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Encapsulation and controlled release formulations of 5-fluorouracil from natural *Lycopodium clavatum* spores

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ABSTRACT

Cost effective, uniform-size multiparticulate formulations of the chemotherapeutic agent 5-fluorouracil (5-FU) loaded in natural *Lycopodium clavatum* spores were developed by three different encapsulation techniques: passive, compression and vacuum loading. The surface morphology, and micromeritic properties of 5-FU spore formulations were characterized by scanning electron microscopy and dynamic image particle analysis, respectively. The encapsulation efficiency of spores by vacuum-assisted loading was higher (49%) compared to passive and compression loading techniques. The vacuum-loaded formulation was selected for further development with a Eudragit RS 100 (EUD) coating that enabled controlled 5-FU release in simulated gastric (pH 1.2) and intestinal (pH 7.4) conditions. The surface morphology analysis after EUD coating at two different EUD concentrations (2.5% w/v and 10% w/v) indicates that a thin, conformal layer of EUD was deposited on the spore surface. The *in-vitro* release of 5-FU from coated spores exhibited a slower release profile compared to uncoated spores, and was extended for up to 30 h in simulated gastrointestinal conditions. Collectively, the findings demonstrate that EUD coated 5-FU loaded natural *L. clavatum* spores provide a controlled release formulation that would aid treatment options against gastrointestinal cancer and other related maladies.

Introduction

Fluorouracil (5-FU) is extensively used in clinical chemotherapy to treat metastatic carcinomas of breast, stomach, intestine and colon [1–4]. The current mode of treatment with infusions is often inconvenient, costly and repeated doses are required due to the short half-life of 5-FU (10 to 20 min) [5]. In addition, 5-FU is rapidly absorbed through blood capillaries into systemic circulation resulting in low levels of drug near the tumor sites and consequent loss of efficacy along with higher systemic toxicity [6]. The development of 5-FU formulations for controlled oral delivery would be highly beneficial [7,8]. With advances in oral drug delivery research, various approaches to developing controlled 5-FU release formulations have been reported [3,4,9,10]. Among the investigated delivery systems, multiparticulate drug delivery systems are attractive due to dose distribution over a large number of small subunits and the drug release profile can be tailored to meet therapeutic needs [11–14].

Controlled-release multiparticulate delivery systems of 5-FU have been reported by encapsulation into different kinds of polymeric microspheres [4,8,10]. However, the conventional techniques proposed are complex and expensive, and it remains difficult to produce uniform-size multiparticulate formulations. In recent years, plant spores (*Lycopodium clavatum*) and pollen grains are emerging as candidate microspheres for drug encapsulation and exhibit high structural uniformity and a monodisperse micron-scale size distribution [15–17]. The main advantages of *L. clavatum* spores for drug delivery are their large inner cavity surrounded by a hard shell (exine) for encapsulation, uniform size distribution and sustainable availability [16–18]. *L. clavatum* spores are cost-effective and can be readily supplied and processed in industrial scale batches. In addition, *L. clavatum* spores contain a range of therapeutic phytochemicals for gastrointestinal diseases and are widely used in traditional herbal medicine [19–21]. Recently, Diego-Taboada et al. [18,22] demonstrated the use of *L. clavatum* sporopollenin exine capsules (SECs) for encapsulation

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of drugs, proteins and oils. Mundargi et al. [23] have explored natural *L. clavatum* spores for materials encapsulation with bovine serum albumin as model biomacromolecule. Materials encapsulation into natural spores has various benefits: (i) to avoid prolonged processing of natural spores with harsh chemicals at elevated temperatures to extract the SECs; (ii) the potential therapeutic spore constituents can be retained in spores during materials encapsulation; and (iii) natural spores are cost-effective raw materials for a wide range of encapsulation applications. Hence, there is significant potential for exploring the application of natural spores for drug delivery, including further development of material coating strategies to control the release profiles.

Herein, we report the encapsulation and controlled release of 5-FU in natural *L. clavatum* spores, which was achieved by passive, compression and vacuum loading. 5-FU loaded spores were characterized for size uniformity, shape, surface morphology, encapsulation efficiency and *in-vitro* release profiles in simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 7.4). In order to achieve controlled 5-FU release over a longer time scale from vacuum-loaded spores, Eudragit RS 100 (EUD) was used as a coating material due to its wide spread usage as a coating polymer to modify drug release from pharmaceutical dosage forms [24,25].

