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Microencapsulated Engineered *Lactococcus lactis Cells* for Heterologous Protein Delivery: Preparation and In Vitro Analysis

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Abstract This article demonstrates the potential of encapsulated, engineered *Lactococcus lactis* as a vehicle for the oral delivery of therapeutic proteins. Using alginate-poly-*l*-lysine-alginate membrane-encapsulated *L. lactis* engineered to secrete the reporter protein *Staphylococcal aureus* nuclease, we show comparable viability and protein secretion between free and immobilized cells. After 12 h, microcapsules with a cell density of 4.8×10^5 colony forming unit (CFU) ml^{-1} grew to 2.2×10^8 CFU ml^{-1} and released 0.24 arbitrary unit (AU) ml^{-1} of nuclease, producing similar results as free cells, which grew from 3.4×10^5 to 1.9×10^8 CFU ml^{-1} and secreted 0.21 AU ml^{-1} of nuclease. Moreover, encapsulated cells at a density of 4.4×10^7 CFU ml^{-1} grew to 2.2×10^{10} CFU ml^{-1} in 12 h and secreted 15.3 AU ml^{-1} of nuclease although 3.1×10^7 CFU ml^{-1} of free cells reached only 2.3×10^9 CFU ml^{-1} and released 5.6 AU ml^{-1} of nuclease. We also show the sustained stability of the microcapsules during storage at 4°C over 8 weeks.

Keywords *Lactococcus lactis* · Live cell therapy · Microencapsulation · Protein secretion · Staphylococcal nuclease

Introduction

Lactococcus lactis has become recognized as an invaluable bacterium. The noncommensal, food-grade lactic acid bacterium, long recognized for its widespread use in fermented food production, has become revolutionized into a powerful protein expression system [1]. It has

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been engineered for the production and secretion of a range of heterologous proteins of medical interest, among which include cytokines and antigens serving as vaccines [2]. In vivo studies demonstrating the ability for in situ production of proteins and effective treatment of intestinal disorders [3, 4] have fuelled the increasing interest into this cost-effective approach for the oral delivery of de novo therapeutics to the gastrointestinal tract.

Whereas the effectiveness of live cell therapy heavily relies upon cell viability, low cell survival during gastric transit and other host mechanisms targeting foreign organisms are recognized as major limitations [5]. Microencapsulation has been established as a technology to abate these setbacks by immobilizing microorganisms in a protective polymer capsule, and in further restraining them from systemic release. In particular, alginate-poly-*l*-lysine-alginate (APA) has been shown to be effective for live bacterial cell encapsulation and oral delivery [6]. The enclosing membrane permits the bidirectional diffusion of small molecules, allowing substrates, nutrients, products, and wastes to diffuse through the capsules, all the while preventing the passage of large molecules. In this study, we establish APA microcapsules containing *L. lactis* for in vitro heterologous protein production. We investigate this using a nisin-inducible strain engineered to secrete the reporter protein *Staphylococcal aureus* nuclease, a 19-kDa stable, monomeric enzyme. The results serve as a preliminary evaluation of the feasibility of such a system and provide insight into its performance.

Materials and Methods

Cell Culturing

Nisin-inducible engineered *L. lactis* cells secreting staphylococcal nuclease (*L. lactis* NZ9000 [pSEC:LEISSTCDA:Nuc]) and nonengineered control cells (*L. lactis* NZ9000) [7] were kindly provided by Dr. Philippe Langella (National Institute for Agricultural Research). Bacterial cells were cultured at 30°C in M17 broth (Difco) supplemented with 0.5% glucose (GM17) and 10 μg chloramphenicol ml^{-1} for antibiotic selection. Induction of cells was performed using 1 ng nisin ml^{-1} (Sigma).

For the experiments, cells from frozen stocks were cultured overnight and subsequently subcultured with a 2% inoculum to achieve an optical density $(OD)_{600}$ of 0.4–0.6. Cells were induced with nisin for 1 h and harvested by centrifugation (8,000×*g*, 10 min) followed by washing thrice in physiological solution (PS), 0.85% NaCl, before being further used in studies. For free cell studies, bacteria were held on ice in PS for 2 h to simulate the latency occurring during the encapsulation process for immobilized bacteria.

