Spore exines increase vitamin D clinical bioavailability by mucoadhesion and bile triggered release. [Dataset]

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2022

Supplementary Material (SM)

MATERIALS AND METHODS

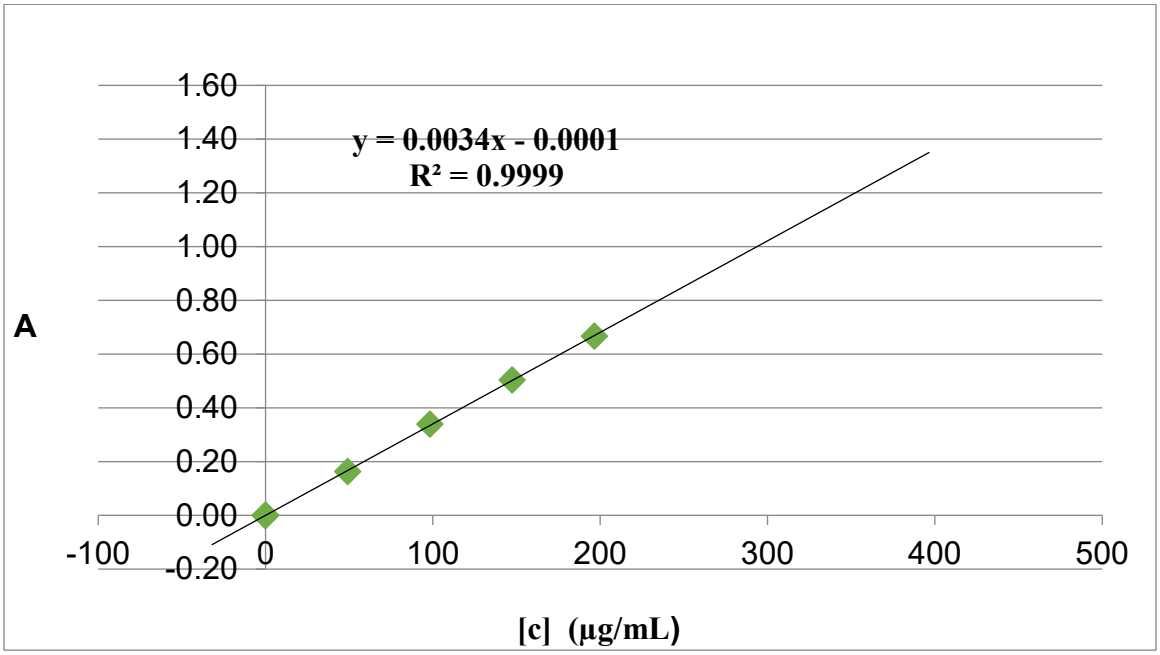
1. Construction of calibration curve for solutions of vitamin D_2

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*]. Vitamin D_2 solutions 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. Vitamin D_2 absorbance (A) at 238 nm *vs* vitamin D_2 concentration [c]

[c] ug/mL	UV			Average A
	A1	A2	A3	
24.56	0.1641	0.1622	0.1631	0.1631
49.12	0.3374	0.3395	0.3403	0.3391
73.68	0.5042	0.5029	0.5034	0.5035
98.24	0.6654	0.6654	0.6685	0.6664

Figure S1. Calibration curve of absorbance of vitamin D_2 (*A*) at 238 nm *vs* vitamin D_2 concentration [*c*]



2. Determination of loading efficiency

The loading efficiency of vitamin D_2 in SpECs was determined by stirring *ca* 20 mg (precise amounts given in Table S2) 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 equation (Table S2) to be 254.10 ± 4.9 mg/g (n = 3). Therefore, based on the mass of vitamin D_2 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.

Table S2. Percentage loading of vitamin D_2 in SpECs using data given in Table S1 and Figure S1

Exines	EtOH	UV			Average	[c]	vit D	Loading
w/mg	mL	A1	A2	A3	A	ug/mL	w/mg	mg/g
24.6	50	1.3435	1.351	1.3553	1.3499	122.61	6130.61	249.21
22.4	50	1.2501	1.2232	1.2871	1.2535	113.85	5692.27	254.12
21.1	50	1.2032	1.204	1.2032	1.2035	109.30	5464.85	259.00
							Average	254.11
							estimated	283.2
							efficiency	90 ± 2 %

3. Data collection and processing for oral delivery of vitamin D_2 trial in human volunteers

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%. 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 number of subjects was small, potentially jeopardising the strong 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).

