IN-VITRO CHARACTERIZATION OF APA MICROCAPSULES FOR ORAL DELIVERY OF LIVE BACTERIAL CELLS

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Abstract: The suitability of the alginate-poly-L-lysine-alginate (APA) microcapsules for gastrointestinal (GI) applications was evaluated using a simulated human GI microbial ecosystem (SHIME). The viability of the encapsulated *Lactobacillus* cells in simulated gastric solutions (pH=1.2 and 2.0) was investigated. Results shows that the APA membrane was morphologically stable in the simulated stomach. However, when exposed to the simulated GI system for 3 days, it was unable to retain integrity. Studies of cell resistance to gastric acidity also demonstrated the limitations of the currently obtainable APA membrane for live bacterial cell oral delivery. The APA microcapsule system may, therefore, require significant improvement to withstand biological impediments and ensure adequate protection of the live cells when used orally.

INTRODUCTION

The interest in the use of live recombinant bacterial cells for therapeutic purposes has been on the rise over the last years[1-2]. The certainty that these cells can be engineered to synthesize potentially therapeutic products has generated considerable attention and excitement among clinicians and health professionals. A major limitation in the use of these bacterial cells is the complexity in delivering the products to the target tissues. Oral delivery may be the easiest method of administration, but the bacterial cells are primarily incapable of surviving their passage through the GI tract[3]. A potential solution is the use of the encapsulation process[4] to provide a physical barrier against adverse environmental conditions. The capsular membrane allows free exchange of nutrients and selected therapeutic substances between the encapsulated cells and body fluids, whereas large substances such as cells, immunocytes and antibodies are excluded. Previous studies[5-6] have shown that oral administration of encapsulated bacteria has potentials as an alternative therapy for several diseases.

The APA microcapsule system[7], formed by electrostatic complexation between polyanionic alginate and polycationic PLL, has been widely used for live cell encapsulation mainly due to the mild conditions of process suitable for preserving cell viability. Intended for oral delivery, it is critical to understand their resistance to relevant conditions similar to human GI transit. The present work involves the investigation of the APA microcapsules GI suitability in-vitro using a dynamic simulated human gastrointestinal system, encapsulation of live *Lactobacillus* plantarum 80 and their survivability in-vitro simulated gastric solutions.

MATERIALS AND METHODS

Sodium alginate (low viscosity) and poly-L lysine (PLL) (MW 27,400) were purchased from Sigma-Aldrich, Canada. *Lactobacillus* plantarum (LP80) with recombinant bile salt hydrolase genes (BSH) (pCBH1) were obtained from LabMET, University of Gent, Belgium. All other reagents and solvents were of reagent grade and used as received without further purification.

Human Gastrointestinal (GI) microbial ecosystem[8]

The human GI tract model used in this study is simulated by means of a series of bioreactors (Fig. 1). Each reactor corresponds to a specific stage of digestion. The whole system is maintained under anaerobic conditions by flushing the headspace of each vessel with N_2 and the temperature is kept constant at 37˚C. The vessels representing large intestines were inoculated with samples of human fecal slurries and thus contain the normal human GI bacteria. This model is sustained by feeding carbohydrate-based nutrition to the first vessel 3 times a day. After feeding, the stomach acidification (0.2N HCl) occurred, followed by the neutralization (0.2N NaOH) and addition of simulated pancreactic juice in

the second vessel. The suspension is then transferred to the simulated ascending colon, the transverse colon, and the descending colon for further interactions, and finally excreted as effluent. All of the process, including the conditions of pH, volume and retention time at each stage is simulated under computer control.

Preparation of simulated gastric fluids (SGF)

SGF were prepared in accordance with USP XXII[9] by dissolving 2 g NaCl and 3.2 g pepsin (Sigma, Canada) in 7 ml hydrochloric acid. The volume was adjusted to 1 L by H_2O to obtain a pH about 1.2.

