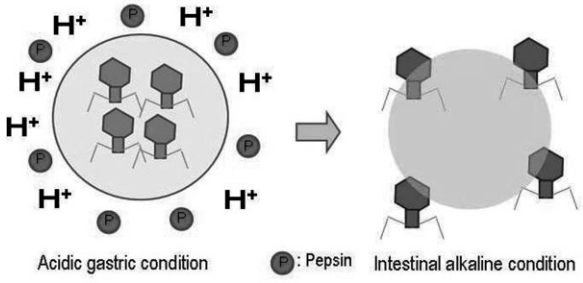
Novel Biopolymer Matrices for Microencapsulation of Phages: Enhanced Protection Against Acidity and Protease Activity

Cecilia Dini,* Germán A. Islan, Patricio J. de Urraza, Guillermo R. Castro

Phage therapy by oral administration requires enhanced resistance of phages to the harsh gastric conditions. The aim of this work is the microencapsulation of phages in natural biopolymeric matrices as a protective barrier against the gastric environment. Alginate and pectin are used as base polymers. Further emulsification with oleic acid or coating with a different biopolymer is also studied. Emulsified pectin shows the maximum encapsulation efficiency and the highest protection against acidity, leaving more than 10^3 active phages after 30 min exposure at pH = 1.6, and protects phage from pepsin activity (4.2 mg mL^{-1}). Non-encapsulated phages are fully inactivated at pH = 1.6 or with pepsin (0.5 mg mL^{-1}) after 10 min.



1. Introduction

The use of bacteriophages as biocontrol agents is becoming relevant since the extensive use of antibiotics for the preservation and control of pathogens in food is promoting the proliferation of multiresistant bacteria.[1] Phage therapy can be used in animals intended for consumption that act as reservoirs of pathogens.[2] The animal stage is highly relevant because it reduces the possibility of the introduction and spread of pathogens into the production and distribution food chains.

Bovines are the main animal reservoir of enterohemorrhagic *Escherichia coli* (EHEC). This bacterial pathogen is considered the major cause of haemolytic-uremic syndrome (HUS) worldwide.[3,4]

Many studies concerning the use of specific phages with ruminants in order to reduce or eliminate EHEC from its reservoir have been published.[5–8] Despite some discrepancies in the results reported so far, oral application seems to be the most practical way of phage administration. However, high doses, in the range of 10^{11} to 10^{13} PFU, are required to allow a considerable number of phages (10^6 PFU or more) to reach the intestine.[9]

In a previous work, phage CA933P had been selected among 20 different EHEC phages isolated in our laboratory.[10] Phage CA933P is highly lytic, and capable of infecting EHEC serotypes O157:H7, O145:H25, O13:H8, and ONT:H12 and other relevant Gram-negative pathogens such as *Shigella flexneri* and *Pseudomonas aeruginosa* species. However, phage CA933P showed high sensitivity to extreme environmental conditions, such as pH values below 4, which constitutes a serious limitation for its oral application.[10] In particular, the phage must withstand the passage through the acid gastric system to be of therapeutic use.

Dr. C. Dini, Prof. P. J. de Urraza
CIDCA (CONICET-UNLP) CCT La Plata, 1900, La Plata, Argentina
Dr. C. Dini, Prof. P. J. de Urraza
Cátedra de Microbiología, Facultad de Ciencias Exactas, UNLP., 1900, La Plata, Argentina
E-mail: cdini@biol.unlp.edu.ar
G. A. Islan, Prof. G. R. Castro
CINDEFI (CONICET-UNLP) CCT La Plata, 1900, La Plata, Argentina

DOI: 10.1002/mabi.201200109

Controlled release technologies are playing a crucial role in overcoming critical environments in which the biological activities of phage are naturally inactivated. In particular, encapsulation in natural biopolymer-derived matrices is gaining increasing attention in recent years.[11] Among the relevant natural biopolymers, promising properties include: synthesis under mild environmental conditions, stereospecific molecular structure, ease of tailorability, and environmental friendliness.[12,13] In addition, some natural biopolymers can be considered 'smart' molecules because they are insensitive to the harsh acidic environmental conditions of the stomach but are responsive to alkaline medium (e.g., the intestinal environment). This property allows the encapsulated molecules and/or biological structures, like phages or sensitive molecules, to be protected against these extreme acid conditions without swelling. On the contrary, the particle cargo can be released from the capsules in the intestine tract having neutral to alkaline environmental conditions by selecting the proper biopolymeric matrix.[12,14,15]

Natural polymers like alginates, pectins, guar gums, or chitosans are, among other common excipients, used in many foods and pharmaceutical formulations.[16,17] In particular, pectin is an inexpensive, non-toxic polysaccharide extracted from higher plant cell walls. Basically, pectin is a partial methyl ester of α-D-galacturonic acid (poly-Gal) interrupted with $(1 \rightarrow 2)$-α-L-rhamnose units and other minor neutral sugars. Low and medium methoxylated pectins with a degree of esterification, roughly less than 40% or between 40 and 60%, respectively, are able to form rigid gels by the action of multivalent cations by crosslinking the galacturonic acid moieties. Meanwhile, high methoxylated pectins, with an esterification degree higher than 60%, are able to from gels by the presence of H^+ and co-solutes.[18]

Alginate is one of the most widely used materials for encapsulation of molecules and cells. Alginates are natural polysaccharides obtained mainly from marine algae and some bacteria. The alginate basic structure consists of linear unbranched polymers containing β-$(1 \rightarrow 4)$-linked D-mannuronic acid (M) and α-$(1 \rightarrow 4)$-linked L-guluronic acid (G) residues. The structure of alginate gels crosslinked with calcium ions is commonly referred to as "egg-box" by analogy. In this structure the divalent calcium ion plays a key role in making the bridges between single alginate chains.[18] Pectins capable of forming gels by ionic gelation with multivalent ions are not able to form egg-box like structures, showing a more random gel network.[19] However, both matrices are suitable for molecule encapsulation, providing major benefits such as high efficiency in protein delivery, control of the amount of protein released, and enhanced protein stability.[20] Despite these benefits, a need for new delivery vehicles providing improved protection of carried molecules against fast acid or proteolytic degradation while also maximizing molecule loading are required. In this way, the development of new and more efficient alternatives for delivery are essential, which can include blends, emulsifications, and surface coating options.

