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ORIGINAL PAPER

Tailoring of alginate–gelatin microspheres properties for oral Ciprofloxacin-controlled release against *Pseudomonas aeruginosa*

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Abstract

Context: Ciprofloxacin (Cip) is a broad spectrum antibiotic frequently used in the treatment of infectious diseases caused by *Pseudomonas aeruginosa*. Cip oral administration is commonly associated with poor drug biodisponibility, gastrointestinal tract irritation, and toxic undesirable side effects.
Objective: The aim of this work is to provide an oral biopolymeric system for controlled release of Cip.
Materials and methods: Alginate–gelatin blend microspheres were crosslinked in the presence of 1,2-propylene glycol, calcium, and glutaraldehyde. Studies of Cip encapsulation and release were performed. Matrix characteristics were studied simultaneously by optical microscopy and Fourier transform infrared spectroscopy (FTIR) using synchrotron light, and by texturometric analysis. Microsphere surface topologies were observed by scanning electron microscopy (SEM), atomic force microscopy (AFM), and epifluorescence microscopy.
Results: Microspheres crosslinked with glutaraldehyde showed about 80% Cip encapsulation and less than 10% Cip release under simulated gastric conditions in 15 min, while a controlled release profile was observed at intestinal environment conditions. Antimicrobial activity against *P. aeruginosa* showed an increasing bacterial growth inhibition in time. Finally, bovine serum albumin (BSA) was used as model protein for binding of macromolecules onto active surface of microspheres, with a consequently modulation of Cip release.
Discussion and conclusions: The results are indicating that alginate/gelatin matrix crosslinked via Ca^{2+} and glutaraldehyde can be tailored by decorating the microsphere surface with biological active molecules useful for targeting, making a potential tool to improve Cip oral administration for infection diseases.

Keywords

AFM, biopolymers, lends, pectin, gelatin

History

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Introduction

Ciprofloxacin (Cip) is a fluoroquinolone with broad antimicrobial spectrum, clinically indicated for the treatment of bacterial diseases such as acute uncomplicated cystitis in females, chronic bacterial prostatitis, urinary and lower respiratory tract infections, acute sinusitis and skin-related infections, bone and joint infections, infectious diarrhea, typhoid fever and uncomplicated cervical and urethra gonorrhea (Appelbaum & Hunter, 2000). Also, it is commonly administered in cystic fibrosis as a control agent against opportunistic *Pseudomonas aeruginosa* (Wood & Ramsey, 1996). The Cip antimicrobial activity is meanly based on the inhibition of DNA gyrase, enzyme necessary to separate bacterial DNA and in consequence inhibiting cell division. However, orally administered Cip is commonly associated to gastric and intestinal problems (Norrby, 1991). Besides, Cip molecules have tendency to stack themselves which is associated to a reduction of biodisponibility and low aqueous solubility under physiological condition (Turel, 2002; Fernandez-Teruel et al., 2013). Additionally, the harsh environmental stomach condition and the antibiotic residence time are points to be considered for Cip inactivation. All these reasons are converging for the development of an effective encapsulation system for Cip oral delivery.

Alginates are linear anionic polysaccharides composed of β-manuronic acid (M units) and α-guluronic acid (G units) linked by 1–4 bounds. Alginates are non-toxic, no immunogenic, biocompatible, and their gelation proceeds by crosslinking with divalent ions forming ''egg box junctions''. Calcium is the preferred biocompatible crosslinker cation for biomedical applications of alginate gels (Rehm, 2009). The alginate crosslinking is made by ionotropic interaction between calcium ions and the free carboxyl groups of guluronic acid residues ($pK_a \approx 3.0$) of two neighboring

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alginate chains, resulting in the formation of stable three-dimensional network (Velings & Mestdagh, 1995).

Gelatin is a natural polymer obtained by a controlled hydrolysis of the fibrous insoluble collagen, protein widely found in the nature. Collagen is the major constituent of skin, bones, and connective tissue. Its biodegradability and biocompatibility properties makes gelatin appropriate in medical industry as plasma expander, wound dressing, adhesive, absorbent pad, and for drug delivery (Kuijpers et al., 2000). Gelatin is the denatured form of collagen with aspartic (0.50 mmol/g, $pK_a = 4.0–4.5$) and glutamic (0.78 mmol/g, $pK_a = 4.5$) as major anionic groups and lysine (0.30 mmol/g, $pK_a = 10.0–10.4$) and arginine (0.53 mmol/g, $pK_a > 12$) as the major cationic side groups. Gelatin type B has an isoelectric point of 4.9, and its pK_a values are $pK_{a1} = 3.6$ and $pK_{a2} = 7.8$. At pH below 5.0, it is positively charged and can make coacervates with negatively charged molecules (e.g. DNA) (Rose, 1990).

Some previous studies have focused on the use of alginate and gelatin coacervates, due the blend advantageous properties for the development of drug release matrices (Klak et al., 2013). Alginate–gelatin films encapsulating Cip were developed for local treatment of infections and for wound dressing (Dong et al., 2006; Saarai et al., 2013). Also, alginate–gelatin tablets were used for controlled release of diltiazem hydrochloride and endosulfan (Tapia et al., 2007; Roy et al., 2009). However, uncoated gelatin capsules containing proteins as cargo disintegrated in the stomach within 15 min of ingestion in human volunteers. Instead, gelatin capsules coated with 20% alginate cross-linked with calcium chloride, remained intact in the stomach and then migrated to the ileocecal region of the intestine and then disintegrated (Narayani & Rao, 1996).

Previous works on drug formulations were focused on chemical crosslinking of either alginate or gelatin with calcium ions for alginates, or formaldehyde, glutaraldehyde, epoxy compounds, and carbodiimides for gelatin. Among these agents, glutaraldehyde is widely used crosslinking agent that reacts with gelatin free non-protonated ε-amino groups (–NH2) of lysine or hydroxylysine through a nucleophilic addition-type reaction (Chan & Heng, 2002).

The aim of the present work was to develop and study an alginate–gelatin matrix for Cip encapsulation in the presence of 1,2-propylene glycol and by double crosslinking via calcium ions for alginate and glutaraldehyde for gelatin. Also, the interaction of macromolecules, using BSA as model, onto matrix active surface was analyzed. Matrix characterization was performed by IR synchrotron beamline, texturometric parameters, scanning electron (SEM), atomic force (AFM), and epifluorescence microscopies. Cip release from the matrices was analyzed by kinetic studies of antimicrobial activity against *P. aeruginosa*.

Materials and methods

Cip (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid) and BSA were purchased from Sigma-Aldrich (Buenos Aires, Argentina). Sodium Alginate was donated by Monsanto (Buenos Aires, Argentina). Gelatin type B ($M_W = 125$, food grade) was kindly provided by Kraft (Buenos Aires, Argentina). 1,2-Propylene glycol ($\delta = 1.036\,g/cm^3$) was purchased from Anedra (Buenos Aires, Argentina). BSA Alexa fluor® 488 conjugate (BSA*) was purchased from Molecular Probes (Invitrogen, Eugene, OR). *P. aeruginosa* ATCC 15442 was used in the experiments. Other reagents and solvents were of analytical grade, and used as received.

Determination of Cip and Biopolymers Interactions

Interaction of Cip with alginate and alginate/gelatin solution blends was studied at different pHs. Alginate and alginate/gelatin (2:1) solutions were prepared by dissolving the weighted powders in buffers at pH 4.0–5.0 (50 mM acetate buffer), 6.3, 6.8, 7.4 (25 mM phosphate buffer), and 9.0 (25 mM borate buffer) previously prepared in distilled water. Four hundred ten microliters of the polymer blends were mixed with 90 µl of Cip solution (100 µg/ml) and stirred for one hour at room temperature. Later, the biopolymer complex was precipitated by the addition of 1.0 ml of cold ethanol (96.0%) (Islan et al., 2012). The resulting suspension was centrifuged at 10 000 ×g for 10 min. The remaining Cip in the supernatant was determined spectrophotometrically at 280 nm (Beckman DU640, Euclid, OH) using appropriate calibration curve.

