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Characterization of smart auto-degradative hydrogel matrix containing alginate lyase to enhance levofloxacin delivery against bacterial biofilms

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ABSTRACT

The aim of the present work is the characterization of smart auto-degradable microspheres composed of calcium alginate/high methoxylated pectin containing an alginate lyase (AL) from *Sphingobacterium multivorum* and levofloxacin. Microspheres were prepared by ionotropic gelation containing AL in its inactive form at pH 4.0. Incubation of microspheres in Tris–HCl and PBS buffers at pH 7.40 allowed to establish the effect of ion-chelating phosphate on matrix erodability and suggested an intrinsically activation of AL by turning the pH close to neutrality. Scanning electron and optical microscopies revealed the presence of holes and surface changes in AL containing microspheres. Furthermore, texturometric parameters, DSC profiles and swelling properties were showing strong changes in microspheres properties. Encapsulation of levofloxacin into microspheres containing AL showed 70% efficiency and 35% enhancement of antimicrobial activity against *Pseudomonas aeruginosa* biofilm. Levofloxacin release from microspheres was not changed at acidic pH, but was modified at neutral pH in presence of AL. Advantageously, only gel matrix debris were detectable after overnight incubation, indicating an autodegradative gel process activated by the pH. Absence of matrix cytotoxicity and a reduction of the levofloxacin toxicity after encapsulation were observed in mammalian CHO-K1 cell cultures. These properties make the system a potent and versatile tool for antibiotic oral delivery targeted to intestine, enhancing the drug bioavailability to eradicate bacterial biofilm and avoiding possible intestinal obstructions.

1. Introduction

Since the last decades, biopolymers are playing a major role for the development of novel technologies in many fields and particularly in the pharmaceutical and medical fields (Skaugrud et al., 1999; Kalia and Avérous, 2011). There is a real need in development of novel medical devices with increasingly functional properties such as durability, flexibility, and strength, in addition to improved biocompatibility, non-toxic and low costs. Consequently, the development of novel smart devices based in natural polymers, coacervates, and hybrid materials are currently been explored in our laboratory (Islan et al., 2012, 2013, 2014).

Among biopolymers, alginates have been long considered as matrices for drug or cells encapsulation (Goh et al., 2012) and for other applications in controlled transdermal or transmucosal drug delivery of active substances (Sachan et al., 2009). Alginates are linear anionic polysaccharides linked by 1–4 bounds containing varying proportions of ß-manuronic acid (M units) and α-gulur-onic acid (G units). Their properties of not being toxic, not immunogenic, biodegradability and biocompatibility, added to a "green" gelation in presence of divalent ions by "egg box junctions" make them very suitable hydrogels for medical applications (Lee and Mooney, 2012).

Another interesting group of biopolymers to be considered for drug delivery applications is pectins. They are water-soluble

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polysaccharides extracted from plant cell wall and made of linear residues of poly-α-(1,4)-D-galacturonic acids with different esterification degrees (ED). Pectins can be classified as low methoxylated (LMP: ED below of 40%), medium methoxylated (MMP: ED 40–60%), and high methoxylated pectins (HMP: ED higher than 60%). It has been reported that pectins played a key role in interaction with antibiotics of the fluoroquinolones family, improving the encapsulation of the drug and providing a controlled release profile (Islan et al., 2012).

Fluoroquinolones like ciprofloxacin and levofloxacin are very common antibiotics working as inhibitors of DNA gyrase and topoisomerase IV causing bacterial death. Despite their effectiveness, these antibiotics are commonly associated to undesirable side effects (Paton and Reeves, 1991; Carbon, 2001), mainly due to a tendency to aromatic stack among themselves under physiological conditions that reduce their bioavailability and become toxic (Turcu and Bogdan, 2012). In this sense, encapsulation in smart nano- and micro-biopolymeric devices are a novel technologies potentially useful to provide effective controlled release of the drugs and therefore reducing their toxic concentrations.

The combination of fluoroquinolones with therapeutic lytic enzymes showed synergic effects on antibiotic diffusion and represents an alternative to improve microbial infection therapies as recently reported (Zhu and Yin, 2015). Particularly, alginate lyase (AL) could play an active role in detaching pathogens immersed into biofilms composed of bacterial alginate (Boyd and Chakrabarty, 1994). AL acts over β-1,4-glycosidic linkages of alginate via β-elimination reaction to produce oligosaccharides. The AL biocatalytic activity could enhance the treatment of infections caused by the biofilm-making *Pseudomonas aeruginosa*, an opportunistic human pathogen usually found in several illnesses and very difficult to eradicate once established. The most common case is cystic fibrosis in which the bacteria are colonizing the lungs and intestine walls producing strong biofilm architecture (May et al., 1991; Cutting, 2015). Behind these circumstances, effective devices for drug delivery are required showing enhanced antimicrobial activity and particularly with the ability of displaying autodegradative properties in order to avoid possible additional intestinal obstructions by the matrix.

A few degradable matrices for molecular controlled release have been reported previously addressing cell migration (Zhao et al., 2005) or in cancer treatment (Ishida et al., 2008), but smart autodegradative microspheres able to be activated under certain environmental conditions and therefore modulate the release profile of the bio-active molecules are still in their infancy.

In a previous work from our laboratory, microspheres composed of alginate and HMP gels were able to co-encapsulate ciprofloxacin and alginate lyase for potential oral treatment of cystic fibrosis (Islan et al., 2013). However, the encapsulation mechanism and the enzymatic behavior under physiological environments were unclear and required further characterizations for the medical application. To our best knowledge only few reports proposed the enzymatic modification of gels and there are no reports of an alginate lyase acting on alginate/HMP matrices (Klak et al., 2013; Liu et al., 2013).

The aim of the present work is to study the auto-degradable properties of a biopolymeric hydrogel made of alginate and high methoxylated pectin containing alginate lyase and levofloxacin. The effect of environmental factors such as pH and phosphate ions on microsphere swelling and enzyme activity were analyzed. Biophysical tests like morphological microsphere kinetic changes and topology was followed by scanning electronic and optical microscopies; texturometric and calorimetric analyses were also performed. The antimicrobial activity of levofloxacin against *P. aeruginosa* biofilm was finally tested in-vitro and the cytotoxicity of microspheres and their components was evaluated in mammalians cells in order to investigate their suitability for use in living organisms.

2. Materials and methods

2.1. Materials

Low-viscosity sodium alginate (Alg, MW_{av} = 120 kDa) was provided by Monsanto (Buenos Aires, Argentina). Levofloxacin (Levo, (S)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid), high methoxyl apple pectin (HMP, MW_{av} = 160 kDa; DE: 70–75%), TRIS (tris(hidroximetil) amino metano) and alginate lyase (AL) from *Sphingobacterium multivorum* were purchased from Sigma–Aldrich (Buenos Aires, Argentina). Ham's F10 cell culture media was from Microvet, Argentina; fetal bovine serum (FBS) was from Internegocios SA Argentina. *P. aeruginosa* ATCC 15442 was used in all experiments. Other reagents were of analytical grade from commercially available sources and used as received from Merck (Darmstadt, Germany) or similar brand.

2.2. Formulation and preparation of alginate based microspheres containing levofloxacin and alginate lyase

Low-viscosity sodium alginate (2.0 wt%) was dissolved in 25 mM acetate buffer (pH 4.0) at room temperature, followed by the addition of 100.0 μg/ml levofloxacin and 40.0 U/ml of AL under gently stirring at 0 °C. Alternatively, alginate (2.0 wt%) was mixed with HMP (1.0 wt%) following the same procedure. Microspheres were prepared by adding 2,0 ml of biopolymeric solution by drop wise via a 20-gauge hypodermic needle connected to a peristaltic pump with a flow rate of 0.2 ml/min (Watson Marlow 101U/R, Cornwall, UK) into 10,0 ml of 500 mM $CaCl_2$ in 1:1 water/1,2-propyleneglycol mixture and stirred at 100 rpm for 20 min (Das and Senapati, 2008; Islan et al., 2013). After that maturation time, microspheres were filtered, washed with distilled water, frozen with liquid nitrogen and lyophilized for further assays.

