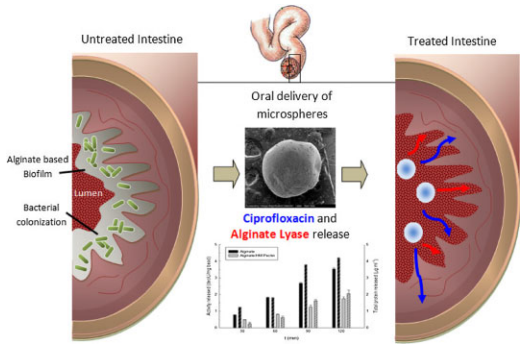
Alginate Lyase and Ciprofloxacin Co-Immobilization on Biopolymeric Microspheres for Cystic Fibrosis Treatment[a]

German A. Islan, Valeria E. Bosio, Guillermo R. Castro*

A new formulation is described based on biopolymeric microspheres containing alginate lyase (AL) and ciprofloxacin (Cip) for sustainable oral delivery in CF patients. Alginate (ALG) and high-methoxyl pectin (HMP) are selected as the biopolymers to develop a composite matrix. ALG microspheres coated with HMP and ALG-HMP blend are gelled in water/organic solvents mixtures, obtaining Cip encapsulations from 46.0 to 100.0%. ALG-HMP shows a Cip sustainable release profile and is able to encapsulate 90.0% of AL, showing 76.0% enzyme activity after release under simulated intestinal conditions. The developed system is a promising delivery carrier to treat chronic infection of *Pseudomonas aeruginosa* and to reduce the viscoelasticity of the mucus accumulated into intestine of CF patients.



1. Introduction

Cystic fibrosis (CF) is an inherited autosomal disease produced by defective chloride transport and is the most common lethal genetic disease amongst caucasians. Chronic lung infection by opportunistic pathogens is affecting more than 90% of all CF patients.[1] *Pseudomonas aeruginosa* is the main colonizing bacteria in CF pathologies and produced considerable amounts of the exopolysaccharide alginate.[2,3] Alginate is not able to be degraded by mammalian cells and continue to be accumulated increasing viscoelastic properties of pulmonary and intestinal secretions. Mucoid strains are capable of surviving aggressive antibiotic therapies and once established becomes extremely difficult to eradicate.[4] The knowledge of CF pathologies is suggesting that alginate plays a relevant role to facilitate biofilm formation, enhancing bacterial survival and adherence to the epithelial layer of the lung, acting as a free-radical scavenger, reducing the phagocytosis process by macrophages, and retarding antibiotic diffusion.[5] Also, the microorganism found in the sputum on the infected lungs acts as a reservoir spreading the bacteria into the all body: colonizing the intestinal tract and clogging the pathways from pancreas, interfering with proper digestion and even, trough the stool, producing cross-infection to susceptible patients.[6]

Quinolones are inhibitors of DNA gyrase and topoisomerase IV causing bacterial death.[7] Among then, Cip is a second generation quinolone, commonly used across Europe for the CF treatment nowadays.[8] However, Cip is an antibiotic commonly associated to gastric and intestinal

G. A. Islan, V. E. Bosio, G. R. Castro
Nanobiomaterials Laboratory, Applied Biotechnology Institute (CINDEFI, UNLP-CONICET CCT La Plata) – School of Sciences, Universidad Nacional de La Plata, Calle 47 y 115, CP 1900, La Plata, Argentina
E-mail: grcastro@gmail.com

[a]**Supporting Information** is available from the Wiley Online Library or from the author.

problems when it is administrated orally.[9] Besides, Cip have low solubility in aqueous media and tendency to aromatic stack amongst themselves,[10] which reduce antibiotic biodisponibility. Moreover, chronic treatments with CF at elevated antibiotic doses imply highly toxic concentrations associated to strong undesirable side effects, leading to a reduced half-life and quality of patients. It was also found that the administration of free antibiotic is not effective to diffuse through the alginate biofilm barrier,[11] in consequence co-immobilization of an alginate degrading enzyme with Cip could be a feasible alternative for the improvement of prevalent CF therapies.

Alginate lyase (AL) is an endo-hydrolase class enzyme acting over β-1,4-glycosidic linkages via β-elimination reaction to produce mono-, di-, and tri-oligosaccharides that can be extracted from different microbial sources.[12] The use of AL in CF treatments is an interesting approach since they are able to reduce the viscosity of purulent mucus of alginate produced by mucoid *Pseudomonas aeruginosa* strains present in the lung and the intestine of the patients. Previous work demonstrated that AL promotes diffusion of aminoglycosides through the extracellular polysaccharide of mucoid strains.[13] Considering that alginate concentration in the sputum was found ranging 78–-192 $\mu g\,mL^{-1}$ in CF patients,[14] only few units of AL would be effectively necessary for reducing the viscosity of the purulent exacerbations.[15]

Natural polymers like alginates, pectins, guar gums, or chitosans among others, have been frequently used in foods and pharmaceutical applications like immobilization and encapsulation of biological active molecules.[16,17] Alginates (ALG) are linear anionic polysaccharides widely used in food and pharmaceutical industries and made of β-manuronic acid (M units) and α-guluronic acid (G units) linked by 1–4 bounds. Their properties of not being toxic, not showing immunologic responses, biocompatibility, and easy gelation in presence of divalent ions by "egg box junctions",[18,19] made them very suitable gels for the oral delivery. Another interesting biopolymer is pectin, a water-soluble polysaccharide present in the plant cell wall, which is not degraded by intestinal enzymes but degradable by the intestinal flora. Pectins are composed of linear polysaccharides of partially methoxylated poly[α-(1,4)-D-galacturonic acids]. Pectins can be grouped into low-methoxylated (LMP) with esterification degree (ED) below of 40%, medium-methoxylated (MMP) with ED range between 40 and 60%, and high-methoxylated pectins (HMPs) with ED higher than 60%. LM and MM pectins can be gelled by multivalent cations, meanwhile HM only at acid pH and in presence of solutes.[20,21]