Table S3 a. Normalised data

Time /min	Volunteer 1 (vitamin D_2 in nmol/L)		Volunteer 2 (vitamin D_2 in nmol/L)		Volunteer 3 (vitamin D_2 in nmol/L)		Volunteer 4 (vitamin D_2 in nmol/L)		Volunteer 5 (vitamin D_2 in nmol/L)		Volunteer 6 (vitamin D_2 in nmol/L)	
	No SpECs	With SpECs	No SpECs	With SpECs	No SpECs	With SpECs	No SpECs	With SpECs	No SpECs	With SpECs	No SpECs	With SpECs
0	0	0	0	0	0	0	0	0	0	0	0	0
15	-2	3.8	15	4.6	na	na	na	na	na	na	15	-5.7
30	-3	0.5	10	5	na	na	na	na	na	na	10	8.5
45	-1	-2.5	6	10	na	na	na	na	na	na	6	13.1
60	12	2.3	-12	9	1	-1	0.4	19	-5.8	17	-12	2
120	10	11.9	11	42.6	3.8	16.3	4.3	18	-9.3	57	11	22.2
180	4	33.1	-23	103.6	-2.6	33	8.6	67	-7.8	223	-23	46.2
240	16	53.9	-4	161.6	5.5	44.6	12.9	85	-6.2	223	-4	88.6

Table S3 b Normalised averages and errors bars from Table 3b

Time/min	Volunteers 1-6 No SpECs Average (vitamin D_2 in nmol/L)	Volunteers 1-6 No SpECs Normalised error bar (SD/√n) (vitamin D_2 in nmol/L)	Volunteers 1-6 with SpECs Average (vitamin D_2 in nmol/L)	Volunteers 1-6 with SpECs Normalised error bar (SD/√n) (vitamin D_2 in nmol/L)
0	0	0	0	0
15	9.3	4.6	0.9	2.7
30	5.7	3.5	4.7	1.9
45	3.7	1.9	6.9	3.9
60	-2.7	3.4	8.1	3.1
120	5.1	2.9	28.0	6.6
180	-7.3	5.0	56.6	11.9
240	3.4	3.6	86.7	18.4

4. *In vitro* release of 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 (*ca* 20 mg; precise amounts given in Tables S4-S6) were stirred (37 °C for 60 min) accordingly, in: 10 mL of an aqueous solution with composition corresponding to phosphate buffer saline (PBS) pH 7.4 (Table S4); 10 mL of buffer pH 9.0 (Table S5); 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). In each case the suspension was 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.

Table S4. *In vitro* release of vitamin D_2 from loaded SpECs at 37 °C in PBS at pH 7.4 for 60 min

Time	SpECs	PBS (pH 7.4)	EtOH	UV			Ave	[c]	vit D	Loading	Ave	Ave	SD
min	w/mg	mL	mL	A1	A2	A3	A	ug/mL	w/mg	mg/g	mg/g	%	(SE)
	26.7	10	50	1.368	1.399	1.386	1.384	125.8	6289	6784	7	7	0.38 (0.17)
	26.9	10	50	1.378	1.400	1.399	1.393	126.5	6324	6835	7		
60	27.2	10	50	1.419	1.424	1.412	1.418	128.9	6444	6912	7		

Table S5. *In vitro* release of vitamin D_2 from loaded SpECs at 37 °C in buffer at pH 9.0 for 60 min.

Time	SpECs	PBS (pH 9)	EtOH	UV			Ave	[c]	vit D	Loading	Ave	Ave	SD
min	w/mg	mL	mL	A1	A2	A3	A	ug/mL	w/mg	mg/g	mg/g	%	(SE)
	21.1	10	50	1.1457	1.1495	1.1548	1.150	104.43	5221.8	5361.5	3		4.04 (1.81)
	20.9	10	50	1.1444	1.3993	1.1541	1.2328	111.96	5598.0	5310.7	-5		
60	14.1	10	50	0.7946	0.7919	0.7929	0.793	71.993	3599.7	3582.8	0	-1	

Table S6. *In vitro* release of vitamin D_2 from loaded SpECs at 37 °C in SGF at pH 1.5 for 60 min.

Time	SpECs	SGF	EtOH	UV			Ave	[c]	vit D	Loading	Ave	Ave	SD
min	w/mg	mL	mL	A1	A2	A3	A	ug/mL	w/mg	mg/g	mg/g	%	(SE)
	22.8	10	50	1.203	1.216	1.214	1.211	109.99	5499.5	5793.48	5	1	3.54 (2.04)
	21.3	10	50	1.183	1.999	1.200	1.1943	108.5	5440.0	5412.33	-1		
60	19.5	10	50	1.105	1.109		1.107	100.57	5028.8	4954.95	-1		

5. Counting SpECs *versus* synthetic black ChromoSphere™ Dry Dyed polymer beads remaining on rat intestinal mucosa following flushing with PBS at different volumes and flush intensities. (Figures S3-S6).