The experimental conditions selected for microspheres preparation were based on ionotropic gelation method in presence of calcium ion dissolved in 1,2-propylene glycol–water (1:1) solution to enhance the antibiotic encapsulation as previously reported (Islan et al., 2013). The pH of the formulation was set at 4.0 because of AL showed reversible inactivation under this experimental condition and consequently the enzyme was not able to hydrolyze the alginate before crosslinking. Also, it was established that the AL is highly stable at pH 4.0 and 0 °C during particle synthesis (Fig. S1a and b). Another advantage of working at low temperature is related to increase the matrix gelation process and reduce molecular diffusion during encapsulation (Islan et al., 2012).

2.3. Evaluation of AL activity and stability studies

Alginate lyase (AL) activity was measured by mixing 75 μl of enzyme solution (40.0 U/ml) with 1.925 ml of 1.0 wt% alginate (in phosphate buffer 25 mM pH 7.4) and incubated at 37 °C for 30 min. The reaction was stopped by the addition of 2.0 ml of 100 mM NaOH and the resulting absorbance was measured at 233 nm. One AL unit was defined as the amount of enzyme capable of increase 1 unit of absorbance at 233 nm per min × ml of sodium alginate at pH 7.4 and 37 °C.

The AL activity into the microspheres was calculated considering the initial amount of enzymatic units added to the formulation and the activity determined after immobilization and subsequent dissolution of the matrix in 100 mM phosphate buffer (pH 7.40).

The stability of AL was studied as function of pH and temperature in order to optimize the parameters for microspheres

preparation. For the effect of pH, soluble AL (4.0 U/ml) was incubated with 1.0 ml of 50 mM KCl/HCl buffer (pH 1.2 and 2.0), 25 mM citrate buffer (pH 3.0), acetate buffer (pH 4.0 and 5.0) and 25 mM phosphate buffer (pH 6.3, 6.8, 7.4 and 8.2) at 37 °C for 30 min. For thermal stability, AL (4.0 U/ml) was incubated at pH 7.4 (25 mM phosphate buffer) and at different temperatures (5, 20, 32, 37 and 42 °C) for 30 min. In both cases, after incubation, the AL activity was assayed at optimum conditions as previously described. The 100% of activity was set by AL at 37 °C and pH 7.4.

2.4. Measurement of total protein content

Protein content was measured using fluorescamine as follow: 50 μl of sample was mixed with 350 μl of 12.5 mM borate buffer (pH 9.0) and 125 μl of fluorescamine (300 μg/ml). After 2 min, fluorescence was measured at λ_{ex} = 390 nm and λ_{em} = 478 nm (PerkinElmer, LS 50B spectrofluorimeter) using proper calibration curve.

2.5. Determination of levofloxacin and AL encapsulated into alginate based microspheres

Microspheres were separated by filtration and the Levo concentration was spectrophotometrically determined in the filtrate solution at 295 nm, using an appropriate calibration curve (UV–vis Beckman DU640 spectrophotometer). The encapsulation percentage was calculated as follows:

$$\text{Encapsul } (\%) = \frac{[(Q_0 - (\text{Cr} \times V)) \times 100]}{Q_0} \quad (1)$$

where Q_0 = initial Levo amount (μg), Cr = Levo concentration in the filtrated solution (μg/ml), and V = volume of filtrated solution (ml).

The same Eq. (1) was used to estimate the AL activity and total protein content of filtrated solutions, but redefining the parameters as follow: Q_0 = initial AL units (U)/initial total protein amount (μg) and Cr = activity of AL (U/ml)/concentration of total protein (μg/ml) in the filtrated solution.

Alternatively, the encapsulation percentage was determined by quantifying the antibiotic release after total matrix degradation. The microspheres (100.0 mg) were weighed and incubated to complete disintegration in 1.5 ml of 100 mM phosphate buffer (pH 7.4) following by centrifugation (10,000 × *g* for 10 min). The antibiotic and protein concentrations were determined in the supernatant by absorbance or fluorescence respectively as mentioned before.

2.6. Scanning electron microscopy (SEM) images

Freeze-dried microspheres were prepared for SEM observation by sputtering the surface with gold using Balzers SCD 030 metalizer and obtaining layer thickness between 15 and 20 nm. Surface and morphology of microspheres were observed using Philips SEM 505 model (Rochester, USA), and processed by an image digitalizer program (Soft Imaging System ADDA II (SIS)).

2.7. Roughness analysis

SEM images were analyzed by ImageJ software (NIH, USA). The roughness of the surface was reflected by the standard variation of the gray values of all the pixels on the image. First, the SEM image files were converted to 8-bit images. Then, all the pixels were selected and statistically measured with the software. The less the standard variation value is, the smoother the surface is. Histograms were performed by duplicate of SEM images at 710× magnification.

2.8. Differential scanning calorimetry (DSC) analysis

The DSC profile of the samples was obtained using a TA-Instrument DSC Q2000. Briefly, 5.0 mg of lyophilized microspheres were placed in a standard aluminum pan, hermetically closed and heated at a constant rate of 10 °C/min from room temperature to 225 °C, under 20 ml/min nitrogen purging. All samples were run in triplicate.

2.9. Swelling kinetics

Vials containing weighed freeze-dried microspheres (100 mg) were incubated in 1.0 ml of TRIS buffer saline (TBS: 150 mM NaCl, 10 mM TRIS; pH 7.4) or 1.0 ml of phosphate buffer saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4; pH 7.4) at 37 °C. Every 1 h, one vial was taken and the liquid media was removed, while microspheres were immersed into distilled water for 10 s to wash out the buffers. Later, they were placed on absorbent paper to remove the excess of liquid from the surface, weighed and kept in capped tubes for further texturometric assays. Swelling was expressed as percentage of microspheres weight increment. The swelling degree with a value of 0% indicates that microspheres are totally disintegrated.

The removed liquid media was spectrophotometrically analyzed to determine AL activity at each measurement point.

2.10. Optical microscopy

The microsphere swelling and erosion determined in presence of TBS and PBS buffers were observed at different intervals of time in an optical microscope (Leica DM 2500, Germany).

2.11. Measurement of texturometric parameters

Microsphere textures was analyzed in a TAXT 2i Texture Analyzer (Stable Micro Systems Ltd., UK) equipped with a loading cell of 25 kg and a cylindrical metal compression plate of 75 mm diameter (p75). The texture profile analysis (TPA) consisted in two compression cycles at 0.6 mm/seg to 30% of the original microspheres height (Dini et al., 2014). In each assay, three microspheres were simultaneously compressed. Data was processed with the Texture Expert® software and the textural parameters (hardness/firmness) were calculated from the TPA curve of force (*N*) vs time (seconds). Hardness (*N*) is defined as the peak force during the first compression cycle and is expressed in Newton units. The results represent the average means of at least triplicate tests.

2.12. Release studies of levofloxacin from alginate based microspheres

Freeze-dried microspheres (100 mg) were placed in a 3-ml glass vial containing 1.5 ml of 50 mM KCl/HCl buffer (pH 1.20, simulated gastric conditions) or 40 mM phosphate buffer (pH 7.40, simulated intestinal environment) and incubated in a thermostatted orbital shaker at 100 rpm and 37 °C. Samples (0.5 ml) were taken out at different times and absorbance was measured at the Levo maximum wavelength corresponding to each buffer (293 and 286 nm at pH 1.20 and 7.40, respectively). Finally, 0.5 ml of fresh media was added back to refill the reaction volume and the vials were placed back.

2.13. Antimicrobial assay of levofloxacin (Levo) containing microspheres against artificial biofilm of P. aeruginosa

Levo activity was tested against *P.aeruginosa* ATCC 15442 using modified disk diffusion method, replacing disks for microspheres. Briefly, five colonies of the microorganism were taken and

resuspended in sterile physiological solution (154 mM NaCl) to adjust the turbidity of the culture to less than 3.0×10^8 CFU/ml (0.5 McFarland scale). Agar plates were loaded with 25 ml of Mueller–Hinton medium in 100 mm diameter Petri dishes and inoculated within 15 min after adjusting the turbidity of the inoculum suspension, using sterile cotton swab dipped into it. After that, a thin layer of sodium alginate (1.0 wt%) was added to cover the whole plate surface (the pH of the mixture was kept around 7.0 by the agar medium and considering that *P. aeruginosa* is non-fermentative bacteria growing mainly using oxidative metabolism). Twenty minutes later, the microspheres were placed on the surface and incubated at 37 °C for 24 h. Then inhibition zones were determined.

