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Characterization and Stability Analysis of Biopolymeric Matrices Designed for Phage-Controlled Release

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Abstract Alginate and low methoxylated pectin gel matrices emulsified with oleic acid were studied for phage oral delivery. Matrix structural analysis revealed that emulsified pectin (EP) gel microbeads were harder and more cohesive than those of emulsified alginate (EA). EP showed high swelling capacity and slower matrix degradation in aqueous media, suggesting that oleic acid is mainly located on the surface of EP microbeads. EA and EP matrices having p-nitrophenyl palmitate (C-16 ester) as tracer dissolved into oleic acid and in the presence of lipase confirmed this hypothesis which is consistent with EP better phage protective capability. Surface analysis of gel microbeads by scanning electron microscopy revealed strong differences between EP and EA gel microbeads. Phage release kinetics was tested using semi-empirical mathematical models. Experimental curve best fitted the Korsmeyer–Peppas model, predicting transport mechanisms according to the high swelling and degradation of EP. The proposed encapsulation model represents an innovative technology for phage therapy, which can be extrapolated to other therapeutic purposes, using a simple environmentally friendly synthesis procedure and cheap food-grade raw materials.

Keywords Modeling · Pectin · Alginate · Biopolymers · Microencapsulation · Phage therapy

Introduction

The microencapsulation of active molecules is a technique widely used in the pharmaceutical, cosmetic, and food industries [1]. Carbohydrate biopolymers used as matrices for microencapsulation are non-toxic and biodegradable materials capable of forming gels. Among them,

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alginate and pectins are commonly used in many industrial applications [2, 3]. Alginate is a complex copolymer extracted mainly from marine algae and one of the most widely used materials for encapsulation of molecules and cells. Alginates are linear biopolymers composed of beta-mannuronic acid (M units) and alpha-guluronic acid (G units) linked by 1–4 bonds with a pK_a of 3.38 and 3.65, respectively. Alginate hydrogels are formed by ionotropic gelation by multivalent cations (e.g., calcium, zinc), which cooperatively interact forming ionic bridges between different polymer chains. Gelation of alginate are due to the stacking of the G units present in alginate making a similar structure commonly known as "egg box" by analogy with egg containers [3]. In between two crosslinking points, the mannuronic (M) units remain mostly free and the degree of ionization of carboxylate residues depends on external pH. At pH 1.2, the carboxylate of M unit is in the acid form (non-polar form, pK_a=3.38). Meanwhile, at pH 6.8 to 8.0, the M carboxylate residue remains as anionic form [3].

Pectin is a predominantly linear glycan consisting of α-D-galacturonic acid units, some of which are present as methyl esters, branched with (1→2)-α-L-rhamnose units and other minor neutral sugars extracted from the cell wall of higher plants. Classification of pectin is based on to their esterification degree as low methoxylated (LM, below 40 % esterification), medium methoxylated (MM, 40–60 % esterification) or high methoxylated (HM, 60 % or high esterification) pectins. Disregarding of the methoxylation degree, the pectin pK_as are ranging from 3.5 to 4.1 [4]. Low and medium methoxylated pectins are able to make gels in the presence of divalent cations. However, pectin gel structures are showing more disordered structure than alginate [2, 5].

In general, when gels made by ionotropic crosslinking are exposed to acid environmental conditions below to the pK_a of carboxyl groups, the ionic interaction is lost and the gel structure collapses. Additionally, ionotropic gels are unstable in the presence of cation-chelating agents such as phosphate or citric acid and/or competing non-gelling cations such as sodium or potassium which are present in biological fluids [2, 3].

The search for more specific and efficient molecular carriers for particular applications led to the development of complex and more sophisticated matrix formulations. New formulations such as nanostructured particles, double network hydrogels, ultradeformable systems, and multi-task hydrogels capable of responding to multiple triggers and/or forming mechanically strong hydrogels, which are allowing to tailor properties such as mechanical stability and release kinetics for the desired application like cell adhesiveness, tissue engineering, and drug delivery, among others [6]. Typical examples are the advances in the treatment of some cancer pathologies (e.g., brain, colon, and bone tumors) and parasitic and microbial diseases (e.g., trypanosomiasis, leishmaniasis, and malaria, and *Klebsiella* spp., *Staphylococcus* spp., *Neisseria* spp.) [6–9].

In this sense, formulations including blends, emulsifications, and coating, are technical options to be explored in more details. Advanced formulations are requiring fine tuning of the matrix properties according to the environmental conditions (e.g., smart matrix behavior), high molecular payload, predictable kinetics, and specific targeting among others [6–9]. Biopolymers capable of modifying their swelling behavior according to the changes in the environmental conditions (such as pH or temperature) are named "smart hydrogels." Some smart hydrogels are particularly attractive for biomedical applications because they are exhibiting minimal swelling at the acid pH of the stomach (therefore, producing a minimal loss of the loaded drug) while an increase in pH, such as in the intestinal condition, produces an enhancement of gel swelling and thus enables drug release [7].

In previous works, phage CA933P has been proved to be a promising therapeutic agent for the biocontrol of enterohemorrhagic *E. coli* and other microbial pathogens in cattle [10]. However, oral administration is limited by low phage tolerance to a pH below 4.0. Phage

encapsulation in microbeads was studied as an alternative for oral delivery administration taken into account acid gastric conditions. Different empiric strategies using pectins and alginates combined with blends, coatings, and emulsification with oleic acid were analyzed [11]. Microbeads of low methoxylated (LM) pectin emulsified with oleic acid showed the highest encapsulation phage efficiency and a better protective capability against acidity among other developed formulations. On the contrary, emulsified alginate showed the lowest protection against acidity, but with acceptable encapsulation efficiency [11].

