. Image (D–F) represent the three different microencapsulation techniques: passive, compression, and vacuum loading, respectively. For the aforementioned three techniques, the spores are incubated in a macromolecule-containing solution, with additional external forces being applied in the compression and vacuum processes for the encapsulation of macromolecules.

We have investigated natural spores with respect to uniformity of size, surface morphology, and physical changes arising during the course of the encapsulation process. In order to characterize natural spores, we employed dynamic imaging particle analysis (DIPA) based on images captured using a high-resolution digital camera (see the Experimental Section). The size and morphology of natural spores are obtained based on digital signal processing of well-focused images. In addition to size analysis, the spore images allow us to obtain additional information including edge gradient, circularity, and the shape of spore particles. Size, edge gradient, and circularity analysis by FlowCam was performed with 1000 well-focused spores. **Figure 1** shows representative histogram data with Gaussian curve fitting of equivalent spherical diameter (ESD) versus frequency, with an average ESD of 30.31 ± 1.87 μm for A) natural spores, and an ESD of 30.63 ± 1.92, 30.61 ± 1.92, and 30.56 ± 1.88 μm, respectively, for B) passive, C) compression, and D) vacuum loaded spores. Hence, these experimental data and observations suggest no change in the diameter of spores after BSA loading.[16]

The morphological uniformity of natural spores was supported by circularity data before and after macromolecule loading. The data are represented by curve fitting to histograms of circularity versus frequency as shown in Figure 1. The shape of spores before and after BSA-loading are considered non-circular due to the tripartite structure and microridges which are characteristic of *L. clavatum* spores with the resulting circularity value < 1 (ideal circle = 1). The quality of the images used for data analysis is evident from the edge gradient versus frequency data which indicates that well-focused spore formulations were used during DIPA. In addition to these micromeritic data, Figure 1E–H suggests the structural similarity of spores



Scheme 1. Schematic of natural *L. clavatum spores* and processing techniques to encapsulate macromolecules. A) Spore microstructure depicting uniform ridges distributed on the surface with natural sporoplasm constituents contained inside, these spores originate from a vascular plant with spirally arranged leaves. B) Natural spores suspended in a macromolecule-containing solution for the uptake of macromolecules, the enlarged inset depicts macromolecule entry via nanochannels located within the *L. clavatum* microstructure. C) Spores encapsulating macromolecules indicated as green along with the natural sporoplasm constituents. D) Passive macromolecule loading technique involving the incubation of natural spores in the aqueous macromolecule solution at 4 °C under stirring at 500 rpm. E) Compression technique involving the compression of a dry spore powder and incubating the resulting spore tablet in the macromolecule solution for the uptake of macromolecules by the spores. F) Vacuum loading technique involving the application of a vacuum to a suspension containing natural spores and macromolecules, whereby the macromolecules enter the spores through the nanochannels located within the surface microstructures of natural spores.

before loading, as well as after passive, compression, and vacuum loading techniques, respectively.

In the present study, natural spores are explored as a novel encapsulating material due to the potential benefits in utilizing these natural biomaterials for encapsulation research as well as being cost-effective raw materials for large-scale industrial production. These spores provide distinct benefits compared to conventional materials for several main reasons. (i) Uniform size and high monodispersity with well-defined micromeritic properties with no need for the formation of particles. (ii) Large-scale raw material availability with reliable uniformity in an international market at a cost-effective price of 25 USD kg^{-1}. (iii) Proven track record of human consumption of *L. clavatum* spores and their constituents for various therapeutic benefits. (iv) Robust capsule structure which has been shown to be stable under elevated temperatures, high pressures, and exposure to solvents, allowing for diverse encapsulation strategies of different molecules. (v) Ready to use particles for encapsulation without the need for use of toxic organic solvents and which can be tailored for different bioactive release applications. In the past, encapsulation research has gained widespread importance due to the commercial potential of therapeutic molecules using various encapsulation materials such as natural polymer particles based mainly on chitosan, gelatin, starch, and sodium hyaluronate, and synthetic polymeric particles based mainly on poly(D,L-lactide-*co*-glycolide), polycaprolactone, polyanhydrides, and polyacrylates.[26] In many natural based polymers, the use of solvents and toxic cross-linking agents is inevitable and may lead to toxic effects due to residual hazardous components. Natural based polymers are also predominantly of animal origin, which leads to relatively higher processing costs to obtain raw materials. In addition, microspheres of these conventional polymeric materials typically lack uniformity as well as defined microstructures. In the case of synthetic polymers, the use of solvents such as dichloromethane is a main prerequisite to develop particulate formulations and there is also a very high raw materials cost of 3000–10 000 USD kg^{-1} in the international market. A further concern is that the acidic degradation products of PLGA based microspheres could potentially lead to deterioration of proteins, including denaturation, aggregation, and even chemical degradation. In addition, in the gastrointestinal tract a decrease in the pH value of the adjacent microenvironment may induce side effects such as inflammation.[33–35] Hence, it is essential to explore new biomaterials with versatile encapsulation strategies to meet the ever increasing demand for encapsulating biomaterials.