Crosslinking of alginate/gelatin microspheres containing Cip

A Cip solution (36.0 µg/ml) was mixed with alginate–gelatin (2.0–1.0%, w/v) and pH adjusted to 4.0 (50 mM acetate buffer). Microspheres were prepared by dropping the Cip–biopolymer solution into 500 mM $CaCl_2$ dissolved in 1,2-propylene glycol–water (1:1 v/v), which was demonstrated to increase the Cip incorporation on biopolymeric matrices (Islan et al., 2013). The solution was gently stirred in an ice cooled bath for 20 min. Later, the microspheres were filtrated and washed twice with sodium bisulfite aqueous solution, followed by air dried for 10 min and stored at 5 °C until use. In the case of alginate/gelatin microspheres crosslinked by glutaraldehyde, the reagent was added to the calcium solution at 2.5% (w/v) final concentration, in order to produce gelatin crosslinking.

After synthesis, microsphere size distributions of wet and dried samples were determined by optical microscopy. The average diameter was determined in 100 microspheres of each sample randomly selected.

Measurement of Cip encapsulation in alginate–gelatin microspheres

Cip was determined in solution before and after biopolymer gelation followed by filtration. The difference between total and residual Cip was considered as the encapsulated antibiotic.

Synchrotron light analysis

Freeze-dried biopolymer samples were cut and put into the analysis chamber of SMIS infrared beamline (Soleil, French National Synchrotron Facility, Saint-Aubin, France). Analyses of samples were simultaneously performed by optical microscopy and Fourier transform infrared spectroscopy (FTIR).

SEM

Sample analysis by SEM was carried out using freeze-dried microspheres. The samples were prepared by sputtering the surface with gold using a Balzers SCD 030 metalizer (Blazers Co., Saskatchewan, Canada) obtaining a layer thickness of between 15 and 20 nm. Microsphere surfaces and morphologies were observed using Philips SEM 505 model (Rochester, NY), and processed by an image digitalizer program (Soft Imaging System ADDA II (SIS), Lakewood, CO).

SEM images were analyzed by Image J software (NIH, Madison, WI). The roughness of the surface was reflected by the standard deviation of the gray values of all the pixels on the image. First, image files were opened by the software and converted to an 8-bit image. Then all the pixels on the image were selected and statistically measured by a computer equipped with the software. The surface smoothness is proportional to the inverse of the sample standard deviation.

AFM

All images were obtained in ambient conditions using a Multimode-Nanoscope V (Veeco, Santa Barbara, CA) operating in tapping mode with an etched silicon Probe model Arrow-NCR-50 Nano World (cantilever resonance frequency: 258 kHz, force constant 42 N/m; tip radius 5–10 nm). Typical scan rates were in the range of 1.0–1.5 Hz. AFM samples were prepared by using a piece of glass (1 cm × 1 cm) immersed into alginate–gelatin (2:1) solution. Later, the glass was wringed and immersed into a solution containing 500 mM $CaCl_2$ in 1,2-propylene glycol–water (1:1) without (a) or with (b) glutaraldehyde (2.5%, w/v). The glasses were freeze dried and later observed by AFM. Images were analyzed by Nanoscope 7.30 software (Veeco, Santa Barbara, CA).

Measurement of texturometric parameters

Microspheres texture was analyzed in a TAXT 2i Texture Analyzer (Stable Micro Systems Ltd, Godalming, 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 in two compression cycles at 0.6 mms^{-1} to 30% of the original microspheres height. In each assay, three microspheres were simultaneously compressed. Data were processed with the Texture Expert® (Texture Expert, Hamilton, MA) software and the textural parameters (hardness and cohesiveness) were calculated from the TPA curve of force (N) versus time (s) with the following definitions (Bourne & Comstock, 1981): 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 (A_2/A_1). The results represent the average means of at least triplicate tests.

Comparison between alginate/gelatin microspheres treated and untreated with glutaraldehyde was performed. Stability of both matrices was determined by incubation of 20 microspheres at pH 1.2 (50 mM Clark and Lubs buffer) and pH 7.4 (40 mM phosphate buffer) at 37 °C for two hours. Microspheres were filtered and washed, later the hardness and cohesiveness were measured as previously described.

Kinetic release of Cip from alginate microspheres

Wet microspheres (200 mg) were suspended in aqueous buffers on rotary shaker at 10 pm and 37 °C. Cip kinetic release *in vitro* were performed under gastric conditions in 50 mM KCl/HCl buffer (pH = 1.2) and at simulate intestinal environment in 40 mM phosphate buffer solution (pH = 7.4). At defined time intervals, 1.0 ml samples were taken off and the absorbance was measured at the maximum wavelength of Cip in each buffer (277 and 270 nm at pH = 1.2 and 7.4, respectively). Fresh media (1.0 ml) were added to keep a constant volume.

Antimicrobial activity of Cip formulation against *P. aeruginosa*

The disc diffusion method (DDM) was modified by using microspheres containing Cip. Alginate–gelatin microspheres with and without glutaraldehyde crosslinking procedure were tested against *P. aeruginosa* using modified DDM according to the National Committee for Clinical Laboratory Standards (NCCLS, Wayne, PA) Briefly, 4–5 colonies of the microorganism were taken and resuspended in sterile physiological solution (154 mM NaCl) and the turbidity of the culture was adjusted to 0.5 McFarland scale (McFarland, 1907). Petri dishes containing 25 ml of Mueller–Hinton agar were inoculated using sterile cotton swab dipped in the inoculum suspension. Later, microspheres were placed on the surface of the inoculated agar plates and incubated at 37 °C. Cip release kinetic was followed by removing one microsphere at each time (20, 40, 60, 90, 120, and 180 min). Finally, plates were left in incubation for 24 h. Then, inhibition zones were determined and measured to the nearest whole millimeter from the back of the inverted Petri dishes using sliding calipers. The assays were performed in duplicate.

Coating of microspheres with BSA

BSA as a model protein was used to test the binding onto surface of alginate/gelatin microspheres previously activated by the glutaraldehyde treatment. Briefly, microspheres synthesized in the presence of 1,2-propylene glycol and glutaraldehyde were filtered, washed, and placed in contact with a cold solution of BSA (10.0 mg/ml) at pH = 4.0 for 10 min. Later, the microspheres were washed and stored at 5 °C. Effect on surface was analyzed by SEM images and also, the effect on Cip release was evaluated.

To confirm BSA binding to microspheres and discard possible changes on surface by degradation, the previous protocol was repeated by using BSA Alexa fluor 488 conjugate. Glutaraldehyde-treated and -untreated microspheres were observed at Leica DM 2500 (Leica Microsystems, Buffalo Grove, IL) epifluorescence microscope at visible and fluorescence mode with a blue excitation filter (450–490 nm).

