Spore exines increase vitamin D clinical bioavailability by mucoadhesion and bile triggered release

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(Dedicated to Professor Gordon Shaw who pioneered early studies on the structural characteristics of sporopollenin)

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Abstract: Sporopollenin exine capsules (SpECs) are microcapsules derived from the outer shells (exines) of plant spore and pollen grains. This work reports the first clinical study on healthy volunteers to show enhanced bioavailability of vitamin D encapsulated in SpECs from *Lycopodium clavatum* L. spore grains *vs* vitamin D alone, and the first evidence (*in vitro*, *ex vivo* and in *vivo*) of mechanisms to account for the enhancement and release of the active in the small intestine. Evidence for mucoadhesion of the SpECs contributing to the mechanism of the enhancement is based on: (i) release profile over time of vitamin D in a double blind cross-over human study showing significant release in the small intestine; (ii) *in vivo* particle counting data in rat showing preferred retention of SpECs *vs* synthetic beads; (iii) *ex vivo* ^{99m}Tc labelling and counting data using rat small intestine sections showing preferred retention of SpECs *vs*

synthetic beads; (iv) *in vitro* mucoadhesion data. Triggered release by bile in the small intestine was shown *in vitro* using solid state NMR and HPLC.

1. Introduction

Vitamin D_2 (ergocalciferol) and D_3 (cholecalciferol) have an important function in calcium and bone metabolism, as well as being neuroprotective agents and important for mitigating inflammation.[1] There has been recent interest supplementing vitamin D for the treatment of COVID-19 patients, with vitamin D combined with melatonin being suggested as a treatment for pulmonary infection by COVID-19 by strengthening the immune system.[2, 3]. In addition, it has been advocated that vitamin D supplementation should be administered to both multiple sclerosis (MS) patients and those with clinically isolated syndrome (CIS), where signs of disease may be delayed, and likelihood of new demyelination changes lowered based on MRI examinations.[4] More generally, it has been suggested that there is a need to identify effective vehicles to address public health strategies for vitamin D deficiency that remains as a global problem, particularly where there are limitations on sunlight exposure, whole body coverage such as in the Middle East. or the relative scarcity of vitamin D in diets.[5] The recommended intake is 600-800 IU/day of vitamin D, but it is reported to be lower at 204 to 276 IU/day, generally.[6] Oral intake of vitamin D is preferred since it is convenient, painless and does not require sterilised conditions, but complicated due to the differences in the potency of current-over-the-counter vitamin D formulations.[7]; hence, why parenteral vitamin D administration may be given.[8, 9] Therefore, a vehicle that could enhance bioavailability *via* the oral route is attractive and could have clinical applications. Despite this perceived advantage, to date only relatively modest enhancements in bioavailability have been reported for oral formulations. In a clinical study involving children suffering from severe chronic cholestasis, micelles capable of stabilising vitamin D prepared using a water-soluble form of vitamin E (tocopheryl succinate polyethylene glycol-1000) showed *ca* 3 % enhanced absorption compared to vitamin D alone.

[10] A 2.5-fold enhanced absorption of vitamin D in rats was found using a β-cyclodextrin formulation, compared to vitamin D alone.[11] Alternative methods of vitamin D delivery include nano-encapsulation in casein micelles,[12] giving good stability upon storage and a marginal improvement in bioavailability in humans.[13] A bioavailability study in rat showed a 25% enhanced bioavailability with microencapsulation of vitamin D compared to other oil-based systems over several days.[14]

In the study reported here using healthy human volunteers, a microencapsulated form of vitamin D was investigated using sporopollenin exine capsules (SpECs) extracted from *L. clavatum* (club moss) spores. This choice was made because ~~in~~ an earlier human study showed a very significant (×10) bio-enhancement for the oral delivery of the lipophilic eicosapentaenoic acid (EPA) ethyl ester when loaded in the same type of SpECs.[15] The use of a microencapsulated form is more attractive than the use of nanotechnology since there are increasing concerns about safety of nanoparticles.[16] SpECs are round (*ca* 33 μm diameter) of uniform morphology.[17] The spores from which they are extracted are readily available in bulk quantities and marketed mostly as herbal remedies and for pyrotechnics. The adaptability of *L. clavatum* SpECs for oral delivery has potential since their fabric is of the polymer sporopollenin, which is amphiphilic and highly stable to strong acid and strong base; hence, sufficiently stable to withstand the acidic and basic condition in the different section of the gastrointestinal tract (GIT). The polymer is composed largely of carbon, hydrogen, and oxygen. In the case of SpECs from *L. clavatum*, it has been proposed, based on mass spectrometric studies, that the structure of sporopollenin constitutes a backbone of macrocyclic oligomer and/or polymer polyhydroxylated tetraketide-like monomeric units and a poly(hydroxy acid) network in which the hydroxyl end groups are covalently attached by ether links to the hydroxylated macrocyclic

backbone.[18] Importantly, in the case of SpECs from *L. clavatum* the relatively harsh extraction procedure renders them protein free and non-allergenic.[19] It is of note that *L. clavatum* SpECs have also been extracted by a wide range of digestive enzymes indicating their potential stability to a gastrointestinal environment.[20-23] In addition, Cho *et al* described *L. clavatum* SpECs as being the most robust of the groups of species tested when incubated in simulated gastric fluid (SGF), but noted some holes and fractures in them by dynamic image particle analysis (DIPA) after such exposure.[24] Also, some minor degradation has been reported when SpECs of different species are exposed to blood.[25, 26] The walls of *L. clavatum* SpECs are porous with nano diameter-sized channels[27] that give access to a wide range of materials to be encapsulated within their chamber.[28] The channels also allow encapsulated material to be released depending on the nature of the surrounding environment[29, 30] and the type of functional groups that are attached to the surface of the SpECs.[30]

Further support for the potential of SpECs for use in oral delivery has been indicated by several *in vitro* [19, 26, 31-43] and *in vivo* studies. For example, SpECs coated with shellac were shown to enable oral delivery of the water soluble 3,4-diaminopyridine to treat botulinum neurotoxin A intoxication in mice without any ill effects being observed to the animals.[44] In another *in vivo* study it was demonstrated that metformin, used for type 2 diabetes loaded into a formulation of excipient (alginate) coated sporopollenin microcapsules extracted from *L. clavatum*, in combination with raw pollen from and *Phoenix dactylifera*, could deliver the drug *via* the oral route with an enhanced bioavailability of 1.215 times higher compared to pure drug alone.[45] Finally, *L. clavatum* SpECs have been shown to be: (i) non-toxic to human umbilical vein endothelial cells (HUVEC) using a variety of assays all of which showed that SpECs did

not reduce the proliferation or induce cell death or cell damage *in vitro* at concentrations between 100 ng/mL-10 μg/mL; (ii) effective in taste masking conducted in human taste masking trials involving fish oils and ibuprofen respectively, also indicating their non-toxicity.[19, 46]

2. Material and methods

2.1. Spores, chemicals and instrumentation

Lycopodium clavatum (club moss) spores were purchased from Tibrewala International (Nepal). The spore grains homogeneous from the one species and were devoid of extraneous plant material (Figure 1).

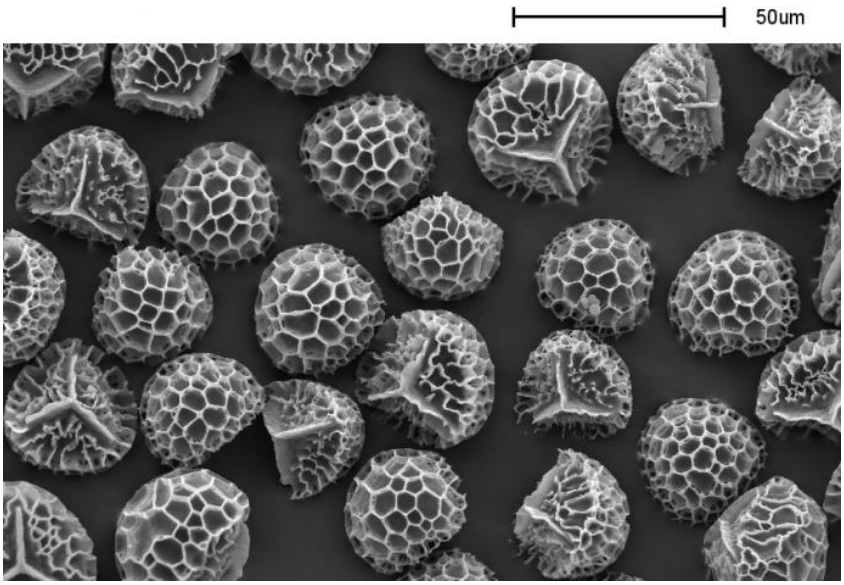

Figure 1: SEM micrograph of *Lycopodium clavatum'* spores as received from Tibrewala International, Kathmandu, Nepal.

