Supporting Information


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

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Supporting Information

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1. Methods

1.1. Microencapsulation techniques to load macromolecule into natural *L. clavatum* spores:

1.1.1. Passive loading technique: 75 mg of BSA was dissolved in 0.6 mL of purified water in a 1.5 mL polypropylene tube and 150 mg natural spores were suspended in the BSA solution (50 wt.% based on spore wt.). The suspension was vortexed (VWR, Singapore) for 5 min and the tube was transferred to a thermoshaker (*Hangzhou Allsheng Inst. Singapore*) set at 4°C, 500 rpm for 2 h incubation. The BSA-loaded spores were collected by centrifugation at 12000 rpm for 4 min. The spores were washed quickly using 0.5 ml water and centrifuged to remove surface adhered BSA. The spores were placed in a freezer at -70°C for 30 min and freeze-dried for 24 h. The resultant macromolecule loaded spores were stored at -20°C until characterization. The placebo spores were prepared using the same procedure without BSA.

1.1.2. Compression loading technique: 150 mg natural spores were filled in a 13 mm die used to form FTIR pellets and compressed to form a tablet under hydraulic press to provide a 5 ton load for 20 s (die diameter 13 mm; area 132.75 mm²; 370 MPa). The dimensions of the spore tablet are mentioned in TableS1 and the tablet was soaked in 0.6 mL of 75 mg BSA containing aqueous solution in a 20 mL flat glass bottle for 2 h at 4°C to allow for the uptake of BSA molecules. The process was stopped and BSA-loaded spores were collected by centrifugation at 12000 rpm for 4 min. The spores were quickly washed using 0.5 ml water and centrifuged to remove surface bound BSA. The spores were placed in a freezer at -70°C for 30 min and freeze-dried for 24 h. The resultant spores were stored at -20°C until characterization. The placebo spores were prepared with the same procedure without BSA.

1.1.3. Vacuum loading technique: 75 mg of BSA was dissolved in 0.6 of mL purified water in a 1.5 mL centrifuge tube and 150 mg spores were suspended in the BSA solution (50 wt.% based on spore wt.). The suspension was vortexed (VWR, Singapore) for 5 min. The sample was placed in a freeze-drier and a 2 mbar vacuum was applied. The process was stopped and the BSA-loaded *L. clavatum* spores were collected by centrifugation at 12000 rpm for 4 min.
The spores were washed quickly using 0.5 ml water and centrifuged to remove surface bound BSA. The spores were placed in a freezer at -70°C for 30 min and freeze-dried for 24 h. The resultant particles were stored at -20°C until characterization. The placebo spores were prepared with the same procedure but without BSA.

1.1.4. FITC-conjugated BSA loading: In order to predict localization of BSA in natural *L. clavatum* spores, FITC-conjugated BSA was encapsulated using the three different techniques as mentioned in section 1.1.

1.2. Characterizations of natural and macromolecule-loaded natural *L. clavatum* spores

1.2.1. Dynamic imaging particle analysis (DIPA) by FlowCam®: Bench top system (FlowCamVS, Fluid Imaging Technologies, Maine, USA) was equipped with a 200 μm flow cell (FC-200), a 20X magnification lens (Olympus®, Japan), and controlled by the visual spreadsheet software version 3.4.11. The system was flushed with 1 mL deionized water (Millipore, Singapore) at a flow rate of 0.5 ml/min and flow cell cleanliness was monitored visually before each sample run. Natural *L. clavatum* spores and macromolecule-loaded spores (2 mg/ml) with a pre-run volume of 0.5 mL were primed manually into the flow cell and were analyzed with a flow rate of 0.1 ml/min and camera rate of 10 frames/s leading to a sampling efficiency of about 9 %. A minimum of 10,000 particles were fixed as the count for each measurement and three separate measurements were performed and data analysis was carried out using 1000 well-focused spores segregated by edge gradient. The instrument was calibrated using polystyrene microspheres (50 ± 1 μm, Thermoscientific, USA). Representative histogram data was fitted with a Gaussian curve and plotted and, values are reported with standard deviations (Figure S3 and Table S2).

1.2.2. Surface morphology evaluation by scanning electron microscopy (SEM): SEM imaging was performed using a FESEM 7600F (JEOL, Japan). Samples were coated with platinum at a thickness of 10 nm by using JFC-1600 (JEOL, Japan) (20mA, 60 sec) and images were recorded by employing FESEM with an acceleration voltage of 5.00 kV at different magnifications to obtain SEM micrographs for morphological evaluation.

1.2.3. Confocal laser scanning microscopy analysis of natural and macromolecule loaded natural spores: Confocal laser scanning micrographic analysis were performed using a Carl Zeiss LSM700 (Germany) confocal microscope equipped with three spectral reflected/fluorescence detection channels, six laser lines (405/458/488/514/543/633 nm), and
connected to a Z1 inverted microscope (Carl Zeiss, Germany). Natural and macromolecule-loaded spores were mounted on sticky slides (Ibidi, Germany), a drop of mounting medium (Vectashield®) was added, and spore particles were covered with another sticky slide. Images were collected immediately under the following conditions: laser excitation lines 405 nm (6.5%), 488 nm (6%) and 633 nm (6%) with DIC in an EC Plan-Neofluar 100X 1.3 oil objective M27 lens. Fluorescence signals from natural and macromolecule loaded spores were collected in photomultiplier tubes equipped with the following emission filters; 416-477, 498-550, 572-620. The laser scan speed was set at 67 sec per each phase (1024 x 1024; 84.94 µm² sizes) and plane mode scanning was performed with a 3.15 µsec pixel dwell. The iris was set as optimal for the sample conditions and all images were captured at the mid region of the particle (optical section) and other settings were fixed the same for all samples. At least three images were captured for each different sample and all images were processed with the same conditions using ZESS 2008 software (ZEISS, Germany).

