JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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Enhanced Survival of Probiotic *Lactobacillus acidophilus* by Encapsulation with Nanostructured Polyelectrolyte Layers through Layer-by-Layer Approach

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ABSTRACT: The encapsulation of probiotic *Lactobacillus acidophilus* through layer-by-layer self-assembly of polyelectrolytes (PE) chitosan (CHI) and carboxymethyl cellulose (CMC) has been investigated to enhance its survival in adverse conditions encountered in the GI tract. The survival of encapsulated cells in simulated gastric (SGF) and intestinal fluids (SIF) is significant when compared to nonencapsulated cells. On sequential exposure to SGF and SIF for 120 min, almost complete death of free cells is observed. However, for cells coated with three nanolayers of PEs (CHI/CMC/CHI), about 33 log % of the cells (6 log cfu/500 mg) survived under the same conditions. The enhanced survival rate of encapsulated *L. acidophilus* can be attributed to the impermeability of polyelectrolyte nanolayers to large enzyme molecules like pepsin and pancreatin that cause proteolysis and to the stability of the polyelectrolyte nanolayers in gastric and intestinal pH. The PE coating also serves to reduce viability losses during freezing and freeze- drying. About 73 and 92 log % of uncoated and coated cells survived after freeze-drying, and the losses occurring between freezing and freeze-drying were found to be lower for the coated cells.

KEYWORDS: probiotics, layer-by-layer, encapsulation, survival, polyelectrolytes

INTRODUCTION

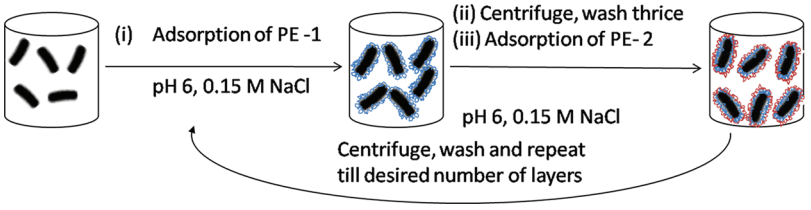
Probiotics, in recent times, have received increased attention because of the multitude of health benefits they incur on the host system when taken in adequate amounts.[1,2] However, the required stability of the microencapsulated organism is not always achieved and the number of viable bacteria becomes deficient to attain the intended effect. The minimum number of bacteria needed to provide any desired effect must be about 10^6-10^7 cfu/mL according to the Food and Agriculture Organization−World Health Organization (FAO-WHO). The method of choice to increase the survival of probiotics is encapsulation or immobilization in a matrix. This is usually done by spray drying, emulsion, extrusion, and gel techniques.[3,4] Encapsulation involves the coating of the microorganism, forming a shell around it or confining and enclosing it within a larger matrix.

Many encapsulation techniques have been devised to protect the bacteria from adverse environmental and processing conditions and also in in vivo conditions. Encapsulation is preferred over immobilization of bacteria because it completely covers the cell, thus giving maximum protection against deleterious conditions. The shell can also be made semipermeable, have sufficient mechanical integrity, and reduce mass transfer limitations.[5−8] However, the challenges with encapsulating microorganisms are multifold. (i) The cells must be viable after coating. (ii) The operating conditions such as pH and ionic strength during encapsulation must be mild. (iii) The encapsulating layers should be robust enough to be able to sustain detrimental packaging materials. (iv) The mechanical integrity of the coating should be able to overcome pressure variations in vivo. (v) It should serve as a barrier to oxygen (for anaerobic species). (vi) The sensory properties should not be altered. (vii) The cells should survive the postfermentation conditions happening during long-term storage. In spite of the challenges associated with encapsulating probiotics, there are several benefits that can be obtained by having a camouflaging shell on the outside. The shell protects the cell from other contaminants like yeast and bacteriophages, provides stability during freezing, facilitates recovery of oxygen sensitive and centrifugation sensitive cultures, and increases survivability in the GI tract.[3]

Encapsulation in microspheres using various polymers has been shown to provide improved survival in gastric/bile solutions.[9−13] In all of the above studies, the reduction in the number of cells was about 3−4 log cycles for the encapsulated cells when compared to about 6−8 log cycles for the nonencapsulated ones. Alginate is a widely used encapsulating material that forms a gel in the presence of divalent cations. It also provides a stable matrix and allows easy processing conditions. It does not disrupt physiological functions, reduces immunogenic responses, and cushions the stress caused by pressure variations in vivo.[4] The limitation of using alginate is its inability to withstand low pH encountered in the stomach. Hence, researchers have tried to overcome this problem by using an outer chitosan coating to protect the microcapsule from harsh gastric conditions.[2] Resistant starch was also found to impart robustness during processing while ensuring good adherence of the bacteria to the starch.[14] Reports also suggest increased survival of encapsulated cells on freezing, heating, and storage under refrigerated and nonrefrigerated conditions and in dairy products when compared to free cells.[15−18] When trehalose (along with the type of alginate used for encapsulation) was used as the cryo-protective sugar, about 85−97 log % of cells survived after freeze-drying in the presence

