SUPPORTING INFORMATION

Lycopodium clavatum Exine Microcapsules Enable Safe Oral Delivery of 3,4-Diaminopyridine for Treatment of Botulinum Neurotoxin A Intoxication

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In vitro methods

Preparation of sporopollenin LEMs

Allergens, cytoplasm, lipids, and other unwanted compounds were extracted from *L. clavatum* spores (Tibrewala International, Nepal) by methods previously described (S. Barrier. *LWT - Food Science and Technology* 2010, **43**, 73.) The spores (200 g) were refluxed in acetone (800 mL) for 4 h, and then filtered and dried overnight at room temperature. The dry defatted spores were heated at 80 °C in aq. potassium hydroxide (54 g in 900 mL) for 12 h, and the solution was renewed after 6 h. The base hydrolysed spores were then filtered and washed with hot water (5 x 300 mL), hot ethanol (5 x 300 mL), and then dried overnight at room temperature. To complete the extraction, the subsequent spores were heated at 60 °C for 5 days in *ortho*-phosphoric acid (900 mL), filtered, washed with water (5 x 300 mL), 2M aq. sodium hydroxide (1 x 300 mL), water (5 x 300 mL), ethanol (1 x 300 mL), and acetone (1 x 300 mL), then dried at 60 °C.

Encapsulation of 3,4-diaminopyridine

To ensure the release of 3,4-diaminopyridine (Sigma-Aldrich) in a controlled manner, we encapsulated the compound within the LEMs using shellac (Sigma-Aldrich) as a co-encapsulate. A mixture of 3,4-DAP (249.8 mg) and shellac (208.8 mg) in methanol (3 mL) was added to prepared LEMs (1.20 g). This mixture was stirred at room temperature for 30 min, and the solvent was evaporated under reduced pressure. A solution of shellac (402.3 mg) in methanol (1 mL) was added, stirred for 30 minutes, and the solvent was evaporated under vacuum; the process was then repeated once more. Upon drying under vacuum, the LEMs containing 10.1% 3,4-DAP were collected as a fine brown powder. This procedure was used with varying amounts of 3,4-DAP to generate LEMs loaded with 2.3, 4.9, 7.4, and 10.1% of 3,4-DAP.

LC/MS analysis

All experiments were performed on an Agilent MSD 1100 Series electrospray ionization mass spectrometer (Column: SeQuant ZIC-HILIC, 150 x 1, 3.5 μm). Analytes were separated using a 30%-90% gradient of MeCN in 5 mM aqueous NH_4OAc, pH 4.0 over 7 minutes. All data were analyzed using Agilent MassHunter Qualitative Analysis software. Individual calibration curves were prepared for each compound by determining the peak area from the respective extracted ion chromatograms and plotting against a known concentration of the compound being analyzed.

Release studies in PBS and SGF

The release profile of the 3,4-DAP:LEM composites in phosphate buffered saline (PBS) pH 7.4 or simulated gastric fluid (SGF) was measured *via* LC/MS. SGF was made using materials purchased from Sigma-Aldrich and prepared as previously described (N. Polovic, Journal of the Serbian Chemical Society 2004, 69, 533.) The loaded LEMs (5 mg of 2.3, 4.9, 7.4, or 10.1% 3,4-DAP) were added to several vials containing either 1 mL PBS or SGF. At the assigned time point, samples were centrifuged for 2 min at 14,000 rpm. Next, 30 μL of supernatant was added to 200 μL of acetonitrile containing 3,4,5-triaminopyridine (10 μM) as an internal standard. Finally, 50 μL of each sample was analyzed by LC/MS.

Scanning electron microscopy images

A 9 mm diameter, double-sided carbon adhesive disc was stuck to an aluminum scanning electron microscope stub (12.5 mm diameter). LEMs (unloaded, loaded with 3,4-DAP, or loaded with 3,4-DAP and shellac) were carefully sprinkled onto the adhesive surface of the carbon disc and any loosely adhered particles were carefully shaken off. A 25 mm length of a strongly adhesive tape was pressed onto the LEMs that had adhered to the carbon disc. The tape was then slowly peeled off with the intention of fracturing a number of the LEMs for examination. The samples were then placed in a gold sputter coater (Edwards S150B) and gold coated (15 nm thickness), then examined using a Stereoscan 360 scanning electron microscope (Cambridge Instruments).

