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Biophysical Chemistry 120 (2006) 154 – 159

Biophysical Chemistry

http://www.elsevier.com/locate/biophyschem

On the dielectric behaviour of collagen–algal sulfated polysaccharide blends: Effect of glutaraldehyde crosslinking

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Received 15 September 2005; received in revised form 1 November 2005; accepted 2 November 2005
Available online 5 December 2005

Abstract

In this paper, impedance measurements in the frequency range from 10^{-2} to 10^{6} Hz are presented for collagen and algal sulfated polysaccharide crosslinked films. We are considering the development of new biomaterials which have potential applications in coating of cardiovascular prostheses, support for cellular growth and in systems for controlled drug delivery. The effect of crosslink sulfated polysaccharide on the physical chemical properties of collagen was studied using FT-infrared spectroscopy, differential scanning calorimetry (DSC), dielectric spectroscopy. The resulting films crosslinked with glutaraldehyde (GA) in concentrations of 0.001% and 0.05% when analysed by DSC, showed that the GA treatment not only left the thermal stability of the collagen unaffected, but it also decreased the thermal transition energy. Dielectric spectroscopy shows that the effect of the crosslink on the blend film was associated to the decrease and stabilization of the dielectric permittivity at low frequencies and decreased its conductivity.

Keywords: Collagen; Sulfated polyssacharide; Glutaraldehyde; Dielectric property

1. Introduction

Blending has acquired importance in improving the performance of the polymeric materials. It has become an economical and versatile way to obtain materials with a wide range of desirable properties [1]. Blends of collagen [2], a biological material applied as biomaterial in the medicine field, with synthetic or natural polymer, can find interesting applications for developing useful materials that require some level of electrical conductivity such as in promoting osteoconductivity, nerve regeneration or hemocompatibility of biomaterials by surface modification [3]. To enhance the biocompatibility of surfaces is one of the most important aims for the development and use of artificial biomaterial and is a topic that had been studied intensively. Therefore, various surface modification attempts have been made to optimize these interfacial biological responses with an attempt to develop antithrombogenic materials [4]. The collagen have been known to platelet adhesion and it have been suggested its association with negatively charged proteoglycans to overcome these effects when is necessary antithrombogenic surfaces [5].

Collagen molecules (molecular weight 300.000) are rod-like triple helices, which are 300 nm in length and 1.5 nm in diameter. Collagen fibers possess a high degree of axial alignment of collagen molecules and are characterized by a regular stagger of approximately 1/4 of a rod length between each molecule and its axially aligned neighbor [6]. Collagen allows its structure to be modified using relatively simple techniques. Chemical modifications may include some classical treatments such as alkaline treatment. This process causes hydrolysis of carboxyamides of asparagine and glutamine residues from collagen in carboxylics groups [7]. Besides the increased negative sites content, the presence of extra carboxylic groups enhances the dielectric properties of collagen [8].

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doi:10.1016/j.bpc.2005.11.001

Whereas native collagen tissue posseses significant strength, this is lost when collagen products are made from soluble collagen. These reconstituted products may therefore require chemical treatment with crosslinking agents, so as to retain adequate strength for particular applications. Glutaraldehyde is the preferred reagent in the biomedical field and has been used extensively as a crosslinking agent for proteins and polysaccharides [9]. Cross-linking of collagen sample with glutaraldehyde involves the reaction of the free amine groups of lysine or hydroxylysine amino acids residues of the polypeptide chains with the aldehyde groups of the glutaraldehyde. The reaction that will take place is the formation of a Schiff base, thereafter a large variety of subsequent reactions may be involved in the cross-linking material.

Anticoagulant and antithrombotic activities are among the most widely studied properties of sulphated polysaccharides, occurring in marine algae and in a great variety of other organisms. In marine algae, they are present as sulphate fucose (fucoidans) and as sulphate galactans (carragennans and agars) [10]. Recently, there has been a scientific interest in systematic screening of biological activity of sulphated polysaccharide isolated from marine algae [11,12]. Red algal galactans are sulfated polysaccharides having usually a linear backbone built up of alternating 3-linked β-D-galactopyranose and 4-linked-αgalactopyranose residues. *Gracilaria* spp. that belongs to the Gracilariaceae family, is a red seaweed widely distributed in tropical Atlantic waters and one of the principal sources of commercial agars. The agar extracted by alkali treatment from *Gracilaria cornea* from Yucatan, Mexico, was studied in relation to the effect of season on the agar content and chemical characteristics and to the influence of alkali treatment [13–15].