The aim of the present work was to establish and compare the main properties of emulsified low methoxylated pectin (EP) and alginate (EA) microbeads in order to rationalize the design of biopolymeric matrices for the oral delivery of phages. Scanning electronic microscopy followed by image analysis (roughness), texturometric analysis (hardness and cohesiveness), swelling kinetics, and water content were tools used to analyze and compare both matrices with and without emulsification. Location of oleic acid patches in EP and EA gel microbeads using p-nitrophenyl palmitate (p-NPP) as hydrophobic tracer in the presence of lipase was determined. Also, the effect of environmental parameters such as temperature and pH on emulsified matrices are reported. The kinetic of phage release at three temperatures was tested on five semi-empirical mathematical models and correlated with the structural and matrix stability results. Finally, a structural model of both matrices based on the interpretation of the different behaviors of emulsified alginate and pectin on phage stability under harsh environmental conditions was proposed.

Materials and Methods

Materials

Sodium alginate (MW_{av}=120 kDa) and low methoxylated pectin (ED, 33 %, MW_{av}= 156 kDa.) were kindly provided by Monsanto (Buenos Aires, Argentina) and CP Kelco (Buenos Aires, Argentina), respectively. *Candida rugosa* lipase (1,600 enzyme unit (EU) mg^{-1} of solid, MW≈65 kDa.) was purchased from Sigma-Aldrich Co. (Buenos Aires, Argentina). All of the reagents were of analytical grade from Sigma (Buenos Aires, Argentina), Merck (Darmstadt, Germany) and Anedra (Buenos Aires, Argentina).

Microbeads Synthesis

Microbeads were synthesized as previously described [11]. Briefly, biopolymers were dissolved in aqueous solutions: 2.0 % (*w*/*v*) sodium alginate or 3.0 % (*w*/*v*) low methoxylated (LM) pectin (33 % esterification degree) adjusted to pH 6.5 with 50.0 mM MES (2-(N-morpholino)ethanesulfonic acid) buffer. Emulsified formulations were made by mixing 790 μl of each polymer solution with 10.0 μl of Tween 20. The polymer mixture was homogenized by stirring; and them oleic acid was added to reach a final concentration of 10.0 % (*v*/*v*). Gel microbeads were made by dropping the biopolymeric solution into 500 mM $CaCl_2$ solution containing 50.0 mM MES buffer (pH=6.0) at 0 °C with continuous stirring and left in $CaCl_2$ solution at 4 °C for 12 h. Finally, microbeads were filtered and stored at 4 °C for further assays.

Measurement of Texturometric Parameters

Microbeads texture was analyzed in a TA.XT2i Texture Analyzer (Stable Micro Systems Ltd., UK) equipped with a load cell of 25 kg and a cylindrical metal compression plate of 75 mm

diameter (p75). The texture profile analysis (TPA) consisted of two compression cycles at 0.6 mm s^{-1} to 30 % of the original microbeads height with a 2 s gap between cycles. In each assay, three microbeads were simultaneously compressed. Data was processed with the Texture Expert® software and the textural parameters (hardness and cohesiveness) were calculated from the TPA curve of force (N) vs. time (s) with the following definitions [12]: Hardness (N) is defined as the peak force during the first compression cycle; Cohesiveness (dimensionless) as the ratio of the areas under the second and first compression (A2/A1). The results represent the means of at least triplicate tests.

Water Content and Water Activity Values of Fresh and Freeze-Dried Beads

Moisture content of fresh beads and residual moisture content of freeze-dried microbeads were determined by gravimetric method. Samples were dried at 105 °C until constant weight and the weight difference was determined using a Mettler-AE240 digital balance, accurate to 10 ng. For fresh beads, the water content was expressed in wet basis (w.b., amount of water related to the total amount of sample). Instead, for freeze-dried beads, the water content was expressed in dry basis (d.b., amount of water related to the dried matter).

Water activity (a_w) of fresh and lyophilized beads was measured at 25 °C in an AquaLab Series 3 Water Activity Meter (Decagon Devices Inc., USA).

Swelling Kinetics

Vials containing weighted air-dried microbeads were incubated in 1.0 ml of phosphate saline buffer (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4; pH=7.2) at temperatures 19, 30, and 37 °C for emulsified pectin and emulsified alginate microbeads up to 5 h. Every 30 min, microbeads were transferred to test tubes containing distilled H_2O and left for 10 s to wash out the buffers. Later, microbeads were placed over absorbent paper to remove the liquid excess from surface, weighed, and kept in capped tubes for further texturometric assays. Swelling was expressed as percentage of microbeads weight increment.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) analysis was performed on freeze-dried biopolymeric microbeads (emulsified alginate and emulsified LM pectin). Samples were sputtered with gold using a Balzers SCD 030 metalizer allowing a metal layer thickness of 15–20 nm. The microbead surface and morphology were determined using a scanning electron microscope (Philips SEM 505), and processed by an image digitalizer program (Soft Imaging System ADDA II, SIS).

The SEM pictures were analyzed by ImageJ software (NIH, USA). The roughness of the surface was reflected by the standard variation of the gray values of the pixels on a section of the image of the entire microbead. First, the SEM image files were opened by the software and converted to an 8-bit image. The scale bar was adjusted with the pixels of the image. Then, a section of 200×300 μm on the image was selected and statistically measured by a computer equipped with the software. Low standard variation values indicate smooth surfaces in the histograms analysis.

Effect of Lipase on Emulsified Matrices

Emulsified alginate and LM pectin microbeads containing p-nitrophenyl palmitate (p-NPP) as a tracer were made as described above with a slight modification: p-NPP was dissolved in the

oleic acid. Briefly, 790 μl of polymer solution, alginate, or low methoxylated pectin, plus 10.0 μl of Tween 20 were mixed and stirred; later, oleic acid containing the 1.0 mM p-NPP was added to the solution in order to reach a final concentration of 10.0 % (*v*/*v*). Gel microbeads were made as previously described under the same experimental conditions [11]. Microbeads of emulsified biopolymers were weighted and put in contact with *C. rugosa* lipase solution (1.0 mg ml^{-1}; 1,600 EU mg^{-1} of solid, MW≈65 KDa.) at pH=7.4 (phosphate buffer saline) and 37 °C under stirring. Lipase activity was measured tracing the release of p-nitrophenol from p-NPP and assayed spectrophotometrically at 405 nm after 30- and 60-min incubation with appropriate calibration curve. Microbeads were weighted after lipase treatment to determine matrix swelling. Controls were performed in absence of lipase. Hydrolytic activity of the stock lipase solution was measured with emulsified p-NPP. One enzyme unit (EU) was the amount of enzyme liberating one micromole of p-nitrophenol per minute in PBS buffer at 37 °C [13].