The coated spores were characterized for surface morphology and *in-vitro* release in SGF and SIF for up to 30 h at 37 °C. Taken together, the work achieved in this study contributes to the development of natural spores for controlled release of encapsulated small molecule pharmaceutical drugs.

Experimental

Materials

Natural *L. clavatum* spores, 5-fluorouracil (5-FU), ammonium hydroxide and ethanol were purchased from Sigma (Singapore). Polystyrene microspheres (50 ± 1 μm) were purchased from Thermoscientific (CA, USA). Eudragit RS100 was procured from Evonik Industries (Essen, Germany) and perfluoroalkoxy polymer (PFA) flasks were procured from Vitlab (Grossostheim, Germany). Stainless steel casted pellet press die (13 mm) was procured from Specac (Kent, UK).

Encapsulation of 5-FU into natural L. clavatum *spores by passive loading technique*

5-Fluorouracil solution was prepared by dissolving 75 mg of drug in a 1.8 mL mixture of ethanol and 1 N ammonium hydroxide (1:1) solution. Natural *L. clavatum* spores (150 mg) were suspended in the prepared solution. The suspension was vortexed for 5 min and the tube was transferred to a thermoshaker (Hangzhou Allsheng Inst. Singapore) set at 500 rpm for 2 h incubation at room temperature. The 5-FU loaded spores were collected by centrifugation at 4500 rpm for 3 min. The spores were washed using 4 mL deionized water and centrifuged to remove surface adhered 5-FU. The 5-FU loaded spores were placed in a freezer at −70 °C for 30 min and freeze-dried for 24 h. The resulting 5-FU loaded spores were stored in a dry cabinet at room temperature until further characterization. The placebo passive-loaded spores without 5-FU were prepared by using the same procedure as described above.

Encapsulation of 5-FU into natural L. clavatum *spores by compression loading technique*

150 mg of *L. clavatum* spores were filled in a 13 mm pellet press die and compressed to form a tablet under a hydraulic press with a 5 t load for 20 s (die diameter 13 mm; area 132.75 mm^2; 370 MPa) [15]. The dimensions of the spore tablet are described in the Supporting Information (Table S1) and the tablet was soaked in a 1.8 mL 5-FU solution in a 20 mL flat glass bottle for 2 h to allow for the uptake of 5-FU. The 5-FU loaded spores were collected by centrifugation at 4500 rpm for 3 min. The spores were washed using 4 mL deionized water and centrifuged to remove surface bound 5-FU. The spores were placed in a freezer at −70 °C for 30 min and freeze-dried for 24 h. The resulting spores were stored in a dry cabinet until further characterization. The placebo compression-loaded spores without 5-FU were prepared by using the same procedure as described above.

Encapsulation of 5-FU into natural L. clavatum *spores by vacuum loading technique*

Vacuum-assisted 5-FU loading was performed by suspending 150 mg of *L. clavatum* spores in 1.8 mL of 5-FU solution. The suspension was vortexed for 5 min. The sample was placed in a freeze-drier (Lanconco, USA) and a 1 mbar vacuum was applied for 2 h. The process was stopped and the 5-FU loaded *L. clavatum* spores were washed using 4 mL water and centrifuged to remove surface bound drug. The spores were placed in a freezer at −70 °C for 30 min and freeze-dried for 24 h. The resulting spore particles were stored in a dry cabinet until further characterization. The placebo vacuum-loaded spores without 5-FU were prepared by using the same procedure without 5-FU as described above.

Surface morphology evaluation by scanning electron microscopy (SEM)

The evaluation of the surface morphology of the 5-FU loaded spores was conducted using a FESEM 7600F (JEOL, Japan). A platinum coating of 10 nm thickness was deposited on each sample by using an auto fine coater JFC-1600 (JEOL, Japan) at 20 mA for 60 s. Images were taken with an acceleration voltage of 5 kV at various magnifications.