Further, to characterize representative batches of spores, we utilized scanning electron microscopy (SEM) to examine any structural and morphological variations, and these images are displayed as **Figure 2**A–D, respectively, for spores before loading, as well as after passive, compression, and vacuum loading techniques. Structural and morphological observations show that natural spore formulations have maintained their structural integrity without any denaturation, and exhibit size uniformity after macromolecule encapsulation using the three different microencapsulation techniques. Of note, as an encapsulation material, it is of the utmost importance to retain structural integrity after material processing at ambient temperatures.[36,37] Interestingly, the surface of the natural

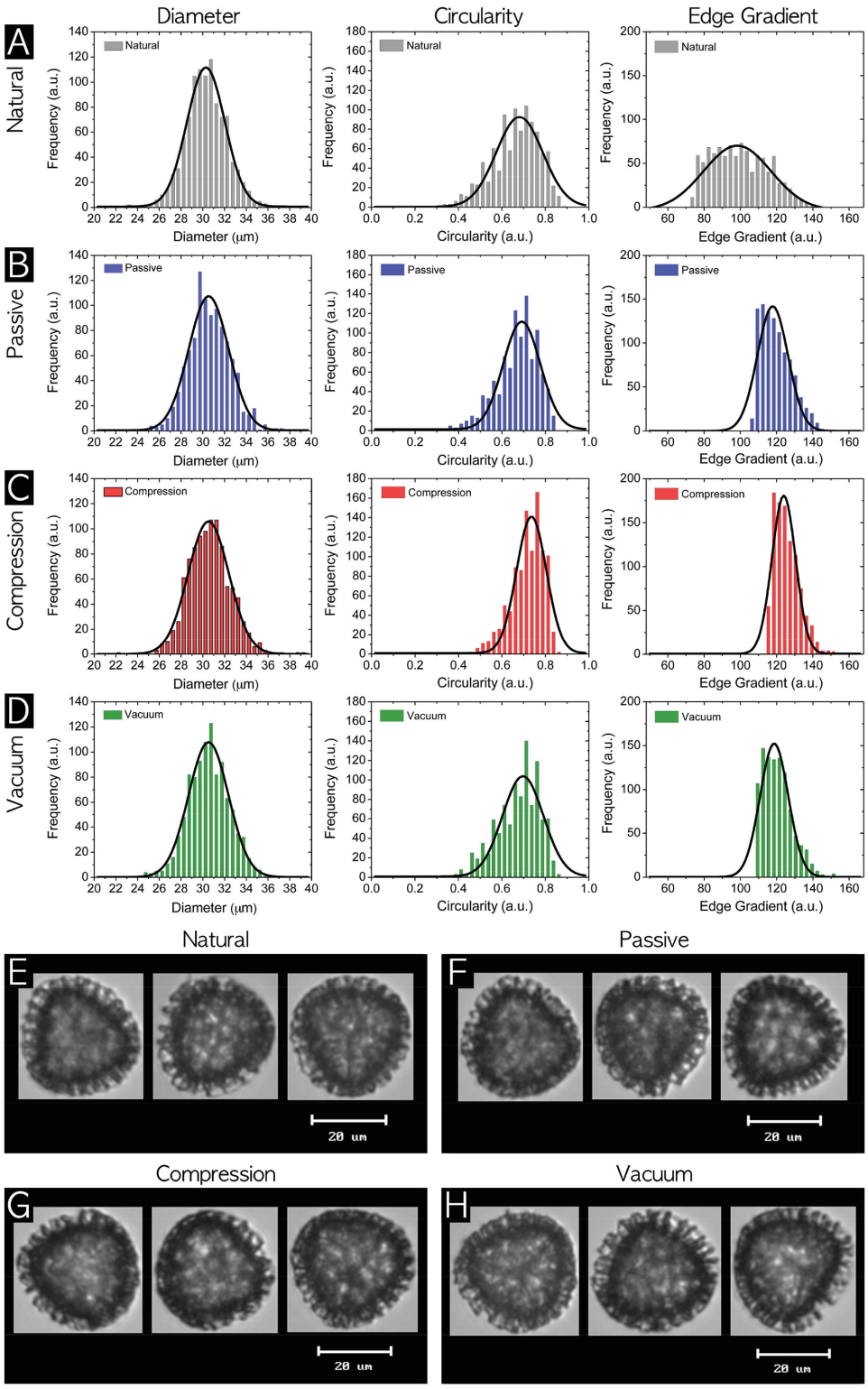


Figure 1. Characterization of natural *L. clavatum* spores before and after macromolecule loading by dynamic imaging particle analysis (DIPA, FlowCam): Size and circularity by DIPA with a particle count of 1000 spores before and after BSA-loading. Representative graphs from curve fitting to histograms for equivalent spherical diameter, circularity, and edge gradient for spores before and after encapsulation of macromolecules are presented as A) natural spores, B) passive loading, C) compression loading, and D) vacuum loading techniques. Images (E–H) represent natural spores before loading, as well as, passive, compression, and vacuum loading techniques captured by FlowCam at 20× magnification, respectively.