Effect of pH on Emulsified Pectin Matrix Stability and Swelling

Destabilization and swelling of the emulsified LM pectin matrix exposed 30 min to low pH values (simulated gastric condition) were analyzed as previously reported [11]. Microbeads were weighted, incubated in HCl (pH=1.2) for 30 min at 37 °C, weighted again for determination of swelling and subjected to TPA analysis for the determination of the texturometric parameters.

For equilibrium swelling assays, emulsified LM pectin microbeads were weighed and incubated at pH 1.2 in 50 mM Clark and Lubs buffer, and pH from 3.0 to 9.0 in 25 mM MES-Tris-Glycine buffer for 12 h at 37 °C. Microbeads were weighted after incubation to determine the equilibrium swelling degree.

Mathematical Modeling of Phage Release Kinetics

Experimental data previously obtained for phage release kinetics at 19, 30, and 37 °C [11] was analyzed by semi-empirical and theoretical models developed for drug release profiles from polymeric matrices. The zero and first-order models of Baker–Lonsdale, Higuchi, Hixson–Crowell, Hopfenberg, and Korsmeyer–Peppas models were tested [14] and analyzed with the SYSTAT 10.0 program (SYSTAT Inc., Evanston, IL, USA).

The correlation coefficient (r^2) values were used as discriminatory parameter and the models with correlation factors lower than 0.5 were discarded.

Statistical Analysis

Two independent experiments were carried out at least in duplicates. Comparisons of mean values 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 $p<0.05$.

Results and Discussion

Analysis of Matrices Stability by Texturometric Assays

Stability of pectin and alginate gel microbeads emulsified or not with oleic acid was determined by texturometric analysis. Texturometric parameters such as hardness (N) gives information about the resistance of the gel matrix to compression (also referred to as gel "firmness")

[15–17] and cohesiveness (dimensionless) provides measures of the degree of difficulty in breaking down the gel's internal structure [18]. Alginate and pectin matrices showed no significant differences in hardness ($p>0.05$). Besides, pectin gel structure was more cohesive ($p<0.05$) compared to alginate one (Fig. 1) and can be associated to the internal structure differences of both biopolymeric matrices [19]. The high randomness of pectin gels compared to the egg box alginate ones allowed get more inter-crossed bridges among different polymeric chains [5], and consequently, high cohesiveness is observed in the first one. On the contrary, emulsification of both biopolymer matrices gives opposite results for alginate and pectin beads. Addition of oleic acid to pectin significantly increased gel firmness of microbeads ($p<0.05$) meanwhile, emulsification of alginate resulted in a significant reduction of hardness with respect to those of alginate alone ($p<0.05$). These findings are confirming the hypothesis displayed in our previous work about the better protective capability against acidity of emulsified pectin matrix compared to emulsified alginate gels due to a more compatible distribution of the hydrophobic phase into the gel matrix [11]. The results could be attributed to different levels of matrix distortion and the respective location of the oleic acid in the biopolymer structure of pectin and alginate gels. In the alginate matrix, the destabilization of the egg box network produced by the addition of oleic acid is expected to be more pronounced compared to the emulsified pectin formulation, since pectin gel has more randomness structure compared to the alginate one [2, 3, 5]. Furthermore, the increase of pectin gel stability by the addition of oleic acid could be attributed to the distribution of the hydrophobic phase within the entire microbead structure. Moreover, the presence of methoxylated regions on pectin chains is playing a key role in stabilizing the oleic acid phase into the hydrophilic gel structures. Addition of oleic acid during the ionic gelation process of the biopolymers could reduce matrix stability only if fatty acids are capable of interfering with the biopolymer complexation with divalent cations such as calcium [20]. Meanwhile, if the oleic acid is able to make a coating on the microbead surface, the ionotropic crosslinking of the biopolymer will be not affected and a hydrophobic barrier between the gel beads and the aqueous environment will protect also the gel matrix structure form degradation, consequently, the effect would be the enhancement of the cargo protection by the increase of matrix stability similarly as it was previously reported [20]. These considerations are in agreement with the similar microbeads cohesiveness ($p>0.05$) found in alginate and pectin gel matrices emulsified or not with oleic acid, indicating similar interactions between the different polymer chains of the biopolymers in presence of oleic acid (Fig. 1) [17].

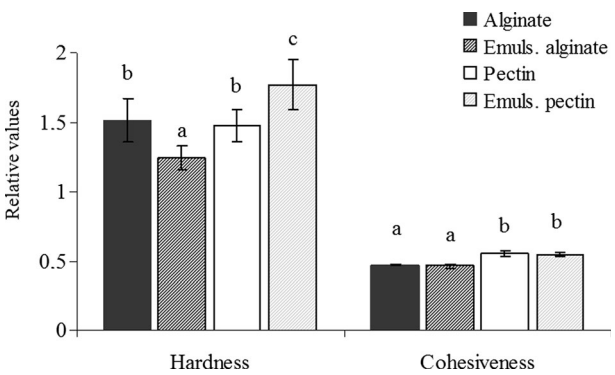

Fig. 1 Texturometric parameters of alginate and pectin matrices with and without emulsification with oleic acid. Different *letters above the bars* indicate significantly different values ($p<0.05$)