Acetone was from Aldrich UK, and potassium hydroxide, ethanol, orthophosphoric acid, hydrochloric acid, and sodium hydroxide were from Thermo Scientific™. 9000 Series Glass

Particle Standards was from Fisher Scientific UK Ltd. Combustion elemental analysis was conducted using a Fisons Instruments Carlo Erba EA 100 C H N S analyser. Vitamin D_2 (ergocalciferol) was purchased from Merck. Scanning electron micrographs were obtained using a Leica Cambridge Stereoscan 360 Scanning Electron Microscope. Samples were mounted on a metal stub with an adhesive and coated under vacuum with carbon before being coated with 200 Å gold film. X-ray diffraction measurements were made using a PANalytical CubiX PRO diffractometer (Amelo, Netherlands) at a wavelength of 1.5418 Å generated by a copper focus tube operated at 45 kV and 40 mA. Samples were mounted on a silicon wafer mount and analysed in reflection geometry in $\theta - 2\theta$ configuration over the scan range 2° to 40° 2θ with 1.905 seconds exposure per 0.0025067° increment. Adhesion measurements were made using a Stable Micro Systems (Surrey, UK) Texture Analyser TA.Xtplus. HPLC system comprised a Shimadzu 20A series degasser, quaternary pump, autosampler, variable wavelength absorbance detector, and 10A series column oven (Shimadzu Corporation, Kyoto, Japan) and was controlled and data processed by Shimadzu LabSolutions software.

2.2. Extraction of SpECs from L. clavatum spores

Lycopodium clavatum (club moss) spores were used as purchased. Scanning electron microscopy showed the spore grains to be devoid of extraneous particles (Figure 1). Sporopollenin exine capsules (SpECs) were extracted from *L. clavatum* spores as follows: Spores (300 g) were heated at 80 °C for 12 h in an aqueous solution of potassium hydroxide (54 g in 900 mL), the solution being renewed after 6 h, filtered, washed with water (5 × 300 mL) and ethanol (5 × 300 mL), and dried overnight in open air. The particles were heated at 60 °C for 5 days in *ortho*-phosphoric acid 85% (900 mL), filtered, washed with water (5 × 300

mL), 2 M NaOH (2 × 300 mL), water (6 × 500 mL), PBS (2 × 250 mL), water (2 × 500 mL), ethanol (2 × 250 mL) then heated in ethanol (900 mL) at reflux for 4 h, filtered and washed with ethanol (2 × 250 mL). After this, the SpECs were suspended in acetone (500−700 mL) and sonicated for 30 min, filtered, and dried in air to afford SpECs (102 g). Typical elemental analysis of sporopollenin of the SpECs (g/100 g) was C, 68.90; H, 7.90; N 0.00 %. Moisture (w/w, loss on drying @ 100 °C 18h), <0.10%; Ash 0.6 ±0.1%; protein (by MALDI-TOF/TOF-MS) < 0.01 ppm. ICP-OES analysis of the inorganic elements in dry mass % w/w of *L. clavatum* SpECs: Al, <0.002; Ca, <0.35; Cu, <0.005; Fe, <0.005; Mg, <0.0146; Mn, <0.0025; Na, <0.003; Ni, <0.002; P, <0.10; Si, <0.08; Zn, <0.0025; S, <0.015 Values of <0.001 ppm by were obtained by ICP-OES for Ag, As, Au, B, Ba, Be, Bi, Cd, Ce, Co, Cr, Cs, Cu, Dy, Er, Eu, Ga, Gd, Ge, Hf, Hg, Ho, In, Ir, K, La, Li, Lu, Mo, Nd, Pb, Pd, Pr, Pt, Rb, Re, Rh, Ru, S, Sb, Sc, Se, Si, Sm, Sn, Sr, Ta, Tb, Te, Th, Tm, U, V, W, Y, Yb, Zr. Values of <0.001 ppm by were obtained by ICP-OES for Ag, As, Au, B, Ba, Be, Bi, Cd, Ce, Co, Cr, Cs, Cu, Dy, Er, Eu, Ga, Gd, Ge, Hf, Hg, Ho, In, Ir, K, La, Li, Lu, Mo, Nd, Pb, Pd, Pr, Pt, Rb, Re, Rh, Ru, S, Sb, Sc, Se, Si, Sm, Sn, Sr, Ta, Tb, Te, Th, Tm, U, V, W, Y, Yb, Zr.

2.3. Encapsulation of vitamin D_2 into SpECs, initial, loading and efficiency of loading

Encapsulation was performed by mixing SpECs (536.7 mg) with an ethanolic solution (0.5 mL) of vitamin D_2 (212 mg) to give a homogeneous mixture with gentle stirring, which was then subjected to a vacuum (*ca* 2 kPa) for 2 h to facilitate loading of the vitamin into the SpECs through the nano-porous wall of the microcapsules. The *initial* loading of 283.2 mg/g (28.3 % w/w) was determined by dividing the mass of vitamin D used (212 mg) by the total mass (748.7 mg), i.e. the encapsulated vitamin D (212 mg) plus the mass of *L. clavatum* SpECs (536.7 mg).

A calibration curve was constructed from working standard solutions of vitamin D_2 in ethanol over the concentration range 24–100 µg/ml by plotting absorbance of vitamin D_2 (*A*), obtained by UV-vis spectroscopy at 238 nm, *versus* vitamin D_2 concentration [*c*]. The vitamin D_2 samples were prepared in six different concentrations. Linear regression analysis of *A versus* [*c*] was attained using Microsoft Excel® and gave the equation $A = 0.0034[c] - 0.0001$ (Table S1 and Figure S1). The loading efficiency of vitamin D_2 in SpECs was determined by stirring 20 mg of the vitamin D_2-loaded SpECs in ethanol (10 mL) at room temperature for 10 min and subsequent filtration (sinter porosity grade 3) of the suspension. This extraction was repeated using a separate sample; however, the remaining SpECs were not dried and re-extracted with ethanol. The filtrate was diluted to 50 ml and the recovered mass of vitamin D_2 was calculated by using the aforementioned equation (Table S2) to be 254.11 ±4.90 mg/g (n = 3). Therefore, based on the mass initially loaded into SpECs (283.2 mg/g) the loading efficiency was determined to be 90 ± 2 %. It is of note that the formulation could be stored at room temperature for several weeks with the same efficiency of recovery.

2.4. Oral delivery of vitamin D_2 trial in humans

Trial structure: Institutional ethics committee approval was granted for the study and six normal healthy volunteers (4 males, 2 females) were recruited after informed consent (approved by the South Humber Research Ethics committee). None of the volunteers was taking any known medication or over-the-counter supplements. The volunteers were randomly allocated (computer-generated randomisation) to take either 200 mg of vitamin D_2 (ergocalciferol) with SpECs encapsulation, or 200 mg of ergocalciferol without spore encapsulation. The mean age of the volunteers was 34.6 (11) years and body mass index was 25.2 (2.1) kg/m^2, and their mean

systolic blood pressure was 132 (11) mm Hg and diastolic pressure was 78 (7) mm Hg. The adjusted calcium was normal 2.42 (0.12) mmol/L. None of the participants had secondary hyperparathyroidism with mean parathyroid hormone level of 52(4) pg/mL. The median 25-hydroxyvitamin D (25OHD) level was 44.38 (32.2) nmol/L. The vitamin D_2 supplement was taken on an empty stomach after participants have fasted for 10 h. Blood samples were withdrawn after fasting (time 0), then at 15, 30, 45, 60, 120, 180 and 240 minutes after taking the supplement. Serum was separated in a refrigerated centrifuge and stored at −80 °C until batch analysis was done. 25OHD was analysed using tandem mass spectrometry, the current gold standard in clinical practice for measurement of 25OHD, with a coefficient of variation of 5%. Participants were then given a 1 month washout period before being asked to take the other supplement and blood sampling was repeated as described above. During the time between blood samples, the volunteers were allowed to move around and perform their daily activities but to avoid sustained exercise.

Statistical analysis: Since this is a proof of concept study, a formal power calculation was not done. Based on a previous study on fish oils[15] and SpECs, the sample size of six individuals was taken considering the cross over design. Area under the curve (AUC0-4h) was used to determine the bioavailability of 25OH vitamin D_2 from the two different ergocalciferol preparations. The mean AUC for 25OHD was calculated using the linear trapezoid method, baseline levels were normalised to 0 (Tables S3 a-b). Since the subjects number was small, potentially jeopardising the assumption of normality a non-parametric statistical test, the Wilcoxon signed rank test, was used to compare the 25OHD from two different supplements using SPSS software version 15. Data were recorded as median (interquartile range).