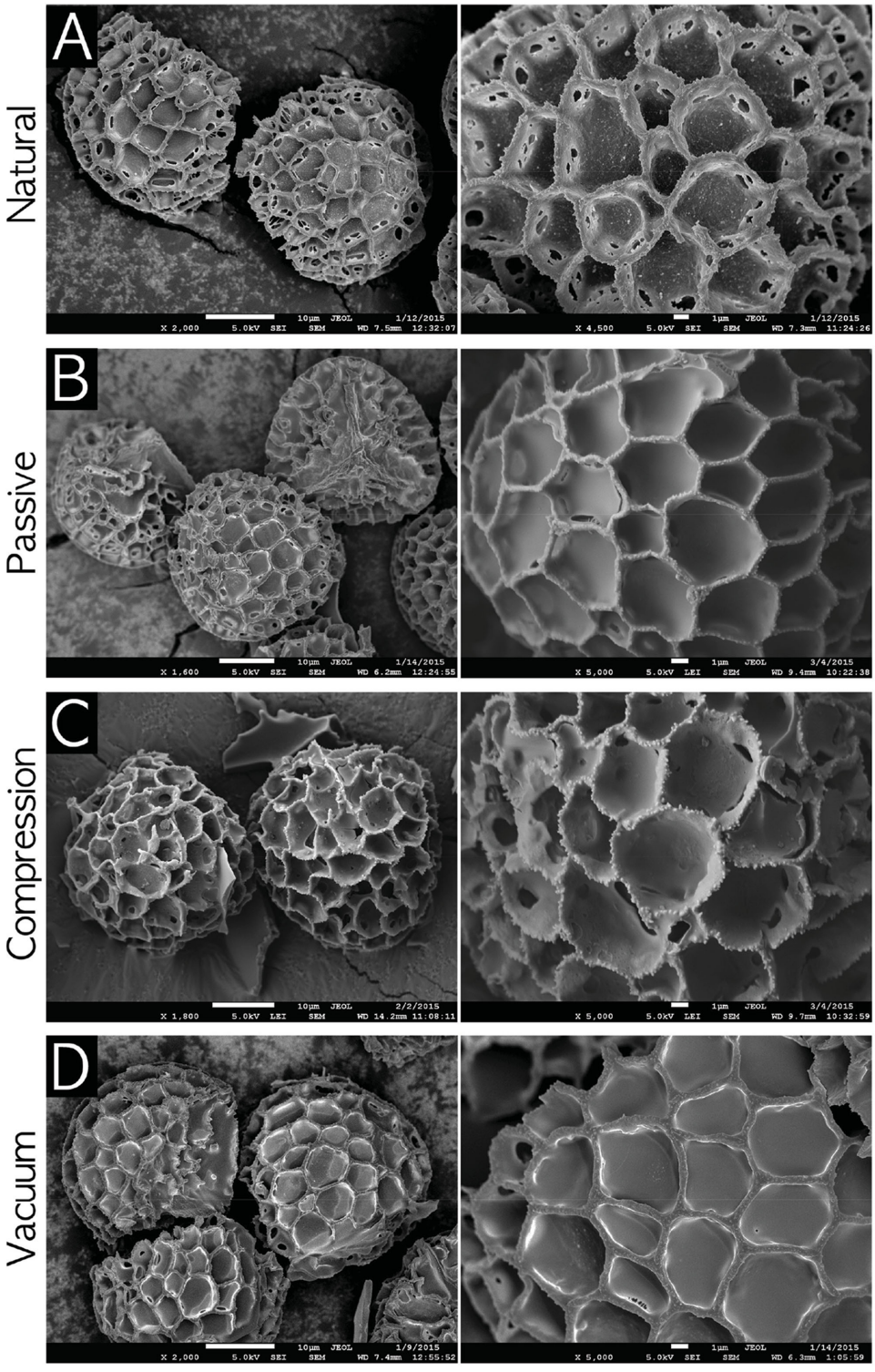


Figure 2. Characterization of natural *L. clavatum* spores before and after macromolecule loading by SEM: SEM images (A–D) represent natural spores before loading, as well as, passive, compression, and vacuum loading techniques captured by FESEM (JEOL, Japan), respectively.

spores before macromolecule loading is characterized by well-defined ornamentation. This ornamentation is categorized with numerous distinct open reticulum chambers walled by the muri (0.5 µm thick) with an approximate depth of 2–3 µm and the floors of the cavities (lumina). It is evident from the SEM images of spores after macromolecule loading that these characteristic features were retained in all the spores without substantial changes in surface ornamentation and morphology.

Table 1. BSA-loaded *L. clavatum* spores: formulation parameters.

L. clavatum spores[a)]	Theoretical BSA loading[b)] [%]	Weight of formulation after lyophilization [mg]	BSA loading [%]	BSA encapsulation efficiency[c)] [%]
Passive filling	50	160.04 ± 7.04	21.3 ± 1.8	42.7 ± 3.7
Compression filling	50	137.31 ± 3.52	21.4 ± 0.5	42.8 ± 1.1
Vacuum filling	50	102.56 ± 4.23	29.6 ± 1.1	59.2 ± 2.2

[a)]Results are the mean of three batches ($n = 3$) with standard deviation; [b)]Theoretical loading is based on 50% weight of natural *L. clavatum* spores; [c)]BSA encapsulation efficiency is determined using 5 mg of BSA-loaded natural spores.

