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

German A. Islan[a], Cecilia Dini[b], Laura C. Bartel[c], Alejandro D. Bolzán[c], Guillermo R. Castro[a,*]

[a] Nanobiomaterials Laboratory, Institute of Applied Biotechnology (CINDEFI, UNLP-CONICET-CCT La Plata), Department of Chemistry, School of Sciences, Universidad Nacional de La Plata (UNLP), Calle 47 y115, C.P. 1900 La Plata, Buenos Aires, Argentina
[b] CIDCA (Centro de Investigación y Desarrollo en Criotecnología de Alimentos, UNLP-CONICET-CCT La Plata), Facultad Ciencias Exactas, Universidad Nacional de La Plata (UNLP), Calle 47 y 117, C.P. 1900 La Plata, Buenos Aires, Argentina
[c] Laboratorio de Citogenética y Mutagénesis, Instituto Multidisciplinario de Biología Celular (IMBICE, CONICET-CCT La Plata—CICPBA), C.C. 403, 1900 La Plata, Buenos Aires, Argentina

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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

* Corresponding author. Fax: +54 221 483 37 94x132/103.
E-mail address: grcastro@gmail.com (G.R. Castro).

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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