Finally, BSA quantification was performed to determine the protein/microsphere mass ratios. Briefly, 50 mg of microspheres were incubated in the presence of 1.0–10.0 mg/ml cold BSA solutions at pH = 4.0 for 10 min. After incubation, microspheres were filtrated. The remnant BSA in the filtered solution was assayed by UV absorption at

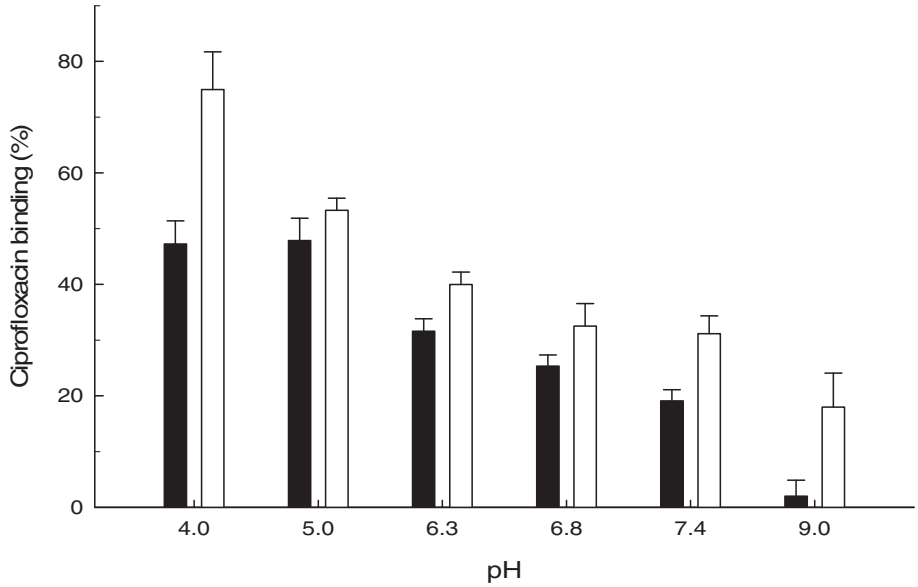

Figure 1. Binding of Ciprofloxacin to 2.0% alginate (■) and alginate–gelatin 2:1 blend (□) at different pHs.

280 nm with proper calibration curve. Difference between the initial and the remainder BSA in the filtered was considered as the amount of adsorbed BSA on surface microspheres.

Considering the absorbance measured and correlating it with a BSA calibration curve, concentration was determined and compare to the initial BSA concentration, in order to calculate the amount of bound BSA to microsphere.

Statistical analysis

All experiments were carried out with at least by two independent experiments run each one by duplicate. 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 a $p < 0.05$.

Results

Cip interactions with alginate and alginate–gelatin coacervate

Interaction between Cip and alginate strongly depends on pH (Figure 1). The binding of Cip with alginate increases when the pH decreases. On one hand, the electrostatic interactions of negative charged alginate with the cationic Cip reached a maximum in the range of pH 4.0–5.0. Below pH 3.5, the alginate solution becomes unstable and precipitates as alginic acid (data not shown). On the other hand, the interaction of Cip and alginate decreased because the pK_a of Cip carboxylate is 6.1 and the piperazinyl group at C-7 is 8.2 at pHs close to 6.0 or higher (Islan et al., 2012).

Incorporation of type B gelatin into the alginate solution increased up to 80% the Cip interaction with the polymer blend at pH = 4.0 (Figure 1). The pK_as of alginate are between 3.4 and 3.7 and for Cip is 6.1, both are far away from the pH 4.0–5.0, in consequence the ionization states of these molecules do not changed and consequently the increase of Cip uptake by the blend that can be attributed only to the presence of B-gelatin, but not by its interaction with alginate. The positive interaction of Cip with B-gelatin at pH 4.0 can be justified by the presence of high levels of aspartic and glutamic acid residues on gelatin with close pK_as to 4.0 (Rose, 1990).

The decrease of Cip binding to alginate–gelatin blend with the increase of pH could be explained mainly by the changes of polymer surface density charges of B-gelatin reaching the Iso Electric Point (IEP), between 4.6 and 5.2 approximately as previously reported (Harrington & Morris, 2009). When the pH of B-gelatin is reaching the IEP, the associative interaction between alginate and gelatin becomes weaker, and has a tendency to be repulsive with phase separation (Antonov et al., 1996). The process is considered as segregative interaction between both polymers by increasing the order in the protein going from extended coil to helix, a more ordered form, triple helix in the case of B-gelatin (Gilsenan et al., 2003). In such case, the amount of B-gelatin ionic residues available for Cip binding was drastically reduced. Also, the increase of pH over the IEP generates the zwiterionic form of B-gelatin increasing the strength of the triple helix configuration (Wüstneck et al., 1988). In addition, the positive-charged Cip molecule at acid pH becomes neutral (or zwiterionic) close to pH 7.4, and consequently the ionic interaction with the anionic polymer blend is reduced.

Evaluation of alginate–gelatin homogeneity in the matrix by FTIR synchrotron analysis

Matrices were analyzed simultaneously by optical microscopy and FTIR synchrotron light (SMIS beamline, Soleil Synchrotron, Saint-Aubin, France). Alginate matrix showed two characteristics peaks attributed to the asymmetric and symmetric stretching vibration modes of R-COO^- group, at 1627 cm^{-1} and 1409 cm^{-1}, respectively (Figure 2, left). Also, the results are confirming our previous determinations using bench FTIR spectrometer (data not shown), but taking advantage of the high synchrotron sensitivity to small band changes produced by the micro-environment. These results

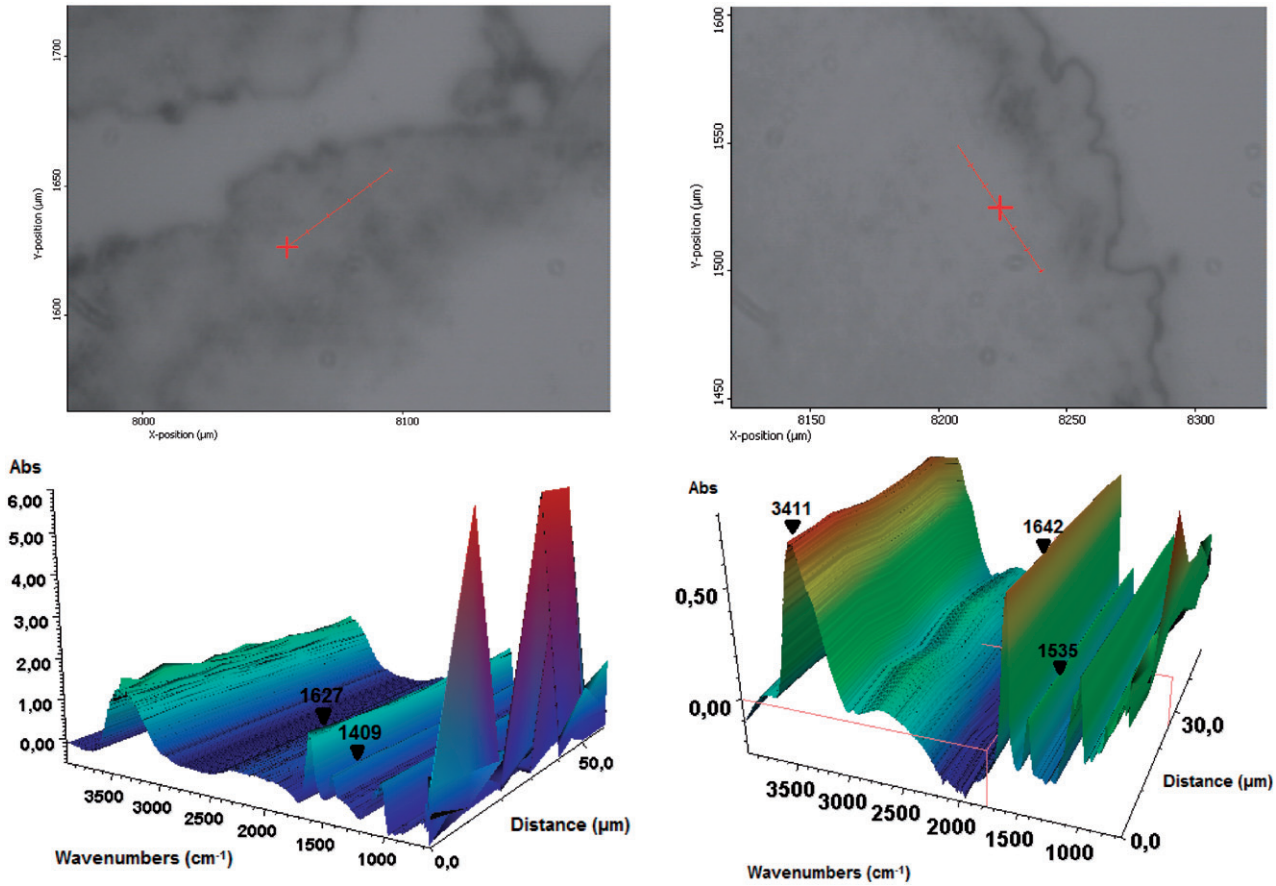

Figure 2. Characterization of alginate and alginate/gelatin matrices by simultaneous optical microscopy and FTIR using SMIS synchrotron light. Left: alginate. Right: alginate/gelatin. Top: optical microscopy of a portion of matrix. Below: 3D-FTIR spectra of red line in the optical microscopy images.

are indicating no effect on alginate gel structure by the presence of 1,2-propylene glycol (50%, v/v) during the ionic gelation process. Similar results of alginate/gelatin matrix gelled in aqueous media using bench FTIR spectrometer were previously reported (Dong et al., 2006).