Dynamic image particle analysis

The benchtop system (FlowCamVS, Fluid Imaging Technologies, Maine, USA) was installed with a visual spreadsheet software version 3.4.11., 200 μm flow cell (FC-200) and a 20× magnification lens (Olympus®, Japan). The flow cell was cleaned by flushing the system with 1 mL of deionized water at a flow rate of 0.5 mL/min. The instrument was calibrated using polystyrene microspheres (50 ± 1 μm) and a pre-run volume of 0.5 mL of natural *L. clavatum* spores and 5-FU loaded spores were primed and transferred into the flow cell. Analysis was carried out at a flow rate of 0.1 mL/min and a frame rate of 10 FPS leading to a sampling efficiency of about 9%. The count for each analysis was fixed at the minimum of 10,000 particles and highly focused particles were selected by edge gradient for data analysis. The representative data reported in this work is an average of triplicate measurements with standard deviation ($n = 3$).

Preparation of Eudragit RS100-coated spore formulations

5-FU loaded *L. clavatum* spores were coated using Eudragit RS100 at two different EUD concentrations (2.50% w/v and 10.0% w/v). The coating solutions were prepared by slowly dissolving Eudragit RS100 in acetone. For the coating process, 150 mg of 5-FU loaded (vacuum method) spores were added to 1.2 mL of Eudragit RS100 solution in a PFA round bottom flask and the solvent was evaporated in a vacuum desiccator for 1 h. Further, spores were dried in vacuum oven (Memmert GnbH, Germany) at 1 mbar for 1 h. The dried spore formulation was then gently powdered using an agate pestle and mortar and stored in a dry cabinet until further characterization.

Encapsulation efficiency

10 mg of 5-FU loaded *L. clavatum* spores were suspended in 10 mL of pH 7.4 phosphate-buffered saline (PBS), mixed using a vortex mixer (IKA, Staufen, Germany) for 5 min, and subjected to probe sonication (Qsonica, Newtown, USA) at room temperature for 15 s at 40% amplitude (3 cycles). The supernatant was collected after centrifugation at 4500 rpm for 3 min and the absorbance at 266 nm was measured using a UV spectrometer (Boeco-S220, Germany) with placebo spores as the blank. The amount of 5-FU present in the natural *L. clavatum* spores was calculated using the following equations:

$$\text{Amount of drug (mg)} = \frac{\text{Absorbance at 266 nm} \times \text{dilution factor}}{\text{Slope(standard curve)} \times 1000}$$

$$\%\text{Drug loading} = \frac{\text{Amount of drug}}{\text{Weight of drug loaded spores}} \times 100$$

$$\%\text{Encapsulation efficiency} = \frac{\text{Practical drug loading}}{\text{Theoretical drug loading}} \times 100$$

In-vitro *drug release in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF)*

In-vitro 5-FU release was performed initially for 2 h in SGF followed by SIF to simulate gastrointestinal conditions. 0.1 M hydrochloric acid solution with a pH value of 1.2 was used as SGF and a PBS buffer with a pH of 7.4 was used as SIF. 10 mg of 5-FU loaded *L. clavatum* spores were suspended in 10 mL of release media and incubated at 37 °C while stirring at 50 rpm in an orbital shaker incubator (LM-450D, Yihder, Taiwan). At predetermined time points, 1 mL of release media was collected and replenished with fresh release media. The absorbance in the release sample was measured using a UV spectrometer (Boeco-S220, Germany) at 266 nm. *In-vitro* drug release from Eudragit RS100-coated 5-FU spore formulations was performed using 30 mg of sample in 5 mL of release media.

Statistical analysis

Statistical analysis was performed using two-tailed *t*-tests and $p < 0.05$ was considered as statistically significant. 5-FU encapsulation with natural spores and *in-vitro* release experiments were repeated at least three times and all data are expressed as mean ± standard deviation (SD).

Results and discussion

Microencapsulation of 5-FU into natural L. clavatum *spores*

In order to encapsulate 5-FU into spores, the solubility of 5-FU was first increased to 50 mg/mL by dissolving the drug in a mixture of ethanol and 1 N ammonium hydroxide (1:1). The higher solubility of 5-FU facilitates higher drug loading into the spores as loading into the spores would be limited by aqueous solubility of the drug [26]. By suspending *L. clavatum* spores in a 5-FU solution, the drug can enter the internal cavity of the spores through nanoscale channels on the spore wall [17]. We optimsed three different encapsulation techniques to load 5-FU into natural *L. clavatum* spores and the data are presented in Table 1. With a theoretical loading capacity of 33% 5-FU, the vacuum-assisted loading results in a significantly higher encapsulation efficiency (EE) of 49% ($p < 0.05$) compared to the passive loading technique. In the case of the compression loading technique, a relatively lower EE was observed in comparison to the vacuum loading technique albeit with no significant difference. These observations are consistent with the fact that the loading of drug molecules is influenced by the external energy supplied during the encapsulation process. In the case of passive loading, no external forces are involved and loading is limited by drug passage into the internal cavity by nanoscale channels located on the spore wall [17,22].