Received: August 25, 2011
Accepted: September 30, 2011
Revised: September 30, 2011
Published: September 30, 2011

Scheme 1. LbL Assembly of Polyelectrolytes (PE) on Bacterial Cell Wall



of pepsin, bile salts, and lysozyme.[19] A recent study also reports higher survivability of cells at low pH using a novel rennet enzyme gelation technique for microencapsulation of probiotic strains, giving higher encapsulation efficiency and smaller capsule size (~60 μm).[20] All of the above studies involved encapsulation by spray drying, extrusion, or other technologies resulting in microspheres of various sizes (30 μm to 3 mm). Most of the above-mentioned methods are mostly immobilization techniques rather than encapsulation technique.

One of the most efficient true encapsulation techniques is perhaps the layer-by-layer (LbL) technique, which was first introduced by Decher[21] in the 1990s. It is a versatile method that was further developed and applied to a variety of substrates including flat surfaces, colloidal particles of different sizes, shapes, and charges. Colloidal particles such as silica, $CaCO_3$, polystyrene latex particles, and even exotic biological substrates like erythrocytes, protein aggregates, and also drug aggregates have been coated by polyelectrolytes.[22−27] Usually, in most cases, the core is dissolved chemically to yield hollow capsules of dimensions comparable to that of the substrate used. These hollow capsules have the potential for applications in pharmaceutical, food, cosmetic, textile, and agricultural industries for delivery of actives in a controlled manner as proved by several studies.[28−30]

The LbL procedure involves the alternative adsorption of oppositely charged polyelectrolytes on surfaces, thereby providing a system with tunable properties. The thickness, permeability, strength, and morphology of the layers can be tailored with precision (by altering the pH, ionic strength, wall materials), providing an ambience with the desired properties. Thus, LbL technique can be exploited for the formation of nanocages on living microorganisms too.

The LbL approach as a new design encapsulation methodology on living cells was first investigated by Diaspro and co-workers.[31,32] Yeast cells were encapsulated in nanolayered polyelectrolyte shells, consisting of alternate layers of polystyrene sodium sulfonate and polyallylamine hydrochloride. They proved the viability of cells after encapsulation and showed that cell division occurs inside the PE shell by means of confocal and two-photon fluorescence microscopy. However, this coating could not protect the cells against the lysosomal enzymes of the *Paramecium*. But this study proved that LbL coating is a simple procedure that has promising applications in biotechnology. Further, study on the morphology change with respect to ionic strength and the elastic modulus of the encapsulated and free cell showed the physical and chemical properties that can be altered by using LbL methodology.[33] The use of LbL technique for biorecognition through DNA hybridization was demonstrated by Hilberg et al.[34] Mesenchymal stem cells from mouse were also successfully encapsulated by LbL technique using polylysine and hyaluronic acid and shown to survive for up to 7 days after encapsulation.[35] The immobilization of nanoparticles by sandwiching them between two PE layers using LbL as a tool to visualize bacteria for surface enhanced Raman spectroscopy measurements has also been tried by other researchers.[36] They have also successfully demonstrated that multicellular assemblies (cellosomes) can be made on rods and rhombohedral templates coated with magnetic nanoparticles for manipulation by external magnetic field.[37] The uptake of sulfide ions and elemental sulfur by bacteria coated with PE layers was shown to vary with the number of layers, showing that the surface properties can be modified according to the requirements.[38]

Unlike other methods, LbL is unique because each individual cell in suspension is coated sequentially, affording complete encapsulation. In our work, we have examined the self-assembly of polymers on probiotic *Lactobacillus acidophilus* surface and tested its survivability in adverse conditions like low pH, in the presence of bile salts and also its protective ability against digestive enzymes like pepsin and pancreatin. We have also examined the survivability before and after freezing and after freeze-drying and as described above in simulated gastric and intestinal conditions in vitro. Our results indicate that the LbL procedure is indeed a promising method to enhance the viability of bacteria during storage, processing, and transit through the GI tract.