The aim of the present work is to develop biopolymer-based matrices as oral delivery systems for the controlled release of bacteriophage CA933P as a biocontrol agent of EHEC in the bovine intestine. In vitro evaluation of the protection capability against acidity and the proteases of the ruminants' digestive tract and the ability to release the encapsulated phages in conditions similar to those expected in the intestine of cattle is performed.

2. Experimental Section

2.1. Bacteriophages, Bacterial Strains, and Media

Bacteriophages CA933P and wild-type isolate 933 of enterohemorrhagic *Escherichia coli* O157:H7 (Children Hospital 'Sor Maria Ludovica', La Plata city, Buenos Aires State, Argentina) were used in all assays.[10] Bacteriophage enrichments and phage titrations were performed in Luria Bertrani (LB) medium composed of 1.0 wt% tryptone and 0.5 wt% yeast extract (Biokar Diagnostics, Alonne, France), and 1.0 wt% NaCl (Anedra, San Fernando, Argentina). Phage titers were determined by the soft agar–overlay method.[21]

Phage lysates were filtered through a 0.22 μm pore size membrane and kept at room temperature for further assays.

2.2. Microsphere Synthesis

Biopolymers were dissolved in aqueous solutions: 2.0 wt% sodium alginate (Monsanto, USA) or 3.0 wt% low-methoxylated (LM) pectin (C.P. Kelco, Lille Skensved, Denmark, 33% esterified) adjusted to pH = 6.5 with 0.050 M 2-(*N*-morpholino)ethanesulfonic acid (MES, Sigma-Aldrich, St. Louis, Mo, USA) buffer. Emulsified formulations were made by mixing 790 μL of each polymer solution with 10.0 μL of Tween 20 (Anedra, San Fernando, Argentina), while the mixture was homogenized by agitation, oleic acid was added to reach a final concentration of 10.0 vol%. Finally, CA933P phage lysate was added to each polymer solution to a final concentration of 1×10^8 PFU mL^{-1}.

Gel microspheres were made by dropping the phage–biopolymer formulation in a 0.500 M $CaCl_2$ solution containing MES buffer (0.050 M, pH = 6.0) at 0 °C with continuous stirring. For polymer-coated microspheres, drops of each polymer formulation were added to the $CaCl_2$-MES buffer solution containing 0.2 wt% of the coating polymer: high-methoxylated pectin (HM, 78% esterified, Sigma-Aldrich, St. Louis, Mo., USA) or Guar Gum (Nutrial, Buenos Aires, Argentina). Gel microspheres were left for 12 h at 4 °C in the $CaCl_2$ solution, and then filtered and stored at 4 °C.

2.3. Acid Resistance of Free Phages and Phages Encapsulated in Different Matrices

The protective effect of matrices to phage CA933P was tested in 1.5 mL vials containing 10 microspheres plus 900 μL of saline

solution (0.154 M NaCl) adjusted to pH = 7.1 (normal saline) or 1.6 with HCl. The vials were incubated at 25 and 37 °C for 30 min, later microspheres exposed to pH = 1.60 were filtered off, rinsed with distilled water and filled with 900 μL of normal saline solution. Furthermore, the microspheres were mechanically broken, mixed by vortexing and left for 1 h at room temperature to allow the diffusion of bacteriophages into the solution. Non-encapsulated phage (free-phage) resistance was performed by adding 3.0 μL of CA933P lysate (1.52×10^{10} PFU mL^{-1}) to 3.0 mL of saline (pH = 1.6) and incubated for 5, 10, and 15 min at 37 °C, and then 180 μL aliquots were extracted and mixed with 820 μL of saline (pH = 9.7) giving a final pH in the range of 7.3–7.5 units.

All mixtures were ten-fold diluted in SM buffer (100 mM NaCl; 8 mM $MgSO_4 \cdot 7H_2O$; 50 mM Tris-HCl (pH 7.5); 0.02% (w/v) gelatin) and bacteriophage titers were determined by the soft agar overlay method.

2.4. Effect of Exposure of Free Phages to Sodium Chloride Solutions

Stability of phage CA933P was assayed by adding 10 μL of phage lysate (1.52×10^{10} PFU mL^{-1}) to 1.5 mL plastic vials containing 990 μL NaCl solution (of 1.2 to 4.5 M). Vials were vortexed and left for 30 min at room temperature. High ionic strength (4.5 M) NaCl solution was also tested at longer incubation times (from 1 to 2 h). After incubation, 100 μL of each mixture was mixed with 9.9 mL of SM buffer and phage titers were determined by the soft agar overlay method in LB medium as mentioned previously.