Microencapsulation of Cells

Microencapsulation was performed using an Inotech Encapsulator IER-20 (Inotech Biosystems International, Inc.). A homogenous solution of cells in 1.65% sodium alginate (low viscosity, Sigma) was extruded from the encapsulator to form droplets that were collected in a stirred, ice-cold calcium chloride solution (0.1M), forming alginate microcapsules. The capsules were left for 30 min to allow for complete gelling. After being washed in PS, the alginate microcapsules were subsequently coated in 0.1% poly-*l*-lysine (MW 27400, Sigma) for 10 min, washed again, and coated in sodium alginate (0.1%) for 6 min to form the APA microcapsules. Capsules were used immediately after preparation.

Determination of Cell Viability

Free bacteria and microcapsules containing an equivalent number of cells were cultured in equal volumes of growth media at 30°C. After sampling, microcapsules were weighed, crushed with a tissue pestle, and suspended in PS. Serial dilutions of these preparations and free bacterial suspensions were evaluated for cell viability through plating on GM17 agar plates, using chloramphenicol (10 μg ml^{-1}) as the selection antibiotic, incubated at 30°C for 48 h. Cell viability for encapsulated cells was expressed as the number of colony forming units (CFU) per milliliter of capsules and for free cells as CFU per milliliter cell suspension.

Determination of Staphylococcal Nuclease Release

For protein release studies, culture media of microencapsulated and free cells were sampled, centrifuged at 8,000×*g* for 10 min and the supernatant collected and stored at −20°C until testing.

Nuclease released into the supernatant solution was quantified based upon its DNAse activity. The enzymatic activity of the protein was measured using the assay described by Heins et al. [8] in which the digestion of DNA forms acid-soluble oligonucleotides that can be detected spectrophotometrically at 260 nm. Five milligrams of salmon sperm DNA per milliliter (Sigma) was used as DNA substrate, and supernatant samples served as the enzyme solution. Nuclease activity was expressed as arbitrary units (AU) per gram of capsules for the case of encapsulated cells and as AU per milliliter of cell suspension in the case of free cells. One AU is defined as a change of 1 OD unit of DNA substrate at 260 nm and 37°C.

Results and Discussion

Viability of Free and Encapsulated *L. Lactis*

A viable and active protein delivery system was established through the encapsulation of engineered *L. lactis*. At the two different cell densities tested (3–5×10^5 and 3–5×10^7 CFU ml^{-1}) the viability of free and encapsulated cells exhibited comparable growth, as summarized in Table 1. By comparing the growth profile of free and encapsulated cells it is apparent that encapsulation did not considerably hinder the growth rate or the proliferation capacity (Fig. 1). In fact, at the high cell load a greater cell concentration is observed in capsules than in free cells; after 12 h the former grew from 7.6 to 10.4 $\log_{10}$ CFU ml^{-1} and the latter 7.5 to 9.4 $\log_{10}$ CFU ml^{-1} (Fig. 1b). At low cell loading the difference is not as appreciable as

Table 1 Summary of *L. lactis* growth and staphylococcal nuclease secretion by microencapsulated and free cells.

Cell preparation	Cell number (CFU ml^{-1})		Nuclease secretion (AU ml^{-1})
	0 h	12 h	12 h
Low density			
Encapsulated	4.8×10^5	2.2×10^3	0.24±0.003
Free	3.4×10^5	1.9×10^3	0.21±0.01
High density			
Encapsulated	4.4×10^7	2.2×10^{10}	15.3±3.6
Free	3.1×10^7	2.3×10^9	5.6±0.1

