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Development and evaluation of thymol-chitosan hydrogels with antimicrobial-antioxidant activity for oral local delivery

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ABSTRACT

Nowadays, the research of innovative drug delivery devices is focused on the design of multiple drug delivery systems, the prevention of drug side effects and the reduction of dosing intervals. Particularly, new mucosal delivery systems for antimicrobials, antioxidants and anti-inflammatory drugs has a growing development, regards to the avoidance of side effects, easy administration and a suitable drug concentration in the mucosa. In this work, chitosan hydrogels are evaluated as a biodegradable scaffold and as a bioactive agent carrier of an antioxidant-antimicrobial compound called thymol.

Throughout the study, swelling behavior, viscoelastic properties and thermal analysis are highlighted to present its advantages for a biomedical application. Furthermore, the *in vitro* results obtained indicate that thymol-chitosan hydrogels are biocompatible when exposed to [3T3] fibroblasts, exhibit antimicrobial activity against *Staphylococcus aureus* and *Streptococcus mutans* for 72 h and antioxidant activity for 24 h. These are desirable properties for a mucosal delivery system for an antimicrobial-antioxidant dual therapy for periodontal disease.

1. Introduction

Today, the development of innovative drug delivery devices is centered in the design of multiple drug delivery systems [1,2], the prevention of drug side effects and the reduction of dosing intervals [3]. Accordingly, one strategy to avoid side effects is the development of local delivery systems [4–7]. Thus, dermal, mucosal, ocular and inhalation administration sometimes help to prevent them [8–10].

Particularly, there are some characteristics of the mucous membrane that influence the design of new mucosal delivery systems. The mucous membranes do not possess a keratinized outer layer as the skin and are therefore much more sensitive to irritants. However, they have a good permeability profile, making them a suitable site for drug delivery.

Additionally, the oral cavity is an environment with a high percentage of moisture with natural secretions like saliva that tend to dilute drugs, so that to achieve a continuous action, multiple applications are required.

Furthermore, usually diseases imply complex and diverse mechanisms in which are needed a variety of molecules to achieve a suitable treatment. Periodontal disease is an example. Bacteria that cause periodontal tissue destruction and inflammation are present throughout every stage of the disease [11,12]. Therefore, antimicrobials, antioxidants, anti-resorptives and anti-inflammatory drugs are required along the treatment [13–18].

Nowadays, some commercial products for periodontal disease include mouthwashes which are applied daily, systemic antibiotics and devices for local delivery of bioactives [19,20].

Taking this into account, thymol has promising characteristics for oral therapy. It has antimicrobial [21–23] and antioxidant properties [24] as well as suitable organoleptic characteristics for oral delivery. Nevertheless, it is slightly soluble in water and volatile. These disadvantages are clearly obstacles to develop an oral delivery device.

Chitosan is a linear polysaccharide constituted by glucosamine and *N*-acetyl-glucosamine derived from crustacean shells that has promising features for biomedical applications. Chitosan amine groups can establish ionic interactions while acetamide groups can participate in hydrophobic interactions, leading to a broad spectrum of candidate

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molecules for drug delivery and high adsorption capacities [25].

Moreover, chitosan functional groups such as hydroxyl and amine groups can be chemically modified [26]. The addition of alkyl and carboxymethyl groups to chitosan changes its physical, chemical and mechanical properties [27]. Interestingly, many advances have been accomplished in chemical and enzymatic modifications. In case of enzymatic modifications, they have demonstrated high specificity while using environmentally friendly reaction conditions [28].

Chitosan has also shown antimicrobial activity [29–31]. Nevertheless, some discrepancies are found in literature. Antimicrobial activity is influenced by polymer factors like deacetylation degree and external factors such as type of microorganism and pH. Additionally, there are several hypotheses of chitosan mechanism of action. Some authors postulate chitosan as a chelating agent, others as a polycationic polymer that binds to microorganisms anionic cell surface proteins [32].

Besides, chitosan cytocompatibility was studied in many types of cells (*i.e.*: osteoblasts, fibroblasts, neural cells) [33–35]. Cells can adhere and proliferate in chitosan matrix showing good biocompatibility. Some authors argue that the homology between chitosan biopolymer and native hyaluronic acid or other extracellular glucosamines could be one of the reasons of its biocompatibility [36]. For these reasons, chitosan biopolymer and composites are considered as good candidates for tissue engineering and wound healing [37–39].

Furthermore, chitosan is a biodegradable polymer. Indeed, lysozyme and some proteinases can degrade chitosan into well known and non-toxic products. Biodegradable scaffolds and delivery devices have promising clinical applications, because they usually show good patient compliance and acceptance [40].