1.3. Encapsulation efficiency: 5 mg of BSA-loaded spores were suspended in 1.4 mL of PBS then vortexed for 5 min and probe sonicated for 10 sec (3 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 using placebo extract as a blank to compute the amount of BSA in the natural spores as below:

\[
\text{Amount of BSA (mg)} = \frac{\text{Absorbance} \times \text{dilution factor}}{\text{Slope (standard curve)} \times 1000}
\]

\[
\% \text{ Loading} = \frac{\text{Amount of BSA} \times 100}{\text{Weight of spores}}
\]

\[
\% \text{ Encapsulation efficiency} = \frac{\text{Practical Loading} \times 100}{\text{Theoretical loading}}
\]

1.4. In-vitro drug release evaluation in simulated gastric fluid (0.1 M HCl, pH 1.2): 5 mg of BSA-loaded spores or placebo were suspended in 1.4 ml media and incubated at 37°C, 50 rpm. 1 ml release samples were collected at specified time intervals by centrifugation at 14000 rpm for 30 s and replenished with 1 ml of fresh release media. The release samples were filtered using PES membrane filters (Agilent, USA) and absorbance was measured at 280 nm using placebo as a baseline. The amount of BSA released was computed using a BSA standard curve.
1.5. *In-vitro* drug release evaluation in simulated intestinal fluid (PBS pH 7.4): 5 mg of BSA-loaded spores or placebo were suspended in 1.4 ml media and incubated at 37°C, 50 rpm. 1 ml release samples were collected at specified time intervals by centrifugation at 14000 rpm for 30 s and replenished with 1 ml of fresh release media. The release samples were filtered using PES membrane filters (Agilent, USA) and the absorbance was measured at 280 nm using placebo as a baseline. The amount of BSA released was computed using a BSA standard curve.
Figure S1. Z-stack images from confocal laser scanning microscopy (CLSM) showing 35 optical sections of an *L. Clavatum* spore (A) After FITC-BSA loading, (B) Before FITC-BSA loading.

Figure S2. CLSM images after FITC-BSA release from natural spores prepared by different techniques in pH 7.4 media, row (A) Passive loading technique, row (B) Compression loading technique, row (C) Vacuum loading technique. (scale bars are 10 µm).

Figure S3. Representative measurement results from dynamic imaging particle analysis (DIPA) using polystyrene microspheres as a standard 50 ± 1 µm (Thermoscientific, USA). (A) Representative histogram of equivalent spherical diameter vs. frequency using 1000 well focused images with ESD of 49.65 ± 0.91 µm. (B) Representative histogram of circularity vs. frequency indicating microspheres very near to ideal circle value (1). (C) Histogram of edge gradient vs. frequency indicating well focused microspheres. (D) Representative image of microspheres at 20X magnification.

Table S1. Details of compressed natural *L. clavatum* spore tablet

Table S2. Equivalent spherical diameter of natural *L. clavatum* spores and BSA-loaded spores
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Table S1. Details of compressed *L. clavatum* spore tablet\(^{(a)}\) used for compression technique

<table>
<thead>
<tr>
<th>Weight of tablet(^{(b)}) (mg)</th>
<th>Diameter of tablet (mm)(^{(c)})</th>
<th>Thickness of tablet (mm)(^{(c)})</th>
</tr>
</thead>
<tbody>
<tr>
<td>126.77 ± 6.44</td>
<td>13.29 ± 0.02</td>
<td>1.47 ± 0.02</td>
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</table>

\(^{(a)}\) Tablets used in BSA-loading by compression technique and results are mean of three batches (n=3) with standard deviation; \(^{(b)}\) Weight determined in a Boeco BBX 22 (Germany) analytical balance; \(^{(c)}\) Diameter and thickness measured using digital vernier caliper.

Table S2. Equivalent spherical diameter of natural spores and BSA-loaded spores

<table>
<thead>
<tr>
<th><em>L. clavatum</em> Spores</th>
<th>Equivalent spherical diameter (ESD, µm ± SD) (^{(a)})</th>
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<tbody>
<tr>
<td>Natural spores before BSA loading</td>
<td>30.31 ± 1.87</td>
</tr>
<tr>
<td>Spores after passive technique</td>
<td>30.63 ± 1.92</td>
</tr>
<tr>
<td>Spores after compression technique</td>
<td>30.61 ± 1.92</td>
</tr>
<tr>
<td>Spores after vacuum technique</td>
<td>30.56 ± 1.88</td>
</tr>
</tbody>
</table>

\(^{(a)}\) DIPA measurements were performed in triplicate and one representative value is reported with standard deviation.