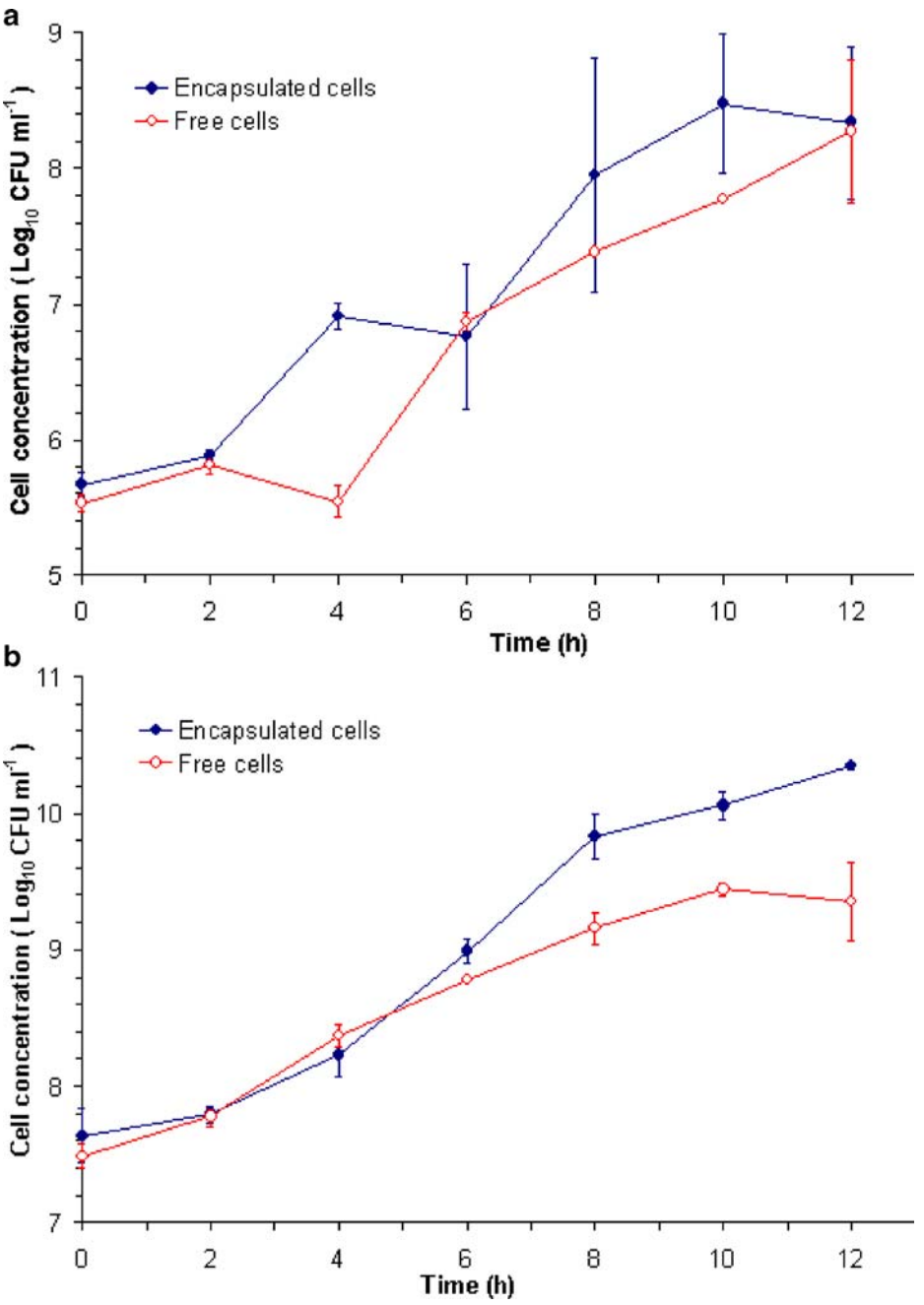

Fig. 1 A comparison between the viability of microencapsulated and free *L. lactis* at low (**a**) and high (**b**) cell densities. Cells were cultured in GM17 media at 30°C

encapsulated cells grew from 5.7 to 8.3 $\log_{10}$ CFU ml^{-1} and free cells from 5.5 to 8.3 $\log_{10}$ CFU ml^{-1} (Fig. 1a). Empty capsules were used as a control and showed no cell growth.

The increased cell proliferation of encapsulated bacteria observed may suggest the presence of growth-encouraging conditions. Cell–cell communication through the production of extracellular signals is known to play a role affecting the growth of *L. lactis* [9] and may differ in an immobilized microenvironment from in free suspension. Other studies have shown that the growth of these facultative anaerobes is adversely affected by the presence of oxygen [10]. Cells enclosed in microcapsules have a slightly reduced oxygen exposure, particularly toward the interior of the capsules where oxygen penetration is the lowest [11]; this may play a factor in their growth. A more prominent effect of these factors is likely observable at higher cell densities and requires further investigation before conclusions can be made.