In vivo methods

Pharmacokinetics

All animal studies were approved by the TSRI IACUC and were performed in accordance with institutional, AAALAC, and USDA guidelines, along with the Animal Welfare Act. To measure the release rate of 3,4-DAP *in vivo*, the loaded LEMs or controls were administered to mice (10 mg spores, $n = 3$). All mice used in this assay were CD-1 mice (30-35 g, 8 weeks old). The LEMs (7.4, or 10.1% 3,4-DAP) and controls were weighed out individually into a 1.5 mL Eppendorf tube. Immediately before administration 0.5 mL of a 20% beta-hydrocyclodextrin/water was added to the tube using a 1 mL syringe. The heterogeneous solution was then mixed to make a suspension and pulled into the syringe. For orally administered compounds, a gavage needle (stainless steel curved animal feeding tube, 18 g, 4 cm length) was affixed to the syringe, any excess air was expelled and 500 µL of solution was administered directly into the stomach. IV injections were given via the tail vein, using a volume of 200 µL. Retro-orbital blood sampling occurred at 0.5, 1, 2, 4, and 8 hrs. All animals exhibiting severe seizure behavior were rapidly euthanized to minimize distress. For each time point, 30 µL of sample was added to 200 µL of acetonitrile containing 3,4,5-triaminopyridine (10 µM) as an internal standard. The sample was thoroughly mixed and then centrifuged at 10,000 rpm for 15 minutes. Finally, 50 µL of supernatant was analyzed by LC/MS using the same conditions as for the *in vitro* release studies. Initial concentrations for the IV condition were approximated from the collected data using a one-compartment model, by extrapolating from the slope of the line during the terminal elimination phase. Half-life and AUC values were calculated using GraphPad Prism v 5.0. Bioavailability was calculated using the formula: $\frac{AUC_{PO}}{AUC_{IV}} \times \frac{Dose_{IV}}{Dose_{PO}} = \mathrm{F}$.

Botulinum neurotoxin mouse lethality assay

Mouse time until death was measured using a lethality assay. All mice used in this assay were female CD-1 mice (25 g, 6 weeks old). A dose of 5 LD_{50} BoNT/A (0.5 mL – 10 LD_{50}/mL) was injected intraperitoneally (IP) for each mouse; this BoNT/A solution was generated from a standard that had been titrated *via* quantal IP bioassay (five dilutions; 30 mice per dilution). Forty-five minutes after BoNT/A administration, LEMs (25 mg/kg of 3,4-DAP) or 3,4-DAP (10 mg/kg) were diluted into 0.5 mL of 20% beta-hydrocyclodextrin/water, then immediately administered to the mice *via* oral gavage.

Statistical analysis

The BoNT/A lethality assay data was analyzed using Prism (ver. 5.02, GraphPad Software Inc.) *via* one-way ANOVA, followed by Tukey's multiple comparison method to determine significance. Error bars on all graphs represent SD values.

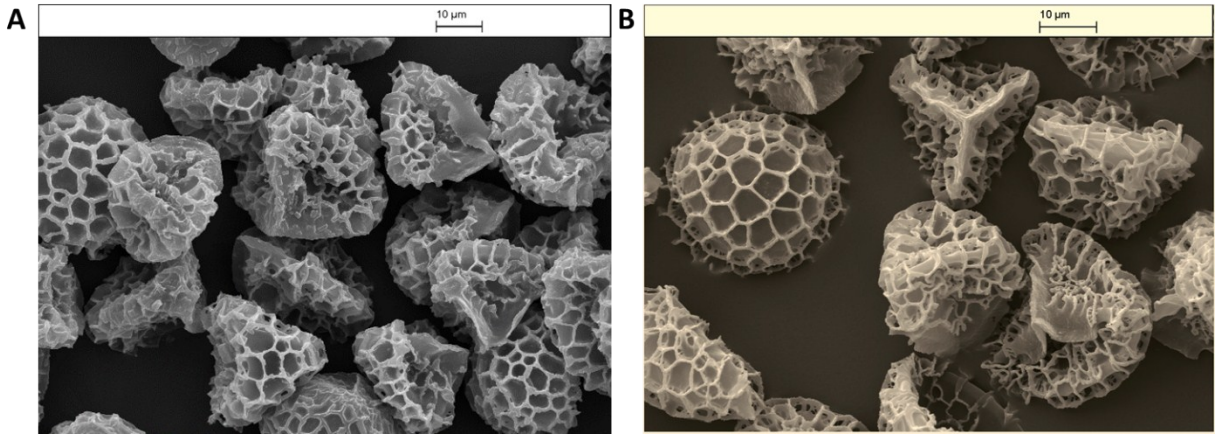

Figure S1. Scanning electron micrograph images of *Lycopodium clavatum* spores that have been co-encapsulated with shellac A) before and B) after incubation in SGF (pH =1.5) and simulated intestinal fluid (pH = 7.4).

	3,4-DAP IV	3,4-DAP PO	3,4-DAP / LEMs / Shellac (%)				
			7.5 / 40 / 52.5	10 / 40 / 50	10 / 50 / 40	10 / 0 / 90	10 / 90 / 0
Dose	2.5	10	18.5	25	25	25	25
AUC	4.29	9.72	5.15	22.99	73.76	0	ND
F	100	56.7	16.2	54.2	(54.2-100)	0	ND
$t_{1/2}$	1.04	1.28	0.61	0.55	0.99	0	ND

Table S1. Pharmacokinetic analysis of 3,4-DAP and 3,4-DAP loaded LEMs in CD-1 mouse plasma (n = 3). Parameters reported are Dose (mg/kg), average F (%), average AUC ($\mu mol \cdot h \cdot L^{-1}$) and $t_{1/2}$ (h). Oral 3,4-DAP given as phosphate salt. ND = Could not be determined due to seizure activity.