MATERIALS AND METHODS

Materials. Chitosan (CHI) (M_w = 70 kDa), carboxymethyl cellulose (CMC) (M_w = 483 kDa), FITC−dextran (M_w = 70 kDa), 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), glycerol, antifade 1, 4-diazabicyclooctane (DABCO), pepsin, NaCl, bile salts, pancreatin, and phosphate buffered saline tablets (PBS) were all purchased from Sigma-Aldrich and used without any further purification. MRS broth was obtained from Hi-Media Labs and used as such. *L. acidophilus* (NCIM 2285) was obtained from National Collection of Industrial Microorganisms (NCIM, Pune, India). The water used in all the experiments was obtained from Milli-Q system with resistivity greater than 18 MΩ cm. All pH adjustments were done with 0.1 M HCl or 0.1 M NaOH. All glass/plastic ware and water used were sterilized in an autoclave at 121 °C for 30 min.

Growth of *L. acidophilus*. *L. acidophilus* was grown in MRS media broth at 37 °C, aerobically for 13 h in a flask kept in a shaker at 220 rpm. After growth, the cells were harvested by centrifugation (Remi, C-24BL) at 8000 rpm for 15 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in distilled deionized water and centrifuged again. This procedure of washing was repeated thrice, and then the cells were used for encapsulation. The coated and uncoated free cells were later lyophilized and stored at 4 °C for 3 weeks and used for further experiments.

Enumeration of Viability. Cell count for the experiments was done by pour plate counts in MRS agar aerobically, and incubation was done at 37 °C for 48 h. Cell counting for cell suspensions was done using a Neubauer hemocytometer. The viability count was expressed as colony forming units (cfu) for powder and cfu/mL or cells/mL for suspended

samples. Serial dilutions were made when required, and all experiments were done in duplicate. The data are reported as mean survival values ± standard deviation.

Encapsulation of *L. acidophilus*. Polyelectrolyte (PE) concentration of 1 mg/mL in 0.15 M NaCl at pH 6 was used consistently for both the PEs. Fresh cells were harvested and washed as mentioned previously. Since the cells are negatively charged, the deposition of first layer was done with CHI at 37 °C under gentle stirring for 20 min until equilibrium was attained. After the deposition of the first layer, the excess polyelectrolyte solution was removed by centrifugation (3500 rpm, 15 min, 4 °C) and the cells were washed thrice in 0.15 M NaCl solution as described above. Then the second layer was made to adsorb by suspending the cells in CMC under similar conditions. Centrifugation and washing was repeated, and the procedure of alternate polymer adsorption was continued until the desired number of layers was obtained as shown in Scheme 1. At the assembly pH, i.e., at pH 6, the amino group of CHI is protonated ($pK_a = 6.5$) and the carboxyl group of CMC ($pK_a = 4.3$) is deprotonated leading to positive and negative charges on the polyelectrolyte chains, thereby facilitating electrostatic self-assembly of layers. The coated cells were freeze-dried using a tabletop lyophilizer and stored for 3 weeks at 4 °C prior to experiments.

Confocal Laser Scanning Microscopy Analysis. The size, integrity, and the permeability of the coated and uncoated *L. acidophilus* were determined using a LEICA TCS SP5 DM 6000 confocal scanning system (Leica, Germany) equipped with a 63× oil immersion objective. The cells were visualized by using two dyes, one macromolecular FITC conjugated to dextran and DAPI, which is a DNA staining dye. About 10^6 cells/mL were suspended in distilled deionized water, and first FITC−dextran was added and allowed to interact for 20 min at room temperature. Then the sample was centrifuged and the pellet was resuspended in DAPI solution for another 20 min at room temperature. The suspension was again pelletized and redispersed and fixed on a glass slide using a solution of 1:1 PBS/glycerol containing 2% DABCO. The excitation wavelength used was 405 and 488 nm for DAPI and FITC−dextran, respectively.

ζ-Potential Studies. The charge on the bacterial surface after the adsorption of consecutive PE layers was monitored using a ζ-sizer (Malvern Zetasizer, Nano-ZS 90). About 10^3 cells/mL were suspended in doubly distilled deionized water, and the ζ-potential was calculated from the mobility measurements. Each data point reported is the average of 12 measurements ± standard deviation.

Freezing and Freeze-Drying. Pellets of coated and uncoated free cells were frozen in liquid N_2 for 1 min at atmospheric pressure and then freeze-dried using a benchtop lyophilizer (Labconco, Freezone 4.5). The cell count before freezing, after freezing (thawed), and after freeze-drying (rehydrated) was done as described previously. After freeze-drying, the samples were stored at 4 °C in a refrigerator.

Simulated Gastric and Intestinal Fluid. Simulated gastric fluid (SGF) was made using 9 g/L NaCl, containing 3 g/L pepsin, and the pH was adjusted to 2 using HCl. Similarly, simulated intestinal fluid (SIF) was made by dissolving pancreatin (1 g/L) and bile salts (3 g/L) in intestinal solution whose composition is as follows: 6.5 g/L NaCl, 0.835 g/L KCl, 0.22 g/L $CaCl_2$, 1.386 g/L $NaHCO_3$.[4] The pH of the solution was 8 and used as such without further pH adjustments. Experiments were done with 500 mg of freeze-dried samples in 10 mL of simulated gastric fluid for 30, 60, 120 min followed by incubation for 1 and 2 h in 10 mL of simulated intestinal fluid. The cell viability was done by pour plate method with appropriate dilutions needed as described in the above section. All experiments and plate counts were done in duplicate.