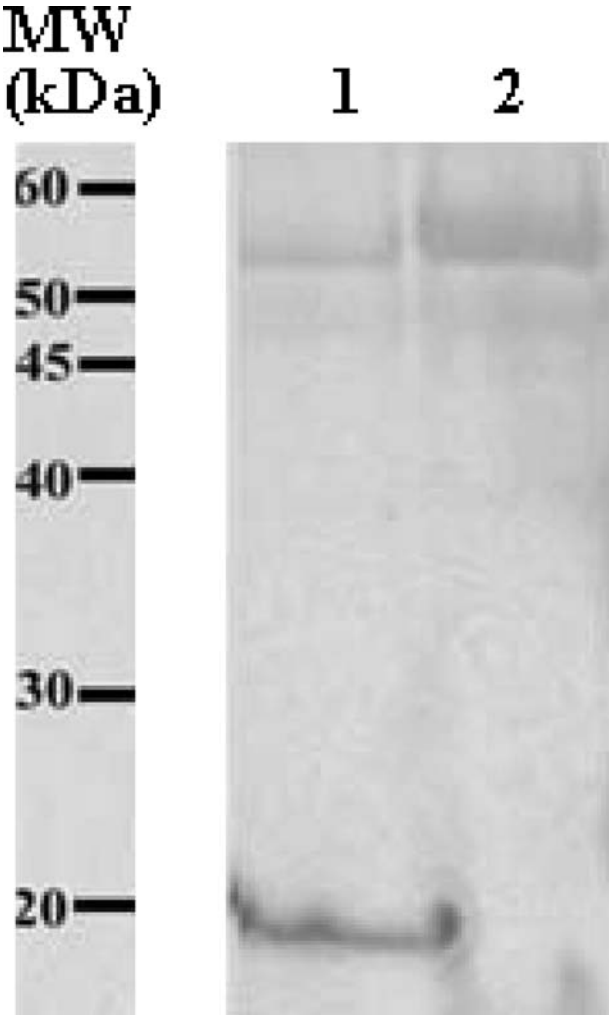

Fig. 2 SDS-PAGE analysis of total protein secreted from encapsulated *L. lactis*. Lane 1: encapsulated engineered *L. lactis* secreting nuclease, Lane 2: encapsulated nonengineered *L. lactis* (negative control). Proteins were run on 10% Bis Tris gel and visualized with silver stain

