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Influence of collagen addition on the thermal and morphological properties of chitosan/xanthan hydrogels

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ABSTRACT

This study investigates the collagen influence on thermal and morphological characteristics of chitosan/xanthan hydrogels for potential tissue engineering applications. Anionic collagen was prepared by selective hydrolysis of type I collagen found in bovine tendons. Chitosan was obtained from the partial deacetylation of squid pen β-chitin and xanthan was acquired from Fluka. The hydrogels were obtained in different ratios and were characterized by thermal and morphological analysis. FT-IR suggested only electrostatic interactions between NH_3^+ groups of chitosan and COO^- groups of xanthan and collagen. Thermogravimetric curves showed that hydrogels contain a great amount of water (above 98%) and the presence of collagen does not change this characteristic. Freezing-bound water transition in DSC curves was shifted to higher values due to the increase of water/polymer interaction, mainly when different ratios of chitosan and xanthan were used. SEM images showed sheet-form structures with the presence of collagen promoting an increase in pore size.

1. Introduction

Chitosan is a linear polymer and the principal derivate of chitin. One of the most important parameters in the characterization of chitosan is the degree of acetylation (DA), defined as the ratio of the number of formed NH_2 groups and the initial number of $NHCOCH_3$ groups present in chitin [1]. Chitosan is biocompatible, biodegradable, and used in a variety of biomedical fields such as drug delivery carriers, surgical thread, and wound healing [2].

Xanthan is an extracellular polysaccharide produced by the bacterium *Xanthomonas campestris*, which is composed of a cellulosic backbone with a three-sugar side chain attached to every second backbone residue. Its chemical structure is composed of glucose, mannose, glucuronic acid acetate and pyruvate and the glucuronic acid gives this polymer a negative charge [3]. Xanthan gels have application in a variety of biomedical areas such as ophthalmology, implantology, tissue engineering and controlled drug release systems [4].

Currently, at least 29 types of collagen have been isolated, varying in the helix length and the nature and size of the nonhelical portions [5]. Type I collagen is predominant in higher order animals, especially in the skin, tendon, and bone. In this study, chemically modified of collagen was performed using an alkaline hydrolysis reaction, which produces a negatively charged anionic collagen matrix at pH 7.4. Increasing the alkaline hydrolysis time of carboxyamide groups of asparagines (Asn) and glutamines (Gln) present in the collagen type I structure increases the number of negative charges [6].

Hydrogels are hydrophilic three-dimensional polymeric networks that can absorb much more water than their own weight [7], hence providing ideal aqueous conditions for biocompatible applications such as tissue engineering and drug delivery systems [8]. Chitosan and xanthan in solutions are polyelectrolytes with potentially ionizable groups (NH_3^+ group of chitosan and COO^- group of xanthan) and when they are mixed in an aqueous solution, a complex is formed because of the electrostatic attraction [9]. Chitosan/xanthan polyelectrolyte complexes have been previously studied [10–15]. Since collagen promotes cell and tissue attachment and growth, this study addresses the preparation of chitosan/xanthan/collagen hydrogels to verify the influence of this protein on chitosan/xanthan hydrogel characteristics.

The addition of collagen enables the improvement of hydrogel properties, e.g., in burn treatments and in addition to the hydration of the wound by chitosan/xanthan hydrogels the presence of the protein can stimulate and promote cellular growth. This study focused on a combination of the best features of the three different

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biopolymers in order to obtain a potential material to use in tissue regeneration.

2. Materials and methods

2.1. Hydrogel components

Pens of *Loligo* sp., 40.0 g were treated with HCl 0.55 mol L^{-1} at room temperature for 2 h. Next, the material was washed with water until neutral and dried at 37 °C. The obtained solid was heated in 0.3 mol L^{-1} sodium hydroxide at 80 °C for 1 h, washed with water until neutral and dried to obtain 14.4 g of a white chitin material. The obtained β-chitin was treated with NaOH 40% (w/w) at 80 °C for 3 h in nitrogen atmosphere [16]. After washing and drying, 11.7 g of chitosan was obtained. For preparing the scaffolds, a chitosan solution of 0.5% concentration was prepared by dissolving the chitosan in 1% acetic acid, with constant stirring for 24 h, at room temperature.

The degree of acetylation of chitosan was determined by conductometric titration [17] and the obtained value was of 10.53% ± 0.03. Molecular weight was determined by gel permeation chromatography (GPC) by dissolving 5.0 mg of chitosan in 2 mL of eluent (0.3 mol L^{-1} acetic acid/0.2 mol L^{-1} sodium acetate) during 24 h. The solution was filtered through a 1 μm filter Acrosdis (fiberglass) – 25 mm/Waters and injected into a liquid chromatograph HP – SEC, Shimadzu. The columns used were a pre-column Shodex OHpak SB-G + 2 columns Shodex OHpak SB (805HQ + 803HQ) with a flow rate of 0.8 mL min^{-1} at 35 °C. Pullulan standards at 2 mg mL^{-1} concentration were used for calibration curve. The molecular weight value obtained for chitosan was 4.28×10^5.