Freshly excised rat small intestine without the duodenum was used immediately, 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) (Figures S3 − S6)

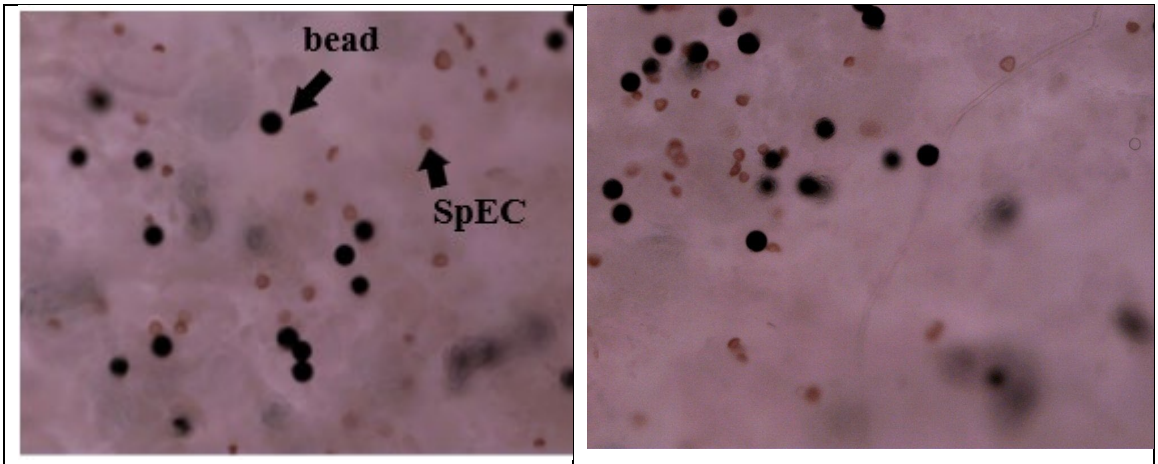


Figure S3. Example of opened segments (40 mm × 40 mm) of excised rat small intestine showing typical counting areas following 5 mL slow elution of PBS (mean SpECs = 31 ±1, mean beads = 21 i.e., SpECs − beads ratio of 2:1

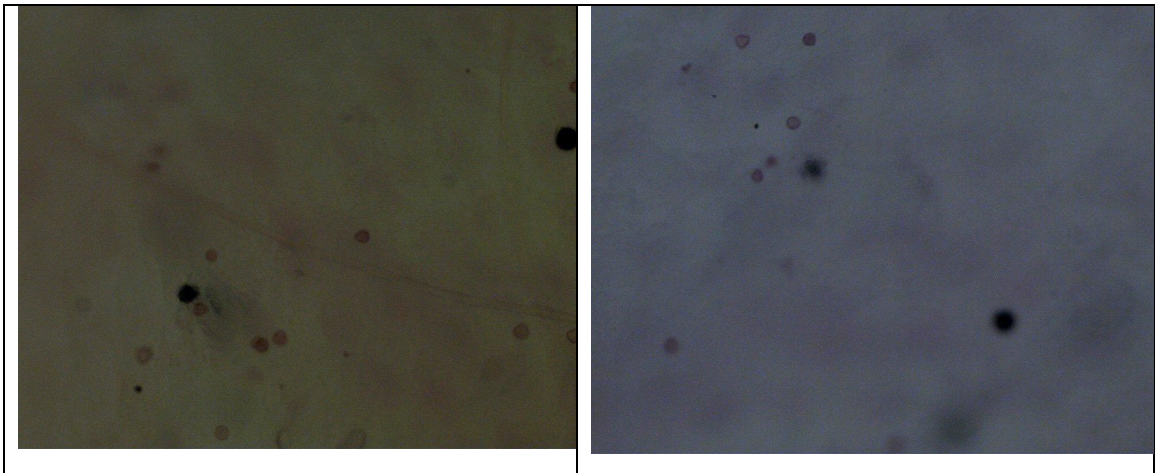


Figure S4. Example of opened segments (40 mm × 40 mm) of excised rat small intestine showing typical counting areas following 5 mL fast elution of PBS (mean SpECs = 11 ±1, mean beads = 2 i.e., SpECs − beads ratio of 6:1