Preparation of the microcapsules

The APA microcapsules were prepared according to the standard protocol[7] with slight modifications. Briefly, a Na-alginate solution (1.5% wt/v) was extruded into a stirred $CaCl_2$ solution (0.1M) using an Encapsulator (Inotech Corp.). The rigid beads were immersed in a PLL solution (0.1% wt/v) for 10 min and further coated by another Na-alginate solution (0.05% wt/v) for 10 min. The resulting APA microcapsules were washed and subject to testing.

Preparation of the microcapsules containing LP80

The LP80-encapsulated beads were made based on our earlier report[6] with a few modifications. BSH isogenic *Lactobacillus plantarum* 80 (pCBH1) were grown at 37˚C in MRS broth supplemented with 100 µg/ml erythromycin. After 10 min of a centrifugation at 10 000x*g*, bacterial pellets (0.94 g CDW) were washed, suspended in 5 ml physiological solution (PS) and mixed with 45 ml of sterile alginate solution (1.5% wt/v), giving cell density of 3.22×10^8 CFU/ml. The subsequent encapsulation, PLL and alginate coating were performed as described above. The LP80 containing microcapsules were stored at 4°C in half broth medium (MRS broth:PS=1:1). The preparation procedure was carried out under a biological containment hood. All the solutions used were either 0.22 µm filtered or autoclaved to ensure sterility.

Resistance of the microcapsules to GI

Plain beads were exposed to SHIME fluids for a time period (Tab. 1) according to estimated maximum period of human GI transit. Microcapsule samples were taken at varied stages of digestion for physical observation using an inverted light microscope (LOMO PC). Microphotographs were taken as records using a digital camera (Canon).

Determination of encapsulated cell viability

0.1 ml of LP80 encapsulated beads were manually ruptured by tissue pestles. Microorganism suspension (0.1 ml) of dilution serials was aseptically plated in duplicate in selective MRS agar plates supplemented with 100 µg/ml of erythromycin. Colonies were enumerated after 3-day incubation at 37˚C in anaerobic jars with the AnaeroGen system (Oxoid Ltd., England). Free LP80 cells and PS served as a positive and negative control, respectively.

Survivability of the encapsulated cells in simulated gastric solutions

0.1 ml of LP80 encapsulated beads were subject to HCl (0.2N, pH1.2), simulated gastric fluids (SGF)[9] and SHIME nutrition with pH adjusted to 1.2 and 2.0. After incubation at 37˚C, 150 rpm for a designated period of time, the remaining live cells in the microcapsules were determined by plate count as described above. Free cells and encapsulated cells incubated in PS and SHIME feed (pH 4.6) served as the controls.

RESULTS AND DISCUSSION

Resistance of the microcapsules to GI condition

The obtained APA microcapsules were spherical and uniform in shape, with narrow size distribution (450 ± 15um) and smooth surface (Fig. 1-a). To evaluate their suitability for oral therapy, it is important to understand the microcapsule behaviors under relevant conditions that represent the digestion course. A dynamic computer-controlled simulated human GI intestinal microbial ecosystem was employed in the current studies. Microcapsules were exposed to the fluids of different vessels for a time period (Tab. 1) according to the human GI transit, as to mimic the experience along the GI course including pH fluctuation, enzymatic stress and GI micro flora effects.

Figure 2 b-e shows the physical changes of microcapsules at various phases of digestion. The microcapsules remained intact in the simulated stomach and became ghost-like when leaving the small intestine. At the phase representing the transverse colon, the beads appeared poorly defined and difficult to visualize under the microscope. The integrity of the microcapsules continued to decline as they passed through the colon; eventually no trace of the microcapsules was recorded at the descending colon. That is, the APA microcapsules maintained physical stability in acidic environment. However they did not uphold structural integrity after 3 days of exposure to SHIME medium, which would be the maximum retention period of food in the human GI transit.