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

5-Fluorouracil loading	Theoretical 5-FU loading[a] (%)	5-FU oading (%)[b]	5-FU Encapsulation efficiency (%)[c]
Passive	33	5.2 ± 0.7	15.5 ± 2.1
Compression	33	9.2 ± 2.0	32.0 ± 5.6
Vacuum	33	16.3 ± 0.6	49.0 ± 1.8

[a] Theoretical loading is based on total initial weight of batch (225 mg).
[b] Results are the mean of three independent batches ($n = 3$) with standard deviation.
[c] 5-FU encapsulation efficiency is determined using 10 mg of 5-FU loaded natural *L. clavatum* spores.

The compressed tablet enables spore to incorporate higher drug concentrations into the internal cavity by virtue of the elastic exine wall [22]. It is notable that compression of spores at 5 t was not detrimental, indicating the robust structure of *L. clavatum* spores. The vacuum-assisted loading of 5-FU into *L. clavatum* spores at 1 mbar facilitates forced passage of drug molecules into the internal cavity of spores. Barrier et al. [18,22] reported similar encapsulation data with drugs and proteins encapsulated in SECs produced from *L. clavatum* spores, and these studies support our higher EE of 5-FU into *L. clavatum* spores by vacuum-assisted loading. In the case of 5-FU encapsulation, previous attempts to load 5-FU into crosslinked natural polymers resulted in EE in the range of 8% to 53% based on the drug-to-polymer ratio. Hence, optimization of 5-FU encapsulation into *L. clavatum* spores by three different encapsulation techniques provides greater insight into how to load low aqueous soluble drugs in order to achieve an optimum encapsulation efficiency by simple loading techniques.

Micromeritic properties of 5-fluorouracil loaded spores

To understand the micromeritic properties of 5-FU loaded *L. clavatum* spores, we have performed dynamic imaging particle analysis (DIPA) on the loaded spores. The results from DIPA are presented in Fig. 1 and it is evident from diameter measurements (Fig. 1(a)) that the spores with a native diameter of 30 ± 0.45 μm remain unchanged after 5-FU encapsulation by all three encapsulation techniques. The diameter of spores before and after 5-FU loading is provided in Table S2. Importantly, the 5-FU loaded spores retain the intact microstructure with uniform size distribution. In order to investigate the uniform shape of 5-FU loaded spores, the circularity and aspect ratio were measured and the data are presented in Fig. 1(b) and (c). It is evident from Fig. 1(b) that the circularity of 5-FU loaded spores is near to circular shape and interestingly, our approach to encapsulate 5-FU into spores by the three different techniques was favorable in retaining the native shape of the spore. To support the shape uniformity of spores, our data for aspect ratio of 5-FU loaded spores also indicates that there is no change in spore microstructure. Edge gradient (Fig. 1(d)) indicates that all the micromeritic properties based on image analysis were obtained using well-focused particles.

The images captured during DIPA are presented in Fig. 2(a)–(d) for spores before 5-FU loading, as well as after loading by passive, compression and vacuum loading techniques, respectively. The DIPA images indicate that all spores after 5-FU loading retain well-defined microstructures supporting the DIPA data for the uniform size distributions.