2.5. Effect of Intestinal Proteases on Free Phages

Trypsin (11 800 U mg^{-1}) and α-chymotrypsin (52 U mg^{-1}), both proteases extracted from bovine pancreas (Sigma-Aldrich, St Louis, Mo, USA), were diluted in normal saline solution (pH = 7.2) to reach final concentrations of 2.5 and 3.0 mg mL^{-1}, respectively. A 10 μL aliquot of phage lysate (1.52×10^{10} PFU mL^{-1}) was added to 1.5 mL vials with 1.0 mL of the respective enzyme or saline solution (pH = 7.2) without enzyme as control. Vials were vortexed and incubated for 24 h at 37 °C. After incubation, 0.1 mL of bovine fetal serum (PAA laboratories, Austria) was added to each vial to stop the reaction. Mixtures were ten-fold diluted in SM buffer and phage titer was determined by the soft agar overlay method.

2.6. Effect of Pepsin on Free Phages and Phages Encapsulated in Emulsified LM Pectin Matrix

Acid protease solution was prepared by dissolving pepsin (from porcine gastric mucosa, 3660 U mg^{-1}, Sigma-Aldrich, USA) in saline solution (pH = 4.4) to a final concentration of 5.0 mg mL^{-1}. A 100 μL aliquot of pepsin solution was added to 900 μL of saline solution (pH = 2.5, 37 °C) and 10 μL of phage lysate (1.52×10^{10} PFU mL^{-1}) (free-phage) or ten microspheres of the emulsified pectin formulation (microencapsulated phage, 1.56×10^{7} PFU per microsphere) were incorporated into the vials. The mixtures were incubated for 10 min at 37 °C. The control pH experiment was performed by using 0.1 mL of saline solution (pH = 4.4) instead of the enzyme solution and proceeding as described above. After incubation, samples from free-phage vials were diluted 1:100 (v/v) in SM buffer and those from microencapsulated-phage vials were filtered off, the beads were rinsed with distilled water and added to 900 μL of SM buffer, after that they were mechanically broken and left for 1 h at room temperature. Mixtures were ten-fold diluted in SM buffer. Free-phage and microencapsulated-phage titers were determined by the soft agar overlay method.

The effect of longer incubation periods with pepsin on encapsulated phages was determined using incubation times from 30 to 180 min. Increased pepsin concentrations on encapsulated phages were tested from 1.5 to 4.2 mg mL^{-1} enzyme solutions and incubated for 3 h as mentioned above.

2.7. Bacteriophage Release from Emulsified Pectin Microspheres

Twenty microspheres of the emulsified pectin formulation were added to individual flasks containing 20 mL of phosphate-buffered saline (PBS, pH = 7.2) and incubated at 19, 30, and 37 °C at 100 rpm in an orbital shaker. Samples of 200 μL were taken at different time points from 30 to 240 min, and replaced with 200 μL of thermostatted PBS. Each sample was ten-fold diluted in SM buffer and titers were determined by the soft agar overlay method.

The total phage cargo in the microspheres was determined by exhaustive extraction of phages on 20 microspheres suspended in 20 mL of PBS and incubated at 37 °C and 100 rpm for 24 h. Beads were completely degraded after incubation, and the resulting homogeneous solution was titrated as described above.

2.8. Rheological Assays

Two samples of 3.0 wt% low-methoxylated pectin plus LB (10.0 vol%) with and without phages were 20 times diluted in buffer MES (0.050 M, pH = 6.50) and the relative viscosity was evaluated at 25 °C with an Ubbelohde viscometer S100 in order to compare changes in viscosity due to possible interactions between the phage and the LM pectin.

2.9. Scanning Electron Microscopy (SEM)

Biopolymeric microspheres were freeze-dried. Microspheres were non-conductive electron samples, and they required preparation by sputtering the surface with gold using a Balzers SCD 030 metalizer. The gold layer thickness was between 150–200 Å. The microsphere surface and morphology were determined using a scanning electron microscope (Philips SEM 505) and processed by an image digitizer program [Soft Imaging System ADDA II (SIS)].

2.10. Statistical Analysis

Experiments were carried out in duplicate. Comparisons of mean bacteriophage titres were performed by the one way analysis of variance (ANOVA) with a significance level of 5.0% ($P < 0.05$) followed by Fisher's least significant difference test at a $P < 0.05$.

3. Results and Discussion

3.1. Encapsulation Efficiency

The number of phages encapsulated by each biopolymer formulation is shown in Figure 1. Alginate/LM pectin matrices were gelled under mild conditions allowing the incorporation of phages without biological inactivation.

Alginate-based beads showed significantly higher encapsulation efficiencies ($P < 0.05$) than those observed for pectin-based formulations, except for the emulsified-pectin matrix, which incorporated a number of phages comparable to that observed for the alginate-based microspheres. High encapsulation capabilities of alginate possibly implies a best phage accommodation in the gel network during gelation, due to a more organized and rapid crosslinking in the presence of calcium ions. On the other hand, LM pectin showed a slower gelation rate than alginate, which leads to an increased diffusion rate of the phages out of the matrix during encapsulation. This result is in agreement with the rheological assays of pectin solutions with and without the addition of phage CA933P. No significant difference ($P > 0.05$) was observed in the relative viscosity of the pectin solution with the addition of phages in LB compared with that obtained with only LB (relative viscosity of 1.15 ± 0.01). These results imply that encapsulated phage CA933P does not considerably interact with the pectin biopolymer, suggesting that the low phage titers obtained for some pectin-based formulations are due to a lower capability of physically trapping phages rather than to a reduced capability of releasing them.