Co-immobilization of an antibiotic and an enzyme for oral delivery is a feasibly alternative with the use of blend composites of natural polymers. In the case of alginate and pectin, blends were used to synthesize capsules,[22] beds,[23] gel microspheres,[24,25] or films[26] as desirable matrices for drug release of molecules. Particularly, microspheres has the advantage of having an intimate and prolonged contact of the drug delivery device with the absorbing membrane, which has the potential to maximize both the rate as well as the extent of drug absorption, prolonging, and controlling gastrointestinal transit of the dosage forms.[27] In order to control the Cip release profile and reduce its toxicity, encapsulation in ALG/HMP blend microspheres was previously reported in our laboratory. The results are suggesting that HMP plays a key role in the Cip release.[24] However, the Cip encapsulation percentage was less than 50% under physiological aqueous conditions, and it should be improved in order to develop a feasible economic process. As only antibiotic is not highly effective in CF treatments, co-immobilization of AL in this matrix would enhanced potential antibiotic killing against pathogens immersed into biofilm. Also, presence of HMP into the composite possibly provide a protective environment for the immobilized AL against gastric acidity in oral delivery, due to HMP gelling properties under acid conditions.[20,21]

In the present paper, experiments were carried out to improve the actual oral delivery systems for CF treatment. Controlled release of Cip from microspheres was controlled by biopolymeric matrices based on different alginate and high-methoxyl pectin compositions. Two main developments were studied: the environmental conditions in which Cip could be encapsulated with high efficiency in alginate-based matrix, using water-miscible organic solvent mixtures (O-A), and the co-immobilization of an AL in order to reduce the mucus viscoelasticity produced by *Pseudomonas aeruginosa* in CF patients, facilitating the antibiotic activity. Scanning electron microscopy (SEM) images to elucidate morphology and surface changes on microspheres were analyzed. The innovative co-immobilizing microspheres for Cip and AL were tested at gastric and intestinal simulated media to elucidate the release and stability of the enzyme and the antibiotic.

2. Experimental Section

2.1. Reagents and Bacteria

Cip (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid), apple pectin (DE: 70–75%) and AL from *Flavobacterium* sp. were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium alginate ($\overline{M}_w = 120$ kDa) was provided by Monsanto (St. Louis, MO, USA). *Pseudomonas 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.

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2.2. Synthesis of Alginate-Based Microspheres Containing Ciprofloxacin and Alginate Lyase

Low-viscosity sodium alginate (2.0 wt%) was made in 25×10^{-3} M acetate buffer (pH = 4.0) containing Cip (36.0 μg mL^{-1}) and AL (40.0 U mL^{-1}) under gentle stirring at 0 °C. Microspheres were made by dropping the biopolymer into 0.500 M $CaCl_2$ solutions. Gelling solutions of $CaCl_2$ were made with 25, 50, 75, and 100 vol% aqueous/solvent mixtures using ethanol, ethylene glycol, 1-propanol, 1,2-propylene glycol (PG), and glycerol to evaluate Cip encapsulation.

Coated microspheres were prepared by dropping the alginate-Cip-AL solution into PG/water (1:1) containing 0.500 M $CaCl_2$ and 0.1 wt% HM pectin at 0 °C with gentle stirring.

After 20 min all microspheres were filtered, washed with distilled water, dried and weighted to perform release assay kinetics.

2.3. Determination of Ciprofloxacin and AL Encapsulated into Alginate Based Microspheres

The resulting microspheres were filtrated and dried at 25 °C for 10 min. Wet microspheres were stored at 5 °C until use. Cip was determined in the filtrate spectrophotometrically in the range of 277–280 nm depending on the solvent composition using appropriate calibration curve (UV-Vis Beckman DU640 spectrophotometer). The encapsulated Cip was calculated as follows:

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

where Q_0 is the initial amount of Cip (μg), Cr the concentration of Cip in the filtrated solution (μg ·ml^{-1}), and V is the volume of filtrated solution (mL).

The same procedure was performed using Equation (1) by measuring AL activity and total protein of filtrated solutions and redefining

Q_0 = initial AL units (U)/initial total protein amount (μg)

Cr = activity of AL (U mL^{-1})/concentration of total protein (μg ·ml^{-1}) in the filtrated solution.

2.4. Scanning Electron Microscopy (SEM) Images

SEM analysis was performed in freeze-dried microspheres for 72 h. Furthermore, samples were prepared by sputtering the surface with gold using (Balzers SCD 030 metalizer) obtaining layer thickness between 15 and 20 nm. Microsphere surfaces and morphologies were observed using Philips SEM 505 model (Rochester, USA), and processed by an image digitizer program [Soft Imaging System ADDA II (SIS)]. Effect of solvents on matrix structure during crosslinking procedure was analyzed.

2.5. 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 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 less the standard variation value is, the smoother the surface is. Histograms were performed by duplicate of SEM images at 710× magnification.

2.6. Saturation of the Alginate-Based Matrix with Ciprofloxacin

Alginate solutions (2.0 wt%) containing 36.0, 100, 200, 500, 1 000, and 2000 μg mL^{-1} of Cip, respectively, were dropped into PG/water (1:1) solution containing 0.500 M $CaCl_2$ to form microspheres. Antibiotic encapsulation was evaluated as stated before.

2.7. Release Studies of Ciprofloxacin and AL from Alginate-Based Microspheres

The microspheres (200 mg) were incubated in 0.050 M KCl/HCl buffer (pH = 1.20) and in 0.040 M phosphate buffer (pH = 7.40) at 37 °C and 100 rpm to simulate gastric acidity and intestinal environment, respectively. Samples (1.0 mL) were taken out at different times and absorbance measured at the maximum Cip wavelength in each buffer (277 and 274 nm at pH = 1.20 and 7.40, respectively). AL activity and total protein content were measured. Finally, 1.0 mL of fresh media was added back to refill the reaction volume.