Further, to evaluate the structure and morphology of 5-FU loaded spores, we have analysed *L. clavatum* spores before and after 5-FU loading by SEM. The SEM images after 5-FU loading by

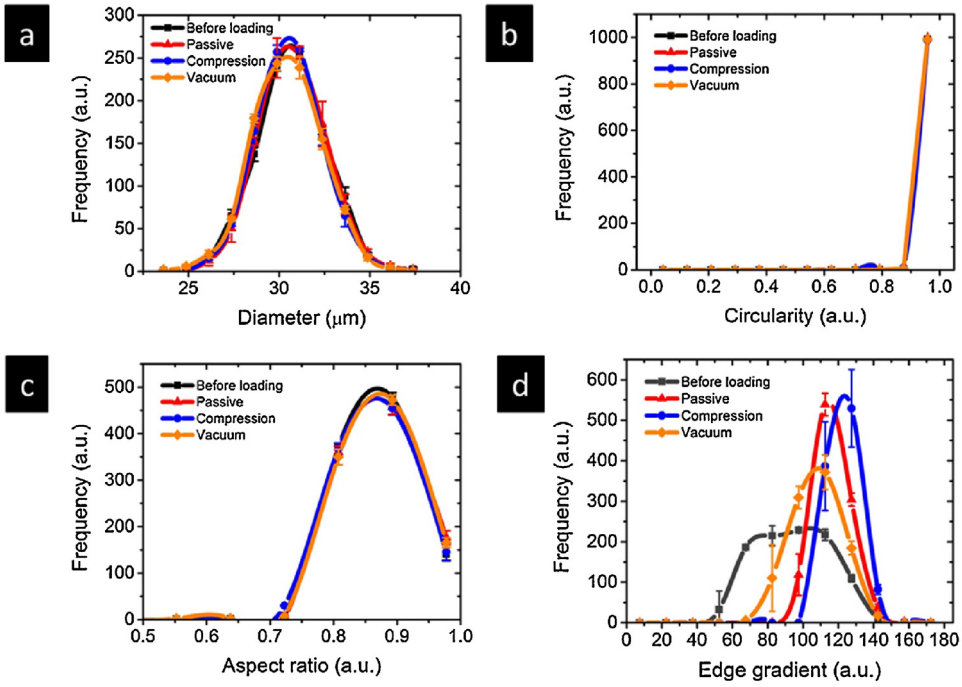

Fig. 1. Characterization of 5-FU loaded *L. clavatum* spore formulations. Diameter, circularity, aspect ratio and edge gradient were analysed by dynamic imaging particle analysis (DIPA) with a 1000 particle count. Representative graphs with standard deviation from three measurements and curve fitting to histograms are presented as (a) diameter *vs.* frequency, (b) circularity *vs.* frequency, (c) aspect ratio *vs.* frequency and (d) edge gradient *vs.* frequency.

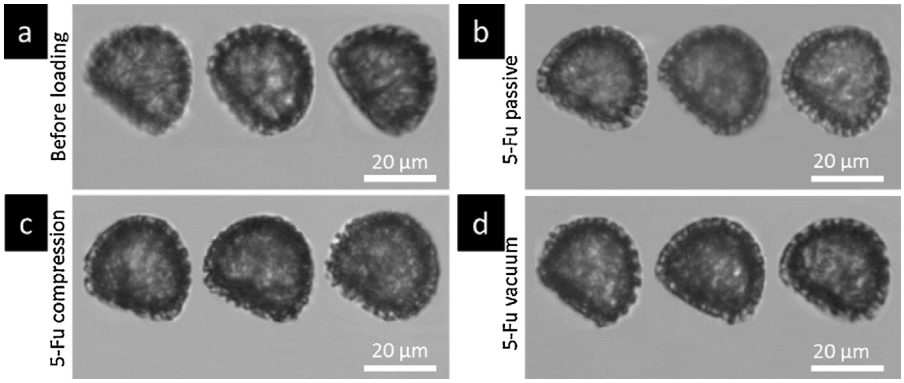

Fig. 2. Dynamic imaging particle analysis images of 5-FU loaded *L. clavatum* spores. Images (a), (b), (c), and (d) represent *L. clavatum* spores before and after 5-FU loading by passive, compression, and vacuum loading techniques, respectively.

passive, compression and vacuum are presented in Fig. 3(a), (b), (c) and (d), respectively. Our structural and morphological data for spores before 5-FU loading indicate characteristic well-defined ornamentation with reticulate structure [23,27] and uniform size distribution. In the case of 5-FU loaded *L. clavatum* spores achieved by the three different encapsulation techniques, the spore's native microstructure and ornamentation is retained. The 5-FU encapsulated spores clearly indicate no detrimental effect to the spore microstructure by drug loading even after the use of external factors such as compression at 5 t and with 1 mbar vacuum. Strikingly, the surface of the 5-FU loaded spore is clean without any evidence of residual drug aggregation suggesting the encapsulated drug is principally inside the spore's internal cavity. Hence, the data for 5-FU loaded spores supports that our simple method to encapsulate 5-FU in natural *L. clavatum* spores offers excellent potential as a multiparticulate oral delivery system with uniform size distribution and well defined surface morphology.