On the other hand, emulsified formulations of alginate or LM pectin showed higher relative phage encapsulation ($P < 0.05$) than those made only with biopolymers. On the contrary, the microsphere coating procedures with HM pectin or guar gum displayed lower encapsulation rates ($P < 0.05$). These differences are slight on alginate based formulations, but much more pronounced on LM pectin ones, as observed in Figure 1.

3.2. Protective Effect of Matrices Against Acidity

The protection provided by each matrix to the bacteriophages against extreme acid environments (pH = 1.6) was assayed at 25 and 37 °C. Remaining phage titers after 30 min exposure of the encapsulated phages to acidity at each temperature are shown in Figure 2.

The degree of protection in vitro offered by the base–biopolymer matrices with or without guar gum or HM pectin coating was the same for all types of microspheres exposed to acid environmental conditions at 25 °C. Meanwhile emulsified pectin showed the highest protection offered to bacteriophages against acidity at 25 °C ($P < 0.05$). On the contrary, the emulsified alginate matrix showed the lowest protection rate under the same experimental conditions ($P < 0.05$). The results for emulsified matrices could be explained based on a high H^+ diffusion rate in aqueous environments inside hydrophilic matrices that are able to penetrate through the physical barriers of a gel network matrix, but are incapable of interacting with the hydrophobic motifs of pectins because of thermodynamically unfavorable interactions. In addition, the main

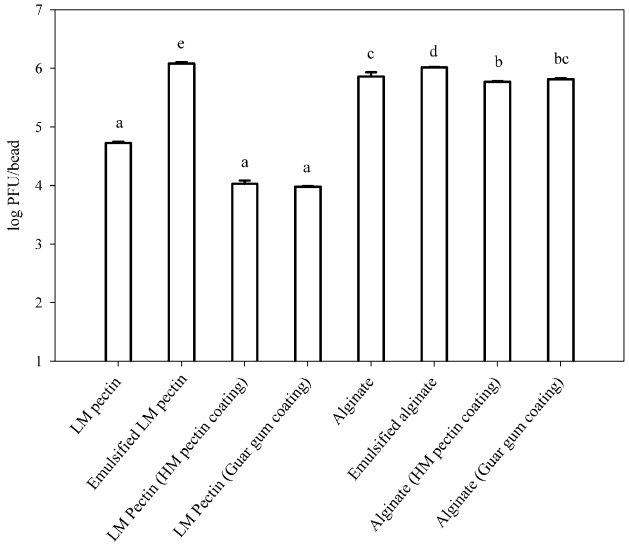


Figure 1. Number of encapsulated phages per bead obtained for each formulation. Different letters above bars indicate significantly different results ($P < 0.05$).

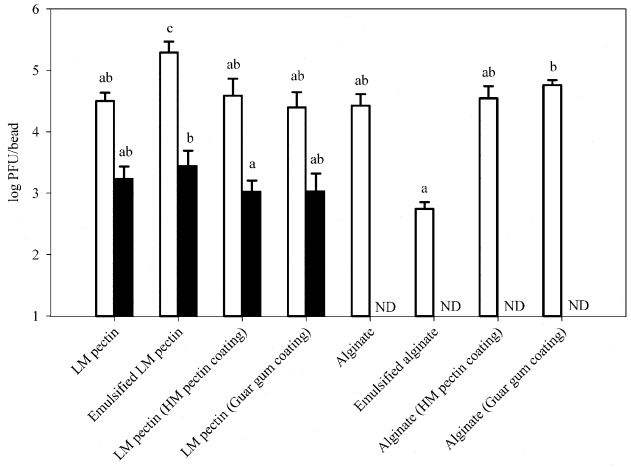


Figure 2. Titer of remaining infective bacteriophages after 30 min incubation at 25 °C (white bars) and 37 °C (black bars) of each formulation at pH = 1.6. ND: no detectable viable phages. Different letters above bars corresponding to the same temperature indicate significantly different results ($P < 0.05$).

differences in the protection offered by the emulsified pectin and alginate matrices can be related to the ionic gelation process of each biogel. It is well known that alginates are able to produce gels in the presence of divalent cations according to the egg-box model.[19] However, the ionic motifs of pectin poly(galacturonic acid) strands do not take the form of the buckled two-fold ribbon necessary for egg-box formation but they are able to form three-fold helices instead.[22] These areas could be methoxylated biopolymeric chain regions forming hydrophobic pockets, cavities in which phages can be located. The distortion of the egg-box model allows the inclusion of phage particles surrounded by oleic acid in hydrophobic patches of the pectin gel microspheres.

Oleic acid is able to bind calcium ions, allowing interference with the crosslinking of the biopolymer chains. The presence of oleic acid in the medium interferes more with the alginate gel formation compared with pectin because of the typical egg-box calcium alginate structure.

Pectin-based microspheres incubated at 37 °C showed a greater phage titer decline than that observed at 25 °C. No significant difference ($P > 0.05$) in the final PFU number among different pectin-based formulations was observed at 37 °C. On the contrary, no viable phage was detected for any alginate-based formulations under the same experimental conditions. This drastic fall in the protection capability observed on alginate-based beads may be related to the ability of phage CA933P to infect *Pseudomonas aeruginosa* and its hydrolytic activity on capsular microbial exopolysaccharides.[10] Considering *Pseudomonas* species as the main microbial alginate producers, it is plausible that CA933P degrades alginate gel microspheres. This hypothesis is supported by a previous report in which *Pseudomonas aeruginosa* phages F116 and GL1 were able to decrease the viscosity of alginate solutions at 37 °C, which was attributed to an enzymatic hydrolysis of the polymer.[23] Progressive alginate degradation by phages reduces the gel matrix integrity. As a consequence, the low performance of alginate-based formulations under acid conditions could be attributed to alginate hydrolysis.