In order to elucidate the effect of maturation time on Cip release, microspheres of alginate-Cip-AL were incubated in 0.500 M $CaCl_2$ solution for 20, 60, and 150 min. Cip release was evaluated at simulated intestinal media at pH = 7.4.

2.8. Evaluation of Alginate Lyase Activity

Alginate lyase (AL) activity was measured mixing 75 μL of enzyme solution with 1.925 mL of 1.0 wt% alginate incubated at 37 °C for 30 min. Reaction was stopped by the addition of 2.0 ml NaOH (0.100 M) and the resulting absorbance was measured at 233 nm.

Effect of pH on AL activity was determined as mentioned before in 0.050 M acetate buffer (pH = 4.0 and 5.0) and in 0.025 M phosphate at pH = 6.3, 6.8, 7.4, and 8.2 at 37 °C.

One AL unit was defined as the amount of enzyme capable of increase 1 unit of absorbance at 233 nm in 1 min mL^{-1} of sodium alginate at pH = 7.4 at 37 °C.

2.9. Measurement of Protein Released

Protein content was measured using fluorescamine as follow: 50 μL of sample was mixed with 350 μL of 0.0125 M borate buffer (pH = 9.0) and 125 μL of fluorescamine (300 μg mL^{-1} in acetone). After 2 min, fluorescence was measured at with a λ_{ex} = 390 nm and λ_{em} = 478 nm (Perkin Elmer LS 50B spectrofluorimeter) with proper calibration curve.

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2.10. Evaluation of AL Activity in Presence of Ciprofloxacin

Microspheres (200 mg) containing AL (40.0 U mL^{-1}) were dissolved in 2.0 mL of 0.050 M phosphate buffer (pH = 7.4). One milliliter sample volume was mixed with 1.0 mL of 2.0 wt% sodium alginate adjusted in 0.025 M buffer phosphate (pH = 7.4) with or without 36.0 μg mL^{-1} Cip and incubated at 37 °C for 30 min.

2.11. Evaluation of Bactericidal Activity of Ciprofloxacin against *Pseudomonas aeruginosa* in the Presence of Different AL Concentrations

Ciprofloxacin activity was tested against *Pseudomonas aeruginosa* ATCC 15442 (non-fermenting Gram-negative bacteria) using modified disk diffusion method according to CLSI/NCCLS, replacing disks for sterile glass cylinders of 8 mm × 6 mm × 10 mm (external and internal diameter, length). Briefly, four or five colonies of the microorganism were taken and resuspended in sterile physiological solution (0.154 M NaCl) to adjust the turbidity of the culture to less than 3×10^8 CFU mL^{-1} (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. The glass cylinders were further placed on the surface of the inoculated agar plate. After their placement, 25 μL of each 10 μg mL^{-1} Cip solutions containing different amounts of AL (0.4, 2.0, 4.0, and 20.0 U mL^{-1}) were placed inside the cylinders and later the plates were incubated at 37 °C for 24 h. Then, inhibition zones were determined and the assays were performed in duplicate.

2.12. Statistical Analysis

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

3. Results and Discussion

3.1. Solvent Screening to Improve Ciprofloxacin Encapsulation

The main criteria for the screening of water-miscible organic solvents was considering Cip encapsulation higher than 50% on alginate gel microspheres in presence of calcium ion as crosslinker. Also, the criteria for solvents selection involved water-miscible polyols with maximum C3 length-chain, different hydroxylation degrees, ability of solubilize $CaCl_2$, and non-toxic when are orally administrated and metabolized by liver (Table 1). Based on these considerations ethanol, ethylene glycol, 1-propanol, and PG were selected. Methanol was discarded because of its metabolic pathway in the mamalian's liver, where alcohol dehydrogenase generates formaldehyde, a highly toxic compound. Also glycerol was not used, due to its elevated viscosity makes difficult the control of the gelation process.

Cip is a very hydrophobic molecule with a log $P = 2.3$, and it is expected that encapsulation of Cip on highly hydrophilic environment will favored. From the list of Table 1 the best solvent candidates are ethylene glycol and 1,2-propylene glycol. Cip encapsulation into alginate biogels higher than 50% in all of water-organic solvents mixtures compared to 43.6 ± 3.2% in water was found (Table 2). This fact could probable due not only to the change of media environment but also to dehydration process of the alginate gel matrix made by low molecular weight hygroscopic alcohols. Also, the calcium interaction between alginate chains shrinking the gel matrix is enhanced by the dehydration process, plus a Cip displacement from ionic environment of the media to nascent gel structure. Besides, no significant changes on Cip encapsulation were observed by increasing solvent concentration in the mixture higher than 50 vol% ($p \geq 0.05$).

Another relevant factor to take in account for Cip encapsulation is the changes of viscosity in the case of some of the tested alcohols (Table 1). For example, solutions containing 25, 50, 75, and 100 vol% ethylene glycol have viscosities roughly 1.9, 5.3, 7.3, and 40 times higher than in water respectively at 0 °C (see Table S1, Supporting Information). A linear relationship between the increase of ethylene glycol/water percentage associated to Cip encapsulation in the matrix was found ($r^2 = 0.96$). The result could be explained in terms of Cip diffusion coefficients in the organic-aqueous solutions which are decreasing with the increase of the viscosity of the organic solvent mixture. In addition, the viscosity in 25, 50, 75, and 100 vol% PG aqueous solutions changed drastically from 4, 12, 45, and 140 times related to water (at 0 °C). In this case was of

Table 1. Properties of solvents used for Cip encapsulation.

Compound	Number of C	Number of –OH	log *P*	Viscocity at 25 °C [cP]	Dipole moment [D]	Dielectric constant	Cip solubility at 20 °C [mg mL^{-1}]
ethanol	2	1	−0.31	1.07	1.71	25.70	0.13
ethylene glycol	2	2	−1.36	14.78	2.28	38.00	45.15
1-propanol	3	1	0.28	1.95	1.68	20.00	0.40
PG	3	2	−0.92	45.70	3.70	32.00	7.41

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Table 2. Screening of encapsulation percentage of Cip in alginate based microspheres using solvents in water mixtures; different superscripts indicate significant differences ($p < 0.05$) in each column.