The results from the quantitative determination of spore formulation yield after batch preparation in terms of loading and encapsulation efficiencies in spores are presented in **Table 1**, and a relatively higher batch yield was observed in the passive loading process due to one-step passive loading as compared with multistep compression and vacuum techniques. However, the encapsulation efficiency (EE) of passive and compression loadings are relatively similar, 42.7 ± 3.7% and 42.8 ± 1.1%, whereas with the vacuum loading process a statistically significant ($p < 0.05$) higher EE of 59.2 ± 2.2% was achieved.

Following the morphological characterization, we investigated the natural spore formulations with the aim to observe the localization of macromolecules within the natural spores. We encapsulated FITC-conjugated BSA into natural spores by the three aforementioned techniques and performed analysis using confocal laser scanning microscopy (CLSM). All the images were captured focusing on the middle section of spores mounted between thin glass slides and embedded within vectashield at 100×. To ensure that the autofluorescence of the natural spores did not interfere with our observations of the fluorescence of the FITC-BSA, we fixed the CLSM settings (see the Experimental Section) such that no autofluorescence was observed for the green channel with the natural spores (**Figure 3**A), and then used the same settings for all FITC-BSA loaded spores. Confocal microscope images of natural *L. clavatum* spores before macromolecule loading are presented in Figure 3A. It is evident from row (A) that there is no autofluorescence of natural spores with these settings, whereas the blue and red channels show strong autofluorescence from the spore constituents which is even more evident from their overlay image. This autofluorescence can be primarily attributed to the presence of sporoplasm constituents in the form of cellular organelles,[16,38,39] and suggests that a large portion of the inner cavity of the natural spores is empty.

In the case of FITC-BSA loading by the passive technique (Figure 3B), a strong green fluorescence was observed, confirming macromolecule loading into the natural spore cavity. It is evident from the overlay of all channels that the FITC-BSA is located in the region where the cellular organelles are not present. In the case of the compression loading of macromolecules (Figure 3C), we also observed a bright green fluorescence. However, it is interesting to note that the structural integrity and spore constituents are retained after compression at 5 ton for 20 s. Relatively higher green channel fluorescence is observed in the case of vacuum loading (Figure 3D) and this supports our assertion that higher encapsulation efficiency may be obtained with the application of an external vacuum force in addition to incubation in a macromolecular solution. Although, there appears to be a small amount of surface adhered FITC-BSA in the passive loading green channel image, and a thin layer of FITC-BSA may be adhered to the microridge structures of all loaded spores, all together, these data confirm that the significant majority of macromolecules are loaded inside the natural spore cavities and not in the reticulum chambers walled by the muri. To further support FITC-BSA localization inside the spore cavity, Z-stack images were provided in Figure S1 (Supporting Information).

Plant based medicines have played a significant role as the main treatment option from ancient times and still now traditional plant-derived medicines play a crucial role as the primary form of healthcare in many developing countries. In addition, this has also led to the discovery of novel drug candidates for a variety of diseases that affect humankind. *L. clavatum*, known as club moss, is the most widespread species in the genus *Lycopodium* of the family *Lycopodiaceae*. Extractions of crushed spores from *L. clavatum* are being used as a plant based medicine for liver dysfunction, as well as urinary and digestive disorders.[13] The medicinal effect of *L. clavatum* is also reported to improve memory function and cerebral blood flow in a memory impaired rat model.[12] *L. clavatum* is also stated to have therapeutic effects on biliary stones and liver failure, and due to its diverse importance, application of these spores is emerging as a potential new treatment modality in health care. These proven therapeutic benefits have led to the commercialization of *L. clavatum* based oral herbal formulations for the treatment of diverse health conditions such as anxiety, albuminuria, constipation, dysentery, gallstones, heartburn, hemorrhoids, impotence, indigestion, irritability, prostatitis, renal colic, and rheumatism.[8,12–15] The therapeutic activity of these spore constituents is supported by bioassay-guided studies on *L. clavatum* indicating that a major component, a *lycopodine* alkaloid, is responsible for the anti-inflammatory activity of the extract for wound-healing applications.[40] As other industrial applications, these spores have also been used as a dry powder in latex gloves and condom manufacturing. From this, it should be noted that there were several reports indicating cases of occupational allergy associated with a risk of sensitization to *L. clavatum* spores. However, it has been proposed that this was probably due to daily prolonged exposure in a place where spores were highly abundant and that the spores may have also been carriers of allergenic latex proteins.[41] Previous reports indicate that *L. clavatum* allergies are very rare and in clinical cases are never diagnosed due to a lack of allergenic symptoms.[42] In particular, a review of 61 clinical cases of rhinitis or skin reactions associated with *L. clavatum* extracts indicates that the majority of cases were weakly positive and without clinical significance.[42] In our study, the *L. clavatum* spore based formulations