Microbeads Swelling and Matrix Stability

Swelling assays of emulsified biopolymeric microbeads in PBS at 37 °C showed first a weight increase of the matrices until 60- and 240-min incubation for alginate and pectin-based microbeads, respectively, where upon a reduction in beads weight was observed (Fig. 2). Emulsified alginate microbeads exhibited a maximum percentage of weight increment of 43.5 ±3.9 %, significantly lower ($p<0.05$) compared to the emulsified pectin microbeads (78.8± 7.0 %). Later, the weight reduction was attributed to a degradation of the microbeads by dissolution of the biopolymers composing the matrix, experimentally observed on naked eyes and correlated to an increased amount of biopolymer precipitated in the vial over the time (data not shown). When the microbeads are placed in the PBS solution, the ionic osmotic pressure between the gel and the solution results in an increase in swelling as previously reported [21]. The diffusion of sodium ions from the solution to inside the gel increase the charged groups in the gel microbeads and partially replace the divalent calcium ions because of the gradient concentration and similar ionic ratios (about 0.1 nm diameter with about 3 % differences). Additionally, the presence of phosphate ions is chelating the calcium ion and forming an insoluble calcium salt ($K_{ps}\approx 2.0\times 10^{-33}$) which is taking out the calcium from the bead surface but also in the whole gel structure and consequently, the matrix structure is destabilized. The resulting effects are the increase of the network hydrophilicity stimulated by the amount of water imbibed in the matrix, with the consequent growing of the bead diameter and the dissolution of the gel matrix into the PBS solution [22].

In the case of emulsified microbeads, the swelling rate was higher for the alginate beads compared to the pectin ones, but the latter reached a maximum percentage of weight increment significantly higher ($p<0.05$) than that of the alginate microbeads (Fig. 2). The last result supports the idea that oleic acid is making a coating in the pectin core microbeads reducing the water diffusion inside the biopolymeric structure. Furthermore, the increment of matrix stability produced by the oleic acid coat might be responsible for the enhanced swelling capability (as structure might tolerate an increased amount of solution within its structure without degradation).

The texturometric assays of emulsified matrices indicated a significant fall ($p<0.05$) in hardness and cohesiveness of both microbeads formulations incubated for 30 and 60 min (Table 1). These reductions are due to the matrix heterogeneities produced inside the gel

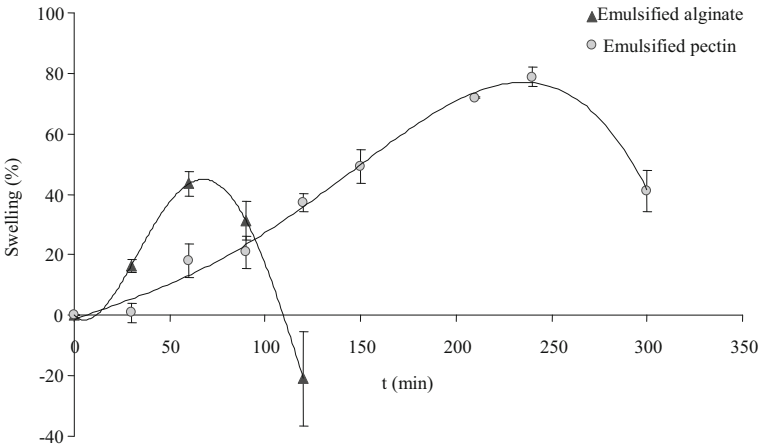

Fig. 2 Swelling kinetics at 37 °C of emulsified alginate and pectin matrices in PBS

Table 1 Texturometric parameters of alginate and pectin microspheres after different times of incubation in PBS at 37 °C

Time (min.)	Emulsified matrices			
	Alginate		Pectin	
	Hardness	Cohesiveness	Hardness	Cohesiveness
0	1.108±0.026a	0.491±0.004a	1.407±0.032a	0.548±0.006a
30	0.465±0.002b	0.205±0.005b	0.706±0.164b	0.357±0.047b
60	ND	ND	0.330±0.098c	0.163±0.001c

Different letters within the same column indicate significant differences ($p<0.05$)

ND not determined (values below the limit detection of the technique).

structure by swelling and by the presence of phosphate ions, which clearly induce the appearance of irregularities and favor the matrix weakness [23]. Besides, no data were obtained by texturometric analysis of alginate and pectin microbeads incubated in PBS after 30 and 60 min because the values were out of the detection range. The absence of oleic acid in their structure does not provide the hydrophobic regions necessary to retard diffusion of phosphate ions inside the matrix, producing the loss of gel structure due to break of calcium bridges among the biopolymeric chains.

Swelling kinetics and TPA analysis of emulsified pectin microbeads showed no significant differences ($p>0.05$) between 19 to 37 °C up to 4-h incubation (data not shown). After that time, the swelling kinetics showed dependence with temperature. The weight of the microbeads incubated at 19 °C was kept constant ($p>0.05$), meanwhile the microbeads incubated at 30 and 37 °C showed a weight reduction of 28.1 and 37.7 %, respectively, between 4 to 5 h ($p<0.05$).

SEM Pictures and Moisture Correlation of Microbeads

Fresh alginate and pectin beads made by emulsification showed similar size with an average diameter size of 3.3 mm. However, after freeze drying process, the emulsified beads suffered a size reduction of around 80 and 40 % for alginate and pectin, respectively, reaching the micrometric scale (Fig. 3).

Incorporation of oleic acid into the biopolymeric matrices increases bead hydrophobicity in both formulations (Table 2). The water content of fresh emulsified microbeads was lower compared with non-emulsified microbeads, and the lowest value was found on emulsified alginate matrix. Similar behavior was observed for both freeze-dried alginate and pectin beads performed by emulsification which are displaying residual moisture content of about 10 % (Table 2).

Despite alginate and pectin-based beads made by emulsification, it showed similar residual moisture contents ($p>0.05$) after freeze drying, the a_w values of pectin microbeads resulted significantly lower compared to the alginate ones. This indicates that emulsified pectin beads have a lower proportion of structural water than emulsified alginate matrix. Meanwhile, higher contents of free (unbound) water is present in the alginate-based microbeads after the lyophilization process compared to emulsified pectin gel beads. This difference can be attributed to the biopolymer structure since emulsification showed no significant changes in a_w values compared to the biopolymers alone. In this sense, pectins polymer chains possess methoxylated regions which are displaying more hydrophobic properties than alginate.