Organic solvent concentration [vol%]	Ciprofloxacin encapsulation [%]				
	Water	Ethanol	Ethylene glycol	1-Propanol	PG
25.0	–	65.5 ± 3.7	51.0 ± 1.5^{a}	59.3 ± 4.0	74.6 ± 3.1^{a}
50.0	–	68.2 ± 1.3	64.0 ± 2.3	58.3 ± 4.5	96.4 ± 3.9
75.0	–	68.7 ± 2.4	65.8 ± 3.5	55.5 ± 4.8	99.9 ± 0.2
100.0	43.6 ± 3.2	65.0 ± 2.7	73.0 ± 1.7^{b}	51.8 ± 4.2	99.0 ± 0.8

particular interest because of the Cip encapsulation in 50:50 PG/water solution was close to 100%, which could be correlated to the increase viscosity as well. In the cases of ethanol and 1-propanol, the viscosity of the aqueous solvent mixtures increases about two and four times maximum compared to water when the concentration of solvents were in the range of 25–50 vol%, respectively. It is interesting to note that the Cip encapsulation increases when the viscosities of organic-aqueous solutions are above two to four times higher compared to water. The effect of enhanced Cip encapsulation could be due to a mixed effect of Cip diffusion from the microsphere to the bulk solution and dehydration of the matrix during the gelation process.

Physicochemical properties of pure polyols such as dipolar moment, dielectric constant, and Cip solubility also were under study (Table 1). An increase of Cip encapsulation was observed as the dipole moment of the solvents rises (propylene glycol posses the maximum value reaching a 100% encapsulation efficiency). As the dipole moment provides information about the distribution of electrical charges of molecules, these results suggest that molecules with high dipole moment can act as bridges between the matrix (with negative charge) and the Cip (with positive charge), retaining most of the drug during encapsulation. On the contrary, it was difficult to establish a relationship between the dielectric constant, parameter that define the solvation, and stabilization capacity of charges. Additionally, no correlation between solubility of Cip in the tested solvents and Cip encapsulation was observed. However, Cip encapsulation percentages were higher than 70% in solvents having dipole moments in the range of 30–40 D.

These results are indicative of the solvent complex effect on the system composed by the polymeric matrix and Cip. The solvent effects could be as a result of changes in Cip solubility, the kinetic of alginate gelling process, molecular solvation of the antibiotic and the matrix, and proton exchange, among others.

The effect of PG at different concentrations during alginate gelation by calcium crosslinking was studied analyzing morphological microsphere surface patterns by SEM images (Figure 1). Extensive dehydration by freeze dry of microspheres made in aqueous media required to get alginate bead SEM images with poor homogeneity and cracks on the shell surface (Figure 1a,b). On the other side, synthesis in presence of 50:50 PG/water solution and calcium chloride, produced microspheres with relative spherical morphology and more homogeneous surface (Figure 1c,d). The microspheres morphology was kept by the increase of organic solvent concentration up to 100%, but folding patterns with non-smooth areas were observed on their surface. By SEM morphological analysis of the beads, the results are showing more efficient chemical dehydration by the solvent compared with physical procedure of freeze drying methods. On the other side, it can be hypothesize that the kinetic of gel matrix dehydration process by PG is faster than the diffusion of calcium ions, the crosslinker, from the bulk solution into the matrix. The effect of retarding in calcium diffusion inside the matrix reduced the crosslinking in the gel and as a result surface folding patterns in surface are shown without partially losing gel spherical topology (Figure 1e,f).

Statistical analysis of SEM pictures by ImageJ software revealed that the roughness of the surface that can be correlated to an increase of the standard variation of the gray values of all the pixels on SEM images (std. dev, Table 3). The lower standard deviation value is correlated to the smoother the surface microsphere.[28] Furthermore, the treatment of alginate microspheres with increasing concentrations of PG, revealed a rise in roughness. The mean values were related with the spatial structure alignment of the polymer chains. However, PG treatment was not affecting the spatial alignment of alginate chains displayed in the histograms of Table 3 in where no significant differences were observed, ($p > 0.05$). These results are confirming the main effect of the PG over the gel matrix which is related to the dehydration of alginate matrix. Consequently, the collapsed gel structure is keeping the Cip inside the gel microsphere.

The developed matrix in presence of 50:50 PG/water was able to incorporate different antibiotic concentrations. The capacity of the gel matrix to encapsulate increasing concentrations of Cip was determined in Table 4. The