Characteristic gelatin IR peaks at 1653 cm^{-1}, 1546 cm^{-1}, and 1236 cm^{-1} were attributed to the bands of: amides, produced by the C=O and C–N stretching vibrational modes, and N–H bending modes. The stretching vibration of O–H bonded to N–H produced a wide absorption band around 3421 cm^{-1}. FTIR spectra alginate–gelatin matrix showed the characteristic absorption bands at 1653 cm^{-1} and 1546 cm^{-1} of gelatin shifted to lower wavenumbers to 1642 cm^{-1} and 1535 cm^{-1} respectively (Figure 2, right). Similarly, the absorption band around 3421 cm^{-1} shifted to a lower wavenumber at 3411 cm^{-1}, suggesting an increase in the hydrogen bonding interaction. These results strongly evidence the intermolecular interactions and compatibility between alginate and gelatin described previously (Dong et al., 2006). Besides the high sensitivity of IR synchrotron beamline, the Cip characteristic IR bands were not elucidated because of low antibiotic concentration present in the formulation. However, as Cip molecules tend to stack themselves producing crystals of 5 µm in size clearly observed by optical microscopy (data not shown) and they probably interfere with FTIR detection, there was evidence that no aggregation of Cip molecules was produced during gel formation. The presence of biopolymers may stabilize Cip molecules.

Microsphere size distribution

Alginate/gelatin and alginate/gelatin/glutaraldehyde microsphere sizes were evaluated by optical microscopy. Spherical microspheres around 900 µm with a narrow size distribution were obtained for both types of matrices in wet state. However, after dehydration (air or freeze dried), the size was reduced to 500 µm, but keeping their morphology, narrow size distribution, and hydrogel properties.

SEM images

The external structure of alginate microspheres synthesized in 1,2-propylene glycol–water (1:1) was improved compared to the aqueous ionic gelation synthesis by the lack of surface cracks and big pores, as previously reported (Islan et al., 2013).

Gelatin incorporation to the alginate matrix and crosslinked only in the presence of calcium ion showed a homogeneous distribution of microspheres with spherical shapes (Figure 3, top), suggesting good compatibility between both biopolymers as previously observed by FTIR synchrotron light. However, the surface morphology of the blended microspheres seems to be more granulated compared to alginate-based matrix. Differences were essentially attributed to the presence of small valleys probably composed of non-crosslinked gelatin on microsphere surface. The microsphere crosslinked in the presence of both calcium ion and glutaraldehyde offers an alternative to produce a double crosslinked matrix in order to

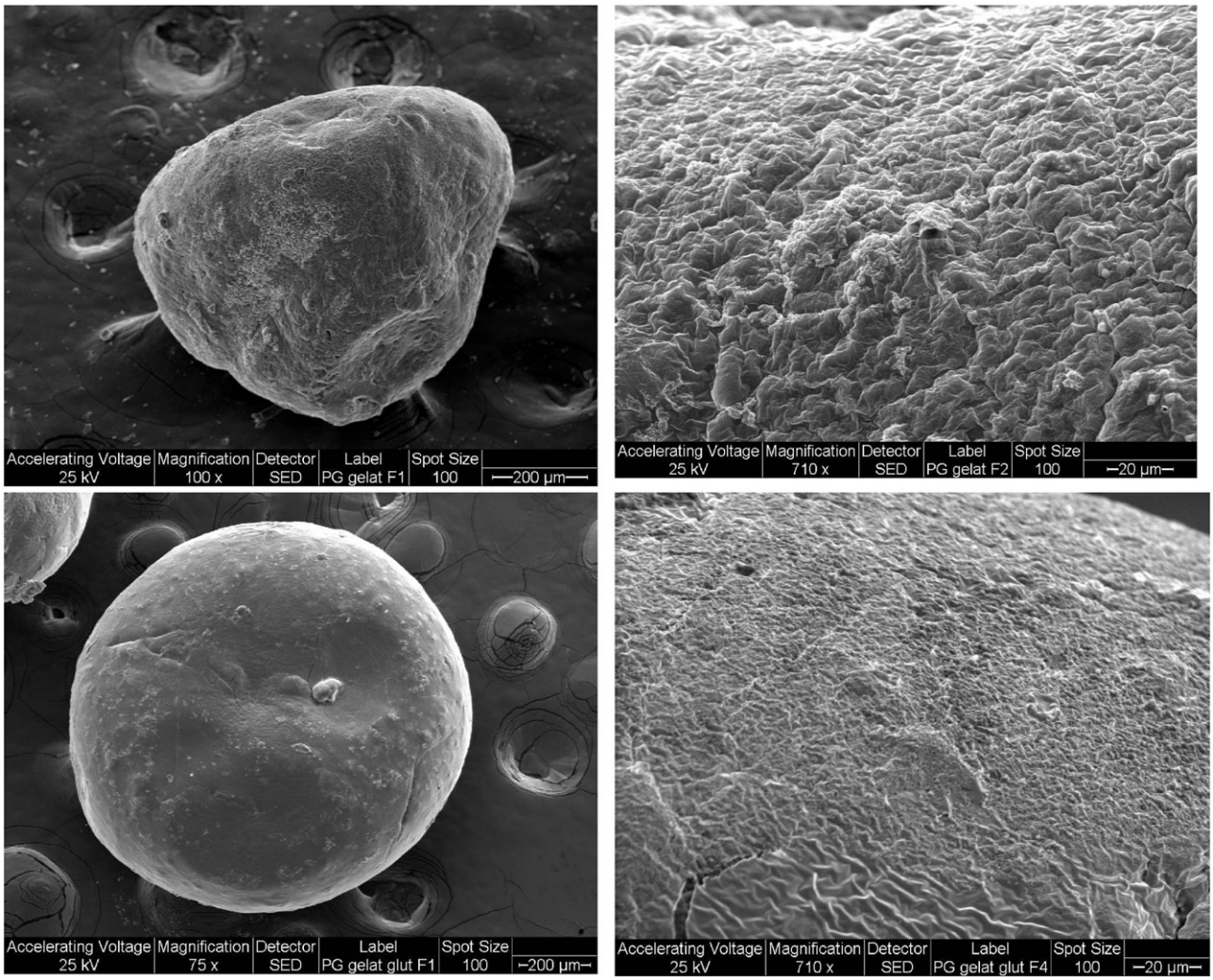

Figure 3. SEM images: top: alginate/gelatin matrix; below: alginate/gelatin matrix crosslinked in the presence of glutaraldehyde.

get more tight surface structure. Alginate–gelatin microspheres treated with glutaraldehyde showed smoother surface compared to the previous ones (Figure 3, below).

SEM microscopy of microspheres is suggesting a thin layer coating over the surface made by glutaraldehyde. Also, microparticle sphericity was kept when a double crosslinking procedure was performed. Images analyzed by Image J software produce a standard deviation of 52 and 35 for blended and glutaraldehyde blended-treated microspheres, respectively, suggesting that the double crosslinking technique increases the smoothness of microspheres and consequently reducing the surface pores concomitantly with Cip diffusion to outside the microspheres. Changes in microspheres surface in both conditions confirm the results observed under naked eyes.