On the contrary, pectin-based gel formulations offered a significant improvement on phage resistance at pH = 1.60 and 37 °C compared with free phages ($P < 0.05$). In the latter case, with an initial concentration of 1.52×10^7 PFU mL^{-1}, no viable phages were detected after 10 min incubation under the same conditions. As previously mentioned, the protection degree provided by all the pectin-based formulations was lower at 37 °C than that obtained at 25 °C ($P < 0.05$). Considering that all the gel matrices seemed to be expanded by increased hydration with the increase of temperature, the gel swelling is also enhanced imparting a less rigid gel structure at high temperatures. The high temperature also facilitates ion exchange and diffusion rates between the gel matrix and the media, reducing the gel matrix stability and consequently accelerating the denaturation process of phage proteins under harsh environmental conditions.

The encapsulation efficiency and the viable phage survival were adopted as selection criteria for choosing the best formulation after incubation at pH = 1.60. Emulsified pectin was selected as potentially better than other matrices for phage administration to cattle because it showed the highest number of remaining infectious phages after incubation at pH = 1.60 and 25 °C and displayed the highest encapsulation efficiency (1.2×10^6 PFU per bead) with protective effect at 37 °C.

3.3. Effect of a High Ionic Strength Medium and Ruminants Intestinal Proteases on Free Phages

The use of pectin-based matrices for phage therapy by oral delivery requires avoiding the passage of the microspheres through the reticulum–rumen chambers present in the digestive tract system of bovines, which could destabilize the microspheres' structure before they reach the abomasum (acid stomach). The destabilization may come from the pH values of the reticulum–rumen chambers (around 6 to 7) and the presence of some bacteria of the rumen microbiota able to hydrolyze pectins (cellulolytic bacteria type), thus reducing its protective effect.

It has been reported that solutions of sodium salts, NaCl (5 wt%) and $NaHCO_3$ (10 wt%), can activate a reflex (naturally present in ruminants in the milk-feeding stage) that ensures fluids and small particles are sent directly to the acid stomach, avoiding the passage through the rumen. Sodium salts were highly effective in the activation of this reflex in cattle of two years of age,[24,25] thus the phage encapsulated beads could be supplied in a 5% sodium chloride solution before slaughtering as a strategy to avoid the passage of the microspheres through the reticulum–rumen chambers. Resistance of the bacteriophage to high ionic strength solutions is required for this administration method. It was observed that the exposure of non-encapsulated phage CA933P to NaCl solution in the range of 1.2 to 4.5 M for 30 to 120 min incubation time showed no changes in phage titer ($P > 0.05$). Also, phages remained fully infective in an environment of extreme ionic strength (4.5 M NaCl) for at least 120 min, which supports the possibility of the implementation of this administration.

In addition, the success of the in vivo treatment lies in the resistance of the phage used as a biocontrol agent to the environmental conditions present in the intestine (where the target pathogen is mainly located). In the presence of intestinal proteases, free-phage activity was not affected, nor with trypsin (2.5 mg mL^{-1}) nor with α-chymotrypsin (3.0 mg mL^{-1}) after 24 h incubation at 37 °C and pH = 7.2, encouraging the possibility of the in vivo application of phage CA933P as EHEC biocontrol agent.

3.4. Effect of Pepsin on Free Phages and Phages Encapsulated in Emulsified LM Pectin Matrix

Total retention of phage titer was observed after 10 min incubation of encapsulated phages at 37 °C and pH = 2.5 without pepsin and with 0.5 mg mL^{-1} of pepsin at the same pH value (Table 1). Incubation of non-encapsulated phages at 37 °C and pH = 2.5 without enzyme reduced the bacteriophage titer by 3.7 logarithmic orders after 10 min, while no viable phages were detected with 0.5 mg mL^{-1} of pepsin under the same experimental conditions (Table 1).

In order to determine the protective effect of this matrix against the proteolytic activity of pepsin at longer exposure periods, encapsulated phages were incubated for 1 and 3 h under the same experimental conditions. No significant reduction on encapsulated phage titer ($P > 0.05$) after exposure to pepsin (0.5 mg mL^{-1}) for 3 h at pH = 2.5 was observed (Table 1). Finally, when microspheres containing phages were incubated with 1.5 to 4.2 mg mL^{-1} of pepsin at 37 °C for 3 h, no significant changes of phage titer were detected. The emulsified pectin matrix fully protected the phages against a pepsin concentration of 4.2 mg mL^{-1} (15 372 U mL^{-1}) at pH = 2.5 for 3 h. The results are summarized in Table 2.

In previous work, Ma et al.[26,27] reported phage microencapsulation on chitosan-coated alginate beads. However, *Staphylococcus aureus* phage K and *Salmonella enterica* phage Felix O1 encapsulated by alginate–chitosan coated microspheres treated with 3.2 mg mL^{-1} of pepsin at pH = 2.4–2.5 were fully inactivated after 1.5 and 2 h of incubation, respectively.[26,27] Also, the encapsulated phage Felix O1 titer was reduced by 0.67 log units when incubated for only 5 min at pH = 2.4 without pepsin.[26,27] It is worth mentioning that naked phages Felix O1 and CA933P are destabilized at pHs below 4.0.[10,27] These results suggest that the microsphere coating is advantageous for encapsulated phage protection only if they are able to reduce H^+ diffusion inside the microspheres, like the hydrophobic shell formed with oleic acid. SEM images obtained for pectin and emulsified pectin microspheres containing phage CA933P (Figure 3) show significant morphological differences in emulsified pectin beads compared with pectin

Table 1. Effect of pepsin activity on free phages and phages encapsulated in emulsified pectin microspheres at 37 °C. N/D: no detectable viable phages. N/A: condition not assayed.

pH and exp. conditions	Time [min]	$\log_{10}$ [Phage titer/PFU mL^{-1}]	
		Free	Encapsulated
2.5	0	6.20 ± 0.01	7.45 ± 0.15
	10	2.50 ± 0.71[a)]	7.63 ± 0.17
2.5 + 0.5 mg mL^{-1} pepsin	10	N/D[a)]	7.63 ± 0.17
	60	N/A	7.25 ± 0.07
	180	N/A	7.16 ± 0.06

[a)]Values significantly different from phage titer at $t = 0$ min. ($P < 0.05$).