2.14. Cytotoxicity of the matrix tested in chinese hamster ovary (CHO) cells

An initial screening of the potential cytotoxicity of the matrix and its components was preliminary assessed by the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, which is based on the ability of living, metabolically active cells to cleave a tetrazolium salt (Weyermann et al., 2005). The assay was developed in the chinese hamster ovary cell line subclone K1 (CHO-K1), obtained from the IMBICE cell repository, which was selected as in vitro model due to its wide use for in vitro toxicity studies and the uniform properties it exhibits.

2.14.1. Sample conditioning

A screening of cell viability in CHO-K1 cells with a wide range of Levo concentrations showed that above 200 μg/ml cellular viability significantly decreased. Based on that, the cytotoxicity of the matrix containing Levo and the free antibiotic was tested at 0, 50 and 200 μg/ml. The microspheres (250 mg) were completely disaggregated in 2.0 ml of 100 mM phosphate buffer (pH 7.40) at 37 °C for 30 min under constant shaking. The resulting suspension was centrifuged 5 min at 9000 × *g* and the cytotoxicity was tested in the supernatant. For free Levo, a stock solution of 10 mg/ml was prepared in 100 mM acetate buffer (pH 4.0) and diluted with sterile distilled water to reach an intermediate concentration which was finally adjusted with cell culture media.

2.14.2. Cell viability

CHO cells were grown in Ham's F10 medium supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C and 5% CO_2 atmosphere. They were seeded on 96-well plates at a density of 1×10^4 cells/well and were allowed to adhere for 24 h. Then, cells were exposed to: free Levo (0, 50 and 200 μg/ml); the empty matrix; the matrix containing the Levo (considering a 70% of antibiotic encapsulation, with a value of 11 μg/mg matrix), matrix containing the AL (encapsulation percentage of 90%) and the matrix with both active components (Levo–AL) for 24 h. Once incubation time was completed, medium was carefully discarded and a volume of 100 μl of MTT solution (0.5 mg/ml in medium without FBS and phenol red) was added to each well. After 3 h of incubation, the presence of violet crystals inside the cells was monitored by optical microscopy. Wells content was discarded, carefully washed with PBS and 100 μl of DMSO was added to extract formazan crystals. After a short incubation time at room temperature, the absorbance at 550 nm was measured. The assay was performed in 3 independent experiments by sextuplicate.

2.15. Statistical analysis

Each experiment was carried out at least in triplicates. Comparisons of the means were performed by analysis of variance

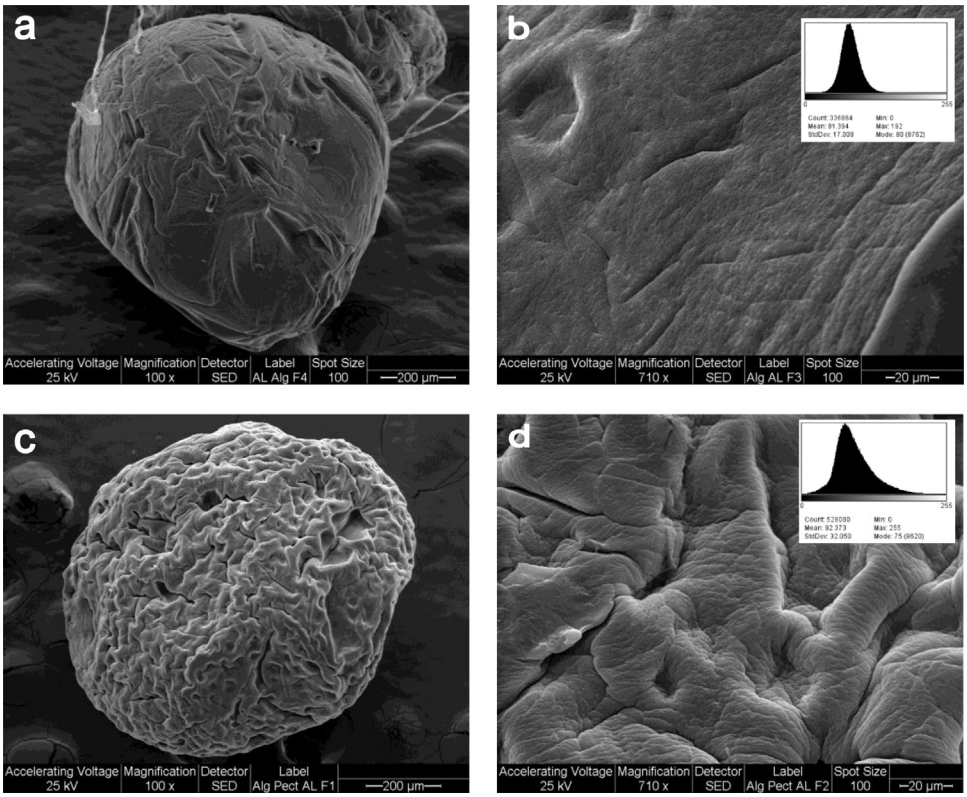

Fig. 1. SEM images of microspheres: alg/AL at 100× (a) and 710× (b); alg-HMP/AL at 100× (c) and 710× (d) magnifications. The insets show the grey scale histograms of surface images analyzed by ImageJ software.

(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. Preparation of microspheres

Two autodegradative gel microsphere formulations based on alginate containing or not HMP were developed. In both formulations the AL entrapment was higher than 90% (determined by total proteins measurement) but showing significant differences in AL activities. The Alg/AL microspheres displayed 90% AL activity after immobilization, meanwhile a 76% enzyme activity was detected in the Alg-HMP/AL formulation. That decrease in the AL enzyme activity could be attributed to the presence of HMP, which possibly reduce the AL contact with the alginate chains and could also interact with the enzyme (Islan et al., 2014).

All the tested formulations produced particles in a narrow size distribution with about 1 mm diameter under native conditions, but the particles shrink to 800 ± 50 μm diameter after freeze-drying process.

3.2. Characterization of microspheres

The microsphere morphology and surface pattern characteristics were analyzed by SEM (Fig. 1). Alg/AL microspheres showed almost spherical shape with some irregularities and holes on surface (Fig. 1a). However, an approach at 710x magnifications revealed a good compatibility of the matrix combining the alginate in presence of the AL in its inactive conformation. A smooth profile was observed through analysis by ImageJ software (Fig. 1b). On the other side, the matrix composed of alg-HMP/AL was characterized by spherical microspheres with a rougher surface based on the standard deviation which was increased from 17 to 32 for alg/AL and alg-HMP/AL formulations respectively. Also, the alg-HMP/AL microspheres were displaying distinctive structural aggregates like knots covering their surface (Fig. 1c and d). The folds on microsphere surface were possibly composed of pectin, and probably partially extruded form the inner alginate gel structure because of lower interchain crosslinking degree in presence of calcium ion due to its high methoxylation degree (Braccini and Pérez, 2001).

Thermal analysis of the microspheres was performed by DSC (Fig. 2). Alginate microspheres showed an endothermic peak at 245 °C meanwhile the alg/AL microspheres exhibited a peak at 239 °C. The 6 °C shift to lower temperature values are indicative of more labile structure and/or porous architecture, reason why water molecules are more exposed and prone to migrate out of the matrix. The results were in concordance with SEM observations, which suggests more irregularities on surface compared with alginate formulations without the enzyme (Islan et al., 2013). In the case of alg-HMP microspheres the DSC profile differs only in 2 °C when the AL was incorporated, suggesting a minor effect of the enzyme on the matrix structure.