In-vitro *release studies*

To confirm, our results on the EUD coating, we performed SEM analysis and the images of EUD-coated spores using 2.5% and 10% EUD concentrations are presented in Fig. 4(a) and (b), respectively. The surface morphology of natural spores after coating indicates that spores are coated with EUD, interestingly the EUD coating is higher in the case of 10% EUD-coated spores. It is important to note that the muri located on the spores are filled with the coating material which acts as a barrier for 5-FU release.

To investigate *in-vitro* release profiles of 5-FU loaded spores, we performed *in-vitro* release studies of 5-FU loaded spores in simulated gastrointestinal conditions. Fig. 5(a) and (b) depict the 5-FU release profiles in SGF (pH 1.2) and SIF (pH 7.4), respectively. High release rates of up to 90% were observed in the initial 10 min and complete 5-FU release was observed within 60 min due to exit *via* the nanochannels in the exine wall [17]. Similarly higher 5-FU release in SGF for stomach-targeted

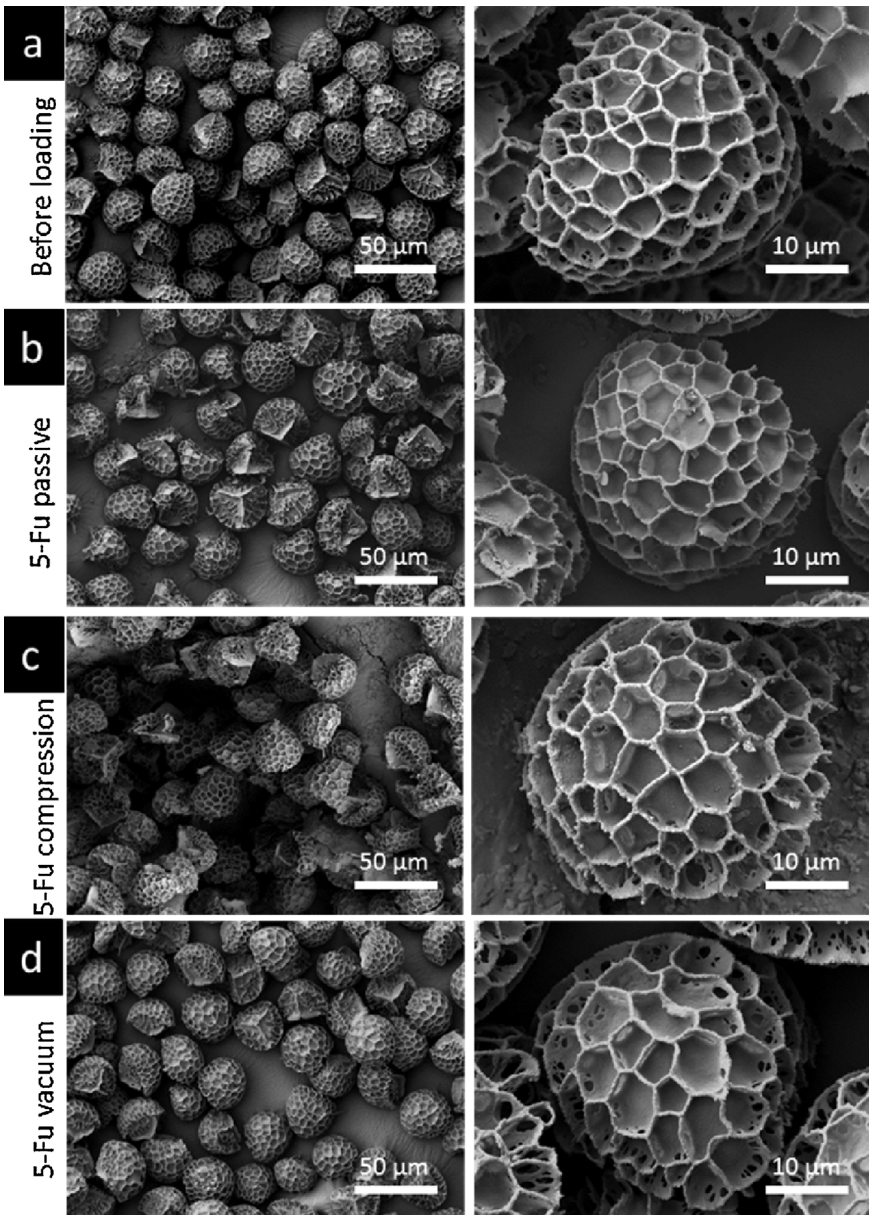