Table 2. Effect of pepsin concentrations on phage activity encapsulated in emulsified pectin microspheres for 3 h at 37 °C and pH = 2.5.

Pepsin concentration		Remaining phage titer[a)] [$\log_{10}$ (titer/PFU mL^{-1})]	Phage titer [%]
[mg mL^{-1}]	[U mL^{-1}]		
0	0	7.22 ± 0.17	100
1.5	5.490	6.97 ± 0.05	96.5 ± 3.1
3.2	11.712	7.20 ± 0.31	99.7 ± 6.6
4.2	15.372	7.47 ± 0.40	103.5 ± 7.9

[a)]Phage titers after incubation of encapsulated phages with different pepsin concentrations. None of these values are significantly different from each other ($P < 0.05$).

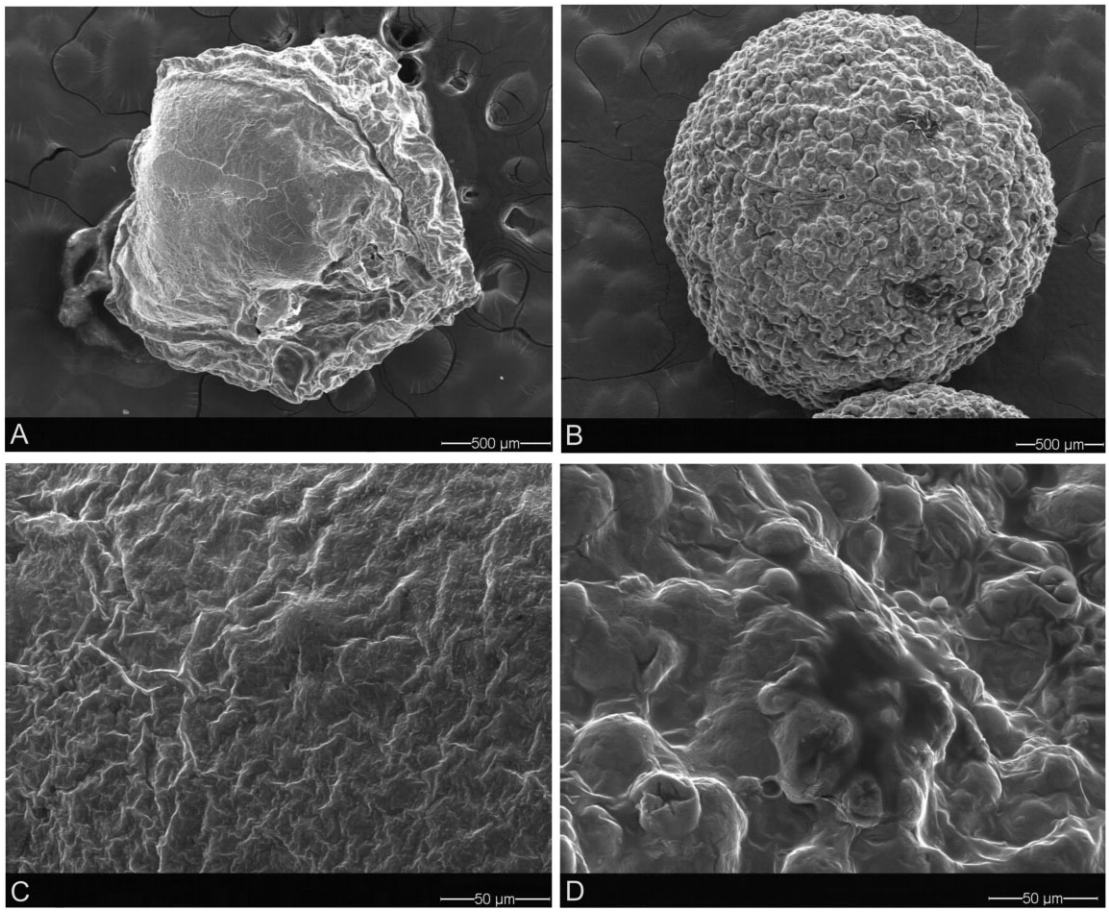


Figure 3. SEM photographs of pectin (A) and emulsified pectin (B) microspheres and surface images of pectin (C) and emulsified pectin (D) beads with 500× magnification.

microspheres. A collapse of the structure of the pectin beads, due to the dehydration process (freeze drying), is observed (Figure 3A), while the emulsified pectin beads conserve their spherical-like shape (Figure 3B). SEM studies clearly revealed significant changes to the microsphere surface properties by emulsification. A rougher surface in comparison with non-emulsified pectin, mainly due to the presence of hydrophobic patches composed of oleic acid, is observed (Figure 3C and D). The loss of the spherical morphology on pectin microspheres could be attributed to the high water content (Figure 3C), while in the emulsified pectin beads, water was repelled from the outer microsphere by oleic acid making the surface more cohesive (Figure 3D) and retaining the spherical structure after the freeze drying process. The hydrophobic environment in the pectin matrix is certainly responsible for the delay of proton diffusion inside the microsphere.[28] This improvement to the matrix features by emulsification is also relevant since it provides a more homogeneous release profile from the surface and a better phage distribution rather than an amorphous microsphere structure. Preservation of the spherical morphology is interesting for system model ling in order to predict the behavior on the phage controlled release.[29]

3.5. Phage Release from Emulsified Pectin Microspheres

In order to determine the effect of temperature on the phage kinetic release from emulsified pectin microspheres, the number of phages released at different time points was assessed in PBS at 19, 30, and 37 °C. The curves obtained at each temperature are shown in Figure 4.