2.5. In vitro *release of vitamin D_2 from loaded SpECs into:*

2.5.1 PBS (pHs 7.4 and 9.0) and simulated gastric fluid (SGF) (pH 1.5), respectively

For the *in vitro* vitamin D_2 release studies, the loaded SpECs (0.02 g) were stirred (37 °C for 60 min) accordingly, in: (i) 10 mL of an aqueous solution with composition corresponding to phosphate buffer saline (PBS) pH 7.4 (Table S4); (ii) 10 mL of buffer pH 9.0 (Table S5) (iii) 10 mL of an aqueous solution with composition corresponding to a simulated gastric fluid (SGF), pH 1.5 at 37 °C for 60 min (Table S6). The suspension, as appropriate, was then filtered at regular intervals and subsequently stirred in ethanol (10 mL) and filtered. This operation was repeated twice, and the filtrate was diluted to 50 mL. Aliquots of the ethanolic solution were taken and the amount of vitamin D_2 remaining was determined by UV-vis spectroscopy. Data as a percentage were based on 100% being the initial loading of vitamin D_2.

2.5.2 Dioleoyl-phosphatidyl-choline (DOPC)

Samples of dioleoyl-phosphatidyl-choline (DOPC) and vitamin D_2 were prepared at mole ratios of 9:1: 10 mg of DOPC was used per sample. Vitamin D_2 pre-partitioned into the lipid bilayer was prepared by co-dissolving DOPC and vitamin D_2 in chloroform/methanol (1:1, v/v). The solvents were evaporated under vacuum, and the resulting lipid cake was suspended in 1 ml of doubly distilled water, frozen in liquid nitrogen and then lyophilized overnight under high vacuum. The dry lipid mixtures were hydrated with 200 ml of appropriate buffer in D_2O for proton NMR and then subjected to three freeze/thaw cycles. Vitamin D_2 partitioning from the SpECs into lipid bilayers was measured by preloading SpECs with vitamin D_2 (50 μg per mg of SpECs) and then incubated them with the appropriate amount of DOPC to give a 9:1 mole ratio. This mixture was suspended in D_2O buffer at pH 4.2 or 7.2. The lipid mixtures were

centrifuged to remove excess water. A 4-mm MAS rotor was filled with the resulting pellets. The samples were incubated at 37 °C for 24 hours before measuring. The NMR experiments were carried out on a Bruker Avance II 500-MHz spectrometer using a 4-mm magic angle spinning (MAS) probe operating at a frequency of 500.1013 MHz. ^{1}H NMR spectra were externally referenced to tetramethylsilane at 0 ppm. Experiments were carried out with an MAS speed of 8 kHz, conducted with a typical $\pi/2$ pulse length of 7 s and a relaxation delay of 4 s. All NMR measurements were performed at 37 °C.

2.5.3 Bile salt solutions at 0.1 mg/mL and 1.0 mg/mL respectively

The *in vitro* vitamin D_2 was analysed by a Shimadzu 20A HPLC. The mobile-phase was isocratic 0.5% formic acid, 4.5% ddH_2O, 95% methanol (v/v), with a flow rate of 1.8 ml/min. Following injection of 15 µl, an Agilent Zorbax Eclipse XDB column (4.6 x × 150 mm, 5 µm particle size; Agilent Technologies, Santa, CA, USA) provided chromatographic separation, and vitamin D_2 was detected by measuring absorbance at 265 nm, eluting as a single peak. Quantification was relative to a standard curve of vitamin D_2 dissolved in PBS, passed through a 0.45 µm PTFE syringe filter and diluted 1:1 in MeOH prior to analysis (v/v).

2.5.4 In vitro *comparison of detachment force and work of adhesion for SpECs, Carbopol®-934 and chitosan*

Test samples of SpECs and the control materials, namely Carbopol®-934 and chitosan, were prepared as powders and as 150 mg tablets (1 cm diameter) with a hardness 40-100. Measurements were made using a Stable Micro Systems Texture Analyser TA.Xtplus. Samples were run in triplicate, were each adhered to the 9 mm probe of the Texture Analyser. The mucin

solution (50 ml containing 5% mucin, 95% water) was dropped over the agar surface and the probe-adhered sample was left to contact with the mucin-agar surface for 1, 2 and 4 minutes before being withdrawn from the mucin. In each case the amount of resistance force related to the strength of the interaction between the samples and mucin was measured.

2.6 Ex vivo *trials to compare the adhesion properties of unfilled SpECs vs control beads in excised rat small intestine using:*

2.6.1 Particle counting

Freshly excised rat small intestine without the duodenum was used immediately from the trial (approved by the AstraZeneca Research Ethics committee). The tissue was warmed in PBS at 37 °C for 20 min., flushed slowly with PBS (2 x 10 ml) at 37 °C (*ca* 1 ml/min) to remove intestinal contents and cut laterally into 4 sections, each 10 cm in length tied off at the bottom with string. Then, each section was dosed and with 1.5 ml of a 1:1 mixture of SpECs and reference ChromoSphere™ black polymer beads (dry, black-dyed polystyrene divinylbenzene) (50 μm) at 37 °C, of SpECs (34 μm) and reference beads (50 μm) in 0.5% Methocel™ and polysorbate 80 vehicle and tied off at the top. The dosed intestine segment incubated at 37 °C in pre-warmed PBS for 20 minutes. Segment contents released and flushed with pre-warmed PBS using different volumes of PBS and different flush applications. Section of each segment taken, cut open and the SpEC and reference beads adhering to the intestinal surface were counted aided by light microscopy (10× magnification) (Table 1). (Figures S3 − S6)

2.6.2 ^{99m}Tc Radiolabeling

^{99m}Tc Radiolabeling of SpECs: $Na^{99m}TcO_4$ (300 µl, 55-65 MBq) and $SnCl_2.2H_2O$ (0.01 M, 100 µL) were added to SpECs (5 mg in 100 µL saline) and shaken at 90 °C for 30 minutes. The reaction was left to cool, taken up in a 1 mL syringe, filtered through a 0.22 µm filter (Merck, USA) and washed with saline (1 mL). The trapped radiolabelled exines were recovered from the filter with PBS (1 mL). The presence of radioactivity in the solution or on the particles was determined by either iTLC (using a Lablogic ScanRam TLC plate reader) or a Capintec CRC-55t Dose calibrator.

^{99m}Tc Radiolabeling of Thermo Scientific™ 9050 Series Glass Particle Standards (49.0 ± 1.4 µm): $Na^{99m}TcO_4$ (300 µL, 55-65 MBq) and $SnCl_2.2H_2O$ (0.01 M, 100 µL) were added to SpECs (5 mg in 100 µL saline) and shaken at 90 °C for 30 minutes. The reaction was left to cool and used directly. A portion was taken up in a 1 mL syringe, filtered through a 0.22 µm filter (Merck, USA) and washed with saline (1 mL) demonstrating quantitative incorporation.

Radiochemical stability of ^{99m}Tc labelled SpECs and Thermo Scientific™ 9000 Series Glass Particle Standards (49.0 ± 1.4 µm): ^{99m}Tc labelled SpECs and Glass Particle Standards were incubated in simulated gastric fluid (SGF, 1 ml) and incubated at 37 °C for 5 hours, after which they were filtered through a 0.22 µm filter (Merck, USA) and washed with SGF (1 ml): no radioactivity was observed in the filtered solution demonstrating that the ^{99m}Tc was fully retained on the exines/ particles under these conditions.

Ex vivo *small intestine retention*

Freshly excised rat small intestine without the duodenum (30 cm) was used immediately from the trial after excision and washed thoroughly with saline at 37 °C to remove intestinal contents using a flow rate of 1 mL/min (Figure S7). Test solutions containing equivalent amounts of radioactive samples namely, "free" technetium ($Na^{99m}TcO_4$), ^{99m}Tc-labelled Glass Microspheres and ^{99m}Tc-labelled SpECs respectively of twice the internal volume of the intestine (*ca.* 70 mL) were passed through the section of intestine. The section was then washed with saline at 1 mL/min flow rate with five times its internal volume (*ca* 400 mL). Radioactivity amounts in syringe, intestine and release solution were compared to determine retention (*3.7.2.*) (Figure S7). (approved by the University of Hull Faculty of Science & Engineering Research Ethics committee):

2.7. *Animal* in vivo *trial to compare the rate of passage of SpECs and reference ChromoSphere™ black polymer beads (50 µm) though rat gastrointestinal tract*

A preliminary *in vivo* trial in mouse was conducted, in which four mice were each fed with empty SpECs (1.4 mg) by gavage and then split into two equal groups after 30 min. Mice in the first group were culled after 2 h, and the second group after 6 h. Their organs (intestines, kidney, spleen, lung and heart) were sectioned and blood, urine and feces were taken and were examined for the presence of SpECs by fluorescent light microscopy

A more detailed second trial was conducted focused on the migration of SpECs *versus* reference ChromoSphere™ black polymer beads (50 µm) though rat gastrointestinal tract (approved by the AstraZeneca Research Ethics committee). However, before the rats were fed with the particles their feces were collected 0-24 h and 24-48h. Neither beads nor SpECs nor were found in any of the samples. Also, for this pre-dosing trial and the subsequent dosing trial,

where rats were fed with beads and SpECs, the feces were pretreated by acetolysis[47] to to remove most of the fecal material and enable SpECs to be viewed more clearly. Rats in this main trial were dosed using approximately 1 million exines and 1 million reference beads to each rat in the study (SpECs: 354 million/g , reference beads: 15 million/g). Rats (Hans Wistar; male, n = 3, 250-300 g weight, fed as normal) were dosed by gavage and feces were collected from rats (Rat 1, Rat 2 and Rat 3) every 8 h for a total period of 40 h after administration and were dried in a freeze-dryer overnight. The trial was repeated twice, as indicated in Table S7, as Run 1 and Run 2. Feces were collected and analysed as follows. Dry feces (130 mg) were suspended in acetic anhydride (4 mL) and sulfuric acid (0.5 mL) and heated at 94 °C for 20 min. The suspension was centrifuged and the solid washed with methanol, 2 M methanolic NaOH, and finally methanol. The solid was collected and solvent removed by evaporation. The remaining solid was resuspended in an aqueous solution of PBS (0.5 mL) and particle counting was preformed with the aid of a haemocytometer. Counting data were given as total number of particles collected per interval, and this was done in two separate runs. The overall count was taken as an average of the particles collected (n = 6) (Table S7).