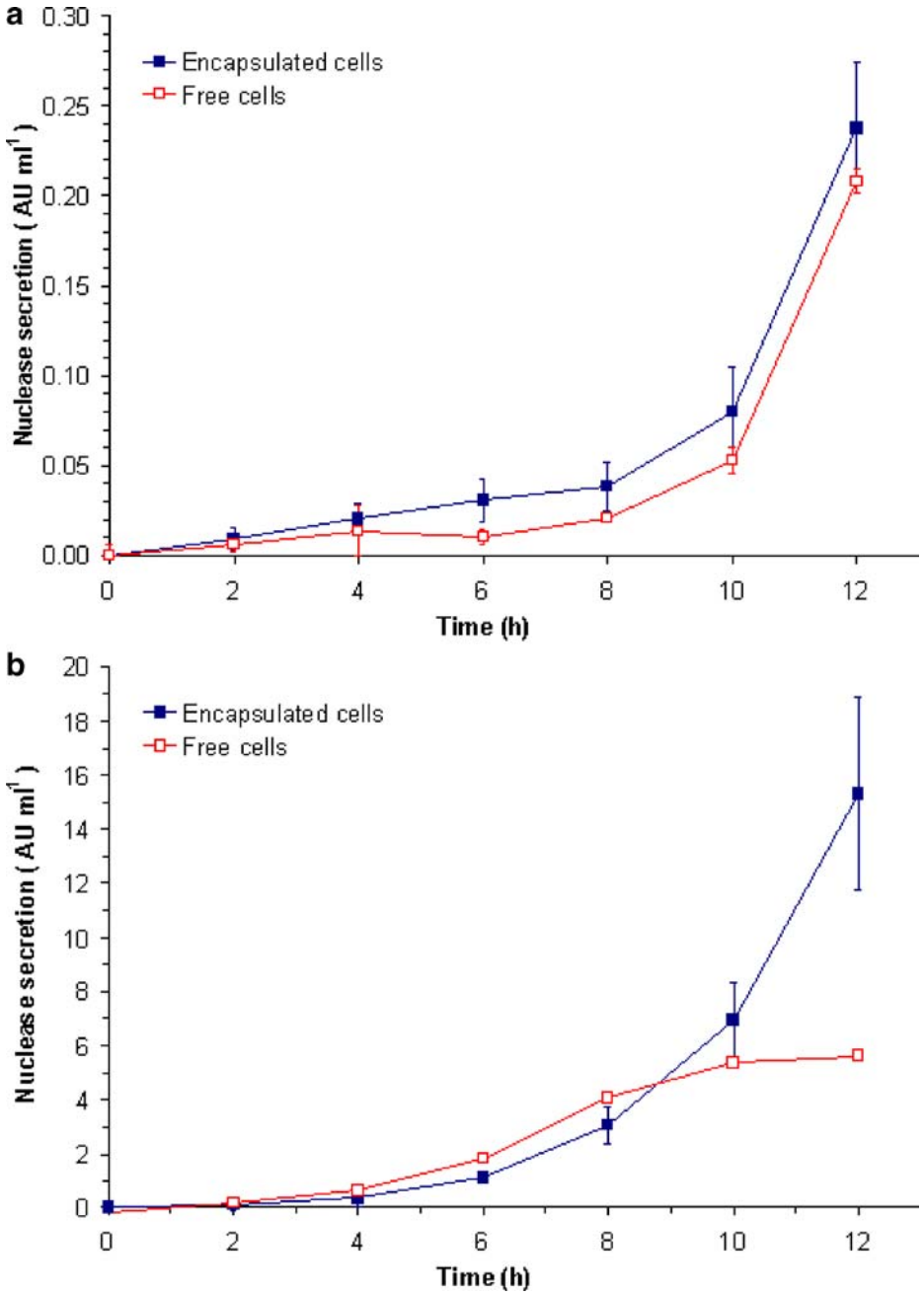

Fig. 3 Comparison of nuclease secretion of encapsulated and free *L. lactis* grown in GM17 media at low cell loads (microcapsules: 4.8×10^5 CFU g^{-1}, free cells: 3.4×10^5 CFU ml^{-1}) (**a**) and high cell loads (microcapsules: 4.4×10^7 CFU g^{-1}, free cells: 3.1×10^7 CFU ml^{-1}) (**b**). Nuclease was quantified based on its enzymatic activity, expressed as arbitrary units (AU)

Nuclease Secretion of Free and Encapsulated *L. Lactis*

Supernatant samples from cultures were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on gels that were silver-stained to reveal the total secreted proteins and to confirm nuclease as the main secreted product. In samples from engineered bacteria the presence of staphylococcal nuclease is clearly detectable as the major expressed protein corresponding to approximately 20 kDa, whereas it is absent in nonengineered control samples (Fig. 2). Two other faint bands are visible in both engineered and control *L. lactis* samples, corresponding to sizes between 50–55 kDa. These are assumed to be forms of Usp45, a protein of unknown function, that is the only detectable protein naturally secreted by *L. lactis* [12].

Studies to determine whether encapsulation limits the secretion of heterologous proteins proved the system to be equally as effective as free cells. The secretion trends mimicked