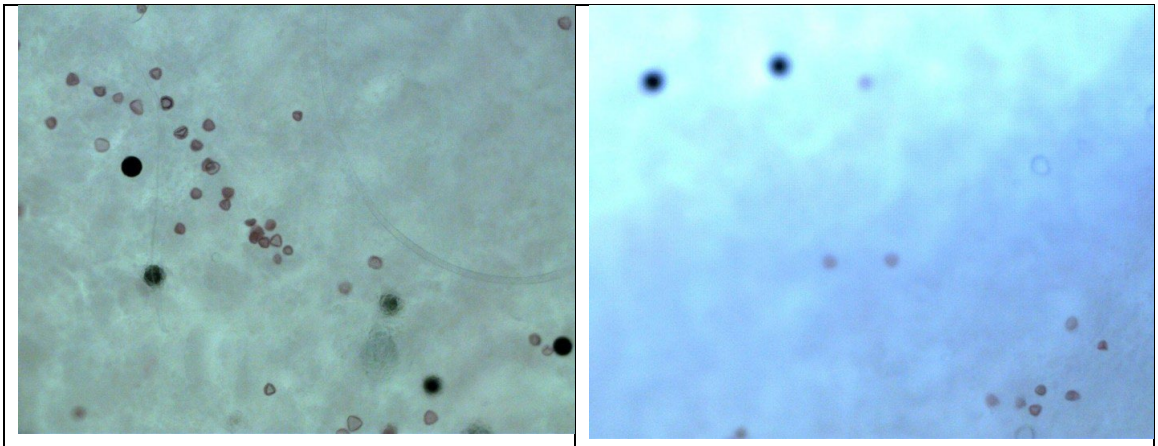


Figure S5. Example of opened segments (40 mm × 40 mm) of excised rat small intestine showing typical counting areas following 15 mL fast elution of PBS (mean SpECs = 12 ±1, mean beads = 2 i.e., SpECs − beads ratio of 6:1 15ml slow SpECs to beads ratio of 6:1 Mean SpECs = 24 Mean beads = 4

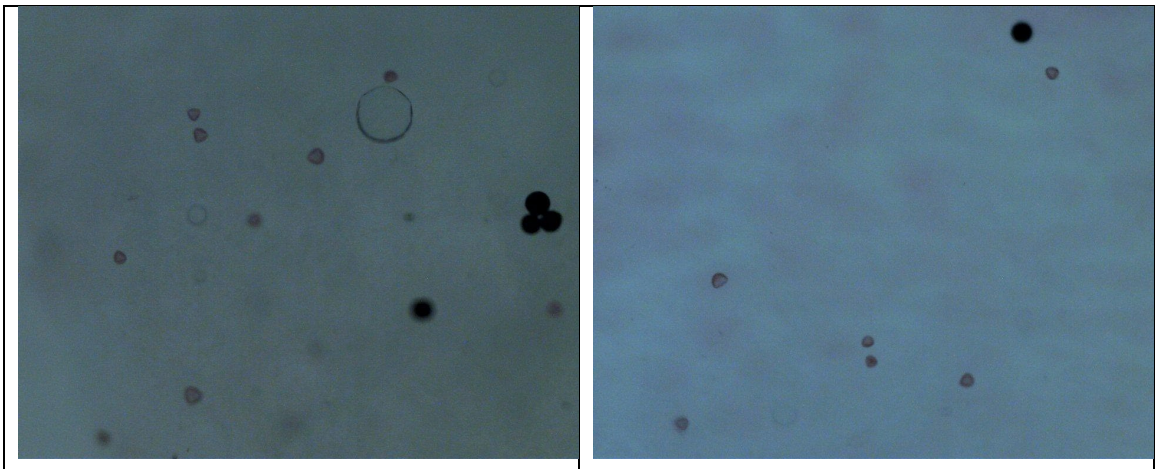


Figure S6. 15 mL fast flush SpECs − beads ratio of 3:1. Mean number SpECs = 9 ±1, mean number beads = 3

6. Passage of 99mTc radiolabeled SpECs, similarly labelled 99mTc radiolabeling glass beads and 'free' radioactive technetium -99m through rat small intestine

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 (Na99mTcO4), 99mTc-labelled Glass Microspheres and 99mTc-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).