3. Results

3.1. Extracting SpECs (exines) from L. clavatum spores and loading with vitamin D_2

SpECs from *L. clavatum* spores were extracted using a modified method reported previously, involving sequential treatment with potassium hydroxide and phosphoric acid (Section *2.2*)[48]. The nano-porous sporopollenin walls[27, 28] of SpECs enable microencapsulation to be performed by subjecting a homogeneous mixture of the empty SpECs and an ethanolic solution of ergocalciferol (vitamin D_2) to a vacuum (*ca* 2 kPa) to give a dry loaded product with a loading of 254.11 ±4.88 mg/g (n = 3) (Section *2.3*). The loading and

efficiency of loading (90 ± 2 %) was determined by extraction of the loaded vitamin D_2 with hot ethanol and assaying the amount extracted by UV/vis spectroscopy (Section *2.3*).

3.2. Characterisation of vitamin D_2 loaded L. clavatum SpECs

Scanning electron microscopy (SEM) micrographs of the SpECs before (Figure 2) and after (Figure 3) encapsulation of the vitamin showed a clean outer surface in each case, indicating that the vitamin was retained within the cavities and/or channels within the walls of the SpECs. This observation was supported by fracturing the vitamin D_2 loaded SpECs to reveal crystalline material deposited in their cavities.

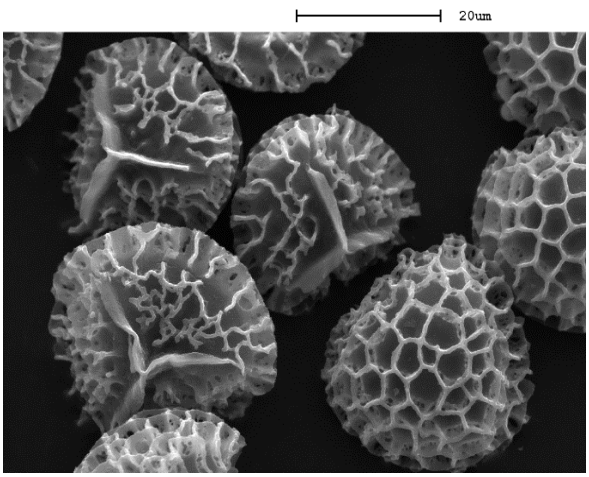

Figure 2. SEM micrograph of empty SpECs extracted from *L. clavatum* spores (Scale bar 20 µm).

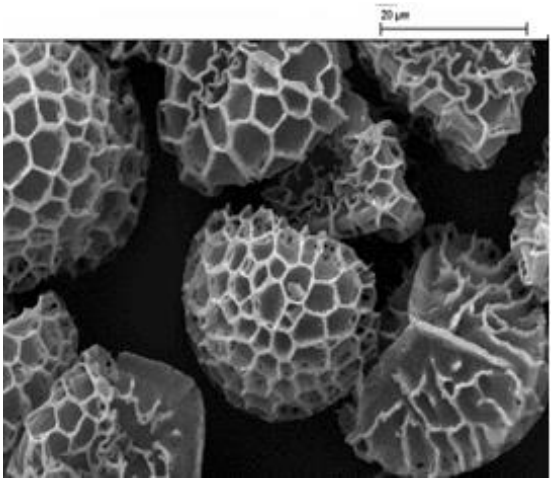

Figure 3. SEM micrograph of SpECs loaded with vitamin D_2 (1:1 w/w) showing a relatively clean surface of the SpECs, devoid of vitamin D_2 crystals (Scale bar 20 µm).

Figures 4 and 5 depict fractured SpECs before and after loading, respectively. Figure 4 depicts an empty chamber within a SpEC and Figure 5 shows the vitamin D_2 to be within the chamber in its crystalline form, which is supported by a comparison of XRD diffractograms of SpECs alone, SpECs loaded with vitamin D_2 and vitamin D_2 alone (Figure 6; traces A, B and C respectively) indicating that the encapsulated vitamin was in a crystalline form. Fourier Transform Infrared (FTIR) spectroscopy of the product demonstrated that the encapsulation process had not altered the chemical structure of either the SpECs or vitamin D_2.

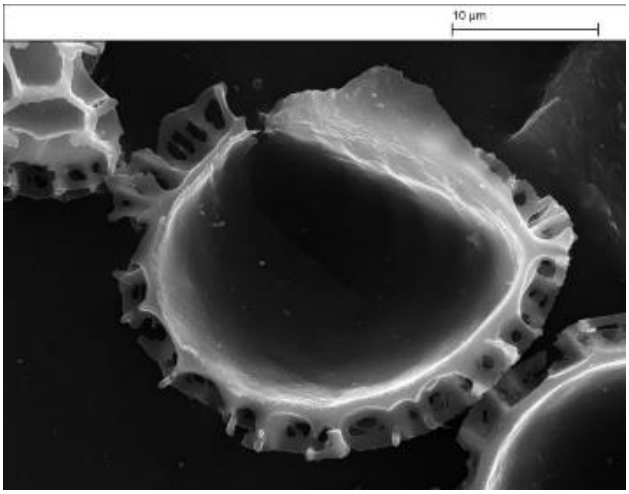

Figure 4. SEM micrograph of a cracked open empty SpEC showing an empty cavity (Scale bar 10 µm).

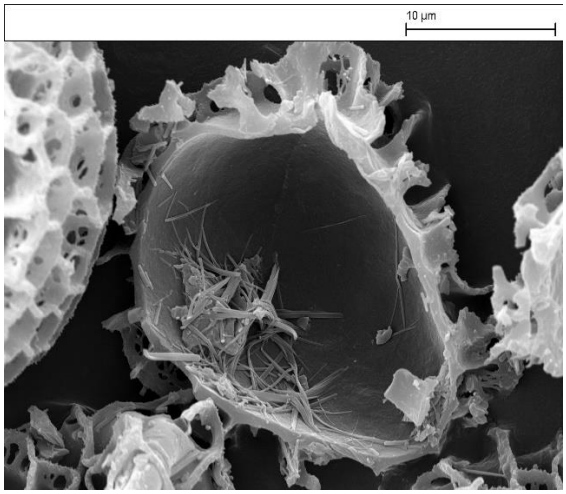

Figure 5. SEM micrograph of a cracked open SpEC loaded with vitamin D_2 (1:1 w/w) showing crystals in the cavity, and a relatively clean outer surface (Scale bar 2 µm).

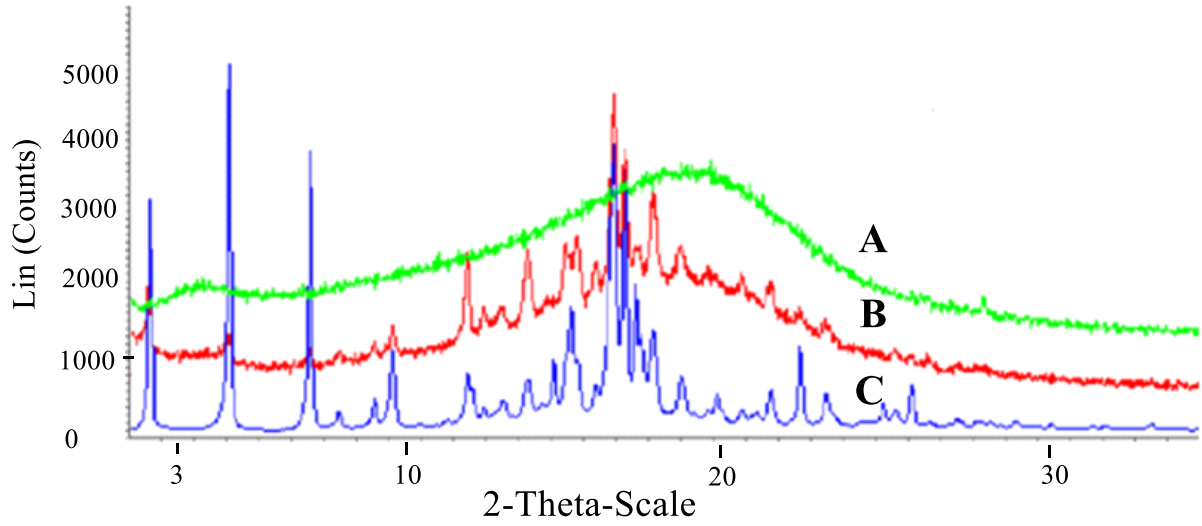

Figure 6. XRD diffractograms of SpECs alone (**A**), SpECs loaded with vitamin D_2 (**B**) and vitamin D_2 alone (**C**) indicated that the encapsulated vitamin is in a crystalline form