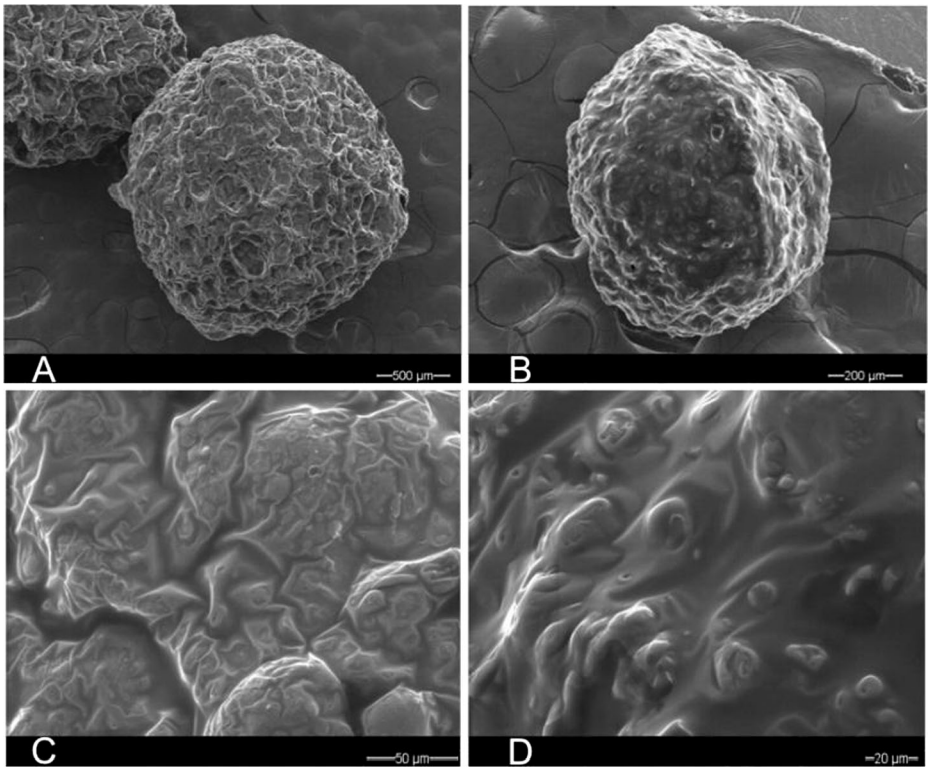

Fig. 3 SEM pictures of emulsified pectin (**a**) and emulsified alginate (**b**) microspheres and surface images of emulsified pectin (**c**) and emulsified alginate (**d**) beads

Meanwhile, the hydrophilic alginate matrix is capable of coordinating more free water molecules with the hydroxyl residues in absence of hydrophobic patches as in the case of pectins. The coordinate water of alginate is lost during freeze drying producing a compacted microbead as it was observed under microscope.

SEM images of both emulsified biopolymer microbeads showed perceptible morphological differences on their surfaces (Fig. 3). The differences on the alginate and pectin gel bead surfaces can be possibly due to the different location of the oleic acid into the hydrophilic gel

Table 2 Water content and a_w values of fresh and freeze-dried beads

Matrix	Fresh beads	Freeze-dried beads	
	Moisture (%, w.b.)	Moisture (%, d.b.)	a_w
Alginate	93.93±0.04a	23.2±4.6b	0.109±0.004ab
Pectin	93.82±0.05a	18.1±4.1ab	0.081±0.022a
Emulsified alginate	81.24±0.23b	10.6±1.8a	0.127±0.005b
Emulsified pectin	84.07±0.28c	11.7±2.4a	0.071±0.008a

Different letters within the same column indicate significantly different results ($p<0.05$)

wb wet basis, *db* dry basis

during ionic crosslinking process. Besides, in both cases the spherical-like shape was preserved (Fig. 3). Alginate gels resulted in a more compacted microbead structure (Fig. 3b); meanwhile, emulsified pectin beads showed a loose surface with holes and folds (Fig. 3a). This roughness in the surface of both emulsified matrices was mainly due to the presence of hydrophobic patches composed of oleic acid (Fig. 3c and d).

The microbead surface roughness was reflected by the standard variation of the gray values of the selected area (200×300 μm) on the SEM images (Fig. 3a and b). The increase of standard variation values is proportional to the roughness of the analyzed surface [24]. The results of roughness analysis are shown in Fig. 4. Surface of emulsified alginate microbeads was at least two times smoother than pectin ones (standard deviation of 16 and 33, respectively). Also, the histograms and mean values provided information about the spatial alignment of the polymer chains. The intensity of the peak signal may be related to the spatial structure alignment of the biopolymer chains. As the peak signal became strong, this implies more regular structure alignment. Emulsified alginate matrix showed a more defined peak with a small range of gray values, reflecting a more regular surface, which is in agreement with the respective SEM images of Fig. 3. Pectin-based microbeads presented a wide range of gray values with a mean value of 91, higher than the alginate ones, certainly due to the distortion of the egg box model and a more loosely folding of the biopolymeric chains (Fig. 4a and b). The surface pattern differences of both matrices were more evident when the SEM images were plotted in 3D graphs (Fig. 4c and d).

Effect of Lipase on Emulsified Matrices Stability

In order to locate the hydrophobic patches made by oleic acid on alginate and pectin matrices a hydrophobic substrate, p-NPP, was used as a tracer in presence and absence of lipase (Table 3).