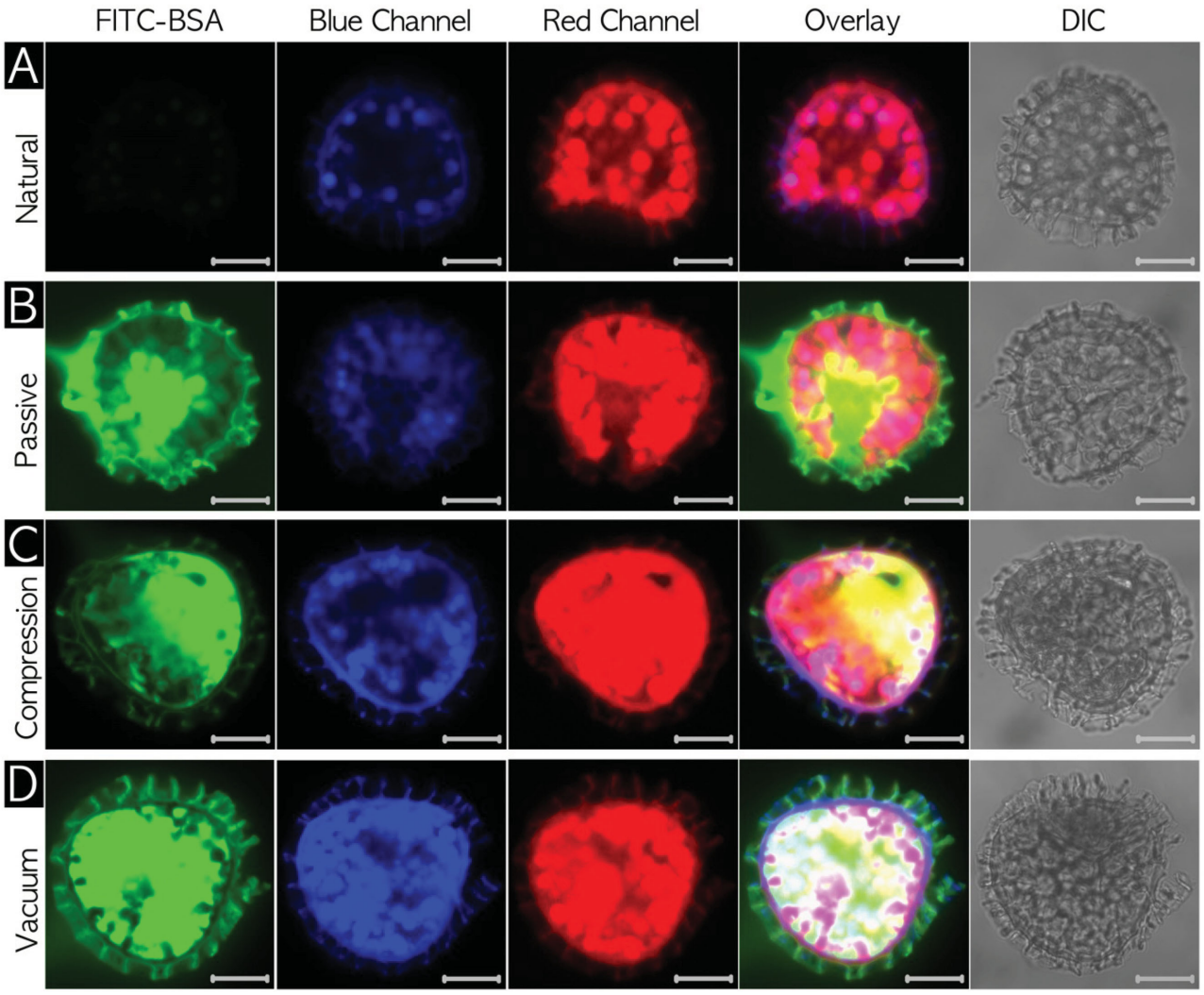


Figure 3. Confocal microscopy analysis of natural *L. clavatum* spores before and after macromolecule loading: A) CLSM images of natural *L. clavatum* spores before BSA-loading. These natural spores exhibit autofluorescence due to the presence of terpenoid, phenolic, and carotenoid molecules. The spore's natural sporoplasm constituents are observed as cellular organelles inside the spore in both the blue and red channel along with the overlaid image of the natural spore without macromolecule loading, and there is a clear absence of any green fluorescence. B) BSA-loaded spores using the passive loading technique. C) BSA-loaded spores using the compression loading technique. D) BSA-loaded spores using the vacuum loading technique. All of these spore microparticles exhibit a green color due to the presence of FITC-BSA in the green channel, and the overlaid images indicate the presence of spore constituents along with encapsulated macromolecules. (Scale bars are 10 μm.)

involve quantities of spores as low as 5 mg for bioactive release as safe oral formulations.

2.2. Modulating Macromolecules Release from Natural Spores

We have demonstrated natural spores as fascinating materials for the encapsulation of various molecules with BSA as a model protein. We further studied in vitro release profiles of BSA from these natural spores separately in simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 7.4) conditions. The in vitro release of BSA in SGF (**Figure 4**A) indicates 90% macromolecules release in the first 5 min and complete release was observed in 30–60 min. There was no significant difference among the release from BSA-loaded spores prepared using different techniques ($p \geq 0.05$). In the case of intestinal conditions (Figure 4B), a similar burst release was observed with spore formulations prepared by three different loading techniques suggesting no significant release differences in simulated gastric and intestinal conditions. The observed release trend indicates fast release in both simulated conditions and is evident due to the high aqueous solubility of BSA resulting in rapid release from nanodomains of natural spores. Our results with natural spores support previous studies using exine capsules, which indicate the lack of a barrier to retard macromolecule release in SGF and SIF for a longer period of time.[7,16,18] Similar drug release profiles were reported with the use of processed sporopollenin microcapsules extracted from *L. clavatum* spores.[16,18] To explore the structural changes after FITC-conjugated BSA release in PBS, we performed CLSM after complete release and