3.3. Double-blind randomised cross over clinical study with encapsulated vitamin D_2

The study involved, six normal healthy volunteers took part (4 males and 2 females). Key results are presented in Figure 7 (see Section *2.4*). Subjects were crossed over to the alternative preparation 4 weeks later and the study repeated. The baseline vitamin D_2 levels did not differ between the two arms (study 1, 87.2 ±21.2 nmol/L, *vs* study 2, 89.4 ±26.2 nmol/L 4 weeks later ($p = 0.68$). It was noted that the adjusted calcium was normal [2.42 (SD 0.12) mmol/L] for the volunteers. The mean area under curve (AUC) of 25OHD (i.e., 25-hydroxyvitamin D, with a coefficient of variation of 5%) from the study involving vitamin D_2 encapsulated in SpECs [M = 10548.9 (SD 32.2)] was calculated using the linear trapezoid method. The baseline levels were normalised to 0. Importantly, there was a 10 (±2)-times higher uptake of vitamin D_2 in the

group when it was encapsulated compared to the group where vitamin D_2 was taken without SpECs encapsulation [M=1049.5 (SD 52.4) nmol/l], where values for the vitamin D levels without encapsulation did not differ compared to baseline over time (Figure 7; Tables S3a and S3b). This relatively large rise over 4 h indicated the effect of the SpECs encapsulation on bioavailability of the vitamin D_2, in comparison to non-encapsulated vitamin D_2.

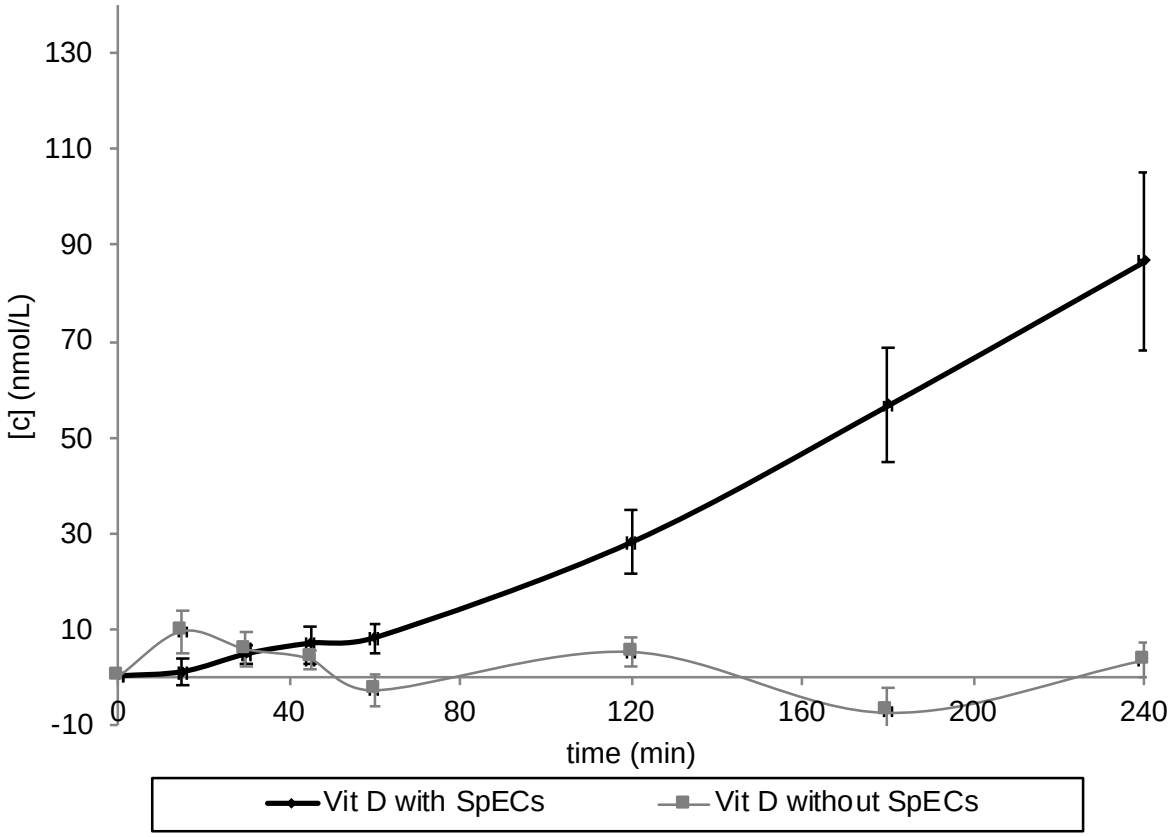

Figure 7. The change in mean vitamin D_2 serum level in human volunteers (n = 6) over time obtained from oral administration of vitamin D_2 alone or encapsulated in SpECs extracted from *L. clavatum* spores. *NB:* The baseline levels were normalised to 0 and it is notable that the vitamin D levels without encapsulation did not differ compared to baseline (Tables S3a and S3b).

3.4. Stability (release) vitamin D_2 in loaded SpECs in PBS (pHs 7.4 and 9.0) and simulated gastric fluid (pH 1.5)

Vitamin D_2−loaded SpECs (25.4 % w/w) were incubated in simulated gastric fluid (SGF) for 1 h at 37 °C during which 99 ± 1 % (n = 3) of the loading was found by UV-vis spectroscopy to be retained in the SpECs. Similar high retention (93 ± 1 % and 99 ± 1 %) (n = 3) was found in parallel experiments over 1 h at 37 °C in phosphate-buffered saline (PBS) at pH 7.4 and 9.0 respectively (Section *2.5.1*) (Tables S3-S5).

3.5. In vitro *release of vitamin D_2 from loaded SpECs into:*

3.5.1 Dioleoyl-phosphatidylcholine (DOPC)−D_2O dispersion

Using magic-angle spinning solid state ^{1}H NMR (ssMAS NMR) it was found that the alkenic CH signal from vitamin D_2 at ~6.2 ppm could only be detected when it was present in the fluid phase of a dioleoyl-phosphatidyl-choline (DOPC)/D_2O dispersion, but not when encapsulated in the SpECs (Section *2.5.2*). Thus, in Figure 8(a) the signal at ~6.2 ppm for the presence of vitamin D_2 was observed indicating that it had partitioned from the loaded SpECs into the fluid phase at pH 7.4. In contrast, when the experiment was repeated in mildly acidic conditions at pH 4.2 (Figure 8(b))**,** the vitamin could not be detected in the fluid phase. It is of note that the signal at ~6.2 ppm from the vitamin could be seen in a sample that had been pre-partitioned into the lipid bilayer (Figures 7 (c)) but was absent when the vitamin had not been added (Figure 8(d)).

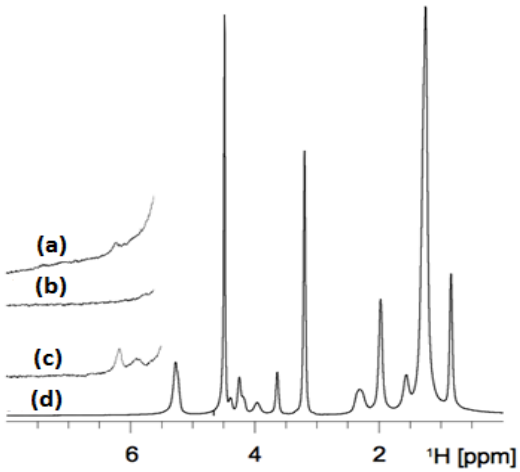

Figure 8. ^{1}H MAS NMR spectra of dioleoyl-phosphatidyl-choline (DOPC)−D_2O dispersions with 10 mol % vitamin D_2 incorporated. Spectra were collected at 318 K with 8 kHz spinning [(a), (b) and (c) are 16-fold expansions of the region above 5.5 ppm.]: (a) is vitamin D_2 encapsulated in SpECs and incubated with DOPC at pH 7.4, 37 °C for 24 h; (b) is vitamin D_2 encapsulated in SpECs and incubated with DOPC at pH 4.2, 37 °C for 24h; (c) is DOPC with the vitamin D_2 pre-partitioned into the lipid bilayer; (d) is DOPC/D_2O in the absence of vitamin D_2.