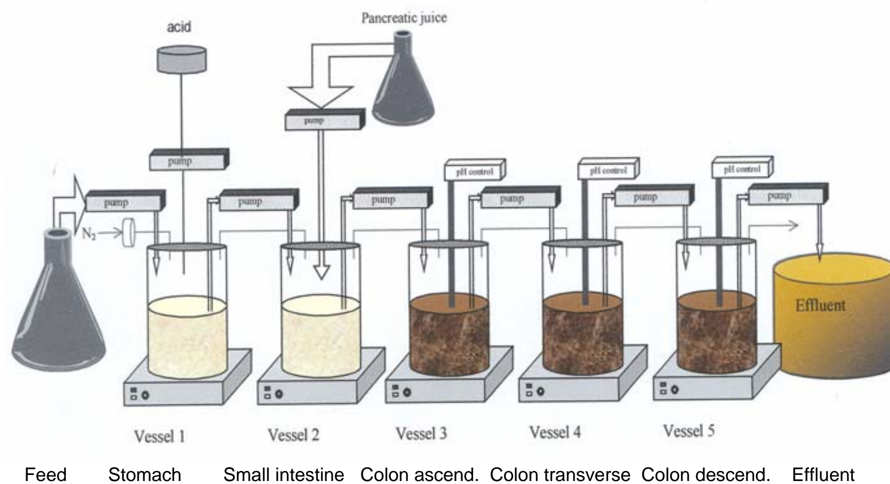
Encapsulation of bacteria LP80 and cell viability post encapsulation

When studied, the morphology of LP80-loaded microcapsules was not much different from the cell free beads except for the apparent change in color. Homogeneous and opaque microspheres were obtained using the same encapsulator. After PLL and

additional alginate coating, these microcapsules were stored in half broth (MRS broth:PS=1:1) at 4˚C to maintain low metabolism of the microorganisms. As one of the most important prerequisites of using live cells is their survival throughout the production process and during storage, the viability of the encapsulated cells post encapsulation was monitored. The initial cell loading was found to be 1.57×10^8 CFU/ml beads, which was comparable to that of alginate-cell mixture (3.22×10^8 CFU/ml). Cell density in the microcapsules slightly declined after a month of storage and maintained the loading of $2 \sim 3 \times 10^7$ CFU/ml beads for at least 3 months (Tab. 2). Although the live cells remain immobilized inside the microcapsules, encapsulation does not appear to hinder their metabolic activities. This demonstrated that the APA microcapsule formulation provided favorable microenvironments for the survival of the LP80 cells and protection against stresses during process and storage.

Survival of encapsulated cells in simulated gastric conditions

Since therapeutic microorganisms are usually required at the target site in the intestine, it is essential that they withstand the host's natural barriers of highly acidic stomach against ingested bacteria. The LP80-loaded microcapsules were exposed to various simulated gastric solutions (pH=1.2 and 2.0) so as to assess the efficiency of the APA system. When challenged for 60 minutes in SGF no severe morphological disintegration of APA microcapsules was found (Fig. 2-f). However, after 15 minutes incubation in solutions at pH below 2.0, cell viable counts decreased considerably (Tab. 3). Less than 0.01% of encapsulated cells survived the acidity of pH 1.2 and 2.0 and no bacterial colonies were detected after 60 minutes of exposure (data not shown). When compared with free cells in similar resistance studies, the APA system was found inadequate to protect enclosed bacterial cells. When exposed to higher pH (SHIME feed at pH 4.6), however, more than 50% of encapsulated bacterial cells survived. In contrast, large percentage of the LP80 cells, either free or encapsulated, remained alive when incubated in physiological solutions.



Feed Stomach Small intestine Colon ascend. Colon transverse Colon descend. Effluent

Fig. 1. Schematic representation of the dynamic simulated human gastrointestinal (GI) microbial ecosystem.

