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Development and characterization of new enzymatic modified hybrid calcium carbonate microparticles to obtain nano-architectured surfaces for enhanced drug loading

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ABSTRACT

Hypothesis: Biopolymer–$CaCO_3$ hybrid microparticles exposed to hydrolytic enzymes can provide new surface tailorable architectures. Soluble Alginate Lyase hydrolyzed alginate chains exposed on microparticle surface are generating considerable matrix changes. The change of porosity and surface to volume ratio is expected to influence absorption of drugs, thereby affecting controlled release profiles. The developed hybrid system potentially shows interesting properties for lung drug administration.

Experimental: Hybrid microparticles were developed by colloidal co-precipitation of $CaCO_3$ in presence of biopolymers: alginate (Alg) or Alg–High Methoxylated Pectin (HMP), followed by treatment with Alginate Lyase (AL). Surface architectures were observed by SEM. The increase in area to volume ratio was confirmed by BET isotherms. Also, enzymatic changes were elucidated by biophysical methods (EDAX, DSC, FTIR, XRD) and determination of the total carbohydrates content. Levofloxacin (a fluoroquinolone antibiotic) as model drug was incorporated by absorption. The drug release profile and the antimicrobial activity of the microparticles were tested against *Pseudomonas aeruginosa*.

Findings: After enzyme treatment, microspheres showed 4 μm diameter and increased porosity. While $CaCO_3$–Alg microspheres resulted in a rougher surface, $CaCO_3$–Alg–HMP ones exhibited "nano-balloon" patterns on surface. Both AL-treated microparticles showed up to 3 and 7 times higher Levofloxacin encapsulation than no treated ones. Microparticles showed controlled drug release profiles and enhanced antimicrobial effect. The present work demonstrates a significant progress in the development of new carriers with potential application for lung infections treatment.

1. Introduction

Calcium carbonate ($CaCO_3$) is one of the most abundant existing minerals in the nature and an important material in the industrial and pharmaceutical fields. Six different polymorphisms of $CaCO_3$ have been reported, but vaterite is the most interesting for microparticles production. Vaterite particles can be synthesized at laboratory scale by two methods: supercritical and chemical synthesis. They showed a spherical geometry with a small crystal size and narrow distribution [1]. Besides size parameters, porous is another special feature to consider in particles formation, as they provide unique properties like enhanced drug absorption and release kinetics, in addition to large surface to volume ratio and low density [2].

These properties make $CaCO_3$ microparticles attractive carriers for pulmonary delivery, as they are inert material and provide sizes around 5 μm that are suitable for nasal administration [3]. It has been demonstrated that particles larger than 5 μm mainly accumulate in the periphery of the lung and escaped through phagocytic clearance mechanisms [4]. Furthermore, pulmonary delivery showed some advantages in comparison with other administration ways. The drug is directly administered in the affected lung and local drug concentration could be easily manipulated by the dose. In the case of lung infections treatments, not only the bacterial killing is enhanced, but also the proliferation of resistant microorganisms is reduced [5]. Inhaled microparticles are effective therapeutic carriers for non-invasive systemic delivery of drugs. They can offer

Abbreviations: AL, Alginate Lyase; Alg, alginate; BET, Brunauer Emmett Teller; BJH, Barrett Joyener Halenda; HMP, High Methoxylated Pectin; DSC, differential scanning calorimetry; EDAX, energy dispersive X-ray spectrometry; FTIR, Fourier transformed infrared spectroscopy; HMP, High Methoxylated Pectin, Levo, Levofloxacin; LMP, low methoxylated pectin; MMP, medium methoxylated pectin; SEM, scanning electron microscopy; XRD, X-ray diffraction.

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a controlled release profile of the drug, prolonging the airways residence time in the lungs and decreasing the dosage and drug side effects in patients. Some reports, describe $CaCO_3$ systems as useful carriers for intranasal delivery of insulin and hydrophilic compounds, because of their easy production and slow biodegradability [3,6–7].

However, $CaCO_3$ matrices showed some limitations in drug delivery, such as stability in physiological environments and low drug encapsulation efficiency. Drugs or bioactive molecules were adsorbed mainly on the surface of the solid $CaCO_3$ particles. As a consequence, the binding of the adsorbed drug to $CaCO_3$ matrix was not strong enough resulting in an insufficient sustained release or targeting [8].

For these reasons, hybrid microparticles become an innovative alternative to overcome their limitations [9]. Aqueous mixtures of biopolymers and $CaCO_3$ under controlled colloidal precipitation conditions produce hybrid particles displaying molecular sustained release properties, biocompatible design and versatility of the matrix [10–11].

In this scenario, alginate and pectins were selected due to their desirable properties for medical applications, such as biocompatibility, lack of toxicity of their degradation products and no immunogenic response. Alginates (Alg) are linear polysaccharides composed of β-manuronic (M) acid and α-guluronic (G) acid linked by 1–4 bounds. They can be easy crosslinked in presence of divalent ions making hydrogels by the so called "egg box junctions" [12]. Pectins are built of linear polysaccharides of partially methoxylated poly[α-(1,4)-D-galacturonic acids]. They showed different esterification degrees (ED) and they are classified in three main groups: low-methoxylated (LMP) with ED < 40%; medium-methoxylated (MMP) with ED between 40% and 60%; and high-methoxylated pectins (HMPs) with ED > 60%. Pectins can be gelled by multivalent cations or at acidic pHs [13].