AFM studies

AFM studies of the biopolimeric blend showed the effect of glutaraldehyde and confirmed the SEM results. Alginate/gelatin microspheres showed homogeneously distributed aggregates on the whole surface, mainly composed of non-crosslinked gelatin chains. Treatment with this crosslinking agent directly affect microsphere surface, by reordering the biopolimeric chain of alginate and gelatin and generating a smother surface (Figure 4). Analysis of AFM glutaraldehyde-treated alginate/gelatin matrix images using Nanoscope 7.30 software evidenced the reduction of surface aggregates (10 nm of height, and less than 500 nm of diameter) and displayed strong differences in the surface profile roughness compared to the untreated matrix.

The relevance of smoothness evaluation is related to the capability of glutaraldehyde to decrease matrix porosity and homogenize microsphere surface, tailoring the matrix properties. This parameter probably has a direct impact on release profile of the encapsulated molecules, as the porosity reduction represents a physical barrier to control the delivery of biological active molecules.

Texturometric assay

Hardness and cohesiveness of microspheres were evaluated. However, no significant hardness differences were found ($p > 0.05$) between alginate/gelatin glutaraldehyde-treated and -untreated microspheres (2.19 ± 0.26 and 2.36 ± 0.35, respectively), indicating that the resistance of the gel matrix to compression is quite similar in both matrices (Table 1). Cohesiveness analysis of samples also gave similar results of

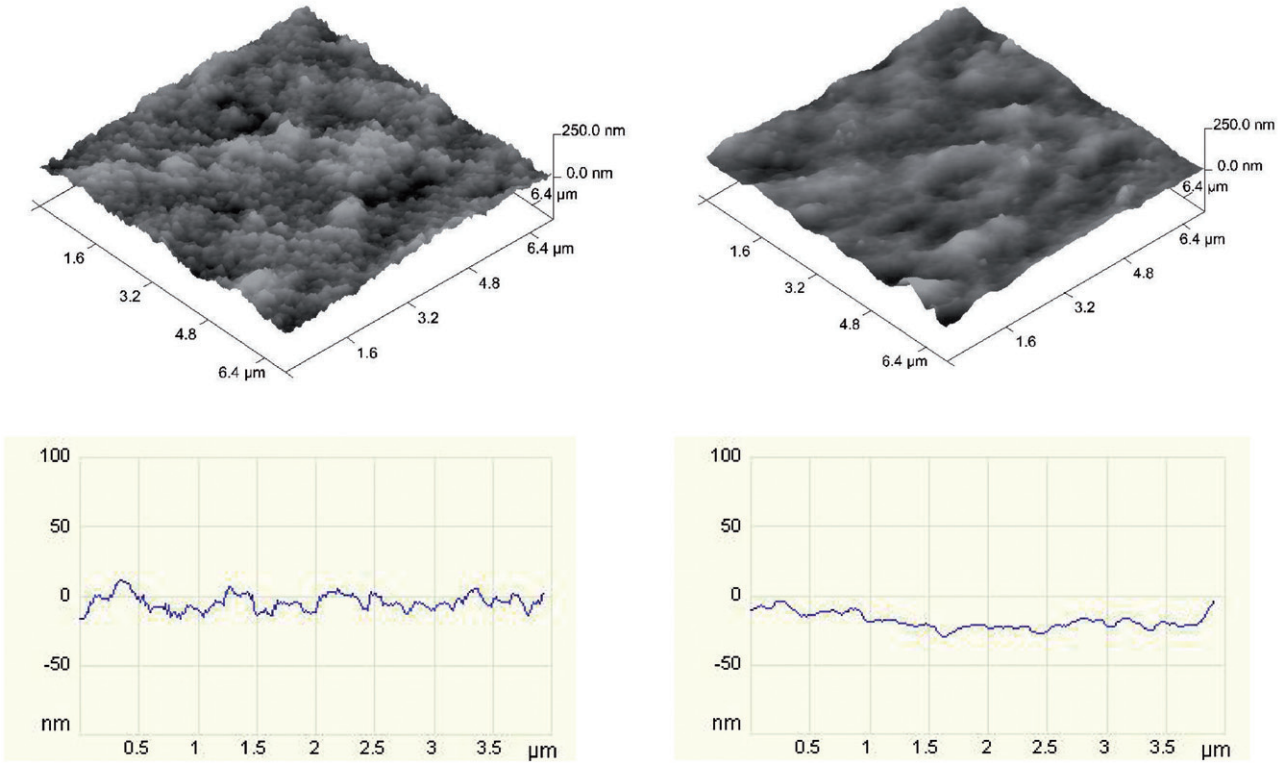

Figure 4. AFM images of alginate/gelatin matrix untreated (left) and treated with glutaraldehyde (right). The corresponding roughness profiles analyzed by Nanoscope 7.30 software are shown.

Table 1. Determination of microspheres stability by texturometric assay without and with glutaraldehyde crosslinking at gastric (1.2) and intestinal (7.4) pHs incubated at 37 °C for two hours.

	Alginate–gelatin		Alginate–gelatin/ glutaraldehyde	
pH	Hardness	Cohesiveness	Hardness	Cohesiveness
Before incubation	2.36 ± 0.35	0.44 ± 0.02	2.19 ± 0.26	0.45 ± 0.02
1.2	1.77 ± 0.14	0.40 ± 0.01	1.81 ± 0.10	0.42 ± 0.02
7.4	ND	ND	0.72 ± 0.05	0.21 ± 0.14

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

0.45 ± 0.02 and 0.44 ± 0.02 for treated and untreated matrices. The cohesiveness results of both matrices are indicating the same degree of difficulty in breaking down the gel's internal structure. Hardness and cohesiveness results are strongly suggesting that glutaraldehyde treatment on the alginate/gelatin matrix mainly produces surface changes without modifying the internal gel structure of the microspheres.

In addition, stability of both matrices was tested in simulated physiological fluids. Alginate/gelatin and glutaraldehyde-crosslinked alginate/gelatin microspheres showed the same texturometric parameters incubated at simulated gastric pH (1.2) for two hours. No significant differences in hardness and cohesiveness ($p > 0.05$) were found, but a decrease of around 25% and 10% respectively was observed in comparison with non-incubated microspheres (Table 1). The fact can be attributed mainly because of the extreme acid pH which probably affects the ionization degree of the crosslinked matrix.

However, significant differences in hardness and cohesiveness ($p < 0.05$) between both types of microspheres incubated at pH 7.4 (simulated intestinal media) for two hours were observed. On one hand, alginate/gelatin microspheres crosslinked only by calcium ion were completely destabilized after incubation at pH 7.4 and the values of hardness and cohesiveness were below the low detection limit of the technique. On the other hand, microspheres double crosslinked with both agents (calcium ion and glutaraldehyde) showed a higher stability to the single crosslink method (Ca^{+2}), preserving 35% of their initial hardness and almost the 50% of the cohesiveness. The results suggest an increase in matrix stability by glutaraldehyde crosslinking, mainly due to a coating surface layer that reduces microsphere erosion.

Cip encapsulation

Cip encapsulation was evaluated in alginate-based microspheres produced by ionic gelation in the presence of calcium ion. The encapsulation percentage was increased around two times when 1,2-propylene glycol was in the synthesis medium (Table 2). However, when 1.0% (w/v) gelatin was incorporated to the alginate (2:1 ratio) a significant decrease of about 20% of Cip encapsulation was observed ($p < 0.05$). The result could be explained considering the isoelectric point of gelatin as follow: at the encapsulation pH of 4.0, the Cip and also the gelatin molecules were positive charged, competing for the negative charged sites of alginate. As gelatin molecule possesses higher M_W than Cip molecule, it has more available binding sites to interact with alginate negative charge chains, excluding Cip from the matrix. However, when surface treatments were carried out in the presence of 50% (v/v)

Table 2. Encapsulation of Ciprofloxacin in different matrices and crosslinking conditions.

