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Kefiran-alginate gel microspheres for oral delivery of ciprofloxacin

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ABSTRACT

Ciprofloxacin is a broad-spectrum antibiotic associated with gastric and intestinal side effects after extended oral administration. Alginate is a biopolymer commonly employed in gel synthesis by ionotropic gelation, but unstable in the presence of biological metal-chelating compounds and/or under dried conditions. Kefiran is a microbial biopolymer able to form gels with the advantage of displaying antimicrobial activity. In the present study, kefiran-alginate gel microspheres were developed to encapsulate ciprofloxacin for antimicrobial controlled release and enhanced bactericidal effect against common pathogens. Scanning electron microscopy (SEM) analysis of the hybrid gel microspheres showed a spherical structure with a smoother surface compared to alginate gel matrices. *In vitro* release of ciprofloxacin from kefiran-alginate microspheres was less than 3.0% and 5.0% at pH 1.2 (stomach), and 5.0% and 25.0% at pH 7.4 (intestine) in 3 and 21 h, respectively. Fourier transform infrared spectroscopy (FTIR) of ciprofloxacin-kefiran showed the displacement of typical bands of ciprofloxacin and kefiran, suggesting a cooperative interaction by hydrogen bridges between both molecules. Additionally, the thermal analysis of ciprofloxacin-kefiran showed a protective effect of the biopolymer against ciprofloxacin degradation at high temperatures. Finally, antimicrobial assays of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhymurium*, and *Staphylococcus aureus* demonstrated the synergic effect between ciprofloxacin and kefiran against the tested microorganisms.

1. Introduction

Ciprofloxacin (Cip) is a broad-spectrum antibiotic belonging to the family of fluoroquinolones. Cip is the fifth generic antibiotic in the world accounting for 24% of sales in the therapeutic market (close to USD 4340 million per year in 2014). Fluoroquinolones are commonly used for the treatment of many microbial infections because DNA gyrase and topoisomerase IV inhibition causes bacterial cell death [1]. On the other hand, Cip is associated with gastric and intestinal disorders in humans when the antibiotic is orally administered during long periods [2]. Additionally, Cip possesses low solubility in physiological aqueous media and propensity to molecular stacking by the π-π interactions because of the presence of the aromatic rings when it is administered at high concentrations, which decreases the antibiotic bioavailability. In order to improve Cip oral delivery, the development of novel systems able to capture, transport, or deliver the molecule is desirable.

Alginate (Alg) is a linear polysaccharide produced by some brown algae (*i.e.*, *Macrocystis pyrifera*, among others) and some bacteria (*i.e.*, *Pseudomonas aeruginosa*). Alg is composed of β-D-mannuronic and α-L-guluronic acids; it can be cross-linked in the presence of multivalent cations such as Ca^{2+}, Zn^{2+}, etc. Alg is used in many biotechnology applications because the gel texture is similar to that of the extracellular matrix and considered GRAS (Generally Regarded as Safe) by the FDA [3,4]. Also, Alg is biocompatible [5], of no thrombogenic nature [4], and low cost [6]. Nevertheless, Alg gels are unstable in the presence of cation chelating molecules such as phosphate, present in biological fluids. Likewise, the alginate tridimensional gel structure is lost after freeze-drying and rehydration in aqueous environments. In order to prevent a drastic Alg gel swelling and to produce matrix structure stabilization, one feasible strategy is to combine Alg with other polymers [7]

Kefiran (Kef) is a water-soluble glucogalactan produced by *Lactobacillus kefiranofaciens* and is present in kefir grains [8]. Kef has

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a Newtonian behavior in diluted solutions, becomes pseudoplastic at high concentration, and it is able to form gels as a result of cryogenic treatment [9]. On the other hand, it has been reported that Kef modulates the gut immune system [10], protecting epithelial cells against *Bacillus cereus* toxin [11], and has many beneficial activities, such as antitumor, antibacterial [12], anti-inflammatory [13], healing [14] and antioxidant [15]. Also, some studies reported the antibiotic activity of kefiran against Gram-positive, Gram-negative bacteria and the yeast *Candida albicans* [9,16].

The aims of the present work were to develop hybrid Kef-Alg gel microspheres loaded with Cip, to examine the interaction and stability of ciprofloxacin in the matrix, in order to evaluate the antimicrobial activity of the formulation and the potential synergic or additive effect between Kef and Cip against pathogenic bacteria. Analyses of the gel microspheres were performed by infrared spectroscopy (FTIR), scanning electron microscopy, thermogravimetry, and antimicrobial tests in agar and liquid media.

2. Materials and methods

2.1. Materials

Cip (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid), low-viscosity sodium alginate, citric acid ($C_6H_8O_7$), sodium citrate ($C_6H_5Na_3O_7$), calcium chloride ($CaCl_2$), potassium chloride (KCl), hydrochloric acid (HCl), potassium phosphate (KH_2PO_4), potassium bromide (KBr), brain heart infusion broth, Mueller-Hinton medium and nutrient broth were purchased from Sigma-Aldrich (St. Louis, Mo, US).