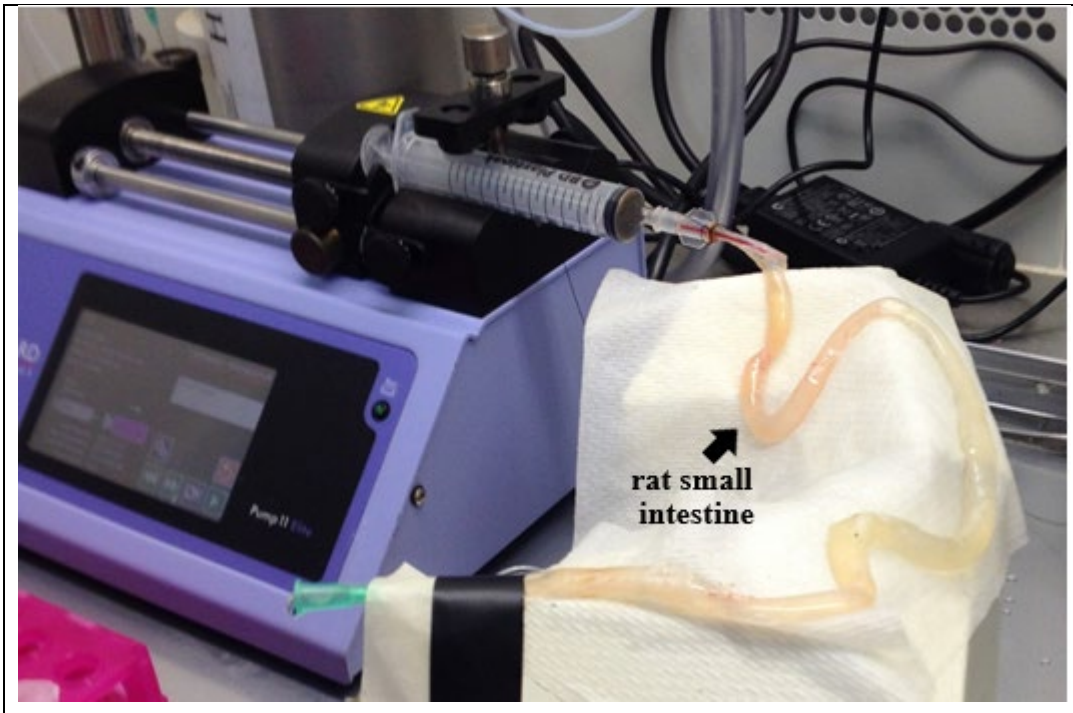


Figure S7. System used to pump test solutions of "free" technetium (Na99mTcO4), 99mTc-labelled Glass Microspheres and 99mTc-labelled SpECs and washings through a section of rat small intestine

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

. 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, as shown in Table S7. The overall count was taken as an average of the particles collected (n = 6) as shown in Table S7.

Table S7 *In vivo* trials comparing passage of unfilled SpECs *vs* control beads through rat gastrointestinal tract (GIT)

Run 1							
	SpECs counted for rats 1-3				ChromoSphere™ black polymer beads (50 µm) counted for rats 1-3		
Times of faeces collection after feeding (h)	Rat 1	Rat 2	Rat 3		Rat 1	Rat 2	Rat 3
0-8	0.00	0.00	0.00		0.00	0.00	15724.49
8-16	360974.76	252407.44	0.00		638647.66	565392.67	450532.79
16-24	0.00	157786.89	19007.89		51620.93	315573.77	31679.81
24-32	0.00	0.00	52825.44		35889.18	10697.62	132063.61
32-40	0.00	0.00	103986.54		45290.63	0.00	118841.76
Total SpECs counted after 40 h	360974.76	410194.33	175819.87	Total black beads counted after 40 h	771448.39	891664.06	748842.46
% of total SpECs recovered	36.10	41.02	17.58	% of total beads recovered	77.14	89.17	74.88

Run 2							
	SpECs counted for rats 1-3				ChromoSphere™ black polymer beads (50 µm) counted for rats 1-3		
Times of faeces collection after feeding (h)	Rat 1	Rat 2	Rat 3		Rat 1	Rat 2	Rat 3
0-8	0.00	0.00	0.00		0.00	0.00	0.00
8-16	326299.78	226178.60	236748.03		498036.50	442076.35	291930.52
16-24	174618.63	133830.76	143837.89		324291.74	316327.25	117685.55
24-32	63210.32	0.00	69384.72		56186.95	13543.25	60711.63
32-40	12043.07	0.00	25999.29		72258.40	16586.09	38998.93
Total SpECs counted after 40 h	576171.80	360009.36	475969.92	Total black beads counted after 40 h	950773.60	788532.95	509326.62
% of total SpECs recovered	57.62	36.00	47.60	% of total beads recovered	95.08	78.85	50.93

Summary data for Runs 1 and 2

	Run 1			Run 2			Average	Standard deviation
	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3		
% of total SpECs recovered	36.10	41.02	17.58	57.62	36.00	47.60	39	15
% of total beads recovered	77.14	89.17	74.88	95.08	78.85	50.93	78	9