		Synthesis media			
Matrix	Biopolymer (w/v, %)	Calcium ion	Glutaraldehyde	1,2-PG[a]	Encapsulation (%)
Alginate–gelatin	2.0	+	−	−	44 ± 3
		+	−	+	96 ± 4
Alginate–gelatin	2.0–1.0	+	−	−	33 ± 5
		+	−	+	77 ± 3
		+	+	+	83 ± 2

[a]1,2-PG, 1,2 propylene glycol.

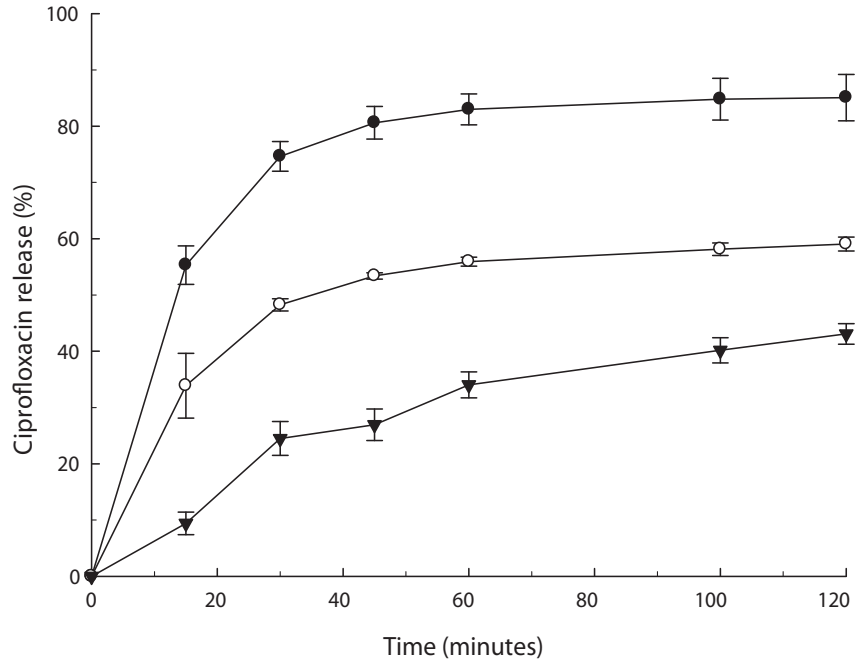

Figure 5. Ciprofloxacin release from gel microspheres composed of 2.0% alginate (●), 2.0% alginate– 1.0% gelatin (○) and 2.0% alginate–1.0% gelatin crosslinked with glutaraldehyde (▼) at pH = 1.2.

1,2-propylene glycol, a rise of at least two times in Cip encapsulation was observed in all cases. Addition of 1,2-propylene glycol in the gelling solution at 0 °C increases the viscosity in about 12 times related to water, which made a drastic reduction of Cip diffusion from the microsphere to the bulk (Curme & Johnston, 1952; Islan et al., 2013). Additionally, the Cip encapsulation increased 6% when blend matrix was double crosslinked with calcium and glutaraldehyde under the same experimental conditions.

Release of Cip

A Cip burst release close to 75% was observed when alginate base microspheres were incubated under simulated stomach conditions at pH = 1.2 and 37 °C for 30 min (Figure 5). A reduction in almost two times of Cip release from the microspheres was found when alginate was blend with 1.0% (w/v) gelatin. The plots had similar biphasic Cip release profiles: fast burst release accounting for 83 and 56% for alginate and alginate–gelatin blend microspheres, respectively, in the first hour. This fact can be attributed to the dissolution of the Cip placed on microsphere surface or those molecules partially embedded just below the surface. The pseudo-plateau phase showing little subsequent Cip release (less than 10%) during the following hours because of the drug more deeply entrapped inside in the matrix. This is a common phenomenon for most matrices in drug delivery systems described as the ''channel effect'' (Huang & Brazel, 2001). Besides, alginate–gelatin microspheres treated with glutaraldehyde released only 25% of the initial drug concentration after 30 min of incubation under the same simulated conditions as mentioned before. Considering that the expected residence time of microspheres into stomach fluids is less than 30 min (for a fasting patient with empty stomach), more than 75% of the Cip will be available at intestine when Cip is dosed encapsulated into microspheres, avoiding potential stomach irritation. The reduction in the cumulative drug release was suggesting the presence of an additional barrier, e.g. a thin layer, generated by glutaraldehyde crosslinking of the biopolymers on the microspheres surface which reduces the porosity of the matrix and consequently the amount of Cip released to the medium. The blended microspheres showed a Cip released profile which best fits fourth grade polynomial equation ($r^2 = 0.99$). Meanwhile glutaraldehyde-treated blend microspheres showed a linear relationship with $r^2 = 0.94$. In both cases, the Cip release could be associated with the diffusional mechanism of the drug from the matrix to the bulk solution.

The Cip release from microspheres under simulated intestinal media (pH = 7.4) was tested to determined if microspheres are capable of release all the encapsulated antibiotic (Figure 6). Alginate-based beads showed a high

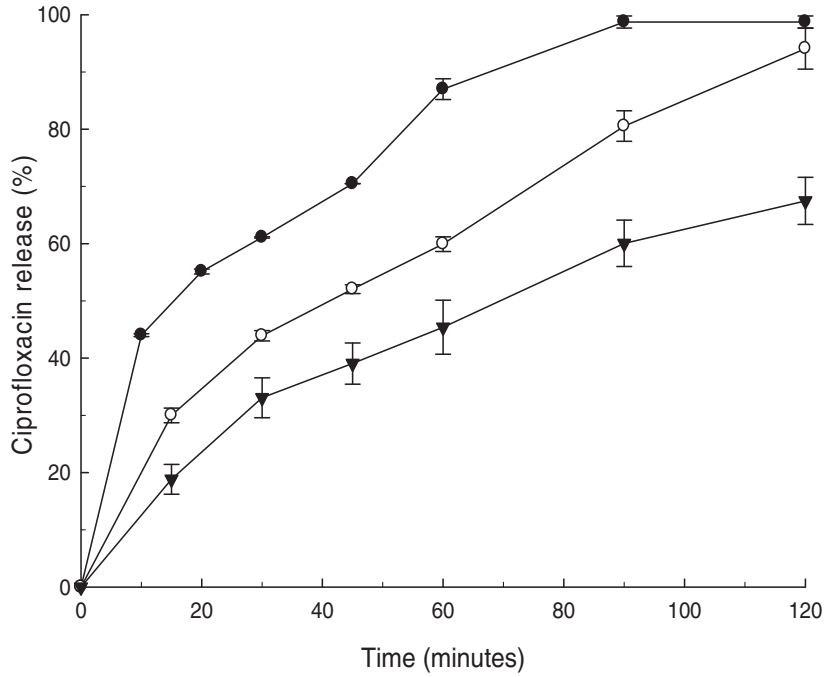

Figure 6. Ciprofloxacin release from gel microspheres composed of 2.0% alginate (●), 2.0% alginate–1.0% gelatin (○) and 2.0% alginate–1.0% gelatin crosslinked with glutaraldehyde (▼) at pH = 7.40.