RESULTS AND DISCUSSION

Cell Viability after Encapsulation. In order to prove the ability of cells to duplicate after coating by polyelectrolytes, we studied the growth kinetics of the coated and uncoated *L. acidophilus* in MRS medium at 37 °C at 220 rpm in a shaker in aerobic conditions. As seen in Figure 1, *L. acidophilus* coated with three and seven PE layers was found to grow as well as the uncoated ones. Similar cell numbers are obtained for both coated and uncoated cells at the end of the growth cycle. However, the lag phase of the coated samples is longer when compared to that of the uncoated samples. Uncoated samples have a lag phase of about 4 h, whereas that of samples coated with three or seven PE layers is about 8 and 15 h, respectively. This is because the time taken for the hydration and nutrition uptake is slower for the coated samples and depends on the number of PE layers. Additional layers increase the lag phase in the growth curve, and the cell division occurs only when sufficient nutrition enters

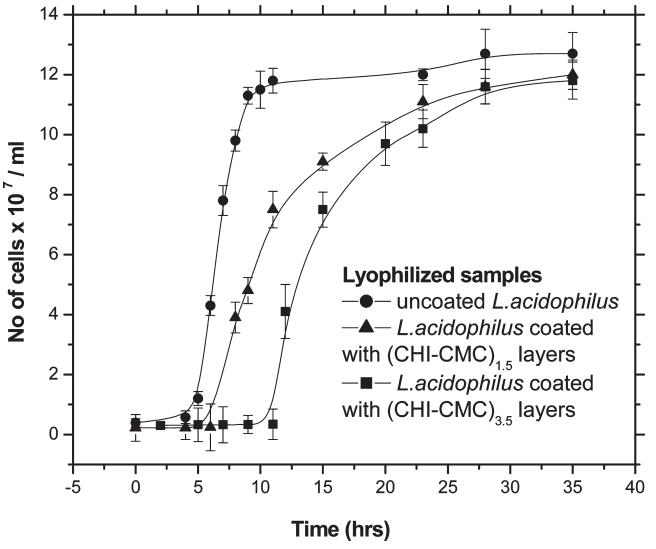


Figure 1. Growth curve of coated and uncoated *L. acidophilus* grown from lyophilized powders in MRS broth at 37 °C at 220 rpm. An amount of 10 mg of the powder was inoculated at zero time (mean ± SD, $n = 2$).

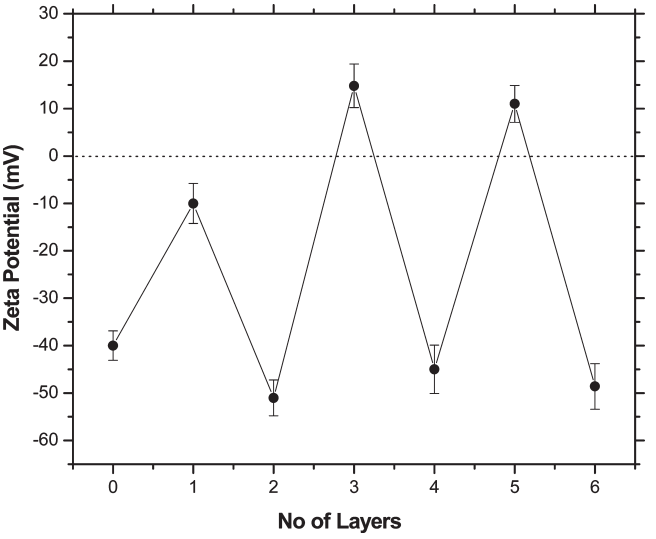


Figure 2. ζ-Potential change with alternate adsorption of chitosan (CHI) and carboxymethyl cellulose (CMC) on the surface of *L. acidophilus* (mean ± SD, $n = 2$).