3.5.2 Bile

To further support the hypothesis, that release of vitamin D_2 from loaded SpECs, could be triggered by a surfactant, the loaded SpECs were agitated in the presence of bile acid salt at concentrations and pHs typically found in the small intestine. Thus, Figure 9 shows release of the vitamin from suspensions at physiologically relevant concentrations (0.1 mg/mL and 1 mg/mL) of bovine bile salts and vitamin D_2 loaded SpECs in PBS (pH 7.4) over 60 min (Section *2.5.3*).

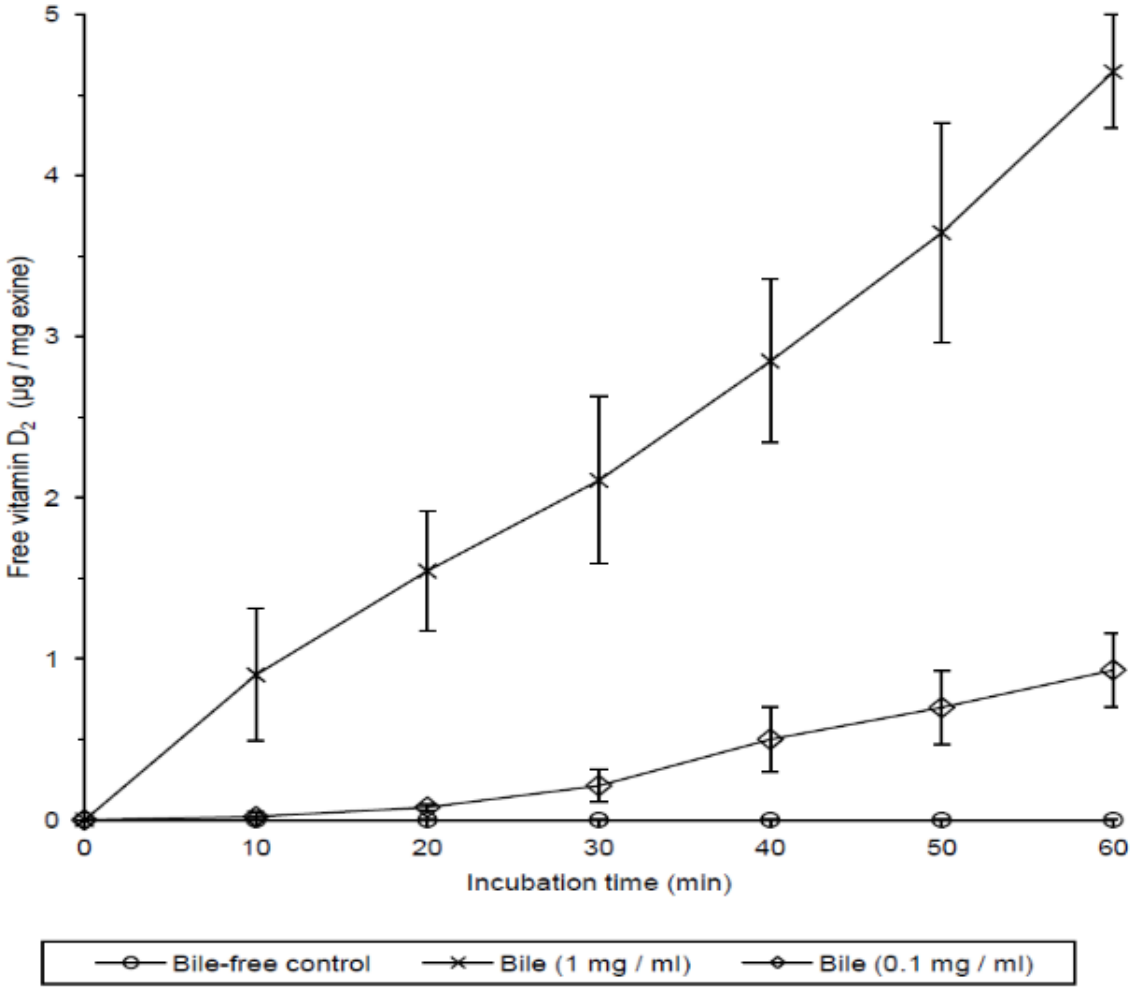

Figure 9. Release of vitamin D_2 release from loaded SpECs in suspensions of PBS (pH 7.4) with bile salt concentrations 0.0, 0.1 and 1.0 mg/mL) over 60 min. The positive increase in release correlated with the bile salt concentration of the surrounding liquid suggesting that gastrointestinal release is likely to occur at or downstream of the duodenum (n = 3, ± 1 S.D.).

3.6 Experiments to investigate mucoadhesion and/or bioadhesion of SpECs

3.6.1. Preliminary in vitro *Detachment Force and Work of Adhesion studies*

The initial *in vitro* experiments to investigate bioadhesive properties of SpECs were to compare them with examples of other known mucoadhesive materials, namely the well-known Carbopol® and chitosan. Therefore, measurements were made of the detachment force and work of adhesion of empty SpECs using a Stable Micro Systems Analyser (Section *2.5.4.*). These parameters are well recognised as preliminary indicators of substances possessing mucoadhesive properties.[49, 50] The parameters were measured for adhesion to mucin, to allow comparison of SpECs with Carbopol®-934 and chitosan, as depicted in Figures 10 (a-d). Overall, either in powder or tablet form, the SpECs showed better adhesive properties than chitosan and marginally less than Carbopol® in most cases. However, in tablet form at 120 seconds, the SpECs appeared to exhibit better adhesion properties than Carbopol® (Figures 10(c) and 10(d)).

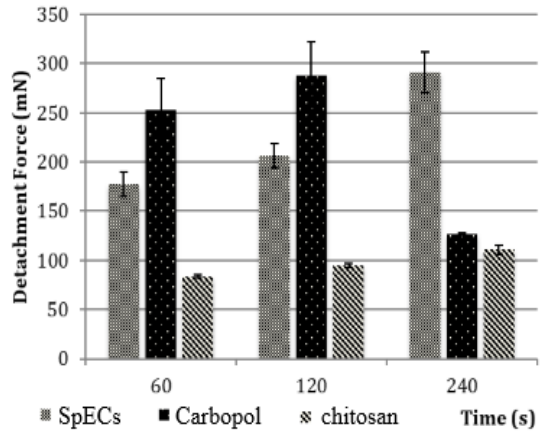

Figure 10(a). Detachment force (DF) *versus* time comparing powders of SpECs, Carbopol® and chitosan (n = 3). (Section *2.5.4.*)

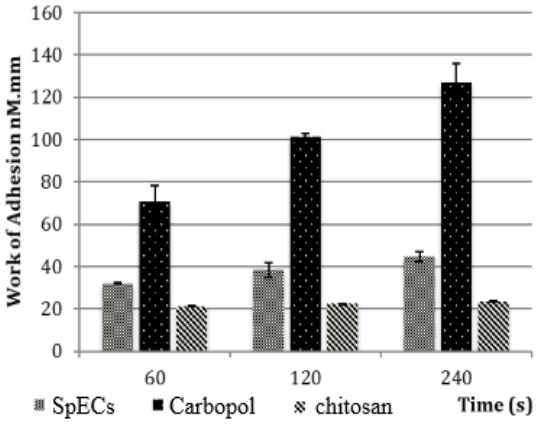

Figure 10(b). Work of adhesion (WoA) *versus* time comparing powders of SpECs, Carbopol® and chitosan (n = 3).

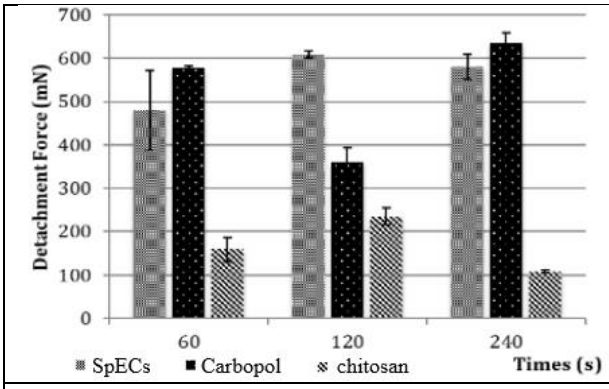

Figure 10(c). DF *versus* time comparing tablets of SpECs, Carbopol® and chitosan (n = 3).

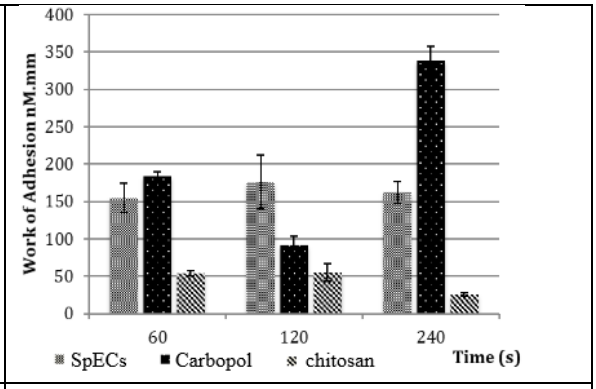

Figure 10(d). WoA *versus* time comparing tablets of SpECs, Carbopol® and chitosan (n = 3).