When microspheres were incubated in a liquid medium and the pH inside the matrix was slowly shifted from acid to neutral values, the AL become active and started to degrade the alginate gel microsphere. The influence of the buffer was studied, considering a calcium-chelating buffer (PBS) and a non-chelating buffer (TBS), keeping the pH at 7.40. It is well known that chelating agents like phosphate are forming complex with the calcium present into the microspheres and breaking the bridges between the alginate chains, and consequently the gel matrix stability is compromised (Almeida and Almeida, 2004). Under these conditions, the swelling properties of microspheres were analyzed (Fig. 3). Alginate gel microspheres incubated in TBS buffer was able to incorporate

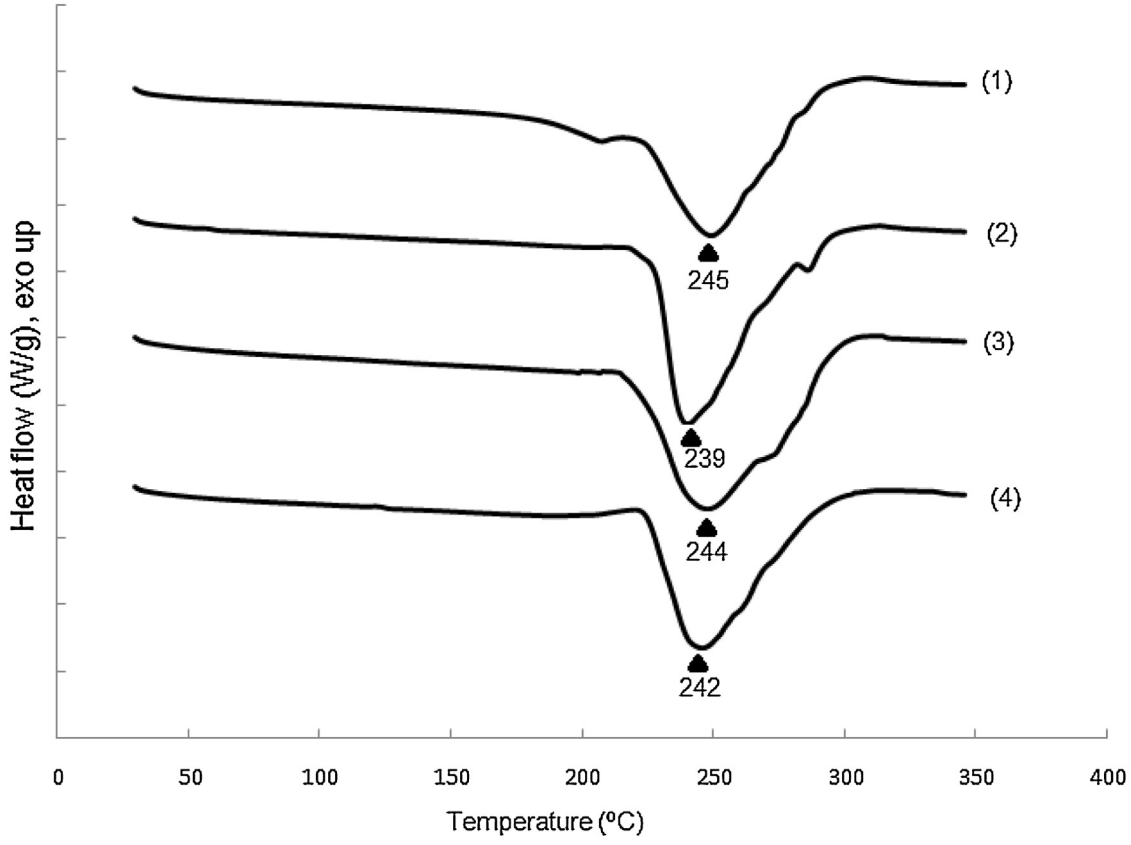

Fig. 2. DSC thermograms of microspheres: (1) alg, (2) alg/AL, (3) alg-HMP and (4) alg-HMP/AL.

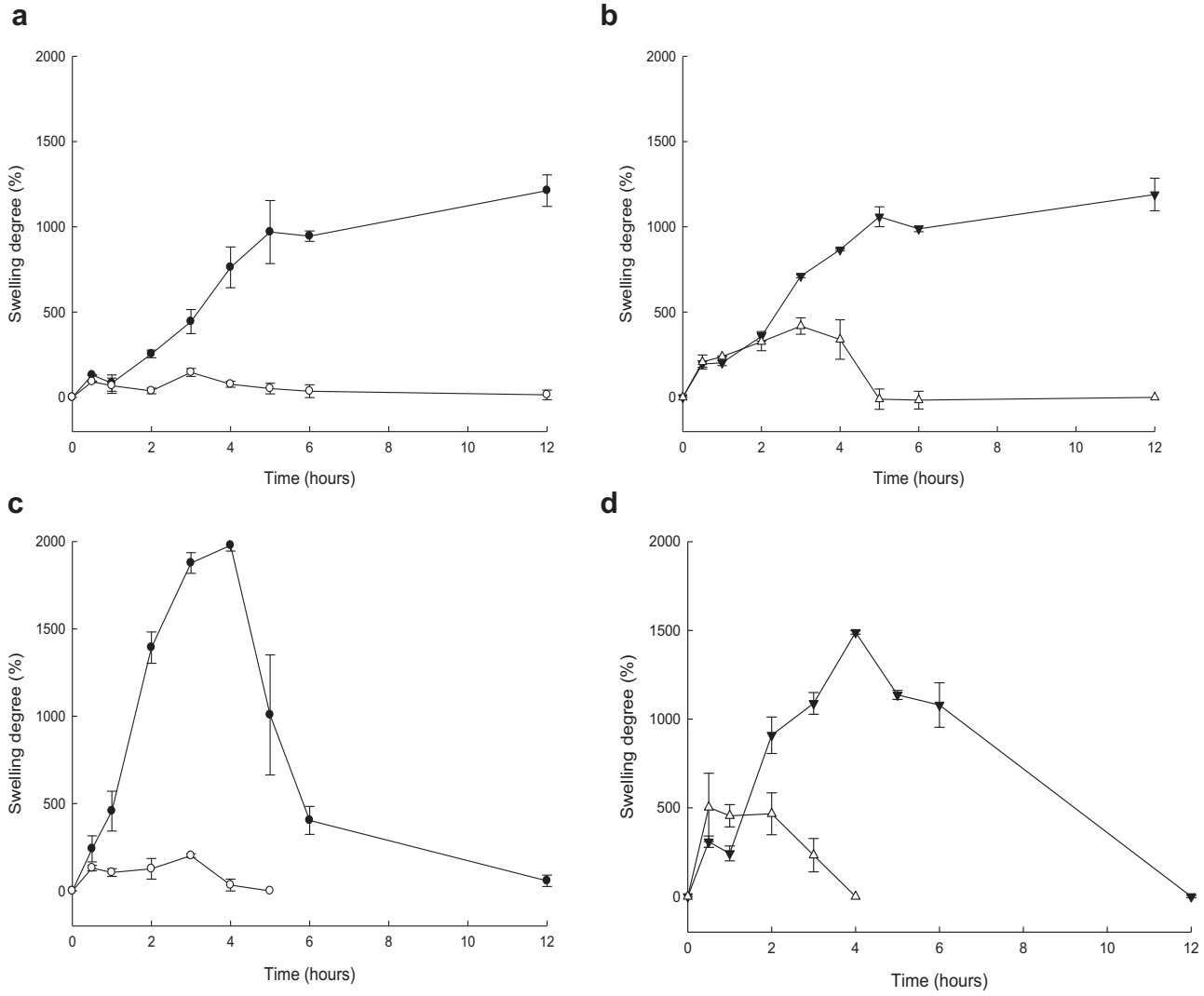

Fig. 3. Swelling of microspheres in TBS (a and b) and PBS (c and d) buffers at pH 7.40 and 37 °C. Symbols: ●, alg; ○, alg/AL; ▼, alg-HMP and △, alg-HMP/AL composites.

increasing amounts of water molecules up to 12 h, which is considered as the water saturation time for this formulation. Instead, the microspheres composed of alg/AL underwent a process of initial swelling up to 3 h, point from which the environmental conditions favored the AL activation (initially inactive at pH 4.0) and started the evident autodegradation of the matrix with a gradual loss of mass follow by complete gel structure disintegration after 5 h of incubation (Fig. 3a).