Table 1. Exposure of the APA microcapsules to SHIME medium

SHIME Stage	Stomach	Small Intestine	Ascend. Colon	Transverse Colon	Descend. Colon	Total
Time (hr)	2	4	18	24	24	72
Morphology	Intact	ghost-like	Un-conformed	Poorly-defined	No trace	

Table 2. Cell density of LP80 cells (CFU/ml) before and after encapsulation

Time (d)	Before	Immediately	30	45	90
CFU/ml beads	3.22E+08	1.57E+08	2.83E+07	2.68E+07	2.50E+07

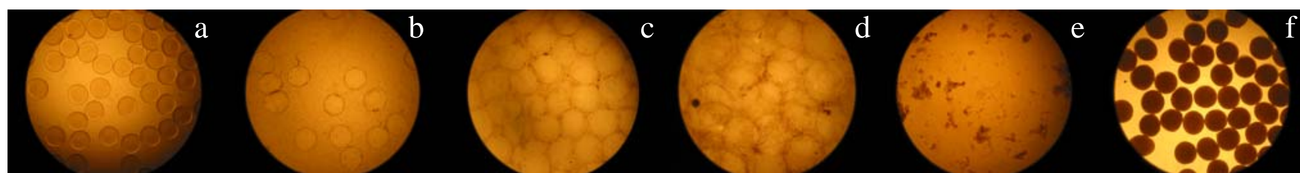


Figure 2. Microphotographs of the APA microcapsules. a, plain beads; b-e, plain beads during transit in SHIME: b, in the stomach; c, in the small intestine; d, in the transverse colon; e, in the descending colon; f, LP80 encapsulated beads after 1-hr incubating in SGF (pH 1.2, 37˚C, 150rpm). Original magnification is 35x.

Table 3. Viability of the encapsulated LP80 cells in simulated gastric solutions (37˚C, 150 rpm)

	LP80 Cells	Time (min)	pH=1.2			pH=2.0	pH=4.6	pH=6.8
			0.2N HCl	SGF	SHIME feed	SHIME feed	SHIME feed	PS
Cell density* (CFU/ml beads)	Encapsulated	0	$1.49X10^7$	$1.49X10^7$	$1.49X10^7$	$2.50X10^7$	$2.50X10^7$	$2.50X10^7$
		15	<50	615	<50	<50	$1.89X10^7$	--
		30	<50	<50	<50	<50	$1.42X10^7$	$1.59X10^7$
Viability (%)		15	<0.01	<0.01	<0.01	<0.01	75.6	--
		30	<0.01	<0.01	<0.01	<0.01	56.8	63.6
Cell density* (CFU/ml)	Free	0	$4.54X10^7$	$4.54X10^7$	$4.54X10^7$	$2.36X10^8$	$2.36X10^8$	$2.36X10^8$
		15	<100	2300	100	200	$2.14X10^8$	$1.89X10^8$
		30	<100	<100	<100	600	$1.35X10^8$	$1.83X10^8$
Viability (%)		15	<0.01	<0.01	<0.01	<0.01	90.7	80.1
		30	<0.01	<0.01	<0.01	<0.01	57.2	77.5

* Mean of duplicate plate counts

CONCLUSION

The APA microcapsule system was used to encapsulate live Lactobacillus plantarum 80 (pCBH1) cells. A dynamic simulated human GI microbial ecosystem was employed to evaluate the GI stability of the APA membrane. Results showed that high cell density was achieved during encapsulation and storage, demonstrating that the APA microcapsule system offered a favorable microenvironment for the growth and proliferation of live cells. The APA membrane was found morphologically stable in acidic conditions. However, it did not uphold integrity for a 3-day exposure in the simulated human GI environment. When exposed to simulated gastric medium (pH=1.2 and 2.0), the encapsulated cells scarcely survived for 15 minutes, indicating that encapsulation by the APA system may not be able to provide adequate protection for the enclosed live bacterial cells for oral delivery applications. However, further in-vivo studies are required.

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