Lastly, chitosan is a mucoadhesive polymer [36,41,42]. Mucoadhesion is a process in which chemical interactions are established between the mucin and the biopolymer. The nature of these interactions differs according to the biopolymer characteristics. The mucoadhesion process can be divided in two different events. Firstly, the polymer gets wet and expands into the mucin network. Secondly, covalent, ionic and hydrogen bonds, Van der Waals forces and electrostatic interactions are established between the polymer and the mucin. In the case of chitosan, amine and hydroxyl groups form hydrogen bonds with mucin and also its linear structure gives good flexibility to the polymer encouraging mucoadhesion [43]. In conclusion, chitosan biopolymer has good qualities for the development of drug delivery and tissue regeneration biomaterials [44,45].

Particularly, chitosan hydrogels can be used as carriers for drug release and as a biodegradable scaffold for periodontal tissue regeneration. In periodontal disease therapy, not only it is important to treat signs and symptoms, but also the recovery of lost tissue. Nowadays, the complete recovery of periodontal tissue still seems too far to achieve. Nevertheless, major advances have been carried out in alveolar bone regeneration thanks to ceramic materials such as silica, hydroxyapatite and calcium phosphate [46,47] given that bioactive ceramic materials encourage bone formation [48]. Additionally, there is a growing development in periodontal soft tissue regeneration regards to organic materials like collagen and chitosan [49,50,51].

Herein, a chitosan hydrogel developed by a spraying method with rheological properties, swelling behavior and a porous structure suitable for drug delivery is presented as an alternative for periodontal disease treatment. We promoted sol-gel transition with an alkali solution spray incorporating thymol, an antimicrobial-antioxidant compound in the resulting hydrogels. Cytocompatibility assays with [3T3] mouse fibroblasts were performed confirming its biocompatibility. In addition, the incorporation of thymol into chitosan polymeric network, its release kinetics as well as its antimicrobial and antioxidant activities were tested in the search of a biomaterial that fulfills the requirements of a biomaterial with dual therapy properties for periodontal disease.

2. Experimental

2.1. Reagents and materials

Low-viscosity chitosan obtained from crab shells, structural viscosity 66 mPas, Deacetylation > 75.0%, lysozyme from chicken egg white (100,000 U/mg) and thiazolyl blue tetrazolium bromide reagent, DPPH (2,2-diphenyl-1-picrylhydrazyl), DAB (diaminobencidine tetrahidrochloride) and epinephrine were purchased from Sigma–Aldrich (St Louis, USA). Folin-Ciocalteu's phenol reagent was obtained from Merck (Darmstadt, Germany). Dulbecco's modified Eagle's medium, fetal calf serum, penicillin and streptomycin were purchased from Gibco. Todd-Hewitt broth was obtained from Britania Lab. All other reagents were of analytical grade.

2.2. Chitosan hydrogel synthesis

A 20 $mg \cdot mL^{-1}$ chitosan solution was prepared dissolving the chitosan powder in 1% (v/v) acetic acid. Stirring for almost 15 min was necessary to obtain a homogenous solution. Afterwards, 1 mL of chitosan solution was poured in each well of a 24-well culture plate, used as a template. Then, a 1 N NaOH solution was sprayed on the template and left for 5 h at room temperature. Finally, the NaOH solution was removed and the hydrogels obtained were washed with deionized water until neutral pH was reached.

2.3. Chitosan hydrogel ultrastructural characterization

Chitosan hydrogels were analysed by scanning electron microscopy (SEM). Samples were fixed with a solution of glutaraldehyde (10% *v*/v in PBS) for 1 h at 4 °C. Following fixation, samples were washed three times with PBS and then freezed at − 80 °C. Finally, the samples were freeze-dried and gold sputter-coated for analysis using a Zeiss SUPRA 40 microscope.

Transmission electron microscope (TEM) analysis was performed in a MET Zeiss 109. Following fixation and wash as described above, the samples were post-fixed using a 5% osmium tetra-oxide in a pH 7.4 cacodylate/saccharose buffer (0.05 M/0.3 M) for 1 h at 4 °C. Afterwards, samples were dehydrated and embedded in epoxy resin. Thin sections were cut with an ultra microtome and contrasted by phosphotungstic acid. Recovered sections were imaged using a Zeiss 109 microscope.

2.4. FTIR characterization

Fourier transform infrared (FTIR) spectra from lyophilized chitosan hydrogels were carried out with an FTIR-Raman Thermo Scientific Nicolet model 50 computer IS, which is coupled with an attenuated total reflection device (ATR). Scanning range was 4000–500 cm^{-1}. Spectra was processed by Thermo Nicolet OMNIC software.

2.5. Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) measurements were performed on a Shimadzu DSC-50 instrument. Temperature was calibrated with In (157 °C, 3.3 J mol^{-1}). Approximately 20 mg of the freeze-dried samples were first equilibrated at 25 °C and then heated from 25 to 240 °C at a constant rate of 10 °C min^{-1}, under a nitrogen flow of 50 $mL \cdot min^{-1}$.

2.6. Swelling studies

Chitosan and thymol-chitosan hydrogels were freeze-dried. Afterwards, they were weighted (W_o) and stored in buffer phosphate saline to allow water uptake. Hydrogels were removed from PBS and weighted (W) at different time points (t) after removing the excess of