On the other hand, the behavior of alg-HMP microspheres in TBS medium was similar. The matrix without the enzyme showed a progressive swelling similarly to alginate microspheres. However, the response in presence of the AL differs in the water absorption. The alg-HMP/AL microspheres also showed a maximum swelling degree in 3 h, but reaching a value of 400% at higher rate. The difference in water incorporation compared to alginate microspheres is possibly due to the presence of HMP, a biopolymer able to bind large quantities of water molecules by hydrogen bonds (Thakur et al., 1997). After 3 h, the alg-HMP/AL matrix began to be degraded by the enzyme and was completely eroded at 5 h (Fig. 3b).

The gel microspheres incubated in calcium chelating buffer (PBS) showed different swelling profiles based on the matrix composition. In the absence of AL, the gel matrix composed of calcium alginate began to swell until 4 h, showing a high swelling degree of 2000%. This fact could be explained considering that free alginate chains are available to bind water molecules after the uptake of calcium ions present in the gel network by the phosphates from PBS (Bajpai and Sharma, 2004). After 4 h, occurred an erosion process due to the loss of calcium bridges within the structure, concomitantly with the dissolution of the matrix. Meanwhile, the autodegradation process of the microspheres is more pronounced in the presence of encapsulated AL from the first time of incubation which is resulting in low rate of swelling and considerable loss of gel matrix mass (Fig. 3c). As similar as previously observed, the alg-HMP microspheres showed high initial swelling. In the case of alg-HMP/AL matrix, the swelling degree was more pronounced compared to alg microspheres, but the total autodegradation of the matrix was also produced after 4 h (Fig. 3d). These results are indicating that AL is able to hydrolize alginate chains from the inside of the matrix, even in presence of other biopolymers, like pectin, structurally similar to alginate which highlights the biocatalytic specificity of the enzyme.

The environmental effect of pH and buffer chelating activity was used to study the microsphere gel stability by tracing the products of Alginate Lyase (AL) activity released from the inside of the matrix (Fig. S2). The microspheres were made by ionotropic gelation using calcium, and two relevant effects over the gel stability were superposed: the pH and calcium concentration

inside the matrix. In this sense, two buffers were considered and used at the same pH to discriminate both effects: Tris–HCl (TBS, non-chelating buffer) and phosphate (PBS, calcium-chelating buffer). Statistical analysis of the AL products released from the microspheres in TBS and PBS buffers are suggesting that the autodegradation of the matrix in both experimental conditions are coming from inside the particles (Fig. S2). However, in the case of TBS, the release of AL increases linearly up to the 2 h and afterwards began to rise slowly. This behavior is probably produced because of AL is mainly acting on the crosslinked alginate chains (e.g., the "*egg box*" structure) and the access of the AL active site to the substrate could be restricted. On the other side, the products of AL activity in time at PBS were increasing linearly until reach the total disintegration of microspheres at 4 h. This fact can be attributed to the action of phosphate ions that are competing for the calcium within the matrix with two consequences: (1) enhancing the matrix erosion; (2) releasing free alginate chains that are more accessible for AL activity. Under these considerations, it is possible to explain the faster disintegration of microspheres in PBS buffer

In order to elucidate changes in hardness of microspheres after incubation in the different buffers, the texturometric parameter was determined at a critical time (in this case, 2 h) (Table 1). The hardness parameter (or firmness) usually gives information about the resistance of the gel matrix to compression (Lau et al., 2000). The hardness of fresh alg/AL microspheres was 25% lower than alginate microspheres, indicating a more labile or porous structure. On the other side, alg-HMP/AL microspheres showed similar hardness values in comparison with alg-HMP microspheres, suggesting that AL is not interfering in the alg-HMP matrix structure. After incubation of microspheres at different buffers, a decrease in hardness with respect to controls (not incubated fresh microspheres) was observed in all cases. A significant change ($P < 0.05$) was elucidated for microspheres containing the enzyme, demonstrating the auto-degradability of the matrix due to AL activation at pH 7.40. The effect is more prominent after incubation of non-containing AL microspheres in PBS buffer because of the matrix erodability was simultaneously enhanced by phosphate ions that are taking out calcium from the matrix. However, in the case of AL containing microspheres, the reduction was independent of the buffer type, indicating that matrix erodability was regulated mostly by enzyme activity.

The swelling behavior of the biopolymeric microspheres containing the AL was corroborated by optical observations in time after incubation in TBS and PBS (Fig. S3). It was seen that alg/AL microspheres were more prone to degradation in TBS than alg-HMP/AL microspheres, with the appearance of holes on surface after incubation for 2 h and loss of their almost entire structure after 4 h incubation. In TBS, both matrices suffered a less autodegradation process and after 4 h incubation the matrix maintain some structure but with cracks on the surface and an evident loss of sphericity.

SEM images confirmed the autodegradation of microspheres (Fig. 4). The alg/AL and alg-HMP/AL microspheres showed holes on their structure and changed their spherical form to a "ring" shape (like donut), indicating that AL was activate and work even from inside of the microspheres at neutral pH for 2 h incubation. Also, both matrices were completely disintegrated with particulate dispersion lower than 5 μm after 4 h incubation.

In summary, the biocatalytic mechanism of microspheres autodegradation is established and schematized in Fig. 5 in order to provide a clear understanding of the process.

3.3. Incorporation of levofloxacin into microspheres and release studies

Based on a previous work from our lab (Islan et al., 2013), encapsulation of levofloxacin was performed into microspheres of alg and alg-HMP (with or without the AL) prepared in a calcium solution containing 1,2-propylene glycol and high encapsulation percentages were obtained. Meanwhile alg microspheres exhibited a efficiency of 75.1 ± 1.6%, the alg-HMP microspheres showed an encapsulation percentage of 70.3 ± 0.2%, which corresponds to 102 μg of Levo per gram of matrix. The encapsulation percentage in presence of AL showed no significant differences ($P \geq 0.05$) in both cases (74.0 ± 3.0% and 68.8 ± 2.0%, respectively).

The Levo release from microspheres was evaluated at two pH conditions: acidic (1.20) and slightly alkaline (7.40), which are the physiological pH's of stomach and intestine respectively (Fig. 6). It was found that the alg-HMP/AL microspheres showed some advantages for oral administration compared to free Levo administration and the alg/AL microspheres (Fig. 6a). The alg-HMP formulation was providing a more controlled release of levofloxacin especially under gastric conditions. The Levo release from alg and alg/AL microspheres showed a burst release at pH 1.2, reaching the 100% in less than two hours and thus, the availability of the drug that hypothetically would reach the intestine was drastically reduced. This is mainly produced because the rapid dissolution of the free Levo at acidic pH's (data not shown). The presence of pectin (HMP) in the formulation plays a key role by interacting with the fluoroquinolone and increases sustainability in the antibiotic release as previously reported by our laboratory (Islan et al., 2012, 2013; Martinez et al., 2012). In agreement, the release of Levo from alg-HMP and alg-HMP/AL was reduced (about 40% in two hours) both with similar values at acid pH, indicating that the AL was inactive (Figure S1) and therefore, no degradation of the matrix had occurred. Nevertheless, taking into account that normal gastric residence times are usually in the range between 5 and 120 min depending on food contain (Mojaverian et al., 1987; Shah et al., 2009), it is possible to consider the administration of the antibiotic dose in the microspheres before ingestion of foods which is reducing the particle residence time in the stomach and allows to reach the intestine approximately in 15 min. In this sense, the success of the alg-HMP and alg-HMP-AL formulations is the low release of levofloxacin from the matrices, only 15% and the 10% respectively, compared with 60% antibiotic release in the alg and alg/AL formulations in 15 min.

Table 1
Hardness of microspheres in Ca^{2+} chelating (PBS) and non-chelating (TBS) buffer at pH 7.40 after 2 h incubation at 37 °C.