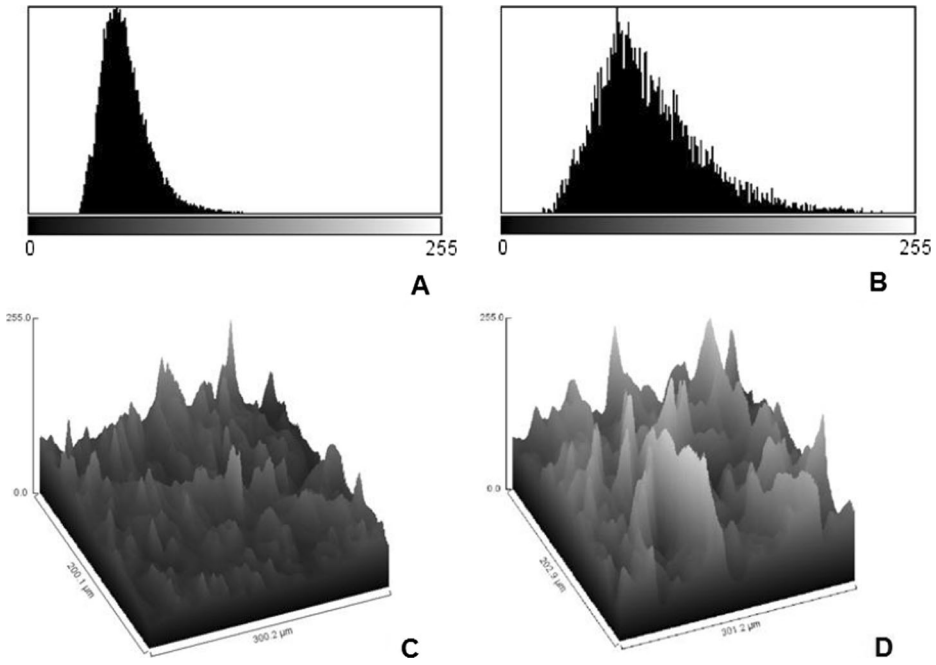

Fig. 4 Roughness analysis of SEM images of emulsified alginate (**a** and **c**) and emulsified pectin (**b** and **d**)

Table 3 Effect of lipase on emulsified matrices

Matrix	Lipase activity (nanomole/min) at different times (min)[a]	
	30	60
Alginate	0.03±0.02	0.02±0.01
Pectin	0.82±0.30	0.88±0.20

[a]Nanomols of hydrolyzed p-nitrophenyl palmitate

The hydrolysis of p-NPP by lipase release palmitic acid and p-nitrophenol which is easily detectable by the characteristic yellow color. If the oleic phase containing p-NPP is placed on the microbead surface it will be available for the enzymatic hydrolysis by the lipase displaying a yellowish color. On the contrary, if the oleic phase containing p-NPP is located inside of the microbead, the substrate will not be available to the enzyme (which is not able to penetrate the matrix, due to its high molecular weight) and the solution remains clear. The results showed that incubation of emulsified alginate and pectin microbeads in presence of lipase showed yellow color only in the case of emulsified pectin. The p-nitrophenol release from emulsified pectin microbeads containing p-NPP showed no significant differences between 30 and 60 min of incubation ($p>0.05$), suggesting an easy accessibility of the enzyme substrate present in the oleic phase by the lipase. This fact can be attributed to the loose structure of emulsified pectin matrix exposing hydrophobic zones of the polymer chain wherein p-NPP molecules can be detected [25]. Meanwhile, the lipase activity on emulsified alginate is negligible after 1-h incubation, indicating poor access to the p-NPP incorporated into the oleic phase because of the high-structured gel network of alginate [3]. The results are in agreement with the more regular surface of alginate microbead images compared to the pectin ones observed by the SEM (Fig. 3). In addition, the presence of an oleic acid coating on pectin-based microbeads was corroborated by swelling assays. While pectin-based microbeads incubated in PBS medium (without the enzyme) showed a swelling degree of 16 % after 30 min, the pectin-based microbeads incubated in PBS with lipase only resulted in a swelling degree of 11.5 %. In the first case, there is a weight increase due to incorporation of water molecules inside the matrix. On the other hand, lipase-treated microbeads showed a lower swelling in comparison with non-treated ones, due to the weight loss associated with enzymatic hydrolysis of the oleic acid from the matrix.

The obtained data was also in agreement with the hypothesis of phage distribution into the matrices proposed in our previous work [11] (Fig. 5). The results provide a strong evidence of the presence of an oleic acid coating structure on pectin-based microbeads.

However, no significant differences were observed in microbeads texturometric firmness and cohesiveness after 60-min incubation with lipase for either of the formulations ($p>0.05$). These results are confirming that the lipase activity is located only on the surface of gel microbeads.

Effect of pH on Emulsified Pectin Matrix Stability

Alginate and pectin microbeads are stable in the pH range from close to the pK_a value of the biopolymers (around pH 3.0 and 3.5, respectively) to slight alkaline pHs (below 9.0) depending on the environmental conditions (e.g., temperature, ionic strength, and solvent) [25, 26]. When the pH of the solution is below the pK_a, the biopolymers precipitate as a neutral molecule because the carboxylate residues are moving to protonated state and are not available to establish bridges with calcium ions. Particularly, the egg box structure in alginate biogels is lost and consequently, the gel matrix collapses [5, 27]. Meanwhile, the hydrolysis of the