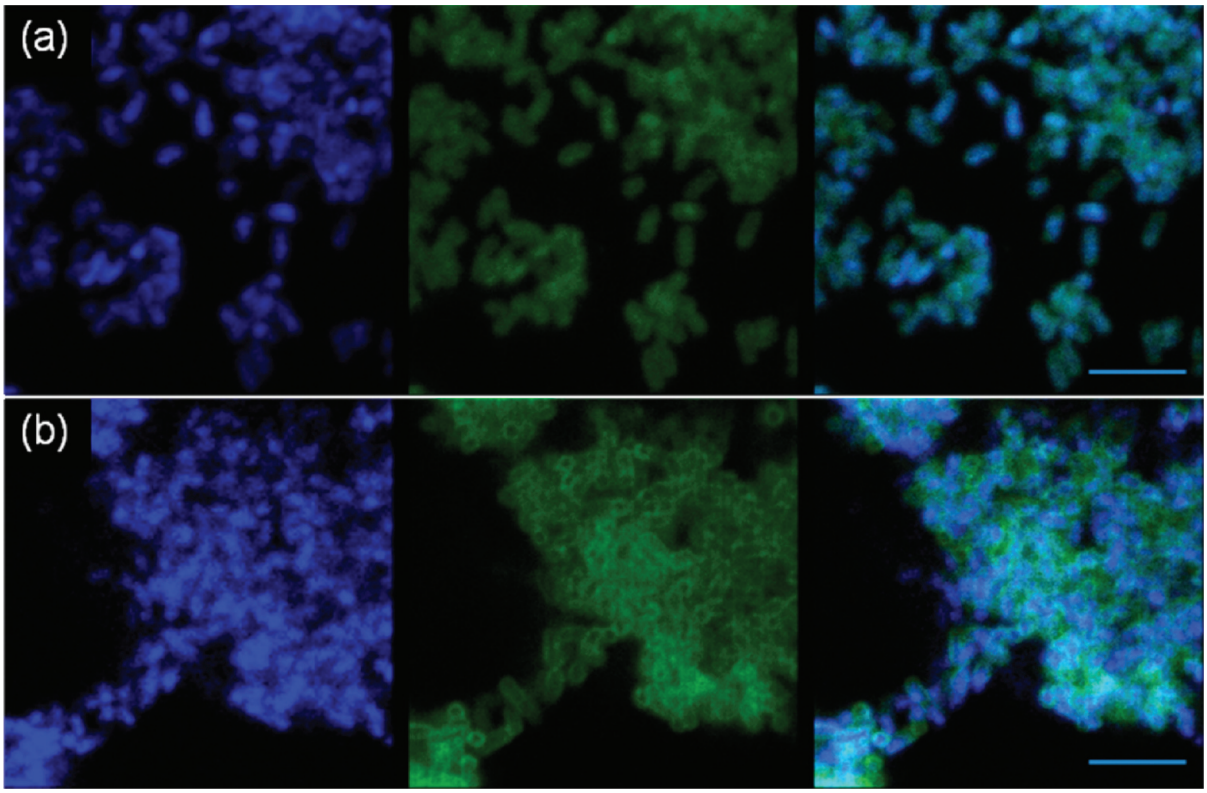


Figure 3. Confocal laser scanning micrographs of (a) uncoated and (b) coated *L. acidophilus* showing permeability to small (DAPI) and large (FITC−dextran) molecules for uncoated cells and only to small molecules for coated cells. The green fluorescence is seen on the outside and the blue fluorescence on the inside for the coated cells, whereas it is seen throughout the cells for the uncoated ones. Scale bar corresponds to 3 μm.

the cells. Thus, encapsulated cells are able to divide and multiply, indicating normal metabolic activity.

ζ-Potential Measurements. The process of LbL assembly on any surface manifests itself as reversal of surface charge after the adsorption of each layer, and this can be observed by measuring the ζ potential after the deposition of each layer. The overall surface charge of the bacteria is negative because of the presence of ionized acid groups on the cell wall. The adsorption of oppositely charged PEs on the cell wall of the bacteria depends on many factors like the type and molecular weight of PE used and the chemical conditions (pH, ionic strength) employed. The negatively charged bacterial surface served as the substrate for the adsorption of polycation as the first layer. The alternate negative and positive ζ potential for each PE layer deposition confirms the LbL self-assembly on the bacterial surface (Figure 2). However, since the bacterial surface is highly negative (−40 mV) and the polyelectrolyte chitosan was deposited from a solution of pH 6 that is closer to its isoelectric point, complete charge compensation does not occur during the deposition of the first layer itself. However, it can be seen that the adsorption of subsequent layers displays alternate negative and positive ζ potential.