2.2. Kefiran purification

The purification procedure was based on the protocol previously described by Piermaria et al. with some modifications [17]. A weighted amount of kefir grains was suspended in boiling water for 30 min with discontinuous stirring, after that, the mixture was centrifuged at 10,000*g* for 20 min at 20 °C to separate the biomass. The supernatant was precipitated by addition of three volumes of cold ethanol (left at −20 °C overnight). Then, the mixture was centrifuged at 10,000*g* for 20 min at 4 °C, and the resulting pellets were dissolved in hot water. The analysis of protein content in the samples was performed by the Coomassie Blue technique using BSA (fraction V) as standard [18]. The precipitation procedure was repeated twice, and no protein content was detected in the samples after purification. Finally, the precipitate was dissolved in hot distilled water and freeze-dried.

2.3. Interaction analyses between ciprofloxacin and kefiran

2.3.1. Binding assay

Based on the previously described protocol with some modifications [19], solutions of 100 μg/mL Cip and 1.0% (w/v) Kef were made separately in citrate buffer (pH = 5.0), then 450 μL of the buffer solution (with or without the polymer (control)) was mixed with 50 μL of Cip solution and stirred for 1 h. Later, 1.0 mL of cold absolute ethanol was added to the aqueous solution, and the samples were centrifuged at 10,000*g* for 20 min. Finally, the absorbance of the supernatant was measured at 279 nm. The binding percentage between Cip and Kef was obtained according to the following equation:

$$\text{Binding}(\%) = \frac{\text{Drug concentration in the control} - \text{Drug concentration in the assay}}{\text{Drug concentration in the control}} \times 100 \quad (1)$$

2.3.2. Viscosity determinations

Kinematic viscosities were measured at 2.0% (w/v) Alg-Kef blends as follows: 0.0% Alg—2.0% Kef, 1.5% Alg—0.5% Kef, 1.0% Alg—1.0% Kef, 0.5% Alg—1.5% Kef in a low temperature viscometer Herzog HVU482 (Integrated Scientific LTD, UK). The assays were performed according to the standard test method for kinematic viscosity of transparent and opaque liquids ASTM D445 at a constant temperature of 40 °C and in a range of 2 mm^2/s to 10,000 mm^2/s.

2.3.3. Fourier transform infrared spectroscopy (FTIR) analyses

The mixture Kef-Cip (1:2) made for FTIR determination was prepared from an aqueous solution of kefiran (1.0%, w/v) and ciprofloxacin (2.0%, w/v) in citrate buffer (pH = 5.0) and kept for 12 h at room temperature under stirring until total dissolution of both components. After that, the sample was frozen and freeze-dried for further analysis. The FTIR spectra were obtained using KBr pellets. Samples were pressed into KBr (0.1%, w/v), and the FTIR spectra were recorded in a BOMEM-Hartmann & Braun MB-series spectrometer (Germany) with resolution of 4 cm^{-1}, 32 scans per minute and transmittance technique. The scanning range was from 400 cm^{-1} to 4000 cm^{-1}. The data obtained were analyzed using the ACD/NMR processor academic edition.

2.4. Thermal properties

The thermal analyses of Kef, Cip and the mixture of Cip-Kef (1:2) samples were carried out using the Netzsch Sta 449 F3 *Jupiter* thermal analyzer (Germany). Five mg of each sample was placed in the equipment and scanned at a heating rate of 10 °C/min at temperatures ranging from 20 °C to 800 °C.

2.5. Preparation of gel microspheres

The ionotropic gelation of 2.0% (w/v) Alg-Kef blends using different polymer ratios were studied. Microspheres were prepared by the jet technique, by dropping 2.0 mL of the blend solutions in 500 mM $CaCl_2$ at 0 °C. Later, the gel microspheres were washed with ultrapure water twice and kept at 5 °C.

Aqueous solutions containing 100 μg/mL Cip, 1.0% (w/v) Kef and 1.0% (w/v) sodium alginate were prepared in citrate buffer (pH = 5.0) in an ice water bath (0 °C), as previously reported [19]. Alternatively, biopolymer blends containing only the antibiotic were made with 1.0% (w/v) sodium alginate or kefiran.

The percentage of encapsulation was calculated with the following equation:

$$\text{Encapsulation}(\%) = \frac{\text{Initial concentration of the drug} - \text{Drug concentration in calcium solution}}{\text{Initial concentration of the drug}} \times 100 \quad (2)$$

The stability of Alg-Kef formulations using different polymer ratios up to 2.0% (w/v) was qualitatively assayed by incubating the gel microspheres in PBS (phosphate buffer saline) solution at room temperature, and determining the time for microsphere total dissolution. The results were expressed as gel strength.

2.6. Scanning electron microscopy (SEM) and roughness analysis

Gel microspheres were freeze-dried for 24 h before SEM observations. Furthermore, samples were prepared by sputtering the sample surface with gold using a Balzers SCD 030 metalizer, obtaining a layer thickness between 15 and 20 nm. Microsphere surfaces and morphologies were observed using Philips SEM 505 (Rochester, USA), and processed by an image digitizer program (Soft Imaging System ADDA II (SIS)).