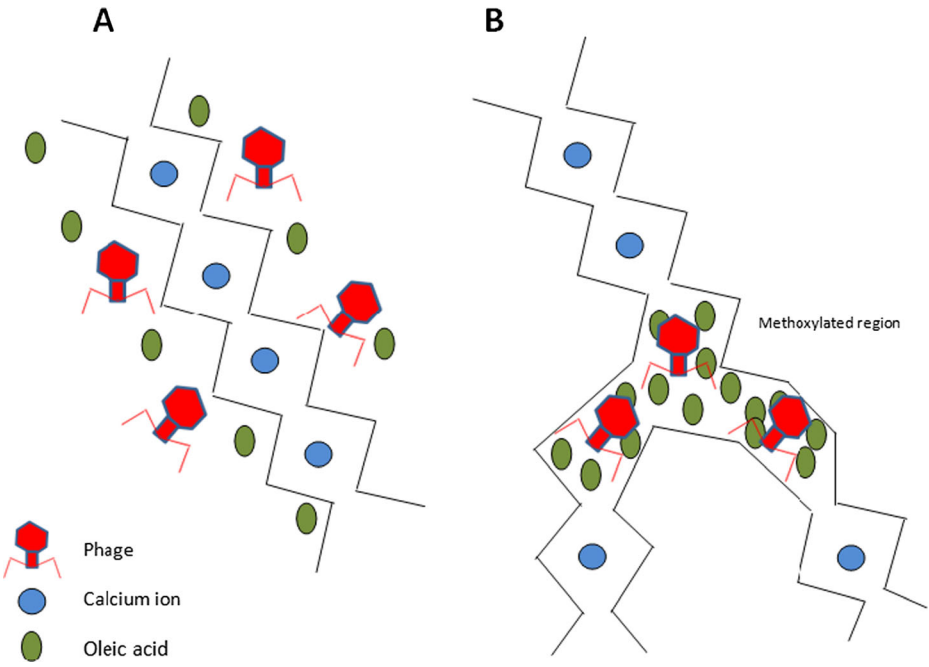

Fig. 5 Structural gelation models produced by ionic crosslinking of alginate (**a**) and low methoxyl pectin (**b**) in the presence of phage particles and oleic acid drops

biopolymer molecules at alkaline pHs higher than 9.0 is conducting to the progressive decrease of the gel matrix structure [28].

In vitro effect of pH on emulsified pectin microbeads stability under gastric conditions was chosen because of its high stability compared emulsified alginate microbeads as previously reported [11].

Swelling rate as well as texturometric parameters of emulsified pectin formulation were determined under simulated gastric condition (HCl; pH=1.2 at 37 °C) after 30 min. Exposure of pectin microbeads to low pH showed a significant decrease in the microbeads hardness and cohesiveness ($p<0.05$), exhibiting values of 0.276±0.008 and 0.180±0.007, respectively, after incubation, which represents reductions of 84.4 and 67.1 % on each respective value compared to the untreated beads. Additionally, a significant reduction ($p<0.05$) in matrix weight was observed (12.7 %) as a result of the exposure to acid. The results obtained are in agreement with those expected for the reaction of pectin-based gels to pH values below the pK_a of pectin, which is approximately 3.5 [29]. At pH values below the pectin pK_a, most of the free carboxyl groups are protonated and calcium ions are displaced from the gel matrix. Therefore, the gel network disruption is directly reflected by the drop of matrix stability (reduction of hardness and cohesiveness). Also, a reduction in matrix weight due to the increased matrix density was observed. The increase of matrix density at pH below the pK_a of pectin could be attributed to the equilibrium displacement of the carboxylate residues of the polymer to carboxylic acid residues losing the coordination of water and getting more compacted gel structure [3].

In order to determine the effect of pH on the stability of emulsified pectin matrix, equilibrium swelling rate, and texturometric parameters of microbeads were measured at 25 °C and pH values from 1.2 to 9.0 after 24-h incubation. Equilibrium swelling assays indicated no significant differences ($p>0.05$) in microbeads weight for all pHs assayed after

24 h, indicating that the incorporation of oleic acid to the matrix provides an hydrophobic coating around microbeads which make them pH independent at hydrophilic environments [30].

The texturometric parameters obtained for each pH value are shown in Fig. 6. No significant differences in the values of gel firmness and cohesiveness were observed in a pH range from 3.0 to 9.0 ($p>0.05$), but a significant reduction in both texturometric parameters was observed at pH 1.2 ($p<0.05$), according to that observed for the HCl solution.

In previous work, it was reported that slight changes in the diet of ruminant/calf may shifted the pH of the true stomach to higher values, ranging from pH 2 to 4 [23]. This technique for raising the pH in the stomach to higher values definitively will increase the gel matrix stability and the protective capability to the cargo, and consequently the efficiency of the phage therapy is enhanced.

Models of Phage Release

Emulsified pectin matrix showed phage release profiles highly dependent on temperature. Raising the temperature of the assays from 19 to 37 °C increased the phage release from 0.3 to 47.5 % after 4 h of incubation with an average rate of 2.67 %/°C [11].

Experimental release profiles for the phage at 19, 30, and 37 °C were modeled by analytical equations used for drug kinetic releases from polymeric matrices previously described [14]. Zero and first-order models of Hixson–Crowell, Higuchi, Baker–Lonsdale, Korsmeyer–Peppas, and Hopfenberg were tested and the r values were estimated (data not shown). The Korsmeyer–Peppas model best fitted experimental data at the three temperatures assayed with r^2 values in the range of 0.98 to 0.993.

$$M/M_{\infty} = kt^{n} \tag{1}$$

For drug release systems with spherical shape, n values above 0.85 is described as super case II transport mechanism in which the release is governed by polymeric relaxation of the crosslinked chain [31–33]. The drug release percentages obtained experimentally were in all cases below

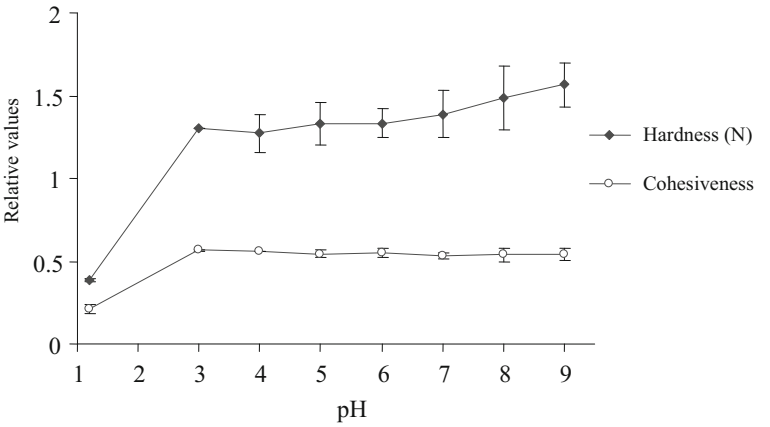

Fig. 6 Texturometric parameters of emulsified pectin matrix after 24-h incubation at different pH values at 25 °C