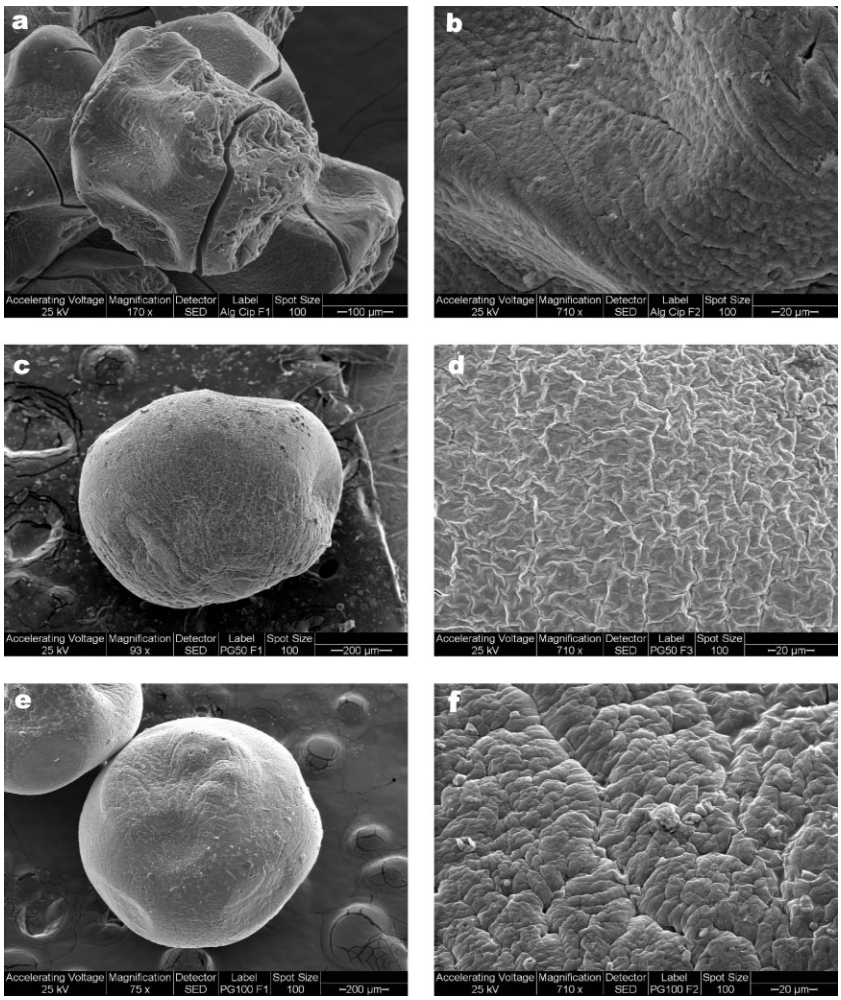

Figure 1. SEM analysis: effect of PG concentration on morphology and surface of alginate microspheres, 0 vol% at a) 170 × and b) 710×; 50 vol% at c) 93× and d) 710×; 100 vol% at e) 75× and f) 710× magnification.

amount of antibiotic encapsulated into the matrix after ionic gelation increased lineally ($r^2 = 0.996$) when the initial antibiotic concentration was raised. However, no saturation point was reach, even at high concentrations up to 2.0 mg mL^{-1} of initial Cip. High amounts of the initial fluoroquinolone led to a high encapsulation, possible due to a molecular π–π stacking process commonly associated to quinolones.[10] This is an interesting advantage of the matrix, because dose can be adjusted by changing the initial Cip concentration in solution getting high encapsulation percentages after gelation.

3.2. Release of Ciprofloxacin from Microspheres

Based on the results of Cip encapsulation and SEM analysis, the synthesis of alginate-based microspheres was performed in 0.500 M $CaCl_2$ and PG/water (1:1).

The Cip release under simulated gastric fluids (pH = 1.2 and 37 °C) from alginate microspheres was 84.8 ± 3.7% when they were synthesized in presence of 50 vol% PG, meanwhile it was 97.5 ± 5.4% for microspheres synthesized on water at 90 min (Figure 2a). In the first approach to prevent the Cip burst release at acid pH and extend the antibiotic inside the gel matrix, microsphere coating procedure with HMP (HM Pectin) was performed. The results showed a reduction of the Cip release in about 20% on PG made-beads coated with 0.1 wt% of HM pectin (the maximum HMP concentration capable of being solubilized in the calcium chloride solution without gelation). The presence of pectin film on microsphere surface prevented the Cip diffusion to the bulk solution. HMP is a good polymer candidate for coating because two main reasons: first of all, interaction between Cip and HMP was found highly favored as previously reported[24] and second, HMP can be gelled under acid conditions, which is reducing the pore size of the microsphere surface under stomach environmental conditions.

Based on the coating results mentioned before, a second approach to reduce Cip release profile consisted in rise the proportion of HMP in the matrix, producing blend microspheres in presence of 1:1 PG-water solutions using different ALG-HMP ratios (Figure 2b). All blend ALG-HMP microspheres showed Cip encapsulation percentages higher than 90%. These results are indicating that ionic gelation process in presence of PG for Cip encapsulation at high amounts is valid for the all biopolymeric mixtures tested. After incubation of microspheres for 2 h at gastric simulated media, it was found Cip release percentages of 71.6 ± 2.9, 44.0 ± 2.9, 48.6 ± 0.8, and 64.7 ± 1.1 for 20, 33, 50, and 66 wt% HMP, respectively. The results are suggesting a major role of HMP on decrease of Cip release until reach concentrations above 50 wt% on the matrix. However, higher HMP ratios led to a destabilization of the matrix and a consequently reduce capacity to retain the Cip. Spherical microsphere morphology was kept up to 50 wt% HMP, but the morphology was lost when the HMP proportion increase to 66 wt% determined by optical microscopy (see Figure S1 in the Supporting Information). The results are in agreement with results of a previous work from our lab.[24]

Table 3. Surface analysis of SEM images (710× magnification) by ImageJ software.

Microsphere treatment	Mean	Standard deviation	Histogram
untreated	97.7 ± 14.6	26.0 ± 0.2	
PG 50%	120.1 ± 25.5	32.2 ± 0.2	
PG 100%	120.5 ± 10.8	45.1 ± 7.4	

The 2:1 wt% ALG-HMP ratio in the matrix showed the best Cip release profile. A biphasic Cip releases plot was observed. First, a burst release phase accounting for 40% of Cip release during the first hour due to the dissolution of the antibiotic covering the bead surface or partially embedded below the surface. The second phase of the Cip release displayed a pseudo-plateau phase with slow antibiotic release (less than 5%) during the next hour, because more deeply embedded Cip molecules were surrounded by the biopolymer blend, difficult to be eroded. A very common phenomenon for most matrix used in drug delivery systems.[29]

Furthermore, Cip release was assayed from microspheres incubated under simulated intestinal media (phosphate buffer, pH = 7.40 and 37 °C). The microspheres made in presence of PG and coated with HM pectin showed 87.0 ± 1.8 and 95.0 ± 0.4% Cip release in 1 h, respectively (Figure 3). On the other side, ALG microspheres made in aqueous environment released about 50% of Cip under the same experimental conditions.

The fast Cip release from microspheres made in 1:1 PG-water in presence of phosphate ions (pH = 7.4) could be attributed to the weak matrix crosslinking degree, due to the delayed diffusion of the calcium ions into the matrix during the gelation produced by the enhanced viscosity of the solvent mixture. The reduced crosslinking of alginate microspheres is facilitating a high erodibility of the matrix made by phosphate ions at simulated intestinal environment.