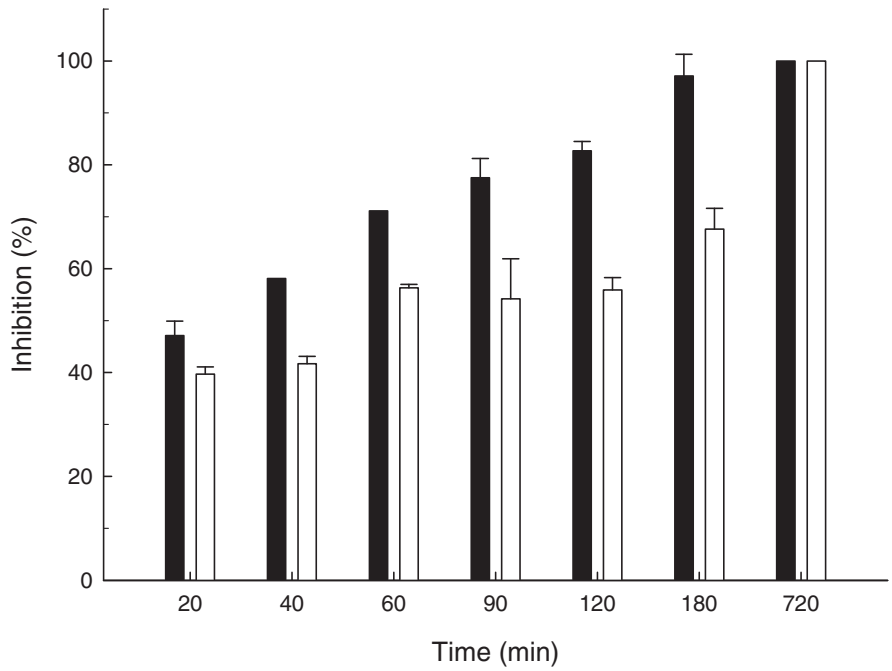

Figure 7. Antimicrobial activity of Ciprofloxacin loaded into microspheres of alginate–gelatin (■) and alginate–gelatin crosslinked with glutaraldheyde (□) against *Pseudomonass aeruginosa.*

release rate, reaching 100% release in 90 min. Alginate–gelatin microspheres decreased the antibiotic release in 20% compared to the alginate microspheres. These results were in agreement with the results obtained for Cip interaction with the matrices previous mentioned (Figure 1). However, the 100% of Cip was released in two hours, mainly produced by completely matrix erosion as it was observed in stability experiments (Table 1) and a non-desirable Cip retention time inside the microspheres was observed. Besides, when the blend microspheres were treated with glutaraldehyde, a decrease in the release profile was found, due to the presence of a thin compacted surface layer that was more resistant to be eroded by the phosphate ions (Ca-quelating compound) and pH. This procedure is providing more stability to the matrix and the encapsulated Cip showed a low release rate to the surrounded medium.

Antimicrobial activity of Cip

Efficacy of Cip released from the microspheres was tested against *P. aeruginosa* (Figure 7). When microspheres were placed in contact with the inoculated agar medium at different times, an increased in the inhibition halo was found. The results are in agreement to the kinetic of Cip release from the blend microspheres performed in liquid medium. Glutaraldehyde-treated Cip containing microspheres showed a microbial inhibition increasing in time, reaching a halo diameter higher than not treated blended microspheres. This effect can be attributed to the higher Cip loading in glutaraldehyde-treated microspheres compared to non-treated microspheres (Table 2). Also, this result is confirming the presence of an additional diffusional barrier made by the glutaraldehyde crosslinking in the microsphere surface.

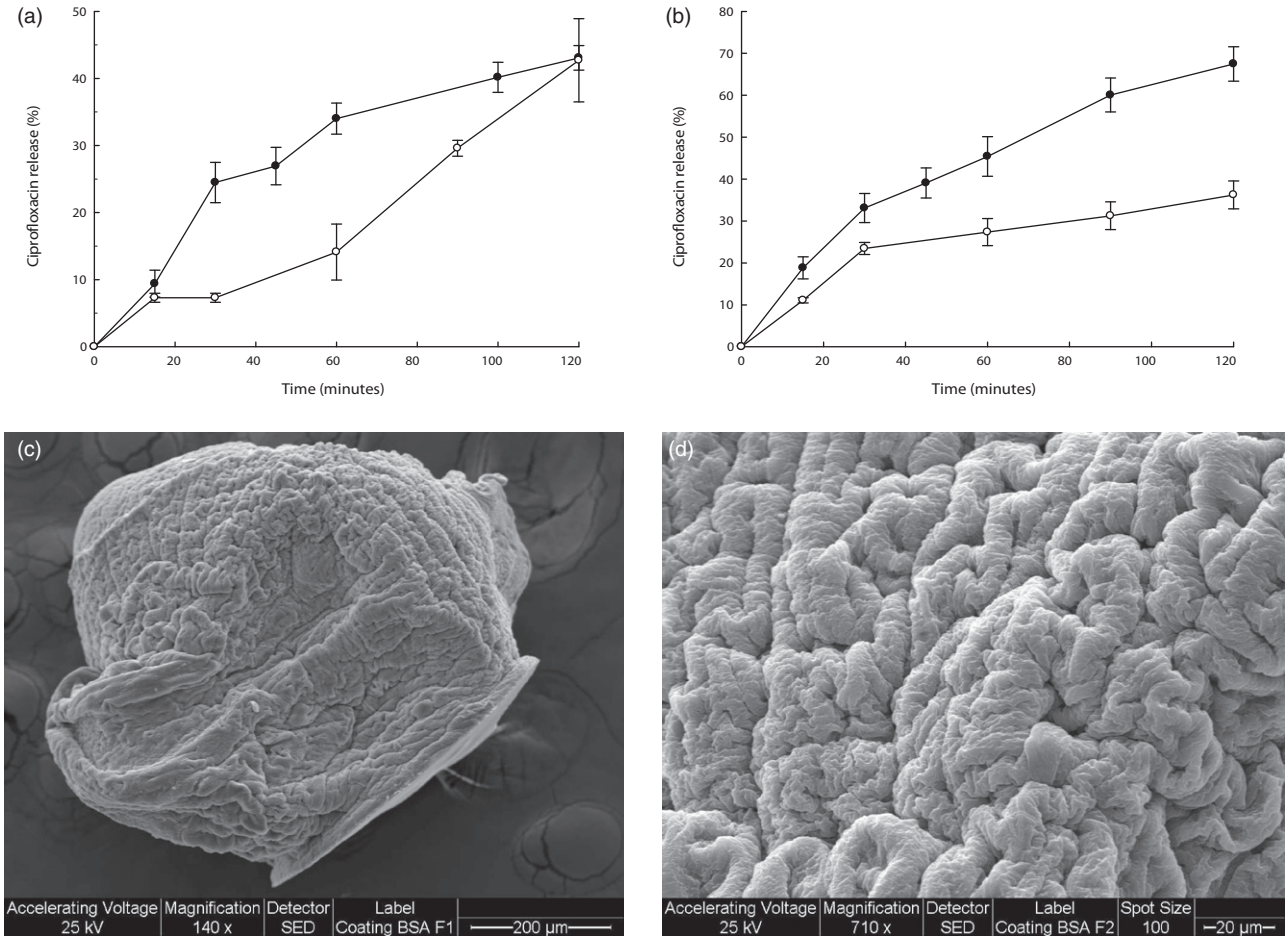

Figure 8. Effect of BSA coating: Ciprofloxacin release under gastric (a) and intestinal pH (b) from microspheres of alginate–gelatin crosslinked with glutaraldheyde (●) and coated with BSA (○), and SEM images of coated microspheres with 140× (c) and 710× (d) magnification.

The microbial inhibition diameter against *P. aeruginosa* was 12.8 ± 0.1 cm and 14.8 ± 1.1 cm for the glutaraldehyde-untreated and -treated blend microspheres, respectively. These values were considered the 100% of inhibition in Figure 7. These results confirm that the Cip released from these biopolymeric matrices was biological active and produced the growth inhibition of the tested microorganism. Besides, the Cip was sustainably released from microspheres as the inhibition halo was growing in time and glutaraldehyde-treated microspheres released the antibiotic in a more controlled profile. The results were in concordance with the previously ones observed in simulated physiological fluids.