CLSM: Study of Bacterial Wall Integrity and Permeability. The integrity and intactness of the bacterial cell wall before and after encapsulation can be evaluated by staining with a nuclear staining dye like DAPI. However, DAPI stains both live and dead cells and is not indicative of viable cells. The permeability of the coated cells to various molecules depends on many factors: the choice of PE, the ionic strength, the number of layers, and the pH of the solution. When the ionic strength is constant during assembly and washing, the pore size in the layers is about a few nanometers.[39] Here, we have studied the penetration of two fluorescent dyes, FITC attached dextran (high molecular weight) and DAPI (low molecular weight). The images in Figure 3 confirm the integrity of the bacterial cells after encapsulation. Distinct bacterial cells with intact membrane are found throughout, proving the ability and the applicability of the method for encapsulation. The uncoated cells take up both DAPI and FITC−dextran easily, showing that the cell membrane is permeable to both lower and higher molecular weight molecules. But when there are three layers of PE coating, the penetration of higher molecular weight FITC−dextran is prevented and only the smaller DAPI is able to enter into the cells. This shows that the layers around the cells create a barrier for the penetration of molecules of larger size (>5 nm), indicating that the pores in the layers are of even smaller magnitude, thereby allowing only DAPI of smaller size (<1 nm). Therefore, the needed nutrient molecules are allowed to pass through freely, while harmful bacteriophages, enzymes, and other macromolecules that cause cell death are prevented from entering the cell wall. Thus, we see the blue fluorescence of DAPI inside the cell and the green fluorescence of FITC outside on the cell wall showing selective permeability to different molecular sizes. In the uncoated cells, both the blue and green fluorescence (mixing of blue and green gives whitish blue) is found throughout the cell, indicating the permeability of the cell wall to large macromolecules.

Viability Losses during Freezing/Freeze-Drying. The loss of viability on freezing occurs mainly because of the injury on the cell membrane and the osmotic imbalance arising out of water crystallization during freezing.[19] Table 1 shows the viability losses of coated and uncoated cells before freezing (BF), after

Table 1. Survivability of Coated and Uncoated Free *L. acidophilus* before Freezing (BF), after Freezing (AF), and after Freeze-Drying (AFD) (Mean ± SD, $n = 2$)[a]

	uncoated free *L. acidophilus*, log cfu/mL	*L. acidophilus* coated with $(CHI/CMC)_{1.5}$ layers, log cfu/mL
before freezing	9.00 ± 0.34^{a}	9.25 ± 0.27^{a}
after freezing	7.25 ± 0.42^{c}	8.60 ± 0.38^{b}
after freeze-drying	6.49 ± 0.31^{d}	8.44 ± 0.26^{b}

[a] Statistical analysis was done by one-way ANOVA, and statistical significance was set at $p < 0.01$. Mean values with different letters are significantly different.

freezing (AF), and after freeze-drying (AFD). The number of viable cells decreased to log 7.25 cfu/mL (80.5%) from log 9 cfu/mL after freezing and further dropped to log 6.49 cfu/mL (73%) after freeze-drying for uncoated cells. The encapsulated cells showed lesser viability losses, and the viable cell count decreased from an initial count of log 9.25 cfu/mL to log 8.6 cfu/mL (93%) and log 8.48 cfu/mL (91.6%) after freezing and freeze-drying, respectively. Statistical analysis by one-way ANOVA indicates that the survivability of the coated *L. acidophilus* is greater than that of free cells after freezing and freeze-drying. For free cells, the difference in mean values is statistically significant after freezing and freeze-drying, whereas for coated cells, it is not significant under the same conditions. This shows that the losses occurring between freezing and freeze-drying are greater for free cells and almost negligible for the coated cells. The nanolayer coating provides protection against damage and injury to the cell membrane that is the main cause of cell death. The polyelectrolytes used also serve as natural carbon resources for maintaining the cell viability and normal metabolic activity.[40,41] When the study was performed with cells coated with seven layers, the plate count method failed to give proper counts in terms of colony forming units, since there are aggregates formed with the increase in number of coated layers. Consequently even with serial dilution, the aggregates could not be disintegrated to give a homogeneous sample suspension. Hence, the error was high when counting cell numbers through the plate count method. Also, when the cell reproduces, it has to grow in size and break off the polyelectrolyte coating before dividing and multiplying. With the increase in layer thickness, this process takes longer time (as evidenced by very long lag phase of 15 h when grown in broth), and hence, it is possible that the agar medium is no longer chemically stable with respect to the optimum conditions required for growth. This in turn affects the growth cycle and the growth rate of the organism.

Survival of Coated and Uncoated *L. acidophilus* in SGF and SIF. The main aim of encapsulation is to protect the bacteria from detrimental enzymes and low pH during its transit through the gastrointestinal tract. Any oral supplement has to pass through the gastric juices, which have a pH of about 1.5−3 and contain pepsin, a proteolytic enzyme that is active only below pH 4. A study on the role of the components of gastric fluids in bacterial killing indicates that pH below 2 and the addition of pepsin caused significant death of *E. coli*.[42] However, the pH in the stomach is not always below 2 and it increases further to 3−3.5 during food uptake. Here, we have examined the survivability of coated and uncoated *L. acidophilus* in gastric environment followed by passage through intestinal