Table 4. Matrix saturation in presence of 50 vol% PG.

Ciprofloxacin concentration [μg mL^{-1}]		Encapsulation efficiency (EC/IC)
Initial (IC)	Encapsulated (EC)	
36	34.7 ± 1.4	0.96
100	71.0 ± 1.7	0.71
200	142.0 ± 1.2	0.71
500	378.5 ± 0.5	0.77
1000	797.0 ± 32.0	0.80
2000	1794.0 ± 136.0	0.90

In order to improve the crosslinking of the matrix, incubation of microspheres for longer period of times in presence of calcium chloride solutions were analyzed. The increase of bead maturation time allows increasing the matrix crosslinking associated to the enhanced matrix stability and lower erodability concomitantly with the decrease of Cip release from the microspheres. Significant differences in the Cip release at different times of incubation in presence of calcium were found between 2 and 20 min ($p < 0.05$) (see Table S2 in the Supporting Information). Microspheres synthesized and kept in 1:1 PG-water solutions (500 mM $CaCl_2$) for 20 min showed Cip release 20% higher than microspheres made in a water environment in simulated intestinal fluids. Besides, the time required to get similar Cip release values is about 7.5 times higher in 1:1 PG-water solution compared to water. Both results are confirming the delay effect of PG in the microsphere gel formation, not only in Cip diffusion during gelation process, but also in calcium penetration into the developing matrix.

Based on the results mentioned before, the blend matrix composed of ALG/HMP (2:1) was selected for the AL immobilization.

3.3. Co-immobilization of Alginate Lyase (AL) into Microspheres

As the microspheres matrix was based on alginate (the natural substrate of the AL) the kinetic parameters of

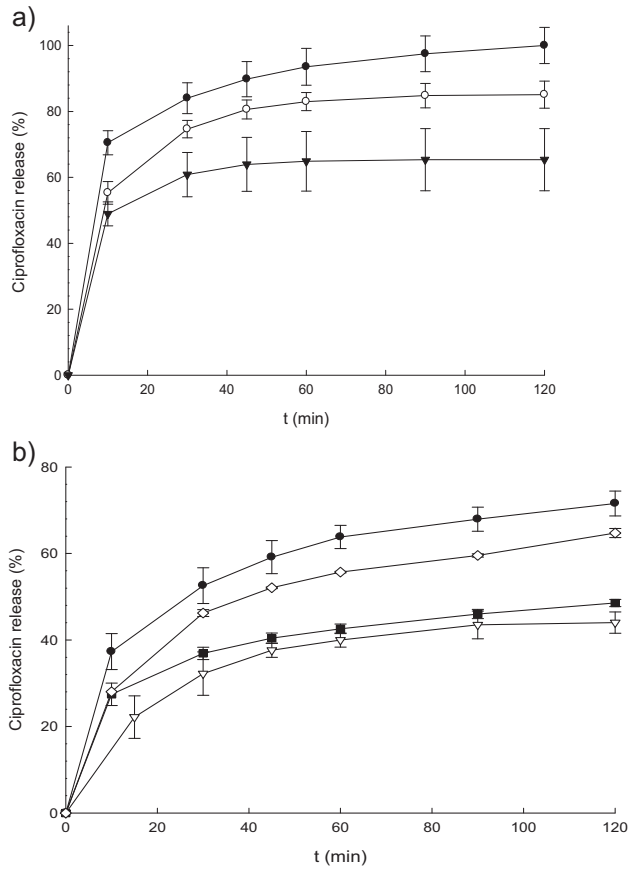

Figure 2. Release profile of Cip at pH = 1.2 (simulated gastric media) from alginate-based microspheres: a) made in different conditions: aqueous media (●); PG (○); PG and 0.1 wt% HMP coating (▼) and b) effect of different ALG-HMP ratios (wt%): 2–0.5 (●); 2–1 (▽); 2–2 (■); 1–2 (◇).

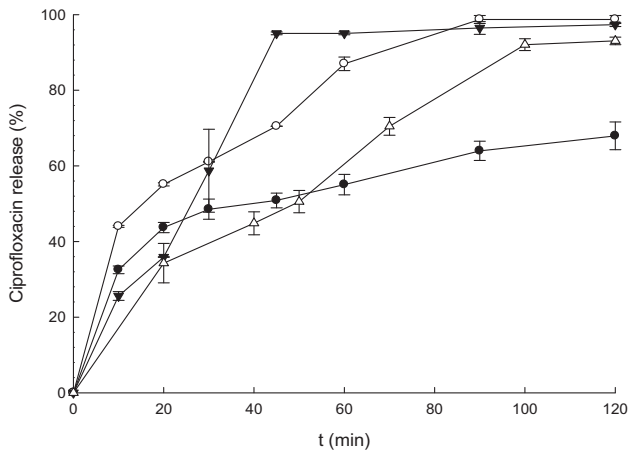

Figure 3. Release profile of Cip at pH = 7.4 (simulated intestinal media) from alginate-based microspheres made in different synthesis conditions: aqueous media (●); PG (○); PG and HMP coating 0.1 wt% (▼); PG and ALG 2 wt%-HMP 1 wt% blend (Δ).

enzyme and especially its activity as function of pH were analyzed in order to establish the conditions for AL co-immobilization (Figure 4). The AL posses practically no activity at pH = 4.0 (Figure 4a), but by shifting to pH = 7.4 the biocatalyst activity is fully recovered. The pH range of activity is providing vital information about the pH of microspheres in which AL was immobilized, avoiding an auto-degradation of the matrix mediated by the enzyme before incubation. Also, saturation of the AL by increases concentrations of aqueous alginate was determined (Figure 4b). A Michaelian enzyme profile was observed. By the Hanes–Hultine equation a linear relation between S/V versus S was obtained ($r^2 = 0.99$) and the kinetic parameters were $K_m = 4.42 \times 10^{-6}$ M and $V_{máx} = 4.42$ U mL^{-1}.[30]