Considering that hybrid biopolymeric microparticles could be easily tailoring by taking into account the chemical composition, a biocatalyst modification was proposed. The use of hydrolytic enzymes with specific activity on their natural substrates could play a major role in providing novel architectures with new properties and capabilities. Changes in porosity, surface patterns and biogel network directly impact on the particle structure and properties. Consequently, the loading efficiency and release of molecules carried as cargo by the microparticles can be tailored [14–15].

Among the biopolymeric hydrolases, Alginate Lyase (AL) was selected as the enzyme to modify the hybrid matrices. The AL is depolymerizing enzyme acting over mannuronate or guluronate residues of alginate via beta-elimination mechanism [15–16]. In addition, it was reported that AL is capable to be active on gelled forms of alginate [17]. However, alginate lyase is unable to hydrolyze pectins composed only by galacturonic residues, reason why its use in alginate–pectin blends could allow to establish a specific tailoring of hybrid particles.

Based on the developed matrices and the purpose modifications, the adsorption of a model drug was proposed. Levofloxacin was a candidate, considering its broad spectrum against pathogens and its application in the treatment of bacterial respiratory infections. An early stage of aggressive antibiotic therapy is essential for preventing bacterial establishment and the consequent biofilm formation. In particular, Levofloxacin is commonly used in treatment of many bacterial infections, including respiratory, urinary tract, gastrointestinal, and abdominal infections. Also, Levofloxacin is considered the safest among the fluoroquinolones, with a low rate of hepatic abnormalities [18]. However, common side effects of the drug are affecting the gastrointestinal tract and other organs producing nausea or vomiting, diarrhea, headache, and constipation. Also, the alteration of the normal flora from the colon usually produces pseudomembranous colitis [19]. Due to these undesirable side effects, a controlled release of the drug is a relevant aspect to be considered. In this sense, the use of the $CaCO_3$ hybrid particles as vehicles for the safe administration of Levo via inhalatory therapy could be a feasible therapeutic alternative in the future. Also, an understanding of the mechanism involved in the enzymatic modifications of the hybrid matrices could bring new avenues for the production of novel engineered materials with potential biomedical applications.

The aim of the present work is to develop and characterize hybrid biopolymer–$CaCO_3$ microparticles enzymatically treated with Alginate lyase, displaying innovative mixed gel surface architectures with a desirable size in a narrow distribution. Levofloxacin was used as a drug model to study the loading and extended release for potential application in pulmonary drug delivery. The changes produced by the enzymatic treatment were analyzed by SEM, EDAX, FTIR, XRD, nitrogen adsorption isotherms and DSC studies. The effect of Levofloxacin loading and drug release from microparticles is also discussed. Finally, the antibacterial capacity of the hybrid microparticles containing Levofloxacin was tested against *Pseudomonas aeruginosa*.

2. Experimental

Levofloxacin (Levo, (*S*)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-7*H*-pyrido[*1,2,3-de*]-1,4-benzoxazine-6-carboxylic acid), apple pectin (DE: 70–75%), Glycine (Gly) and Alginate Lyase (AL) from *Flavobacterium multivorum* able to degrade poly(G) and poly(M/G) [16] were provided by Sigma–Aldrich (cat # A-6973, Buenos Aires, Argentina). Sodium Alginate (MW_{av} = 120 kDa) was purchased from Monsanto (Buenos Aires, 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 (Darmstad, Germany) or similar brand.

2.1. Synthesis of hybrid calcium carbonate/biopolymer microparticles

Hybrid microparticles were synthesized by colloidal crystallization of $CaCO_3$, in presence of Glycine (Gly) buffer and biopolymers: alginate or high methoxyl pectin (HMP) as previously reported [20]. Briefly, 9.0 ml of an aqueous solution of Na_2CO_3 (3.2% w/v) prepared in miliQ water were mixed with 2 ml of the biopolymeric solution of alginate or alginate/HMP at 1.0% (w/v). Then, 9.0 ml of 3.2% (w/v) of $CaCl_2$ in Gly buffer (pH = 10.0) were added and stirred at 1000 rpm in an ice bath for 5 min. The precipitated products were collected by centrifugation at 10,000*g* for 10 min. The resulting precipitate was washed with miliQ water. Later, the samples were resuspended in water, freeze with liquid N_2 and lyophilized. Finally, the obtained light powder was stored in vacuum desiccators at room temperature until further use.

2.2. Modification of microparticles by Alginate Lyase treatment

A mass of 25 mg of hybrid microparticles was weighted and incubated with 1.5 ml of Alginate Lyase solution (1.0 mg/ml; 40 EU/ml in 25 mM phosphate buffer mM, pH = 7.4) at 37 °C for 48 h. Then, microparticles were washed twice with miliQ water and lyophilized. Controls without enzyme were done.

The AL activity was measured by the detection of oligomeric unsaturated residues produced per minute at 37 °C. The rate of double-bond product formation was assayed using 4600 M^{-1} cm^{-1} molar absorptivity at 233 nm by continuous recording in an UV–visible spectrophotometer as previously reported [21]. The degradation rate of AL on $CaCO_3$/Alg and $CaCO_3$/Alg–HMP microparticles