As expected, the phage release was faster as the temperature increased, in agreement with the observation that the acidity protection offered to phages encapsulated in this formulation was lowered at 37 °C compared with 25 °C. Also, an expansion of the microsphere size was observed by microscopy when the temperature was raised (data not shown).

In PBS most of the phage content of the microsphere was released at 37 °C after 4 h incubation, being the total phage

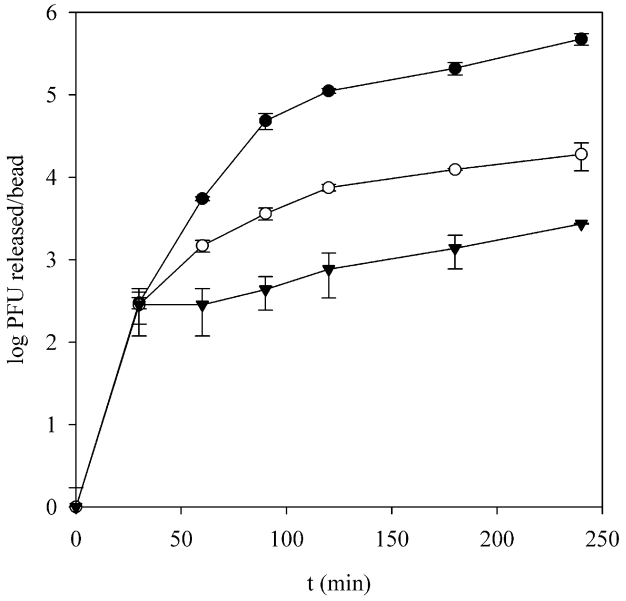


Figure 4. Profiles of phage release in time from emulsified pectin beads (10^6 PFU per bead) at 19, 30, and 37 °C in PBS. Symbols: ●, 37 °C; ○, 30 °C and ▼, 19 °C.

titer of 1.0×10^6 PFU per bead. This is advantageous as the effectiveness of the treatment depends not only on the protection provided against harsh gastric conditions but also on the facility to release the therapeutic agents into the target organ. In a previous work, Ma et al.[26] proposed the incorporation of $CaCO_3$ as a buffering agent into alginate gel beads in order to reduce the pH shift when the spheres come in contact with the stomach acid. This procedure notably increased the number of infective phages remaining after exposure to a 3.2 mg mL^{-1} pepsin solution at pH = 2.5 with respect to alginate beads alone. However, incorporation of $CaCO_3$ to the formulation reduced the ability of microspheres to release encapsulated phages: the percentage of the phages released in simulated intestinal fluid (50×10^{-3} M phosphate, pH = 6.8) was less than 20% after 4.0 h incubation. On the other hand, approximately 50% of the Felix O1 phage content was released from alginate gel beads without $CaCO_3$. The last result is comparable to that obtained for emulsified pectin beads formulated in the present work, which released 4.75×10^5 PFU per bead from an initial count of 1.0×10^6 PFU per bead (release of 47.5% of the initial phage cargo) in PBS at 37 °C after 4 h.

The effect of temperature on CA933P phage release from the emulsified pectin matrix in PBS showed no significant differences in the number of phages released in the first 30 min at 19, 30, and 37 °C (Figure 4). This behavior may be due to desorption of phages attached to the microspheres in the oleic coat, meanwhile bifurcation of the curves could represent the spread of phages from the inside of the matrix after 30 min. The number of adsorbed phages, represented as the number of phages released up to 30 min, constitutes less than 0.00025% of the total number of phages per microsphere, which implies that practically all phage content of the microspheres is inside the gel matrix structure. This hypothesis is supported by the fact that no significant decrease in phage titer was observed when the microspheres were exposed to the proteolytic action of pepsin.

As the temperature increases an expansion of the microsphere matrix can be seen: the increment of pore size leads to a high phage release and penetration of water molecules inside the microspheres. This fact could be the reason why at high temperatures the protection offered by the matrix against acidity is reduced concomitantly with the facilitation of the release of phages from within the structure, as previously reported.[30]

4. Conclusion

Our results strongly suggest that the matrix formed by emulsification of LM pectin with oleic acid is a very promising system for increasing the resistance of bacteriophages to the passage through the abomasum (acid stomach) of bovines for their application as biocontrol agents of EHEC by oral administration. In addition, the lack of the matrix and matrix components' toxicities, the low raw material costs and large market availability, unsophisticated microsphere synthesis procedures, and simple scale up are relevant properties making the proposed emulsified pectin formulation a real alternative to alleviate serious bacterial infections by phage therapy.

Acknowledgements: The Argentine financial support from CONICET (PIP0214) and ANPCyT (PICT14–32491) to Dr. G. R. Castro and ANPCyT (PICT–2006–00479) to Dr. G. L. De Antoni are gratefully acknowledged. The authors also thank Ms Mariela Theiller for her kindly assistance with the SEM pictures (CINDECA, La Plata, Argentina).