3.7. Ex vivo experiments to compare retention of SpECs versus synthetic beads passed over rat small intestinal mucosa

3.7.1. ChromoSphere™ Dry Dyed beads

The possibility of mucoadhesion explaining the enhanced bioavailability was further explored in preliminary *ex vivo* experiments to compare directly the adhesion of SpECs to open sections of rat small intestinal mucosa *versus* the adhesion of synthetic beads, namely, ChromoSphere™ Dry Dyed beads (polystyrene− divinylbenzene copolymer beads)(approved by the AstraZeneca Research Ethics committee). These are slightly larger (50 µm) than the size of SpECs (34 µm), but have a smooth surface. Thus, a mixture of equal numbers of polymer beads and SpECs were introduced onto an opened flat segments (40 mm × 40 mm) of a rat small intestinal mucosa

surface, which were flushed with different volumes of PBS at different intensities of flushing (S3-S6). Following flushing, the remaining polymer beads and SpECs were counted (Table 1), aided by light micrscoopy, to determine the ratio of beads *versus* SpECs that remained. Significantly more SpECs (2 to 6-fold) remained attached on the rat small intestinal mucosa after flushing with PBS (S3-S6), thus indicating a preferred attachment to the intestinal mucosa of SpECs over synthetic beads of a similar size.

Flush volume of PBS (ml)	Intensity of flush with PBS (*ca* ml/min)	Mean ratio of SpECs:beads
5	2	2:1
5	1	6:1
15	2	6:1
15	2	6:1
15	1	3:1

Table 1. Ratio of SpECs to synthetic black ChromoSphere™ Dry Dyed polymer beads that remain attached to rat intestinal mucosa following flushing with PBS at different volumes and flush intensities. (Section *2.6.1.*) (Figures S3-S6).

3.7.2. ^{99m}Tc-labelled glass beads

The experiment *3.7.1* indicated a preferred bioadhesion of SpECs over the Dry Dyed polymer beads over a small flat section of intestine. However, a system was required to provide a more realistic model with a larger surface area to better mimic a living small intestine and

provide a more accurate means of measuring the relative migratory characteristics of the two types of particles over the intestinal surface. Therefore, a section of the small intestine of a mouse was selected and a competition was made between ^{99m}Tc radiolabelled SpECs, similarly labelled ^{99m}Tc radiolabeling glass beads and 'free' radioactive technetium -99m. It was shown that little (5%) 'free' radioactive technetium -99m was retained in the sample of rat intestine, compared to 33% for the ^{99m}Tc-labelled glass beads and 88% ^{99m}Tc for the labelled SpECs (Section *2.6.2*). These data showed SpECs to adhere to the tissue 2.7 ± 0.2 times (n = 2) more tenaciously than glass spheres of a similar size, which was comparable with those found in the first preliminary *ex vivo* trial shown in Table 1.

3.8. In vivo *studies*

Before conducting the more extensive rial, an initial study was conducted in which four mice were each fed with empty SpECs by gavage and then culled after 2 h and after 6 h accordingly. No SpECs were detected by fluorescent light microscopy in any of their organs (intestines, kidney, spleen, lung and heart), blood, or urine; however, some SpECs were found along the small intestine lining (Section *2.7*). This observation was also made in a second more extensive *in vivo* trial involving six rats where SpECs were seen on the surface of all the small intestines excised after 8 h as depicted in the scanning electron micrograph shown in Figure 11 (Section *2.7*). Importantly, neither the SpECs nor beads appeared to be damaged, corroded or partly digested (Figure 12) when examined in the feces. The SpECs and beads were counted after the periods 0-8 h, 8-16 h, 16-24 h, 23-32 h and 32-40 h. Data are provided in Table S7 and summarised in Table 2.

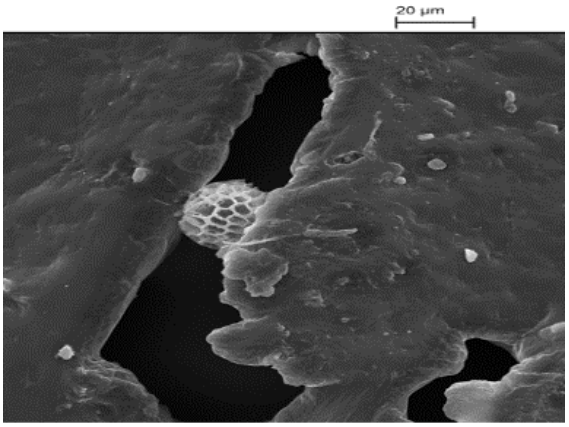

Figure 11. Scanning electron micrographs of a SpEC found lying close to gut villi (Scale bar 20 µm).

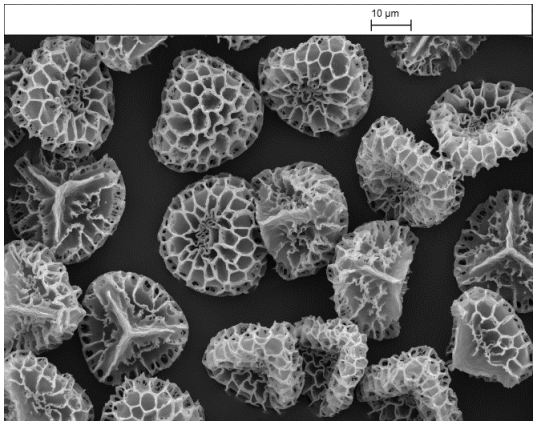

Figure 12. Scanning electron micrographs of SpECs found in feces after cleaning by acetolysis (Scale bar 10 µm).[47]

After 40 h 78% (SD 9%) of the beads ingested were recovered, compared with 39% (SD 15%) of the SpECs, supporting the finding that the beads were passing through the GIT more rapidly.

Rat number (Run number)	SpECs recovered in feces after 40 h (%)	ChromoSphere™ black polymer beads recovered in feces after 40 h (%)
1 (1)	36.10	77.14
2 (1)	41.02	89.17
3 (1)	17.58	74.88
1 (2)	57.62	95.08
2 (2)	36.00	78.85
3 (2)	47.60	50.93
	Average = 39 (SD = 15)	Average = 78 (SD = 9)

Table 2. Summary data of % SpECs *versus* % synthetic beads (ChromoSphere™ black polymer beads (i.e., dry, black dyed polystyrene divinylbenzene, 50 μm) recovered in rat intestinal feces 40 h following dosage of *ca* 1 million of each particle type to each rat. NB Run 2 is a repeat of Run 1 with three separate rats (Table S7) (Section 2.7).

4. Discussion

We have reported that SpECs can enhance the bioavailability of a lipid (EPA)[15] by *ca* 10-times in a preliminary clinical trial with human volunteers, though the mechanism of enhancing bioavailability was unknown. In this study we chose the lipophilic vitamin D_2 for a clinical study because it has a totally different structure to EPA and might indicate a broader scope to the enhancement of bioavailability using the SpECs delivery system. Therefore, as an immediate comparison we chose to undertake a study in the clinic to assess bioavailability

before embarking on preliminary, in *vitro*, *en vivo* or *in vivo* studies to understand any mechanism involved. Important to this study, was that the vitamin was loaded into SpECs with no additional confounding excipients, to accord with the EPA study[15]. Vitamin D_2 was readily encapsulated into SpECs with a 90 ±2 % w/w loading efficiency. The study (Figure 7), showed that the bioavailability of vitamin D_2 (Figure 7), resembled that of the absorption profile and bioavailability (*ca* 10-times using area under curve calculations) observed for EPA.[15] over 4 h. This finding led to investigating a mechanism for the phenomenon of the enhanced bioavailability.[15] Despite the limitation of the small number of participants and the study duration, the 10-times enhancement in bioavailability is to our knowledge the largest increase observed for vitamin D_2 compared to all other modalities explored for this vitamin to date.