Encapsulation of AL in ALG and ALG/HMP microspheres was performed at pH = 4.0, in where the enzyme displayed

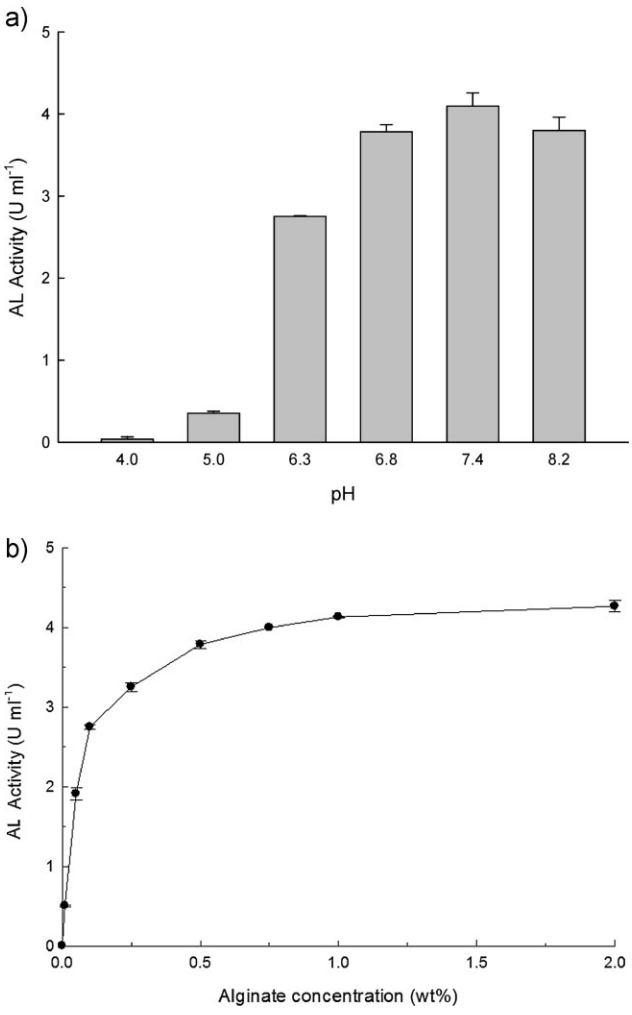

Figure 4. Alginate lyase (AL, 1 U mL^{-1}) activity: a) at different pH b) at different alginate concentrations at 0.025 M buffer phosphate (pH = 7.4) at 37 °C.

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very little activity (Figure 4a). The total protein incorporation into microspheres synthesized in presence of calcium and 50 vol% PG was higher than 90 wt% for both matrices. However, a decrease in activity after immobilization was observed when microspheres were dissolved at neutral pH and AL activity was measured. The remaining AL activity after immobilization was 90 and 76% for ALG and blended microspheres, respectively.

3.4. Release of AL and Cip from the Co-Immobilizer System

Evaluation of free and encapsulated AL activity during passage through gastrointestinal tract was evaluated in order to develop a system for oral delivery. Free AL and AL incorporated into ALG microspheres were completely inactivated by incubation at pH = 1.2 (gastric simulated media), 37 °C for 2 h. However, when the AL was immobilized into ALG/HMP microspheres and incubated under acid conditions, 26% (9.0 U g^{-1} of bead) of the initial AL activity was detected. The fact can be explained based on HM Pectin properties, which tend to produce strong gels in presence of environmental acid conditions.[20] The HMP gel reduces the protons diffusion inside the matrix under acid pH increasing the AL protection.

The profile of AL controlled released in both matrices under simulated intestinal conditions was shown in Figure 5a. The release of AL from ALG/HMP microspheres was two times lower compared with alginate matrix. In both cases, the AL specific activity was constant along the enzyme kinetic release: while the protein was released, the AL activity increased.

ALG-HMP microspheres kept the capacity of providing a controlled release profile of AL at alkaline pH, after being exposed to acid conditions (Figure 5b). Almost 100% of the AL was released from the microspheres (reaching a final activity of 9 U g^{-1} of beads) after approximately 2 h incubation. However, the amount of enzyme release from the microspheres treated under acid pH was lowered by AL inactivation compared to the control experiment (squares in Figure 5b). On the other side, the percentage of AL release was higher after acid treatment, indicating not only the deleterious effect of acid pH on enzyme activity but also on matrix destabilization. Nevertheless, the protective effect of the matrix over AL under acidity is clearly observed, making this system suitable to across the gastric barrier and releasing all enzyme into intestine.

Release profile of Cip was not changed in presence of the enzyme at gastric pH (Figure 6a). However, the Cip release was 90 and 60% in absence and presence of the enzyme in the matrix, respectively, in 2 h at pH = 7.4 (Figure 6b). The reduction of Cip release in presence of AL is suggesting a physical reduction of the main output pathway of Cip from the matrix.