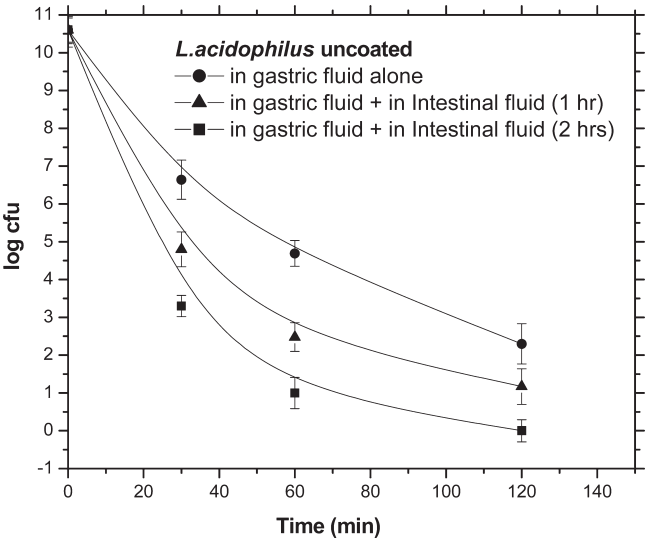


Figure 4. Survivability of uncoated *L. acidophilus* when exposed to simulated gastric and intestinal conditions (mean ± SD, $n = 2$). An amount of 500 mg of the sample was taken initially. The sample was incubated in SGF for different time intervals and transferred to SIF for a period of 1 or 2 h.

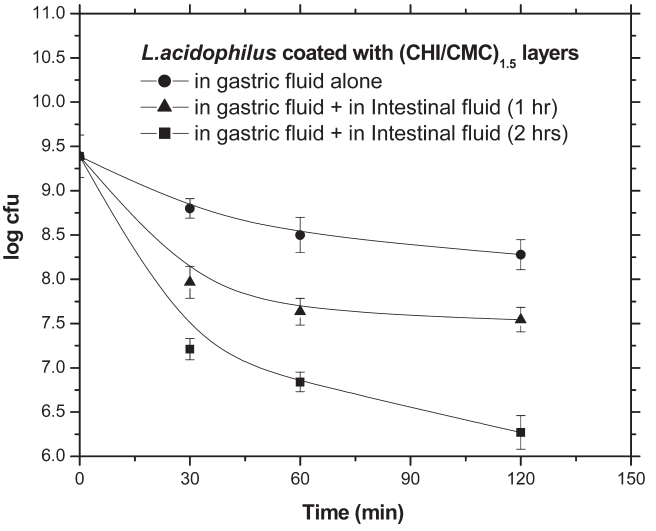


Figure 5. Survivability of *L. acidophilus* coated with $(CHI/CMC)_{1.5}$ layers when exposed to simulated gastric and intestinal conditions (mean ± SD, $n = 2$). An amount of 500 mg of the sample was taken initially. The sample was incubated in SGF for different time intervals and transferred to SIF for a period of 1 or 2 h.

fluid at various time intervals in vitro. We have done all the studies with cells coated with three layers of PEs only due to the problems mentioned in the previous section when more number of layers is used. Figure 4 and Figure 5 gives us a clear indication of the advantage of encapsulating bacteria within a shell. Uncoated cells have low survival in harsh conditions. On incubation in SGF for 120 min, the number of cells declined to 4 log cfu/500 mg from an initial count of 10.4 log cfu/500 mg. For cells coated with $(CHI/CMC)_{1.5}$ layers the decline was significantly less in the same conditions, from an initial count of 9.4 log cfu/500 mg to 8.2 log cfu/500 mg. The enhanced

survival rates of encapsulated *L. acidophilus* are attributed to the following reasons.

(i) The polyelectrolyte CHI/CMC complex is stable in acidic pH unlike the widely used alginate that is unstable at low pH. At the assembly pH of 6, the layers are held firmly, mainly by electrostatic attraction. Once the LbL assembly is formed, the pK_a of the polyanion and polycation gets shifted by about 2−3 pH units because of the attractive potential created by the neighboring charges when compared to isolated groups in pure PEs.[43] In our case, this causes the shift of pK_a of CHI and CMC to higher (>6.5) and lower (<4.5) pH, respectively. When the pH is lowered to 2 in gastric environment, the amino groups of chitosan are protonated whereas the carboxyl groups of CMC remain undissociated/slightly dissociated. There is slight swelling of the layers due to disruption of the electrostatic bonding that occurs at near neutral pH. But this does not lead to the disintegration of the nanolayers at low pH, as hydrogen bonding interactions also operate collectively leading to stability of the layers. The stability of the CHI/CMC complex at lower pH has also been proven by other workers.[44] Also, the layers have the ability to be viscoelastic, retaining their strength and structure under pressure variations and mechanical stresses.[39]

(ii) Furthermore, the selectivity of the layers toward permeability for ions of different molecular weight as shown by CLSM studies helps in the exclusion of the larger enzyme molecule pepsin that causes proteolysis and consequently the death of the bacteria.