Fig. 3. Characterization of 5-FU loaded *L. clavatum* spores by SEM. SEM images (a), (b), (c), and (d), respectively, represent *L. clavatum* spores before loading and after loading by passive, compression, and vacuum loading techniques.

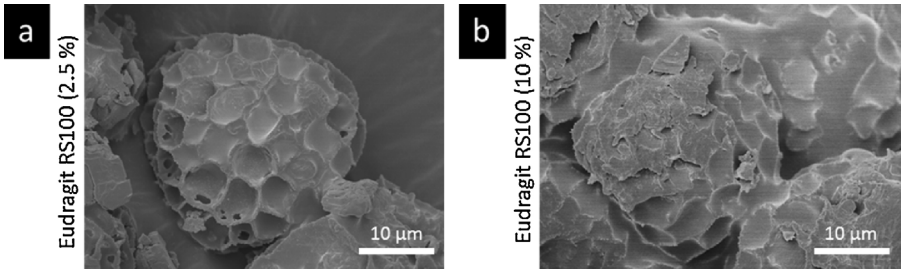

Fig. 4. Characterization of Eudragit RS100-coated *L. clavatum* spores by SEM. (a) 5-FU loaded spores after coating with 2.5% Eudragit RS 100 and (b) 10% Eudragit RS 100.

release was reported by Peeyush et al. [4] using floating microspheres, and recently Diego-Taboada et al. [18] have reported higher ibuprofen release from *L. clavatum* SECs within 1 h in SGF. Hence, the 5-FU release from natural spores indicates that a suitable polymeric coating to retard the drug release in simulated gastrointestinal conditions would be beneficial. We have directed our efforts to modulate 5-FU release from spores by employing a polymethacrylate (Eudragit RS 100) coating. Eudragit RS 100 is a copolymer of ethyl acrylate, methyl methacrylate and is widely used as a coating material to develop controlled release