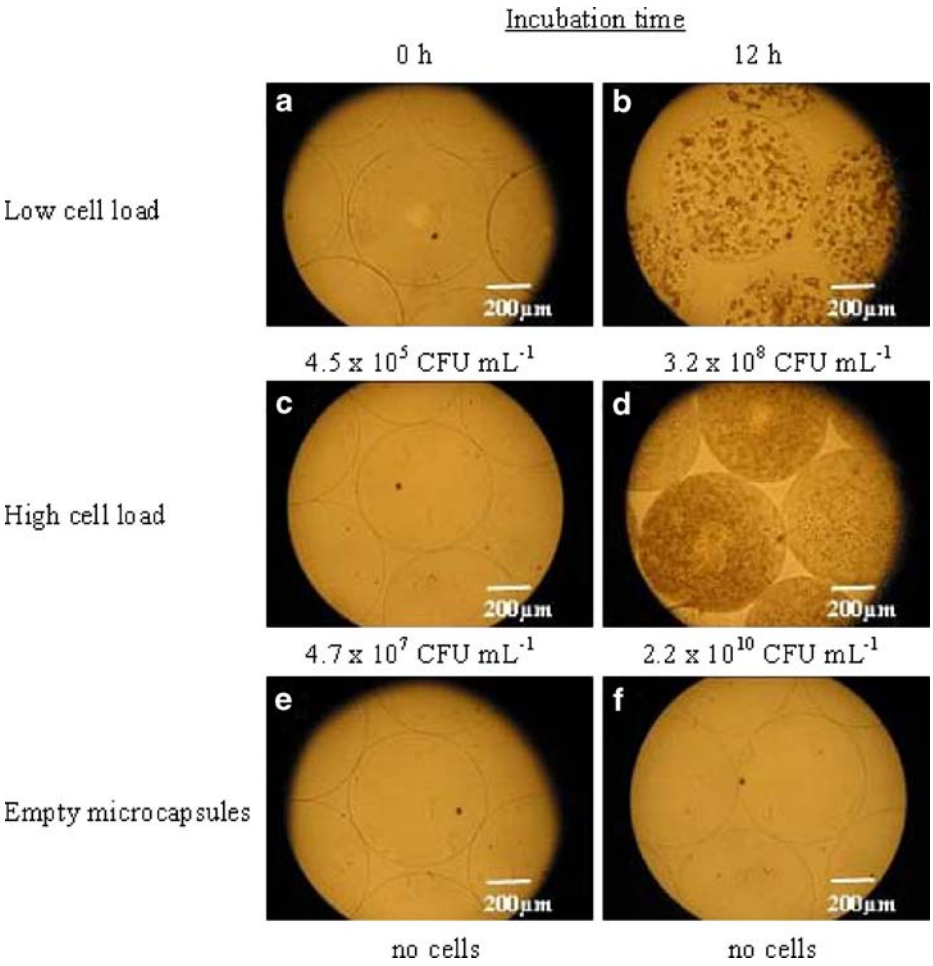

Fig. 4 Photomicrographs of microencapsulated *L. lactis* cells at 0 and 12 h of culturing in GM17 media showing sustained capsule morphology and changes in the physical appearance. Cells were encapsulated at low density (**a**, **b**), and high density (**c**, **d**), whereas empty microcapsules (**e**, **f**) served as a control

those observed for cell viability. At low cell loads the secretion profile for suspension cells was paralleled by microcapsule samples having a slightly higher secretion level (Fig. 3a), attributed to the slightly higher cell concentration in microcapsules previously shown. After 12 h, free cells secreted 0.21 AU nuclease ml^{-1} and encapsulated cells released 0.24 AU nuclease ml^{-1}. At high cell loads, initial protein secretion by encapsulated cells was nearly equivalent to that of free cells. However, beyond 10 h of culturing, a large difference in secreted levels of proteins was observed. After 12 h, encapsulated samples produced 15.3 AU nuclease ml^{-1}, superseding free cells, which produced 5.6 AU nuclease ml^{-1} by nearly three times (Fig. 3b). Largely, this deviation may result from the greater encapsulated cell concentration and comparably lower growth rate for free cells during this time period. Nonengineered *L. lactis* and empty microcapsules were used as negative controls, exhibiting no activity.

A consistent time lag between logarithmic growth and protein secretion was observed with free and encapsulated cells. In all samples tested the logarithmic growth phase occurred after 2–4 h of culturing, although a corresponding increase in the presence of nuclease was detected only after 6–8 h, midway into the logarithmic growth. This consistency of results does not suggest any adversity in microcapsule samples because of diffusional limitations of nutrients, nor insufficient space for bacterial growth. Alginate-poly-*l*-lysine-alginate microcapsules have a reported molecular weight cutoff of 50–70 kDa [13], allowing for their implementation for the delivery of the various therapeutic proteins expressed by *L. lactis*. The size and charge distribution of the expressed protein will affect its permeability and accordingly protein release rate, and this can be modified as desired by adjusting membrane properties such as capsule size and membrane porosity.