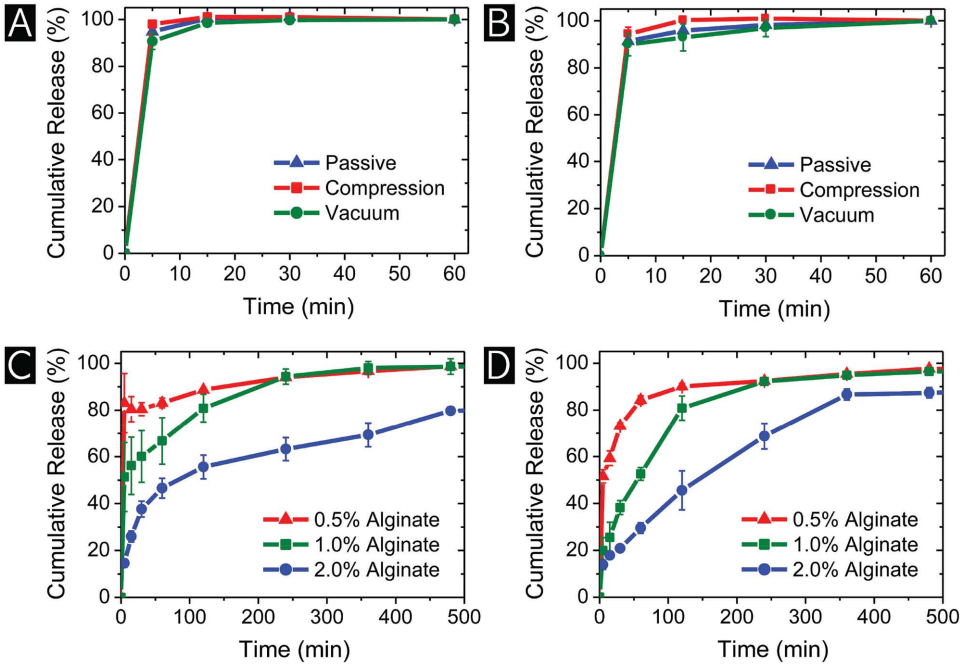


Figure 4. In vitro release profiles of macromolecule-loaded natural *L. clavatum* spores: Cumulative release profiles of BSA-loaded spores by passive, compression, and vacuum loading in A) simulated gastric fluid (SGF), pH 1.2 media, and B) simulated intestinal fluid (SIF), pH 7.4 media. Further optimization of tunable release was achieved using vacuum loaded spores and cumulative release profiles of vacuum loaded spores after different alginate coatings are presented in C) SGF, pH 1.2 media, and D) SIF, pH 7.4 media. All in vitro release studies were performed in triplicate ($n = 3$) and average values with standard deviations are presented.

the results indicate the absence of significant green channel fluorescence within the spore cavity, confirming the complete release of FITC-BSA from natural spores (see Figure S2, Supporting Information). However, a low intensity green color is observed surrounding the exine shell of the spores indicating a small portion of external FITC-BSA binding. Interestingly, the autofluorescence of these natural spores was retained after processing for encapsulation and release. These observations clearly indicate the intact sporoplasmic cellular organelles in the natural spores.

Further, in order to achieve a sustained tunable release, we have investigated the incorporation of macromolecule-loaded natural spores into alginate microbeads. These microbeads were prepared by using vacuum loaded spores in different concentrations of alginate solutions, 0.5%, 1%, and 2% (w/v). We have presented the release profiles from natural spores with varying concentrations of alginate in SGF and SIF in Figure 4C,D, respectively. In the case of SGF, the release profiles from natural spores indicate a systematic release trend based on the concentration of alginate used in the formulations. There was a higher burst release with 0.5% alginate formulations compared to 1% and 2% alginate. Sustained release up to 8 h without any burst effect was achieved using vacuum loaded natural spores in 2% alginate beads. Statistical analysis of macromolecule release from natural spores was performed separately in SGF and SIF, and the results indicate significant differences ($p < 0.05$) among the various alginate concentration formulations. When considering oral formulation applications, it is important to study in vitro release profiles in both simulated gastric and intestinal conditions. Our data indicate that macromolecule release from spores is independent of pH and with further development, such spore based formulations could be highly beneficial in oral dosage formulations for drugs targeting gastrointestinal diseases which involve varied absorption windows and require drug release in different parts of the GI tract.[43,44] In addition, the large internal cavity of these natural spores provides high therapeutic loading and release in the gastrointestinal tract.

In order to evaluate the structure of the microbeads after alginate coating, SEM analysis was performed on 0.5%, 1%, and 2% alginate coated spores, and the results are depicted in **Figure 5**. Interestingly, spores were fused inside alginate microbeads and surface analysis reveals spores were intact with variable layers of coating with 0.5%, 1%, and 2% alginate. These morphological observations further support the concentration-dependent release profiles, which indicate that alginate acts as barrier to retard macromolecule release from spores. Hence, our results provide a way to tune the macromolecule release from natural *L. clavatum* spores by varying the amount of alginate used for coating. The ability to tune compound release profiles is crucial in targeting various parts of human gastrointestinal track[43,45] and to develop new oral therapeutics for disease targets.