Since collagen is constituted by polar repeating units of amino-acid (–CO–CR–NH–) and sulphated polysaccharide contains polar groups (COO^-, OSO_3^-, OH), the dielectric techniques are particularly sensitive to analyze relaxation phenomena. These techniques [16–20] used to study the effects of water and electric field frequencies on the dielectric properties of constituent phases of unmodified collagen. In view of the above, the aim of this work was to study the thermal and dielectric properties of crosslinked sulphated polysaccharide–collagen blends films considering the development of new biomaterials which have potential applications in coating of cardiovascular prostheses.

2. Experimental methods

2.1. Soluble anionic collagen preparation

To hydrolyze amide groups selectively, 50 g of bovine serosa, and in the wet state, were treated at 20 °C from period of 72 h, with an alkaline solution (3 ml of solution/g of tissue), salts (chlorides and sulfate), bases of alkaline (K^+ and Na^+), and alkaline earth metals (Ca^{2+}). The resulting materials were equilibrated with a solution containing Na_2SO_4, NaCl, KCl, and $CaSO_4$ (6 ml of solution/g of tissue) for a period of 12 h and the excess salts removed as described earlier [21]. The materials were suspended in deionized water, the pH adjusted to 3.5 with pure acetic acid and the mixture homogenized in a blender. Concentration of polyanionic collagen gels were adjusted to 10 mg/g as determined by hydroxyproline assay [22].

2.2. Isolation of soluble polysaccharide

Specimens of the red seaweed *Gracilaria cornea* (G) were collected in April, 2000 on the Atlantic coast of Brazil (Fleixeira Beach, Trairi, Ceará) cleaned of epiphytes, washed with distilled water and stored at −20 C. The sulphated polysaccharide was extracted from the dried tissue (100 g) by papain digestion, and partially purified by cetylpyridinium chloride, as described in the literature [23]. About 15.6 g (dry weight) of crude sulphated polysaccharide, denoted as crudeSP, was obtained after these procedures. The crudeSP (1 g) was dispersed in 100 mL of distilled water and stirred for 15 h at room temperature (25–28 °C). The water-soluble fraction (designated as solSP) was separated from the insoluble one (insolSP) by filtration in a sintered-glass plate of fine grade (4–5.5 μm pore size). The fraction was lyophilized and stored. The degree of sulphation detected was DS = 0.21 [14].

2.3. Preparation of the sulphated polysaccharide solution

The solution of the sulphated polysaccharide was obtained by homogenization in acetic acid solution at pH 3.5. The solution was centrifuged at 10,000 rpm for 1 h, and the dry matter of the suspension was determined by heating at 100 °C until constant weight. The result solution was brought to a final concentration of 10 mg/g.

2.4. Preparation of collagen–sulphated polysaccharide films

The films (CG) were prepared by adding the polysaccharide solution on soluble collagen (25% of G). The blends were casted in acrylic molds, and dried in laminar flow of air.

2.5. Crosslinking of films with glutaraldehyde (GA)

In fixation with GA, films pieces of 8 cm^2 were immersed in GA solution with variable concentrations (0%, 0.001% and 0.05%), respectively samples CG00, CG01, CG50 prepared in PB buffer pH 7.4, for 1 h at room temperature. After fixation, the pieces were treated in glycine solution (0.025 M glycine : 0.05 M borate, pH 9.2) for 10 min, washed exhaustively with water, dried in laminar flow of air.

2.6. FT-Infrared spectroscopy

Infrared (IR) spectra were recorded using ATR regime using a SHIMATZU FTIR-283B spectrophotometer in the wave number region 400–4000 cm^{-1}.