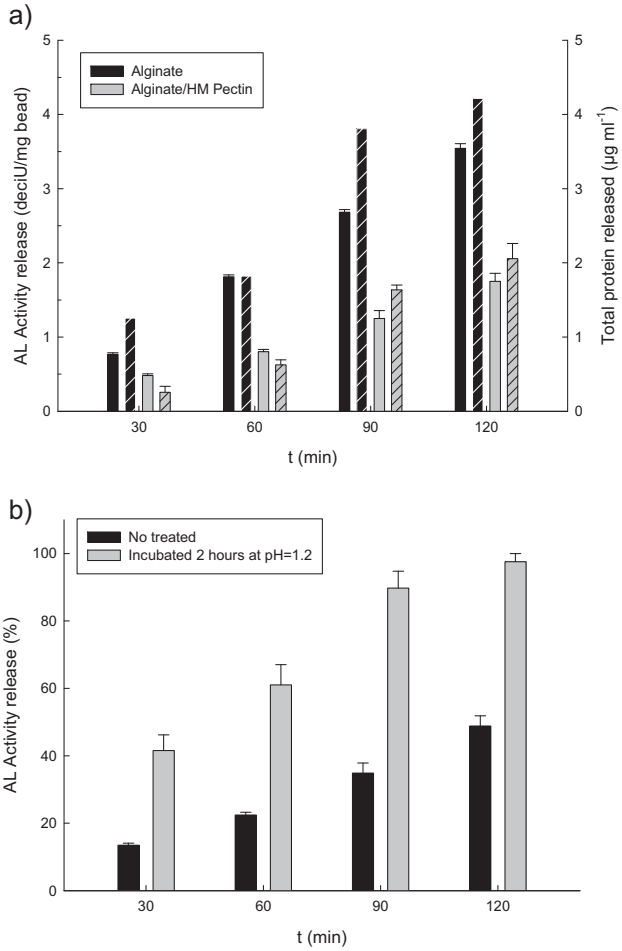

Figure 5. a) Comparison of two matrix formulations in the kinetic release of AL (solid bars) and total protein (striped bars) at pH = 7.4. b) AL release from alginate/HM Pectin microspheres at simulated intestinal media before and after incubation at pH = 1.2. Squares indicate AL activity (U g^{-1} of bead).

3.5. Simultaneous Presence of AL and Cip in the Microspheres

In order to avoid potential interferences on Cip antibacterial activity due to presence of the enzyme and vice versa, some experiments were carried out.

The effect of increasing amounts of AL (from 0.4 to 20 U mL^{-1}) on Cip activity against an opportunistic pathogen commonly associated with CF: *Pseudomonas aeruginosa*, showed that Cip generate an inhibition average halo around the bacteria of 20 mm and the diameter was not changed by the presence of AL, even at concentrations as high as 20 U mL^{-1} (see Figure S2, Supporting Information).

Based on the high interaction between Cip and alginate previously reported,[24] evaluation of AL activity in presence of Cip was performed. In the experiments, AL

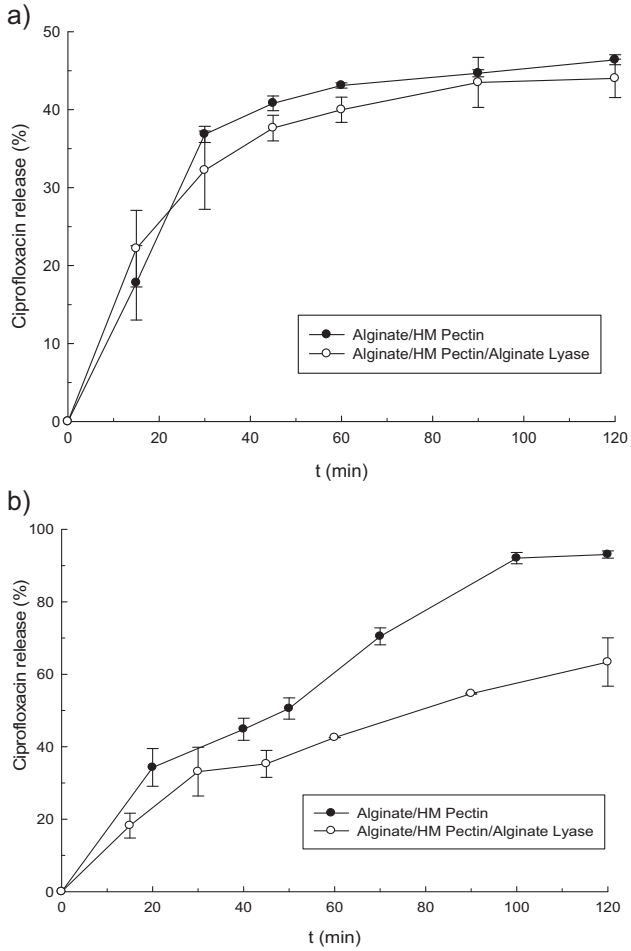

Figure 6. Release of Cip in presence of AL at a) pH = 1.2 and b) pH = 7.4. Symbols: ●, ALG-HMP; ○, ALG-HMP-AL.

(40 U mL^{-1}) and Cip (36 μg mL^{-1}) were co-immobilized in alginate/HM Pectin microspheres. Later, microspheres were dissolved at pH = 7.4 and samples were taken out to determine AL activity, obtaining a value of 76 U g^{-1} of microspheres. Control microspheres without antibiotic showed no significant differences in AL activity ($p > 0.05$), suggesting that Cip was not affecting the AL activity.

Finally, the ratio of enzyme/antibiotic required to reach intestine and to effectively reduce visco-elasticity of the produced mucoid alginate and kill the microbial infection can be dosed according to the pathology grade and the individual characteristic of the patients.

4. Conclusion

An innovative dual system containing Cip and AL co-immobilized into ALG-HMP microspheres with potential for the treatment of CF was developed. It was demonstrated that solvent engineering during ionic gelation process is a feasible method to modify biopolymeric matrices, allowing high encapsulation rates of molecules from low to high molecular weight. The co-immobilization of an antibiotic, Cip, and an enzyme, AL, was achieved without loss of their activities. The blend microspheres showed controlled release profile of active Cip and AL molecules on gastrointestinal simulated environments. Incorporation of HMP into the matrix not only allowed to control the release of both cargo molecules, but also provides a protective mechanism to partially prevent AL inactivation under acid conditions (stomach). Finally, the inclusion of AL in the microsphere biomatrix formulation does not affect Cip antimicrobial activity, but improves its release profile at intestinal simulated conditions. Additionally, controlled release systems based on biopolymers represent a new area of drug release since the matrices are mostly non-toxic, made in aqueous media, biodegrable, and easy to tailor.

The advantages of using simultaneously Cip/AL encapsulated for the treatment of CF allowed to reduce the antibiotic dose, the potential undesirable drug side effects, and avoiding the development of antibiotic resistance mechanism of opportunistic pathogens commonly found in this pathology. The dual characteristic of microspheres will potentially allow to improve the life span and quality CF patients.

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