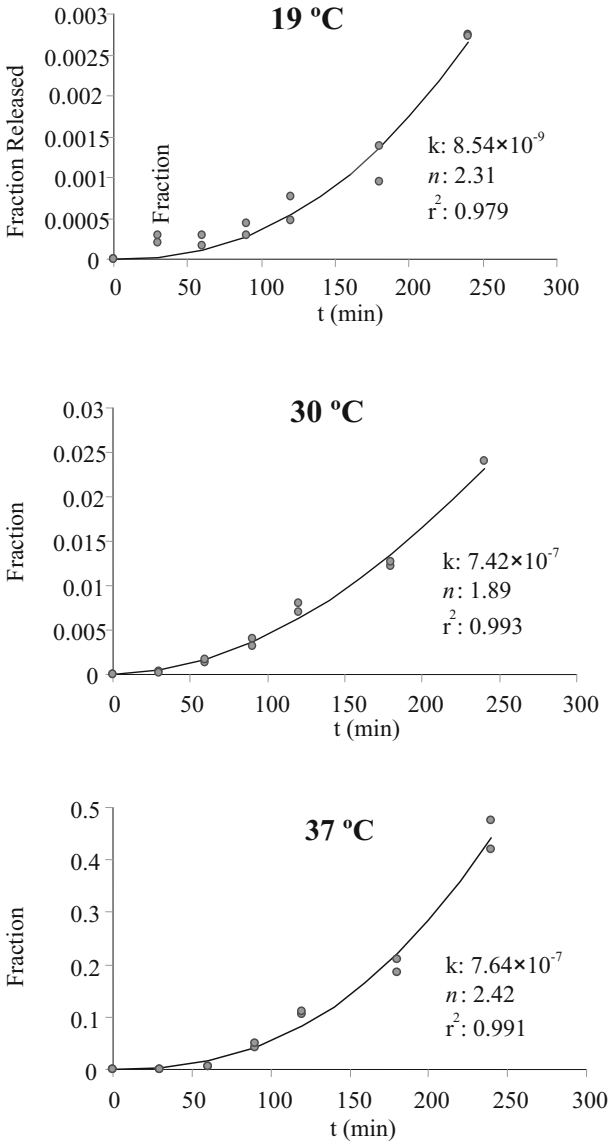

Fig. 7 Effect of temperature on kinetic of phage CA933P release from emulsified pectin microspheres

60 % of the total microbead cargo, as specified for the application of this model [34]. The theoretical curves adjusting experimental data are shown in Fig. 7. This model considers matrix swelling, diffusion of the drug/therapeutic agent as well as dissolution of the polymer matrix [35]. The good correlation of this model with the experimental data is in agreement with both, the high swelling capacity and degradation of the polymer network observed in the swelling kinetics assays for emulsified pectin microbeads. Additionally, the correlation with the Korsmeyer–Peppas model is reflected in the expected release mechanism. The release exponent (n) values is indicative of the drug release mechanism determined by the erosion of the polymer matrix [34]

and the swelling and relaxation of the polymer chains [35]. These results predict that the use of emulsified pectin matrix for phage delivery is suitable for release of phage cargo within the first hours of the microbeads contact within intestinal fluids. Additionally, matrix erosion also assures the total release of the therapeutic agent into the target organ.

Conclusions

The rational of previous findings related to the phage encapsulation in emulsified alginate and pectin matrices was established by SEM, texturometric, and enzymatic assays of the microbeads. The results are indicative of structural strong differences in the both gel biopolymeric matrices making the differences for phage encapsulation.

The higher protective capability to the phage against acidity displayed by emulsified pectin matrix with respect to emulsified alginate could be explained in terms of the hydrophobic methoxylated zones of pectin wherein the oleic acid and the phage can be placed. The use of hydrophobic p-nitrophenyl palmitate as a tracer established different distributions of oleic acid within alginate and pectin-based matrices. In pectin gel matrices, oleic acid coating increases matrix firmness, in agreement with improved isolation of the loaded phage from the external aqueous medium. Additionally, the less ordered structure of pectin compared to alginate allows the incorporation of phage into the hydrophobic cavities surrounded by oleic acid, increasing its protective capability to acid environmental conditions (Fig. 5).

On the other hand, alginate gelation is strong enough to arrange the polymeric chains in the presence of calcium and could not allow the phage to go into the hydrophobic pockets produced by presence of oleic acid (Fig. 5). Alginates gels can be synthesized by crosslinking with divalent cations like Ca (II) and others making a network gel structure named egg box. However, the monosaccharide sequences in the poly-galacturonic acid chain from pectin do not match the form of the buckled twofold ribbon necessary for egg box formation but they are able to form threefold helices instead. This kind of distortion of the egg box model allows the inclusion of phage particles surrounded by oleic acid in specific areas of the crosslinked biopolymer.

The release kinetics of phage is fitting the Korsmeyer–Peppas model with n values according to the high swelling and degradation observed for pectin-based matrices in the present work. The super case II type release, which is associated to the relaxation of polymer chain, will allow tailoring the pectin by changing the ratio of hydrophobic–hydrophilic areas based on the cargo to be carry and the environmental conditions of the release.

Furthermore, the reduction in matrix degradation speed observed at 19 °C compared to that at 37 °C in this work is directly reflected in the reduced release kinetics of phage at lower temperatures previously observed for emulsified pectin microbeads.

Based on the experimental results, an encapsulation model was proposed, which will be very helpful for phage-controlled release therapies and could be extended to other therapeutic purposes. Since phages can be considered as macromolecular protein complexes, this system is plausible to be generalized for controlled release of high MW compounds, drugs, and/or therapeutic agents. Furthermore, a simple and environmentally friendly synthesis procedure using economic raw materials extracted from renewable resources (even from the reutilization of agricultural industry wastes) suggests this encapsulation technology might be easily applied to large-scale.

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