2.7. Thermal analysis

The thermal stability of collagen, polysaccharide and polysaccharide–collagen films, were determined by DSC,

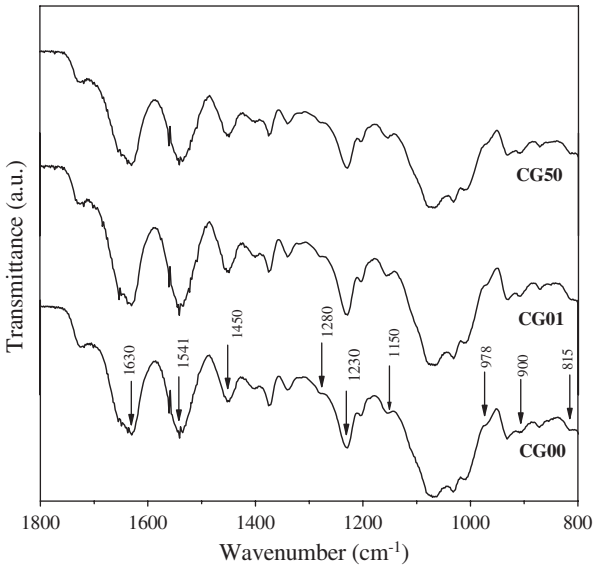

Fig. 1. FT-IR spectrum of collagen–sulphated polysaccharide films (COL–GC 25%) treated with GA (0.0%, 0.001%, 0.05%).

using an equipment Shimadzu DSC-50. Samples were sealed in aluminum cell and heated up with rate of 5 °C/min with N_2 atmosphere.

2.8. Impedance measurements

Impedance measurements were conducted at 25 °C using a Solartron SI 1260 Impedance/Gain Phase Analyzer, which is computer-controlled. In the measurements, an AC (alternating current) amplitude of 100 mV was employed and the AC frequency was in the range of 10 mHz to 1 MHz. The flat faces of the samples, with a size of 2×2 cm and thickness of 90 um, are painted with a circular silver electrode with a diameter of 1 cm. The relative error of the electrical measurements was less than ±1%.

3. Results and discussion

3.1. FT-IR spectroscopy

FT-IR spectroscopy results for collagen–sulphated polysaccharide films when treated with GA are shown in Fig. 1. For all samples the spectrum showed absorptions typical of collagen and sulphated polysaccharide. Collagen was characterized by its amide bands, amide I (C=O) at about 1629 cm^{-1}, a strong amide II (N–H) at 1541 cm^{-1} and a band centered at 1230 cm^{-1}, representing the amide III (C–N) vibrational modes. The absorption band near 1452 cm^{-1} due a stereochemistry of pyrrolidinics rings of proline [24] showed no shift in presence of sulphated polysaccharide. In the same Fig. 1, one can see the most characteristic bands relative to the presence of the sulphated polysaccharide, at 1280 cm^{-1} describing an assimmetrical S=O vibration and others between 815 and 900 cm^{-1} indicating a symmetrical C–O–S vibration associated to a C–O–SO_3 group [14]. Other bands are common for polysaccharides, such as values at 1150 cm^{-1} assigned to bending vibrational modes δ(C–O) due to the pyranose ring. The spectral range 1150–950 cm^{-1} is characterized by the contribution of bending δ(C–OH) modes. The band at 978 cm^{-1} was assigned to deformation of the axial (C–OH) at C-4 [25].

FT-IR spectrum for the films of CG00 when crosslinked with GA 0.001% and 0.05% (CG01 and CG50 receptively), were very similar with small modification in band intensity for highest concentrations of CG50. In all sample spectra, the absorption bands associated with amide I and II of collagen molecule were shifted to lower frequency as shown in literature [24]. It suggests small interaction between collagen and sulphated polysaccharide probably due by the presence of sulphate charge and conformational change in the collagen molecule.

3.2. Thermal analysis

Fig. 2 shows the DSC thermogram of all samples (CG00, CG01 and CG50), for which the denaturation temperatures were 74.8, 70.1 and 73.6 °C, respectively. The results indicate that heating temperature influences the films structure significantly and the increasing of crosslink leads to a moderate variations of the denaturation temperature of the protein. This can be explained by the presence of sulphated groups from the polyssaccharide, which could decrease the reactivity of free amine groups of lysine of collagen with GA. On other hand, denaturation enthalpies increased with increasing degree of crosslinking for both collagen species. For the higher crosslinked films (CG01 and CG50) a higher denaturation enthalpy was found (74.4 and 137.7, respectively) compared with the non-crosslinked film (59.4 J/g).