Lymphatic transport is a major contributor of oral vitamin D_2 bioavailability.[42] After uptake into the enterocyte, vitamin D_2 (LogP = 7.2, [43]) associates with intracellular lipoproteins that are subsequently exocytosed and taken up into the intestinal lymphatic system. In this study, lymphatic transport is possibly assisted by the association of the vitamin D_2–loaded SpECs immediately adjacent to the enterocytes of the gut lumen within the mucus coat. Since the appearance in serum occurs after *ca* 60 minutes (Figure 7), absorption into systemic circulation appears likely in the small intestine. Therefore, *in vitro* tests were made to establish the stability of the vitamin loaded SpECs in solutions of different pHs as would be experienced *en route* to the small intestine. Thus, after 1 h good retention of the vitamin was observed in SGF (98 ± 2 %) and PBS (93 ± 1 % at pH 7.4 and 99 ± 1 % at pH 9.0). Due to the low solubility of the vitamin in water (50 mg/L at 25 °C) it was extrapolated that any further loss of the vitamin over the extra 3 h would be minimal and would not explain the high level of enhanced bioavailability as indicated in Figure 7. Further experiments were explored to view the influence of a surfactant

at different pHs. Initially, experiments with and without dioleoyl-phosphatidylcholine (DOPC) using magic-angle spinning solid state proton nuclear magnetic spectroscopy (^{1}H MAS ssNMR) (Section *3.5.1.*) supported the possibility that the vitamin being retained during transit through the gastric-lipid environment of the stomach and released in the small intestine triggered by a change in pH in combination with a surfactant. This was further supported using bile acid salt at concentrations and pHs typically found in the small intestine (Section *3.5.2.*). The addition of bile to PBS suspensions of loaded SpECs, rather than using a multi-component simulated intestinal fluid, supported the importance of bile concentration alone at pH 7.4 in the release process. These observations indicated that passage through the duodenum environment could initiate the release process in the small intestine and that the vitamin D_2 load was protected by SpECs encapsulation during gastric passage. Consideration was then given to bioadhesion between the SpECs at the gut wall influencing a high concentration of loaded SpECs at intestinal wall. Paunov recently observed that alginate coated sporopollenin microcapsules attached to rat stomach mucosa following oral administration gave a 1.2 times enhancement over pure metformin alone.[45] Indeed, the passage of SpECs across the gut wall into the bloodstream seems unlikely in any large quantity, at least to explain the high- enhanced of bioavailability as in Figure 7 due to their being too large to pass *via* either the paracellular or intracellular routes. Furthermore, the SpECs are robust in strong acid and alkali and were not seen to fragment by light microscopy in the media used use in the studies described in Section 2.3; hence, any breakdown into smaller particles that could traverse the gut wall seems extremely unlikely. Therefore, to explore the potential of bioadhesion playing a role towards enhanced bioavailability standard detachment force and work of adhesion measurements [49, 50] were made comparing SpECs (as powder and tablet) with the well-known mucoadhesive

materials Carbopol® and chitosan. Overall, SpECs showed stronger bioadhesive properties than chitosan and in the case of tablet form at 120 seconds, the SpECs appeared to exhibit better adhesion properties than Carbopol® (Figures 10(c) and 10(d)).

It is perhaps of no surprise that the SpECs show the adhesive properties indicated in Figures 10a-d since the physical[51] and chemical[30, 52, 53] [54] properties of the outer surface of SpECs are in common to those that are required for possessing adhesive properties. SpECs surfaces are amphiphilic due to their possessing cross−linked hydrophobic saturated and unsaturated carbon chains, to which are attached functional groups capable of hydrogen bonding, aromatic or aliphatic alcohols and carboxylic acids.[18, 55] These functional groups are likely to endow SpECs with the ability to attach to epithelial tissue or the mucus coat on a biological surface. Important to bioadhesion is the pronounced surface morphologies of pollen and plant spores [56] that are specific to the individual plant species and are not found on the surfaces of syntheic microparticles. In the case of SpECs from *L. clavatum* spores (Figures 2 and 3), the morphology is characterised by raised features, with sharp edges that increase the surface area in comparison to regular spheres and increase probability of physical attachment.[57, 58] It is of note, for example, that pollen grains from *Helianthus annuu*s having a spiky surface, have attracted attention for their adhesion properties[59, 60]. The investigation of bioadhesion was extended to preliminary *ex vivo* experiments where equal numbers of SpECs and ChromoSphere™ Dry Dyed beads (polystyrene−divinylbenzene copolymer beads; (50 μm)) were applied to sections of rat small intestinal mucosa. The particles that remained following washing with buffer at different rates were counted (Tables S3-S6), It was observed that significantly more SpECs (2 to 6-fold) (Table 1) than beads remained attached on the rat

small intestinal mucosa after flushing with PBS, thus indicating a preferred attachment of SpECs to the intestinal mucosa.

In support of the findings given in Table 1, an additional *ex vivo* study was conducted in which ^{99m}Tc-labelled glass beads (50 μm), in competition with ^{99m}Tc-labelled SpECs and 'free' radioactive technetium ($Na^{99m}TcO_4$) were passed through a 30 cm section of rat small intestine (Section *3.7.2*). The results showed SpECs to adhere 2.7 ± 0.2 times (n = 2) more tenaciously than spheres of a similar size, which was comparable with those found in the first preliminary *ex vivo* trial shown in Table 1.

Further to the adhesion studies an *in vivo* trial was made to discover the location of the SpECs within the mouse GIT and organs shortly after ingestion (Section *3.8*). SpECs were not found in any of the sections (*ca* 400) of organs (intestines, kidney, spleen, lung and heart), and samples of blood, urine and feces. Fluorescent light microscopy was used because SpECs and pollen grains are strongly fluorescent[61] and easily identified according to plant species by their morphology and topography[62]). It is of note that our observations were not in agreement with the *L. clavatum* spores being found in the blood of humans[63] even though they are approximately the same size and have much the same topography of the SpECs extracted from them. Furthermore, both the spores and SpECs are too large to pass into the bloodstream *via* the M-cells of the Peyer's patches;[64] hence, our observations might not seem too surprising. Empty exines from *L. clavatum* spores have been reported to translocate into the intestinal wall of mice following oral ingestion;[65] however, we were unable to find an example to support this finding in our study using either fluorescent microscopy or scanning electron microscopy (SEM). In contrast, SpECs were found within the bulk of material passing through in the intestines with some running very close to the villi, as shown by SEM (Figure 11). In any case,

we propose that it would seem unlikely that the observed enhanced bioavailability, approaching 10-times, could be explained by either transcellular or paracellular mechanisms. Hence, some form of mucoadhesion or bioadhesion enabling a concentration of exines close to the gut wall might seem more likely to be involved. Some SpECs were found in the feces, but in this initial study the collection period for the feces was too short to take full account of the number of SpECs expelled. Therefore, a second preliminary *in vivo* trial was undertaken to count SpECs, aided by a haemocytometer, expelled in the feces of rat over a longer period (40 h) after administration, in competition with synthetic beads, which were of the same 50 µm black ChromoSphere™ Dry Dyed beads used in the foregoing *ex vivo* study (Section *3.7.1.*). Two runs were made where in each of the runs three rats were each fed the same number SpECs as beads by gavage (Section *3.7.2.*). The results showed that over 40 h (Table 2), the beads passed through the GIT at twice the speed of the SpECs, which approximates to results from the two *ex vivo* experiments described in Sections *3.7.1.* and *3.7.2.* respectively. After 40 h few particles of either beads or SpECs were observed; hence, recording was not continued.

Since only 39% of SpECs (*versus* 78 % beads) were recovered after this time, it is perhaps indicative that they are moving slowly through the GIT or being digested by gut bacteria. In favor of the former hypothesis, the SpECs recovered were apparently the same topography, morphology, and size as those fed to animals, as viewed by light microscopy and SEM, although only a limited number were viewed by the latter technique. Interestingly, we did not view any fragments of SpECs in the feces. Also, it of note that not all the beads were recovered after 40 h, with some 28 % being unaccounted for. The robustness of *L. clavatum* SpECs in SGF was in keeping with Cho's earlier reported study.[24] The lack of apparent or significant degradation

might indicate that digestion of the SpECs on transit through the GIT is possibly not taking place; hence, we could be seeing evidence of prolonged migration through the GIT. Interestingly, prolonged transit of exine through the GIT in humans has been reported in studies involving prehistoric feces and coprolites.[66]

Conclusions

We report the first clinical study involving a double-blind cross-over study with healthy human volunteers showing enhanced bioavailability of vitamin D_2 *ca* 10 times higher than vitamin D_2 alone. Furthermore, to our knowledge, it is largest reported increase in bioavailability for this important vitamin *via* all modalities reported to date. It is proposed that the results offered herein indicate the potential of using SpECs for high dose delivery of vitamin-D as an alternative to using parenteral route in treating vitamin-D deficiency,[60] though larger definitive clinical trials are needed. Significantly, this work provides the first evidence, based on a combination of *in vivo*, *en vivo* and *in vivo* data, to indicate that the enhancement in bioavailability is due to bioadhesion of SpECs in the small intestine. Importantly, *in vitro* release of vitamin D_2 from SpECs was not observed following treatment of either SGF or PBS but in contrast was triggered selectively by bile salts adding strong support to their role in triggering release of the active in the small intestinal section of the GIT. It is worthy of emphasizing hat the vitamin D_2 loaded SpECs obtained from *L. clavatum* L were used without any coating or excipients. Therefore, the data presented are not confounded by the inclusion of any properties associated with such materials. Furthermore, SpECs from this inexpensive and bulk available source, could provide a simple, and efficient vector for the delivery of biologically active lipids of various types.

Finally, it is suggested that the data here indicates that SpECs have a prolonged transit time through the GIT with their potential as an extended-release system, delivering actives into the large intestine.

Conflicts of interest

Grahame Mackenzie has an Emeritus position at the University of Hull and is the Technical Director for Sporomex Ltd, which has patents pertinent to the contents of this article.

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