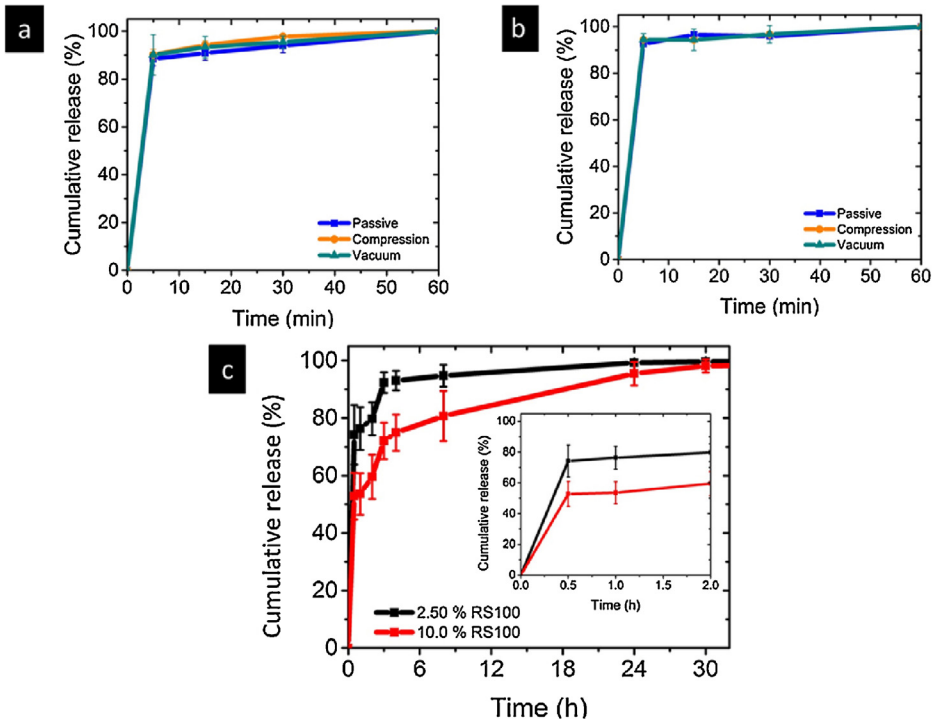

Fig. 5. *In-vitro* release profiles of 5-FU loaded *L. clavatum* spores. Cumulative release profiles of 5-FU loaded spores by passive, compression, and vacuum loading in (a) simulated gastric fluid (SGF pH 1.2 solution) and (b) simulated intestinal fluid (SIF), pH 7.4 phosphate buffer saline. (c) Controlled release of 5-FU from vaccum-loaded spores after Eudragit RS 100 coating in SGF and SIF, and inset indicates initial 5-FU release in SGF in 2 h. All *in-vitro* release studies were performed in triplicate ($n = 3$) and average values with standard deviations are presented.

formulations [24,25]. Our initial coating and *in-vitro* release studies in simulated gastrointestinal conditions using different concentrations of Eudragit RS 100 indicates that coatings with 2.5% w/v and 10% w/v EUD provide a suitable coating on the *L. clavatum* spores. Our data for *in-vitro* release profiles using EUD coated spores are presented in Fig. 5(c) and indicate that the EUD coating significantly ($p < 0.05$) retards 5-FU release in simulated gastrointestinal conditions. The inset (Fig. 4(a)) indicates around 70% of 5-FU is released in the initial 2 h and by increasing the EUD concentration to 10% the 5-FU release is reduced to 50%. Further, *in-vitro* 5-FU release was extended up to 30 h and a significant ($p < 0.05$) difference in 5-FU release was observed with 10% EUD-coated spores in comparison to 2.5% coating, suggesting that 10% EUD coating is beneficial to achieve controlled 5-FU release from spores.

In case of 2.5% and 10% EUD coated spores, the enteric coating covers the spore microstructure, there by closing the nanochannels on the exine wall [17]. The 5-FU release from spores is controlled by the enteric coating on 5-FU loaded spore. There is no lag time observed in 5-FU release from enteric coated spores due to pH independent release behavior of EUD. Further, 5-FU release from EUD coated spores is gradually decreased in controlled fashion based on the EUD concentration used in coating the spores. Our *in-vitro* release data indicates that, 5-FU release from the EUD coated spores is a result of polymer erosion from the surface of spores, as the enteric coating is higher the 5-FU release is lowered during 30 h. Hence, the possible mechanism of 5-FU release from enteric coated spores is a combination of dissolution, diffusion erosion and is consistent with previous finding [25].

We have demonstrated that *in-vitro* release of 5-FU from *L. clavatum* spores can be controlled in gastrointestinal conditions by EUD coating. Similar 5-FU release profiles from modified sodium alginate microspheres were reported by Sanli et al. [28] with controlled release up to 12 h in simulated gastrointestinal conditions. The controlled gastrointestinal release of 5-FU is highly beneficial in the treatment of breast, stomach and colon cancer, and furthermore repeated doses can be avoided. Hence, our results for 5-FU loaded *L. clavatum* spores indicate that spores could be a potential natural material to encapsulate and control the release of 5-FU in gastrointestinal conditions.

Conclusions

The present study reports a cost effective, simple approach to produce oral-controlled release formulations of 5-fluorouracil based on natural *L. clavatum* spores. The vacuum loading technique provides the highest encapsulation efficiency of 49% compared to the passive and compression loading techniques. Micromeritic properties of 5-FU loaded spores confirmed a uniform size distribution, and surface characterization of 5-FU spores verified no evidence of residual 5-FU, indicating encapsulation of 5-FU inside spores. Uniform Eudragit RS 100 coatings on 5-FU loaded spores provide a controlled release of 5-FU for up to 30 h. The demonstrated features of 5-FU loaded spores indicate a potential oral drug delivery system for gastrointestinal cancer treatment.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jiec.2016.01.022.

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