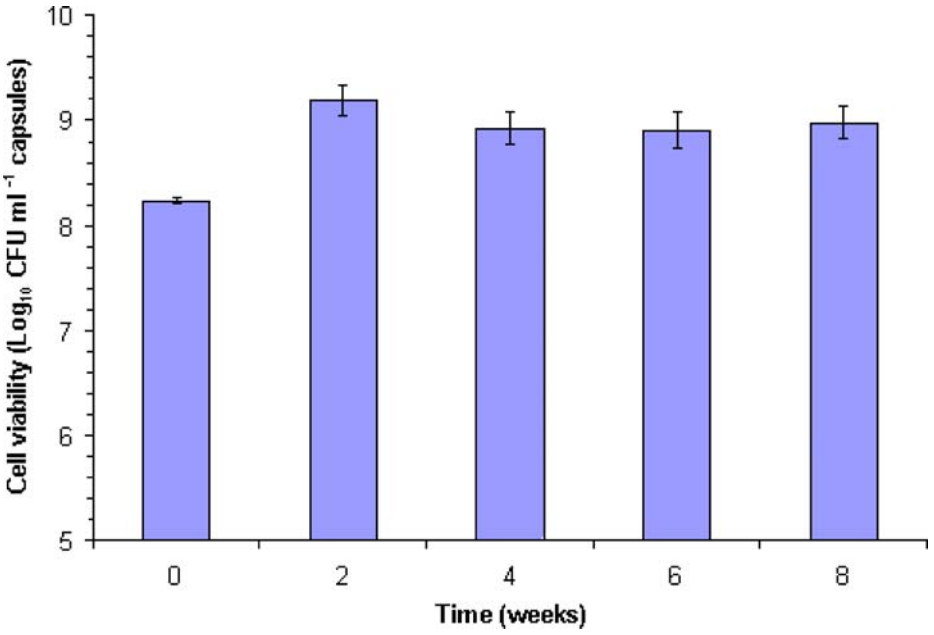

Fig. 5 Survival of APA encapsulated *L. lactis* during storage at 4°C in a solution of GM17 media/PS (1:10), $n=6$

Morphology of Microcapsules

The morphological properties of the microcapsules were investigated during the studies. Uniform spherical APA microcapsules were produced with an average diameter of 625± 91 μm. Figure 4 shows that after 12 h of culturing the relative cell density between the two cell loads is quite distinguished. During cell proliferation and secretion the membrane integrity of the capsules was preserved, maintaining the capsule sphericity and physical stability and consequently retaining the cells enclosed in the polymer matrix. No considerable change in the capsule size was observed.

These physical properties are very important for this approach. Although *L. lactis* are "generally regarded as safe" and noninvasive, the administration of genetically modified organisms for clinical purposes can still produce some concerns. The possibility of the dissemination of the expressed gene or antibiotic resistance genes and their effect on the host microbial flora is an ever present issue of concern [14]. Immobilization of cells in APA has the advantage of providing a precautionary boundary to retain the cells enclosed in microspheres as they pass through the human system before being excreted in the stool.

Storage Stability of Microcapsules

We monitored the survival of encapsulated *L. lactis* cells during storage in a solution of GM17 media/PS (1:10) at 4°C. Microcapsule preparations demonstrated their ability to maintain cell survival over 8 weeks. During the initial 2 weeks, cell viability appeared to increase by nearly 1 log unit (Fig. 5). Further tests performed showed this early, unprompted growth occurred during the initial 4 days of storage (data not shown), presumably a result of supplying a dilute nutrient solution to primed cell preparations. Thereafter, the viability remains relatively stable, decreasing less than 0.3 $\log_{10}$ units. Microscopic analysis revealed these microcapsules remained structurally intact with no visible changes in their size, shape, or cell density (data not shown). This demonstrates that APA encapsulation is a good method to sustain the viability of engineered *L. lactis* for a long term under refrigeration.

Conclusions

Whereas the use of live *L. lactis* for oral protein delivery has been proven successful, encapsulation is an avenue that can provide a resolution to the limitations that exist. Tests performed have demonstrated that encapsulated cells engineered for protein delivery have comparable growth and protein secretion as free cells. During this, cells are retained in the enclosed polymer matrix as the capsules maintain their physical stability. The advantages imparted by microencapsulation highlight this novel system for the oral delivery of heterologous proteins that can be further tested in experimental animals for their clinical significance.

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