Received: March 30, 2012; Published online: July 30, 2012; DOI: 10.1002/mabi.201200109

Keywords: biocontrol; biological applications of polymers; biopolymers; microencapsulation; phages

[1] T. Häusler, "*Viruses vs. Superbugs : A Solution to the Antibiotics Crisis?*", Macmillan, London **2006**, p. xiv.
[2] P. Garcia, B. Martinez, J. M. Obeso, A. Rodriguez, *Lett. Appl. Microbiol.* **2008**, *47*, 479.

[3] M. A. Rivero, N. L. Padola, A. I. Etcheverria, A. E. Parma, *Medicina* **2004**, *64*, 352.
[4] S. Johnson, C. M. Taylor, *Eur. J. Pediatr.* **2008**, *167*, 965.
[5] H. Sheng, H. J. Knecht, I. T. Kudva, C. J. Hovde, *Appl. Environ. Microbiol.* **2006**, *72*, 5359.
[6] S. J. Bach, T. A. McAllister, D. M. Veira, V. P. J. Gannon, R. A. Holley, *Anim. Res.* **2003**, *52*, 89.
[7] R. R. Raya, P. Varey, R. A. Oot, M. R. Dyen, T. R. Callaway, T. S. Edrington, E. M. Kutter, A. D. Brabban, *Appl. Environ. Microbiol.* **2006**, *72*, 6405.
[8] T. R. Callaway, T. S. Edrington, A. D. Brabban, R. C. Anderson, M. L. Rossman, M. J. Engler, M. A. Carr, K. J. Genovese, J. E. Keen, M. L. Looper, E. M. Kutter, D. J. Nisbet, *Foodborne Pathog. Dis.* **2008**, *5*, 183.
[9] R. P. Johnson, C. L. Gyles, W. E. Huff, S. Ojha, G. R. Huff, N. C. Rath, A. M. Donoghue, *Anim. Health Res. Rev.* **2008**, *9*, 201.
[10] C. Dini, P. J. De Urraza, *J. Appl. Microbiol.* **2010**, *109*, 873.
[11] A. Metters, J. Hubbell, *Biomacromolecules* **2005**, *6*, 290.
[12] V. E. Bosio, G. A. Islan, Y. N. Martínez, G. R. Castro, "Control Release Applications in Food Technology", in *Advances in Bioprocesses in Food Industries* (Eds: C. R. Soccol, A. Pandey, V. T. Soccol, C. Laroche), Asiatech Press, New Delhi **2011**, p. 1.
[13] E. Chiellini, P. Cinelli, F. Chiellini, S. H. Imam, *Macromol. Biosci.* **2004**, *4*, 218.
[14] G. A. Islan, I. P. de Verti, S. G. Marchetti, G. R. Castro, *Appl. Biochem. Biotechnol.* **2012**, DOI: 10.1007/s12010-012-9610-2.
[15] G. R. Castro, B. Panilaitis, E. Bora, D. L. Kaplan, *Mol. Pharm.* **2007**, *4*, 33.
[16] A. D. Augst, H. J. Kong, D. J. Mooney, *Macromol. Biosci.* **2006**, *6*, 623.
[17] E. Agulló, M. S. Rodríguez, V. Ramos, L. Albertengo, *Macromol. Biosci.* **2003**, *3*, 521.
[18] S. Rolin, P. Paindavoine, J. Hanocq-Quertier, F. Hanocq, Y. Claes, D. Le Ray, P. Overath, E. Pays, *Mol. Biochem. Parasitol.* **1993**, *61*, 115.
[19] I. Braccini, S. Perez, *Biomacromolecules* **2001**, *2*, 1089.
[20] G. R. Castro, R. R. Kamdar, B. Panilaitis, D. L. Kaplan, *J. Controlled Release* **2005**, *109*, 149.
[21] A. M. Kropinski, A. Mazzocco, T. E. Waddell, E. Lingohr, R. P. Johnson, *Methods Mol. Biol.* **2009**, *501*, 69.
[22] H. Kastner, U. Einhorn-Stoll, B. Senge, *Food Hydrocolloids* **2012**, *27*, 42.
[23] G. W. Hanlon, S. P. Denyer, C. J. Olliff, L. J. Ibrahim, *Appl. Environ. Microbiol.* **2001**, *67*, 2746.
[24] R. F. Riek, *Austr. Vet. J.* **1954**, *30*, 29.
[25] O. M. Radostits, C. C. Gay, D. C. Blood, K. W. Hinchcliff, "*Veterinary Medicine: A Textbook of the Diseases of Cattle, Sheep, Pigs, Goats, and Horses*", 9th ed. edition, Saunders, London **2000**, p. xxvii.
[26] Y. Ma, J. C. Pacan, Q. Wang, P. M. Sabour, X. Huang, Y. Xu, *Food Hydrocolloids* **2012**, *26*, 434.
[27] Y. Ma, J. C. Pacan, Q. Wang, Y. Xu, X. Huang, A. Korenevsky, P. M. Sabour, *Appl. Environ. Microbiol.* **2008**, *74*, 4799.
[28] C. A. Wraight, *Biochim. Biophys. Acta* **2006**, *1757*, 886.
[29] G. Frenning, *J. Controlled Release* **2004**, *95*, 109.
[30] M. L. Koç, Ü. Özdemir, D. Imren, *Chem. Eng. Sci.* **2008**, *63*, 2913.