Anionic type I collagen was obtained by treating the bovine tendon in an aqueous alkaline solution (pH 13), for 24 h using a protocol developed in previous studies in our laboratory [6,18]. In short, the bovine tendon was treated at 20 °C with an alkaline solution (3 mL g^{-1} of tissue) containing salts (chlorides and sulfates) of alkaline (K^+ and Na^+) and alkaline earth metals (Ca^{2+}). The material was suspended in deionized water, the pH was adjusted with acetic acid (pH 3.5) and then stored under refrigeration (4 °C). Molecular weight of collagen was determined in our laboratory [19] by electrophoresis of SDS/polyacrylamide and found that the ratio α1/α2 was close to 2:1, confirming the predominance of collagen type I. The tropocollagen weight is around 300 kDa. The collagen concentration was determined by lyophylization ($n = 3$) and adjusted to 0.5% with acetic acid pH 3.5.

Xanthan was supplied by Fluka (molecular weight up to 6 million Daltons) and used without purification and a solution (0.5%) was prepared by dissolution in deionized water.

2.2. Hydrogel preparation

The chitosan solution was added to the xanthan solution, with constant stirring, in different ratios: 1:2, 1:1 and 2:1 (w/w) and namely (CX12), (CX11) and (CX21), respectively. For chitosan/xanthan/collagen hydrogels, the mixture of chitosan and xanthan was initially prepared, next the collagen solution was added with constant stirring in ratios of (w/w/w): 1:2:0.5 (CX12Col); 1:1:0.5 (CX11Col) and 2:1:0.5 (CX21Col). The hydrogels were dialyzed against McIlvaine buffer (sodium phosphate – citric acid) pH 5.6 for 4 days with constant stirring, washed with deionized water and stored under refrigeration (4 °C) until their use.

2.3. Hydrogel rehydratation

The frozen scaffolds were lyophilized in a freeze-dryer for at least 2 days to completely remove the solvents. They were used in the rehydratation processes that consisted in the centrifugation of freeze-dried hydrogels (5 g) for 30 min at 3000 rpm with 2 g of deionized water. The rehydrated hydrogels were labeled with "_r" which represents the rehydratation process. These hydrogels were characterized by thermogravimetric curves to verify the capacity of network to retain the water in the structure after the lyophilization.

2.4. Fourier transform infrared spectroscopy

FT-IR analysis was performed in films by casting the hydrogels in silicon molds and dried at room temperature. FT-IR spectra were obtained using a Bomem Michelson Series at 400 a 4000 cm^{-1} interval with 4 cm^{-1} resolution.

2.5. Thermogravimetry and differential scanning calorimetric measurements

Differential scanning calorimetric (DSC) was used to measure the temperature transition of the freezing-bound water in the hydrogel. 20 mg of the sample was put in a hermetically sealed aluminum DSC pan to prevent water loss during the scanning. The pan was cooled to −30 °C, and then heated to 120 °C at a heating rate of 10 °C min^{-1}, in nitrogen atmosphere using a DSC-2010 (TA Instruments). The phase transition of water was recorded at the endothermic peak near 0 °C. Thermogravimetric (TG/DTG) curves were obtained using a TGA-2050 module at a heating rate of 10 °C min^{-1} between 25 and 800 °C, in synthetic air atmosphere. The sample sizes were about 30 mg. For the rehydrated hydrogels, thermogravimetric analysis was performed under the same conditions as previously described.

2.6. Scanning electron microscopy (SEM)

The hydrogels' morphology was examined using a Zeiss LEO-440 SEM apparatus. Before the examination, they were freeze-dried and coated with 20 nm of alloy gold–palladium in a Balsers SDC model.

3. Results and discussion

The complexation reaction between chitosan and xanthan occurs due the interaction among the opposite charges present in the biopolymers (NH_3^+ groups of chitosan and COO^- groups of xanthan) [9] and due to the molecular interaction among the polymeric chains. The formation of a 3D network structure has an important advantage since the complexation process increases the mechanical and chemical stability (Fig. 1).

In all cases, after the dialyzing and washing process, the hydrogels showed a rigid appearance, maintaining their form, with a white coloring and gelatinous aspect, independent of the chitosan/xanthan ratio and the presence of collagen (Fig. 2).

3.1. Fourier transform infrared spectroscopy

FT-IR spectra of the hydrogels CX11 and CX11Col (Fig. 3) have the characteristic absorption bands of chitosan, xanthan and collagen, without any additional bands, suggesting that the interactions between polysaccharides are purely electrostatic. Other proportions showed the same behavior and the addition of collagen did not shift the bands.

Characteristic bands of chitosan were observed in FT-IR curves (Fig. 3): at 3420 cm^{-1} relative to the stretching OH groups, 1323 cm^{-1} – group —CO—N— of chitosan. An intense band at 1080 cm^{-1}, referent to the stretching groups C—O in the xanthan structure, was observed in the FT-IR spectra [20]. In addition, collagen bands at 1650 cm^{-1} and 1238 cm^{-1}, referent to amide I and