Binding of protein (BSA as model) onto active surface of microspheres

The capacity of glutaraldehyde-activated microspheres to bind proteins onto surface was evaluated using BSA as model protein (Figure 8). Kinetic release profile of Cip was followed under acid and alkaline conditions (Figure 8a and b). The kinetic of Cip release is suggesting the presence of a diffusional barrier for the antibiotic produced by the presence of the BSA on surface. The Cip release profile from BSA-coated microspheres under acid simulated gastric conditions is showing a decrease of the drug release of roughly 20% in 60 min compared with non-coated BSA microspheres. After about 60 min, the Cip release from both types of microspheres trend to converge, which are suggesting a major role of BSA coating on Cip release. In the case of Cip release kinetic under slight alkaline intestine conditions, the drug release was delayed 30% in two hours. Analysis of microspheres by SEM is confirming the changes on microspheres surface by the presence of aggregates made by the BSA that looks like covered by knots (Figure 8c and d). In addition, epifluorescence microscopy to confirm BSA binding to microspheres surface was carried out. First, alginate/gelatin microspheres (glutaraldehyde treated and untreated) were immersed in 10 mg/ml of fluorescent BSA* (Alexa fluor conjugated), and then filtrated and washed. The resulting microspheres were observed by optical/fluorescence microscopy. Meanwhile untreated microspheres did not show fluorescence (Figure 9, top), glutaraldehyde-treated microspheres showed a high green fluorescence intensity, using filters to excite the BSA* around 497 nm (Figure 9, below). These pictures are in concordance with the SEM images, confirming BSA binding to microsphere surface and discarding possible microsphere degradation after BSA incubation.

Finally, quantification of BSA attached to microsphere surface was performed to obtain the protein/microsphere mass ratios and established their properties as a tool for targeting of biological molecules. When glutaraldehyde-treated microspheres were incubated in 1.0–10.0 mg/ml cold BSA solutions (at $pH = 4.0$ for 10 min) ratios from 1.5 ± 0.1 and 2.3 ± 0.4 µg of BSA per milligram of microsphere were obtained.

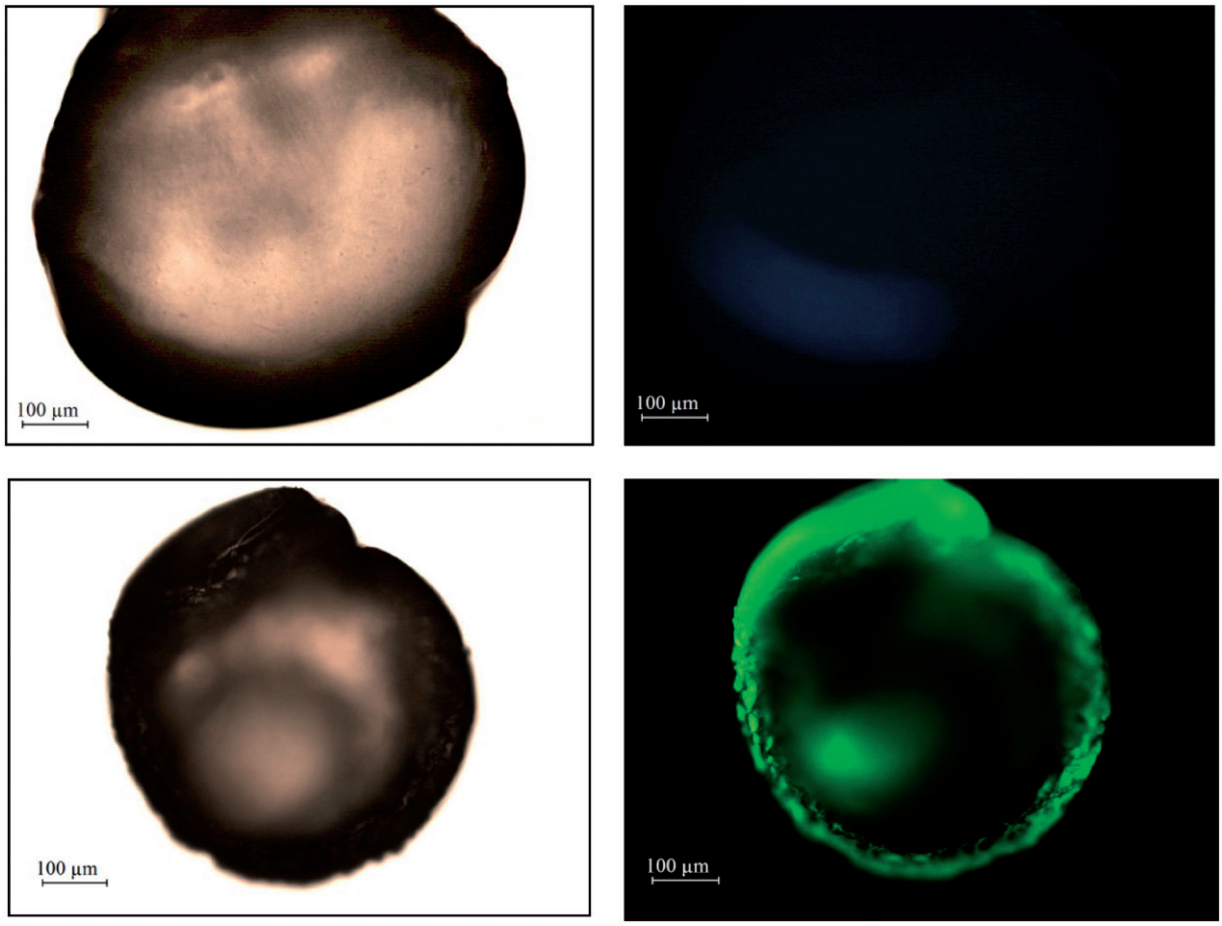

Figure 9. Optical and fluorescence microscopies of alginate/gelatin microspheres incubated with and without BSA conjugated with Alexa fluor (Invitrogen Argentina, Buenos Aires, Argentina). Top: no treated; below: glutaraldehyde treated microsphere. Left: optical; right: fluorescence microscopy ($\lambda_{Exc} = 497$ nm).

Considering the high specific activity (units per milligram of protein) of biological molecules like enzymes, the amount of protein bound to microspheres surface is high enough to deliver functional molecules activities.

Conclusions

Cip encapsulation in alginate–gelatin blend microspheres was performed through a doubled crosslinked method by calcium ion and glutaraldehyde in 1,2-propylene glycol–water solvent mixtures. The developed matrix is showing a modified surface structure for controlled release of the antibiotic. Homogeneity of Cip dispersed into the matrix was confirmed by FTIR spectra obtained by synchrotron light. Characterization of the developed matrix was performed by SEM and AFM images that showed a smooth surface of microspheres when they were treated with glutaraldehyde. Also, microspheres hardness and cohesivity were determined by texturometric assays showing no changes between microspheres synthesized in the absence or the presence of glutaraldehyde without incubation at the assayed pHs. However, significant stability associated to the both parameters in glutaraldheyde crosslinked microspheres at simulated intestinal media (pH 7.4) incubated for two hours were found. These results are suggesting that a thin film produced by glutaraldehyde treatment on the microspheres surface was responsible of the Cip release delay from the matrix. Also, glutaraldehyde-treated microspheres in the presence of BSA as a model protein showed a reduced kinetic Cip release from the microspheres.

The deposition and quantities of BSA adsorbed into the microsphere surfaces was confirmed by SEM and fluorescence microscopies and UV spectrophotometry analyses.

Antimicrobial activity of Cip release from the matrix was kept almost constant over certain a period of time and tested against *P. aeruginosa* showing high *in vitro* inhibition efficiency. The alginate–gelatin matrix could be suitable for oral delivery of Cip and treatment of recurrent infections reducing the antibiotic toxicity at least in a half, which is an advantage for long-term treatment and/or recurrent microbial infections. Also, the matrix has the capability of bind proteins onto surface, which is an attractive tool for attachment of biological molecules with different functional properties, such as antimicrobial peptides, enzymes, or others. The microspheres blend composed by alginate–gelatin is providing a polifuntional ‘‘smart’’ matrix that can be tailored with specific markers for diverse therapeutic purposes. In addition, controlled release devices based on biopolymers is becoming a new area for specifically targeted drug release for highly toxic drugs, easily to scale up, with low cost and non-toxic raw materials. Further experiments in cell cultures to characterize the *in vivo* Cip release, matrix behavior and toxicity are under way in our laboratory.

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Declarations of interest

The authors report no declarations of interest.

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