Glutaraldehyde stabilizes the collagen molecules and leads to a moderate increase of the denaturation temperature [26]. In the spectrum of CG00 blend, without treatment with GA, one can observe a single transition at 74.8 °C, with a denaturation enthalpy of 59.4 J/g. DSC spectras from crosslinked films,

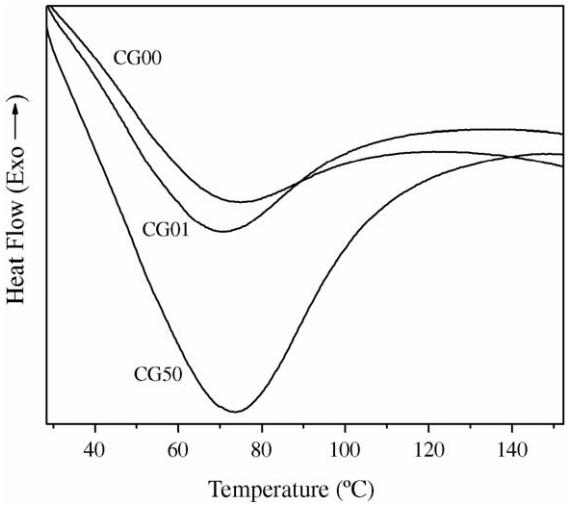

Fig. 2. DSC thermograms of collagen treated with different concentrations of GA.

wherein the denaturation temperatures were 70.1 °C for CG01 film and 73.6 °C for CG50, show that a higher denaturation enthalpy was found for both films, 74.4 and 137.7 J/g, respectively, compared with the non-crosslinked film.

Some authors found that very low degree of cross-linking tended to reduce sample stability and, as the cross-linking agent concentration increased beyond a certain value, there was an increase in the end temperature [27,28]. Since the glutaraldehyde concentration used in this work was quite low (0.05%) and the cross-linking reaction was heterogeneous, one can assume that, in this work, the cross-linking degree was quite low. A possible explanation for the decrease of thermal stability at low degree of cross-linking would be the formation of intra cross-linking reactions between collagen chains, which by its turn interferes with previously existing attractive hydrogen bonds, in those regions where cross-linking occurred. As a consequence, the cross-linked polymer's structure weakened, reducing its thermal stability [29]. This can be also explained by the presence of sulphated groups from the polyssaccharide, which could decrease the reactivity of free amine groups of lysine of collagen with GA. On other hand, denaturation enthalpies increased with increasing degree of crosslinking, indicating that these films differ in their water holding capacity and strength of water–polymer interaction. In the case of the cross-linked CG50 sample, the peak position was shifted to higher temperature, indicating that the water interaction with this network is stronger than with low cross-linked CG01 sample. The higher cross-linked film will have less amino groups available to form hydrogen bonds with water molecules. As a consequence, most of water molecules will be bound to sulf poliss hydroxyl groups instead of amino groups and, since the hydrogen bonds with the hydroxyl groups of polysaccharide are stronger than the ones with the amino groups, one could expect that a higher temperature would be necessary to remove such water molecules [30].

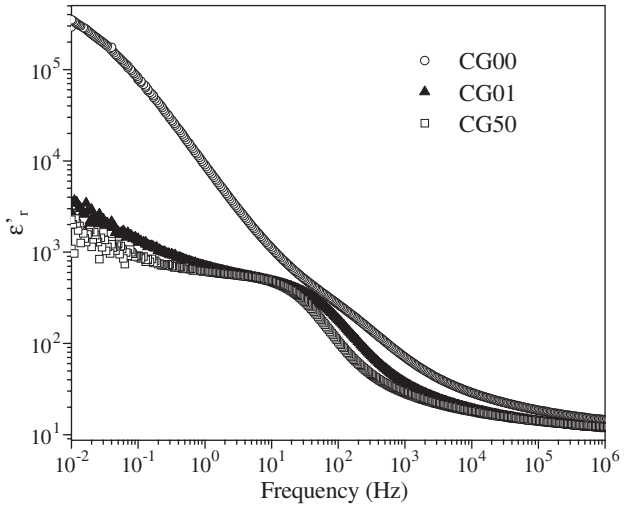

Fig. 3. Plots of the dielectric permittivity (ε'_R) vs. frequency at different concentrations of GA.