Conditions	Hardness of microspheres (Newton)			
	Alg	Alg/AL	Alg-HMP	Alg-HMP/AL
No incubated fresh microspheres	0.56 ± 0.07^{a}	0.41 ± 0.03^{a}	0.62 ± 0.02^{a}	0.61 ± 0.03^{a}
After incubation				
TBS (2 h)	0.43 ± 0.01^{b}	0.31 ± 0.04^{b}	0.52 ± 0.02^{b}	0.40 ± 0.03^{b}
PBS (2 h)	0.37 ± 0.02^{c}	0.32 ± 0.01^{b}	0.41 ± 0.08^{c}	0.39 ± 0.04^{b}

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

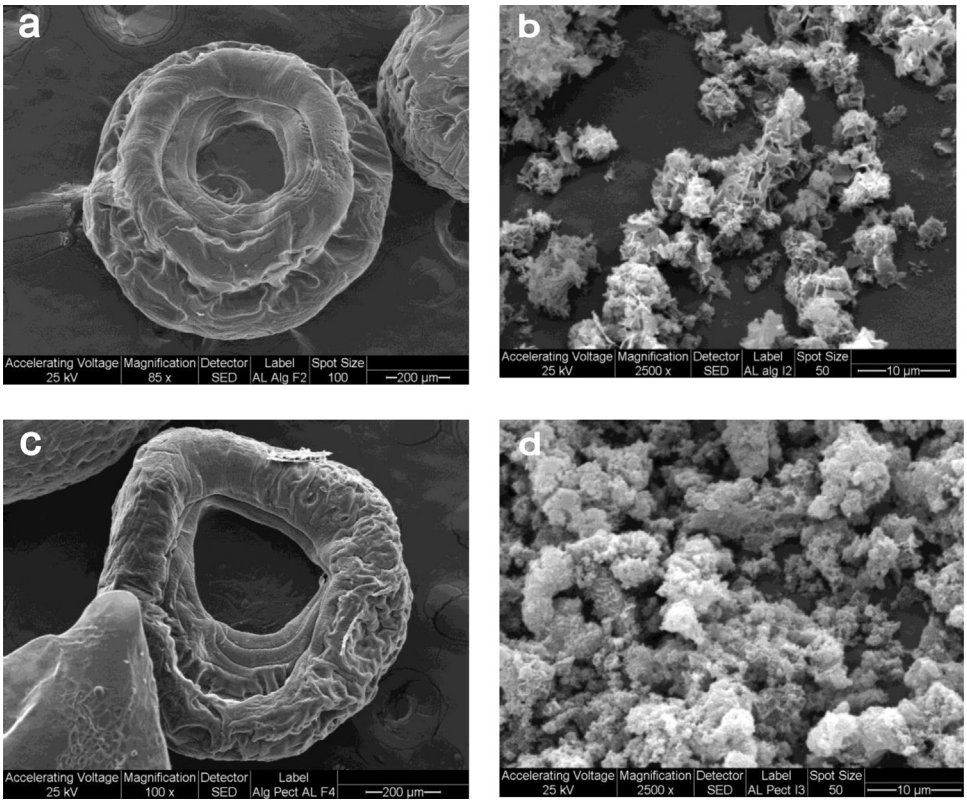

Fig. 4. Autodegradation of matrices after incubation at neutral pH and different times: alg/AL microspheres at 2 h (a) and 4 h (b); alg-HMP/AL microspheres at 2 hs (c) and 4 hs (d).

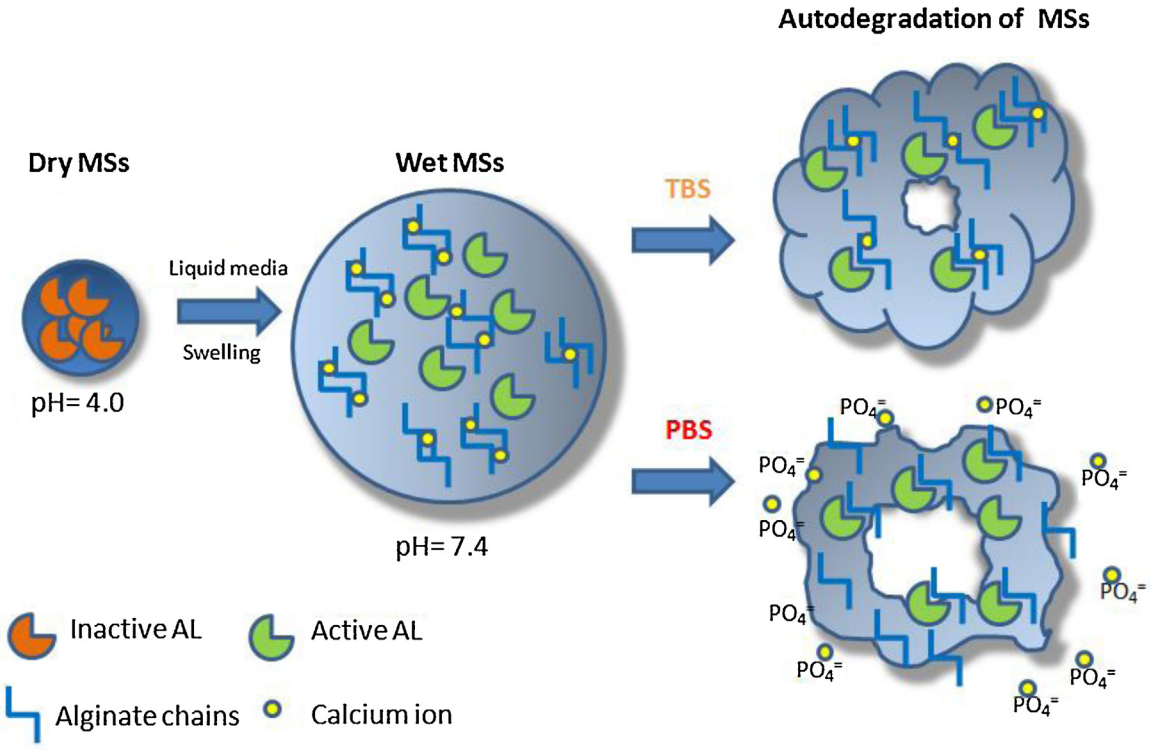

Fig. 5. Scheme of the autodegradation mechanism of microspheres (MSs): first, the AL is encapsulated at pH 4.0 at its inactive form and MPs are freeze dryer; then, at liquid media MSs begin to swell and at pH 7.4 the AL is converted to its active form: in TBS the AL activity is slow because is working on alginate chains mostly crosslinked with calcium; in PBS the AL activity against MSs is higher because phosphate ions are taking the calcium from the network, releasing more free alginate molecules that are more accessible for AL activity.