3. Conclusion

In conclusion, we have successfully demonstrated the encapsulation of macromolecules into natural *L. clavatum* spores by three different microencapsulation techniques, passive, compression, and vacuum loading. The greatest encapsulation was achieved using vacuum loading and the encapsulation of macromolecules

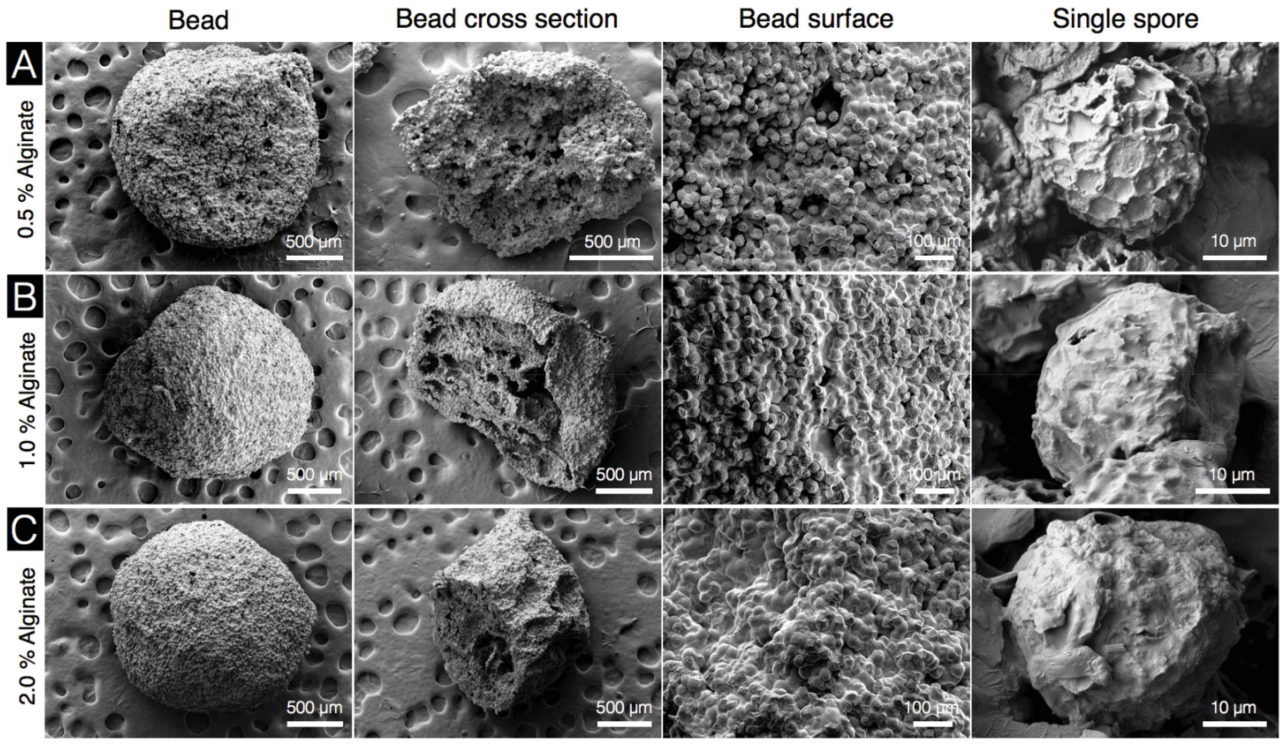


Figure 5. Scanning electron microscopic images of *L. clavatum* spores after alginate coating. Images represent A) 0.5%, B) 1%, and C) 2% alginate coated spores, respectively.

was confirmed by CLSM with localization of FITC-BSA observed to be within the natural spore cavity. The surface characterization and DIPA revealed a high degree of uniformity in the size of all the developed formulations along with well-defined microridges. The demonstrated potential of natural spores for encapsulation applications provides motivation for their further exploration as a delivery vehicle for various compounds. Indeed, different natural spores have unique architectures that are formed by natural bio-templating processes and their high degree of uniformity and robustness make these ideal biomaterials for encapsulation purposes, especially in light of our environmentally friendly encapsulation strategies, which bypass the need for harsh chemical treatments. Importantly, we have also demonstrated that the release of macromolecules from within *L. clavatum* spores can be controlled by forming a biocompatible alginate coating so as to obtain a tunable release profile. In the future, the integration of natural and synthetic biomaterials offers rich opportunities to develop multifunctional delivery platforms for both medical and other biotechnology applications.

4. Experimental Section

Materials and Chemicals: Natural *L. clavatum* spores, BSA, FITC-conjugated BSA, sodium alginate, and other chemicals were purchased from Sigma (Singapore). Vectashield (H-1000) medium was procured from Vector labs (CA, USA) and Sticky-slides, D 263 M Schott glass, No. 1.5H (170 µm, 25 mm × 75 mm) unsterile, were procured from Ibidi GmbH (Munich, Germany).