(iii) A Donnan equilibrium situation arises when a semipermeable membrane excludes larger sized polyions and allows only water and smaller ions to freely permeate between the interior and exterior. In our case, the selective permeability gives rise to a Donnan distribution of permeable (small, i.e., Na^+, Cl^-, H^+, OH^-) and impermeable (large macromolecular pepsin) ions.[45] Pepsin has an IEP at pH 1. Hence, the polyion of pepsin is anionic in the gastric conditions employed. Though overall electroneutrality will be maintained in the suspension, the distribution of the permeable positive and negative ions between the exterior and interior of the encapsulated bacteria is not uniform. The association of the permeable cations with the polyanion reduces the inflow of cations into the interior of the bacteria. Since the permeable ion includes H^+, this leads to a pH difference inside and outside the cells, making the interior slightly more basic than the exterior. Therefore, the pH encountered by cells is higher and this further helps in reducing the mortality rate of the encapsulated bacteria.

As the bacteria pass through the gastric environment of the stomach and reach the intestine, the conditions they are exposed to in the intestine are different and completely reversed in terms of pH. The intestinal pH is around 8, and there is a strong osmomolar difference (due to the presence of many salts) between the bulk and the interior of the encapsulated cells. But in spite of these extreme changes in conditions, the encapsulated cells show better survival when compared to uncoated free ones. Figures 4 and 5 describe the improved survival rates for cells coated with three layers of PEs $(CHI/CMC)_{1.5}$, obtained at different time intervals in simulated gastric fluid followed by sequential incubation in simulated intestinal conditions. Sequential exposure to SIF for 120 min after exposure to SGF (for 120 min) resulted in the survival of only about 1 log cfu/500 mg for the uncoated bacteria. But under the same conditions, about 6.3 log cfu/500 mg of the encapsulated cells were found to survive, which is the minimum amount required for therapeutic effects. However, the loss of viable cells in these conditions is greater than the losses that occurred in gastric fluid. This is due to the enzymatic degradation of polyelectrolyte layers by pepsin present in gastric fluid. Chitosan, though stable at low pH, can be degraded enzymatically by many nonspecific enzymes including pepsin. This degradation depends on the molecular weight, degree of deacetylation, and the concentration of chitosan.[46,47] Thus, the depletion of protective coating makes the cells more susceptible to deleterious intestinal conditions. The osmomolar stress created by the polyelectrolyte layer depletion becomes larger thereby deforming the nanolayers further, exposing the cells to harsh conditions. Also, at higher pH, the layers start losing their integrity (because of repulsion of deprotonated carboxyl groups and lack of other stabilizing counter effects) and get deformed, which is also a cause for increased death of bacteria in intestinal solution.

In conclusion, we have shown that the survival rate of probiotic *L. acidophilus* encapsulated through LbL self-assembly of biocompatible polyelectrolytes chitosan (CHI) and carboxymethyl cellulose (CMC) is enhanced during its gastrointestinal transit. The alternate ζ potential displayed by adsorption of positive and negatively charged CHI and CMC proves that LbL coating has occurred on the bacteria surface. About 10^6 cfu/500 mg (33 log %) of encapsulated cells survive when exposed to simulated gastric fluid for 2 h followed by sequential exposure to simulated intestinal fluid for 2 h, whereas uncoated, unprotected cells were almost completely destroyed in identical conditions. The nanolayers are stable at low pH in the gastric environment and also shelter the cells under intestinal conditions, in spite of the higher pH and osmotic stresses encountered by the bacteria under such conditions. The layers of nanodimensions also exclude larger enzyme molecules like pepsin and pancreatin, preventing proteolysis of the cells, while allowing smaller nutrient molecules to pass through. The viability losses that occur during freezing and freeze-drying are also lesser for the encapsulated cells. About 93 log % of encapsulated cells survived after freeze-drying, whereas only 73 log % of uncoated free cells survived under identical conditions. Thus, nanocoatings engineered through LbL process provide a versatile method to customize and tune properties according to specialized needs.

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Funding Sources

This work was funded by Dow Chemical International Pvt. Ltd., Pune, India.

ACKNOWLEDGMENT

The authors thank the Centre for Imaging Research in Science and Engineering (IRIS), Indian Institute of Science, for the use of

confocal laser scanning microscopy facility. The authors thank Dr. Nimisha Agarwal and Dr. Madhav Kulkarni, Dow Chemical International Pvt. Ltd., Pune, India, for helpful discussions.

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