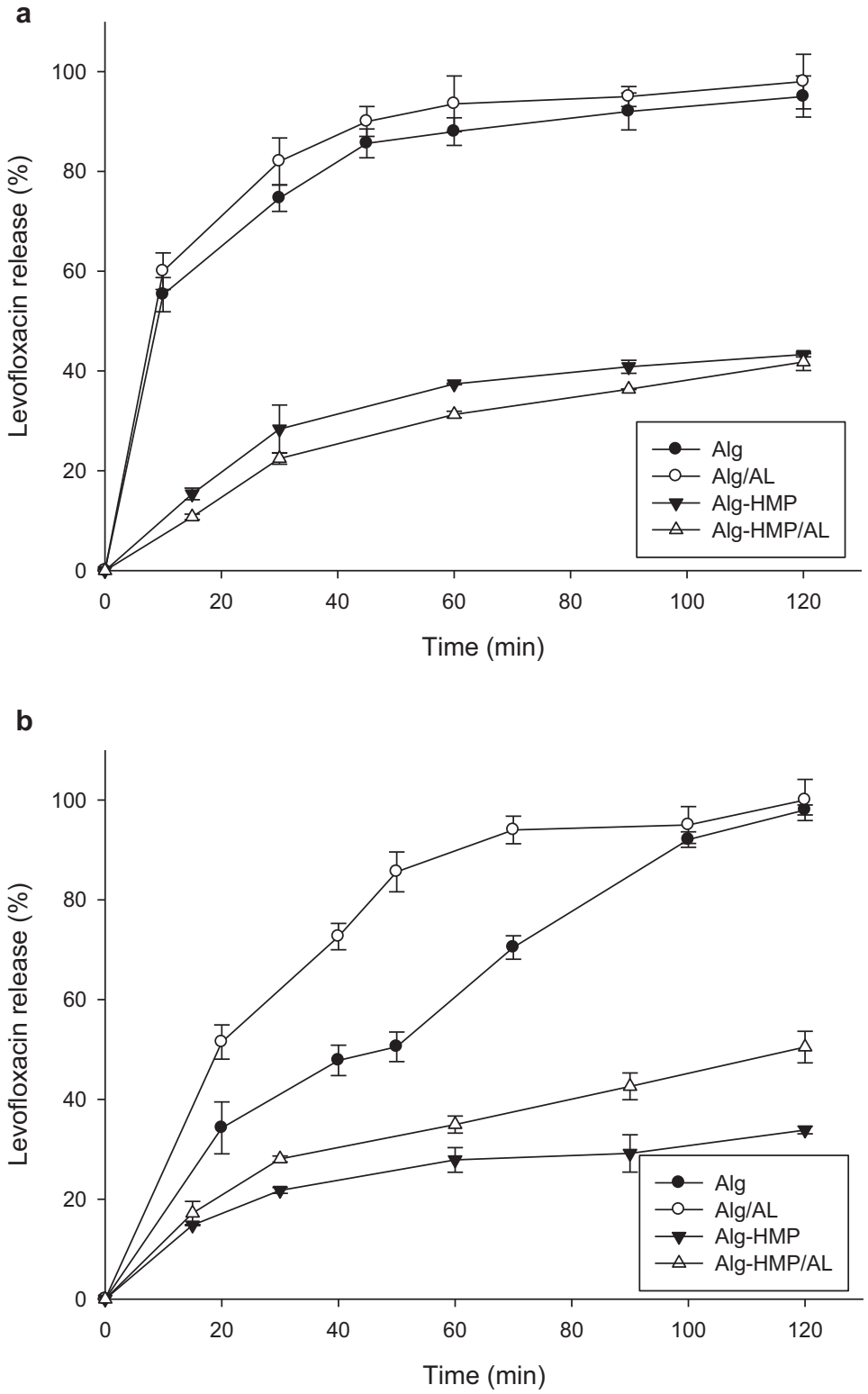

Fig. 6. Release profile of levofloxacin from microspheres at pH 1.20 (a) and pH 7.4 (b). Symbols: ●, alg; ○, alg/AL; ▼, alg-HMP and △, alg-HMP/AL composites. Values shown are the means + SD from triplicate samples ($n = 2$).

Moreover, the presence of HMP in the formulation is required to prevent the fast enzyme inactivation by acid pH (Fig. S1a). The hydrophobic motifs from the HMP structure would provide protection for the enzyme (AL activity) reaching the intestine almost "alive" (active) (Islan et al., 2013). According to this observation, the alg-HMP/AL composite was able to retain the 26% of the initial AL activity at pH 1.2 and 37 °C after 2 h incubation. On the other side, free AL and the alg/AL microspheres completely lost the enzymatic activity under the same experimental conditions.

On the other hand, almost 100% of the Levo was release from alg and alg/AL formulations under intestinal conditions (pH 7.4) in two hours, meanwhile the antibiotic release was reduced to 34% and

51% from the alg-HMP and alg-HMP/AL microspheres respectively at the same incubation time (Fig. 6b). Later, these microspheres will be fully disintegrated at intestine producing the total release of Levo in about 4 h (Fig. 3), in agreement with the human small bowel transit average time of 4.0 ± 1.4 h previously reported (Read et al., 1986; Baluom et al., 1998).

It was possible to observe that in the case of alg-HMP/AL microspheres the Levo kinetic release at initial time-points in pH 1.2 and 7.4 were 30% and 35% in 60 min, respectively, showing minimal statistically differences, because of AL irreversible inactivation at acid pH. Besides, the Levo release from the microspheres were 42% at pH 1.2 and 51% at pH 7.4 after two hours incubation and displaying a 9% difference, which confirmed the AL activation at further times at neutral pH's.

Finally, the ability of alg-HMP/AL microspheres to be auto-degraded at intestinal conditions and release active enzyme is an attractive feature to treat intestinal infections. Particularly in the case of *P. aeruginosa*infections, because the AL will be breaking down the bacterial exogenous alginate matrix that composed the biofilms, making more accessible the antibiotic for acting over the bacteria (Boyd and Chakrabarty, 1994).

Based on the present results, the alg-HMP/AL microspheres will be considered for further studies along the work.

3.4. The antimicrobial activity of microspheres

To assess the antibacterial activity of the encapsulated Levo, alg-HMP and alg-HMP/AL microspheres were put in contact with agar plates containing *P. aeruginosa* growing on surface (Fig. 6). Additionally, the agar plate was covered with a thin layer of alginate 1.0 wt%, to simulate a diffusional barrier for the antibiotic. The results were of great interest and remarked the relevance of the AL into the formulation: they showed how alg-HMP microspheres were capable to produce a bacterial inhibitory halo (14.8 ± 0.1 mm) but a considerable enhanced inhibition (20.0 ± 2.0 mm) was observed with alg-HMP/AL. This result indicates that the enzyme is improving in a 35% the antimicrobial activity of Levo, not only favoring the release from microspheres, but also degrading the external bacterial alginate film and enhancing its diffusion across that barrier.

3.5. Cytotoxicity studies of microspheres

In vitro cytotoxicity of the alg-HMP matrix and the effect of the presence of free and encapsulated Levo were studied in cell cultures. The presence of the AL, encapsulated alone in the matrix as well as together with Levo, was also evaluated (Fig. 7). Free Levo concentrations in the range of therapeutic serum concentrations, 50 and 200 μg/ml, were selected for the tests (Swoboda et al., 2003). Therefore, the amount of Levo containing matrix added to the cell culture medium was adjusted to reach those Levo concentrations and considering the antibiotic release after processing. Also, gel matrixes containing AL without the antibiotic were tested (corresponding to 4.5 and 18.2 mg of matrix/ml). Control in presence of phosphate buffer was also carried out in order to detect any other effect on cell culture viability. Viability controls (untreated cells) and cells treated with the two concentrations of dissolved matrix were performed by the MTT assay (Fig. 7a). No significant differences in cell viability were observed between the control cells and the cells exposed to both matrixes. Similar results were obtained when controls were compared with phosphate and alg/HMP-AL, indicating no deleterious effects of the different matrix components on cell viability. In this regards and considering the potential cytotoxicity of free Levo, as well the combinations of it with the matrixes and the enzyme, the differences were clearly observed. Levo treatment decreased CHO-K1 cell viability in a concentration-dependent manner ($P < 0.01$) as displayed in Fig. 7b. In comparison to the control group, the cell viability at 50 and 200 μg/ml decreased to 87% and 65%, respectively. Considering the molecular characteristics of fluoroquinolones and the intrinsic toxicity of Levo, these results are in agreement with previous literature (Bai et al., 2014; Liu, 2010) and sustain the encapsulation as an alternative to reduce cell toxicity an enhance antibiotic efficacy (Islan et al., 2012, 2013). Interestingly, encapsulated Levo showed reduced effects on cell viability, even at 200 μg/ml. In this sense, no significant differences were observed compared to the control cells at 50 μg/ml ($P > 0.05$), being slightly cytotoxic at 200 μg/ml, where a decrease of 19% was detected. However, not a major difference was observed when comparing free and encapsulated Levo at 50 μg/ml ($P \geq 0.05$), but a significant difference was found at the antibiotic concentration of 200 μg/ml ($P < 0.05$), suggesting a positive tendency to diminish Levo toxicity by the encapsulation procedure. It could be estimated that the presence of dissolved biopolymers would also decrease the toxicity of the drug in mammalian cells. Finally, the co-encapsulation of Levo and AL did not show any significant variation when is compared with encapsulated Levo (Fig. 7b). These results support the hypothesis that AL would not interfere with Levo activity on cell viability, but it is improving the Levo release. In this sense, under the tested conditions, we could conclude that the alg-HMP matrix presents no toxicity in the tested mammalian cells, and the encapsulation of Levo in the alg-HMP gel matrix would reduce the toxic effects associated with the administration of the free antibiotic. The co-encapsulation with the AL enzyme could