Encapsulation of Macromolecules into Natural L. clavatum Spores: 75 mg of BSA was dissolved in 0.6 mL of purified water in a 1.5 mL polypropylene tube and then 150 mg of spores were suspended in the BSA solution (50 wt% based on spore wt.).[26,36] The suspension was vortexed (VWR, Singapore) for 5 min and the tube was transferred to a thermoshaker (Hangzhou Allsheng Instruments, Singapore) at 4 °C and 500 rpm for passive loading. In the case of compression loading, a compressed tablet was prepared by using a hydraulic press at 5 ton pressure for 20 s (die diameter 13 mm; area 132.75 mm^2; 370 MPa). The spore tablet was soaked in a BSA solution to allow for BSA uptake by the spore particles (dimensions of spore tablets are provided in the Supporting Information). For the vacuum loading technique, the BSA and spore particles suspension were placed in a freeze-drier (Labconco, MO, USA) and a 1 mbar vacuum was slowly applied. The quantity of BSA, spore particles, and incubation time (2 h) were constant for all batches, and after incubation the BSA-loaded spore particles were collected by centrifugation at 12 000 rpm for 4 min and washed using 0.5 mL water, and then centrifuged to remove surface adhered BSA. The spores were placed in a freezer at –70 °C for 30 min and then freeze-dried for 24 h. Finally, the final BSA-loaded *L. clavatum* spores were stored at –20 °C until characterization. The placebo spores were prepared with the same procedure but without BSA.

DIPA by FlowCam (VS, Fluid Imaging Technologies, Maine, USA): Natural *L. clavatum* spores and macromolecules-loaded spores with a prerun volume of 0.5 mL (2 mg mL^{-1}) were primed manually into the flow cell. The focused spores were analyzed with a flow rate of 0.1 mL min^{-1} and a camera rate of 10 frames s^{-1} leading to a sampling efficiency of about 9%, and 1000 well-focused spores were segregated by edge gradient ordering and manual processing. Representative histogram data were fitted with a Gaussian curve and plotted, and values are reported with standard deviations (SDs). (For a detailed description and calibration details refer to the Supporting Information.)

Surface Morphology Evaluation by SEM: SEM imaging was performed using an FESEM 7600F (JEOL, Japan). Samples were coated with platinum at a thickness of 10 nm by using a JFC-1600 (JEOL, Japan) (20 mA, 60 s) and images were recorded by employing FESEM with an acceleration voltage of 5.00 kV at different magnifications to provide morphological information.

Confocal Laser Scanning Microscopy Analysis: Confocal laser scanning micrographic analysis was performed using a Carl Zeiss LSM700 (Germany) confocal microscope. Laser excitation lines 405 nm (6.5%), 488 nm (6%), and 633 nm (6%) with DIC in an EC Plan-Neofluar 100× 1.3 oil objective M27 lens were used. Fluorescence from natural and macromolecule loaded *L. clavatum* spores were collected in photomultiplier tubes equipped with the following emission filters: 416–477, 498–550, and 572–620. The laser scan speed was set at 67 s per each phase (1024 × 1024: 84.94 μm^2 sizes) and plane mode scanning with a 3.15 µs pixel dwell was used, and at least three images were captured for each sample. All images were processed and converted under the same conditions using software ZESS 2008 (ZEISS, Germany).

Encapsulation Efficiency: 5 mg BSA-loaded *L. clavatum* spores were suspended in 1.4 mL PBS, vortexed for 5 min, and probe sonicated for 10 s (three cycles, 40% amplitude). The solution was filtered to collect extracted BSA using 0.45 µm PES syringe filters (Agilent, USA). The absorbance was measured at 280 nm (Boeco-S220, Germany) using a placebo extract as a blank to compute the amount of BSA in the spore particles.

In Vitro Drug Release Studies: 5 mg of BSA-loaded *L. clavatum* spores were suspended in pH 1.2 (0.1 M HCl) solution, or phosphate-buffered saline, pH 7.4. Incubation was performed at 37 °C at 50 rpm in an orbital shaker incubator (LM-400-D-220, Yihder, Taiwan). The release samples were collected at specified time intervals by centrifugation at 14 000 rpm for 30 s, and the samples were replenished with fresh release media. The release samples were filtered using PES syringe filters and absorbance was measured at 280 nm. The amount of BSA released was computed using a BSA standard curve.

Formulation of Macromolecule-Loaded Spores for Tunable Release: 150 mg of BSA-loaded (vacuum loading) spores were mixed homogeneously with 1.5 mL of alginate (0.5%, 1%, 2%) in water. The resulting suspension was slowly added to a 10 mL calcium chloride (8%) solution using a blunt stainless steel 18 G needle under magnetic stirring. Stirring was continued for 5 min, and calcium chloride was removed by centrifugation (1500 rpm, 2 min). The beads were washed twice using 1.5 mL water and freeze-dried for 24 h. All formulations were stored at –20 °C until characterization. 10 mg of alginate coated *L. clavatum* spores were used for in vitro release studies.

Statistical Analysis: Statistical analysis was performed using two-tailed *t*-tests and $P < 0.05$ was considered as statistically significant. Encapsulations with natural spores and release experiments were repeated at least three times and all data are expressed as mean ± SD.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This work was supported by the National Research Foundation (NRF-NRFF2011-01) and an NRF Competitive Research Programme (NRF-CRP10-2012-07).

Received: June 8, 2015
Revised: September 21, 2015
Published online: December 7, 2015

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