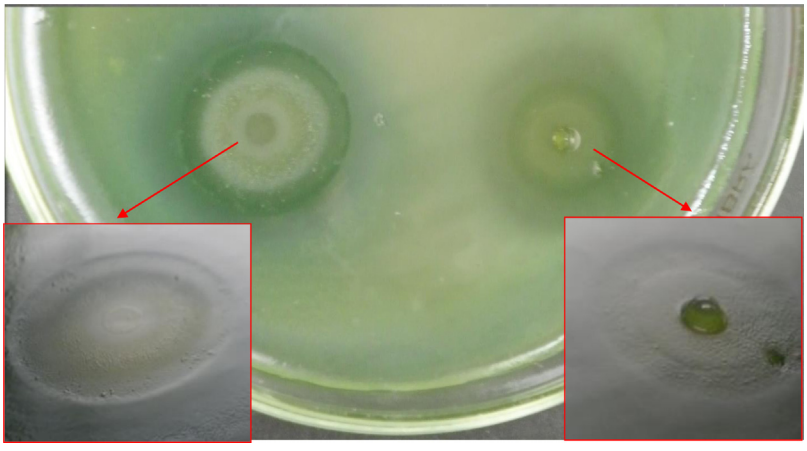

Fig. 7. Antimicrobial activity of microspheres against *Pseudomonas aeruginosa*immersed into an artificial alginate biofilm: left, alg-HMP/AL and right, alg-HMP microspheres.

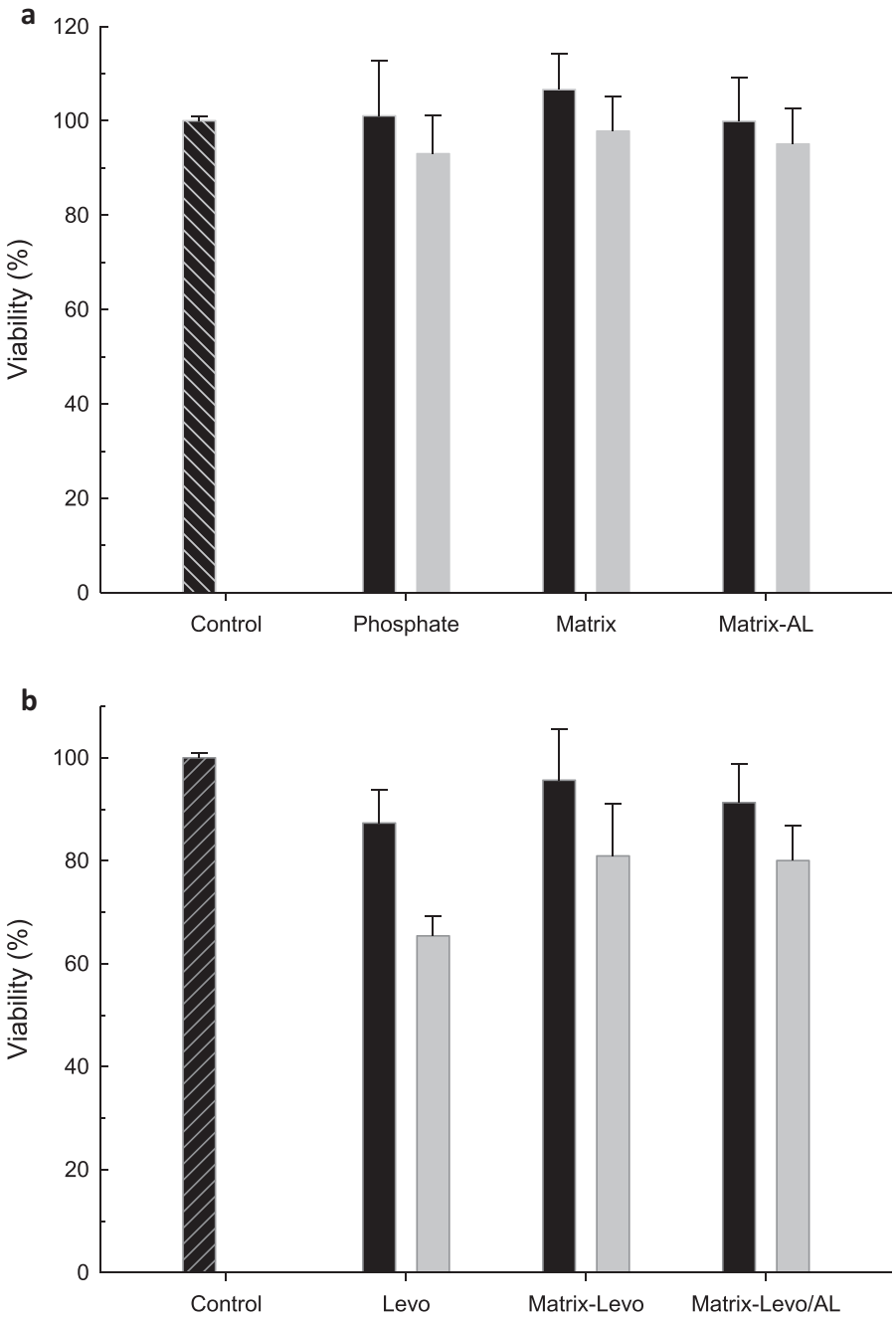

Fig. 8. Cytotoxicity assay by MTT method tested in CHO-K1 cells of: (a) phosphate ions, the matrix of alg-HMP and the AL containing matrix. Two concentrations were evaluated: 4.5 mg/ml (black) and 18.2 mg/ml (grey); (b) free Levo, Levo encapsulated into the matrix and the Levo/AL containing matrix. Two Levo concentrations were tested: 50 μg/ml (black) and 200 μg/ml (grey). No treated cells (control) were indicated as a striped column.

also improve Levo efficacy without the common associated undesirable side effects (Fig. 8).

4. Conclusions

In the present study, the ability of alginate lyase to be immobilized and encapsulated in its inactive form into an alginate based matrix was demonstrated. Furthermore, the matrix shows auto-degradable properties when microspheres reach optimum environmental conditions for AL activity. The incorporation of high methoxylated pectin into the formulation gives interesting properties in swelling capability and morphology of microspheres, but does not affect the AL activity. However, the pectin can be utilized to improve and/or modulate the encapsulation and controlled release of a fluoroquinolone antibiotic (levofloxacin). Moreover, the presence of AL can control and increase the antibiotic release from the matrix, due to the extensive depolymerization of alginate chains. This fact is reflected in a mass loss and the appearance of holes on microspheres surface observed by OM and SEM.

The antimicrobial activity of microspheres against *P. aeruginosa* is enhanced when the alg matrix contains AL, because of AL is increasing the antibiotic diffusion through the biofilm. Surprisingly, no residues of gel matrix are detectable at naked eye after overnight incubation, indicating that the microspheres are totally autodegradable and allow to release the loaded drug.

Widening the application of the system in pharmaceutical and biomedical areas, the microspheres did not show considerable cytotoxic effects on mammalians cells and the presence of biopolymers could play a key role in reducing the toxicity of the Levo.

The advantage toward the existing materials in the market is that the present development of the alginate gel matrix can modulate the release of biological active molecules through the autodegradative microspheres under physiological conditions and without any toxic residue. Particularly, the matrix can be an excellent candidate for cystic fibrosis disease therapy, not only for actively release an antibiotic at intestinal conditions, but also for the capability to be completely degraded and avoid a possible intestinal obstruction. Furthermore, the presence of AL will help the antibiotic diffusion across the biofilm barrier made by *Pseudomonas*spp. Also, based on the characteristic of the hydrogel that can be extensively tailored by the HMP and the AL content, the morphology is not only restricted for production of micro- and nano-particles, but also novel films or 3D structures with smart autodegradative properties could be developed.

Considering these facts, the matrix shows very interesting properties that can be tailored according to the needs of specific biomedical application and/or personalized medicine.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2015.10.050.

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