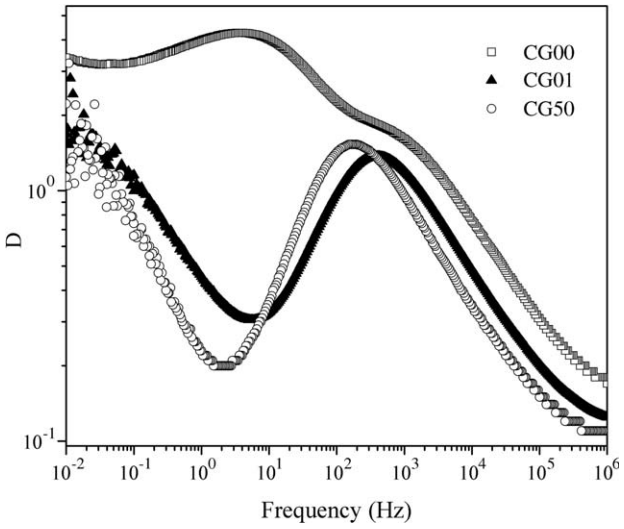

Fig. 4. Plots of loss tangent (D) vs. frequency.

3.3. Impedance measurements

Dielectric permittivity ε' was evaluated by measuring the capacitance (C) of the film [31]

$$\varepsilon'_R = \frac{Cd}{\varepsilon_0 A} = \frac{\varepsilon'}{\varepsilon_0}$$

where d=thickness of the film, A=effective area of the electrode, ε_0=permittivity of free space (8.85×10^{-14} F/cm), ε' is the real part of the dielectric constant and ε'_R denotes the polymer real relative permittivity depending on the angular frequency (ω), and the dissipation factor (D) is given by,

$$D = \tan\delta = \varepsilon''/\varepsilon$$

where ε'' is the imaginary part of the dielectric constant. From the results of the AC impedance spectroscopy, the specific conductivity σ_{ac} of the samples was calculated according to

$$\sigma_{ac} = = \omega\varepsilon'' = \omega\varepsilon_R' \varepsilon_0 \tan\delta$$

Fig. 3 shows the variation of ε'_R with frequency at different crosslinks: 0%, 0.001%, and 0.05%. From the plots, it is observed that the dielectric permittivity decreased with

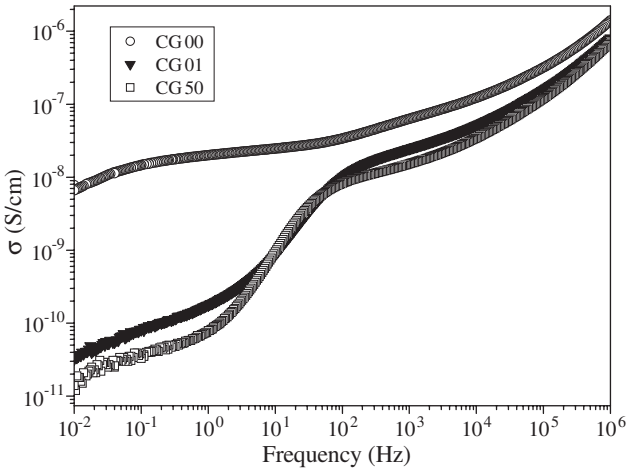

Fig. 5. Plot of the ac conductivity vs. frequency.

increasing frequency and attains a constant value at higher frequencies. Similar behaviour was observed in a number of polymers [32,33]. Verifying the fact that for polar materials the initial value of dielectric permittivity is high, but as the frequency of the field is raised the value begins to drop which could be due to the dipoles not able to follow the field variations at high frequencies and also due to electrode polarization effects. At high frequencies, the periodic reversal of the electric field occurs so fast that there is no excess ion diffusion in the direction of the field. The polarization due to the charge accumulation decreases, leading to the decrease in the value of ε'_R [34].

From Fig. 3, it is also clear that the dielectric permittivity is found to decrease with increasing crosslink at lower frequencies and attains a constant value at higher frequencies. The variation of ε'_R is different for crosslinked and non-crosslinked blends. For non-crosslinked film the ε'_R plots show that the dielectric permittivity decreased monotonically with increasing frequency. The effect of crosslink was to decrease and stabilize the dielectric permittivity at low frequencies, which can be related with the lower molecular mobility due to the crosslinked process.

Fig. 4 shows the variation of D with frequency at different crosslinks. From the plots, it is clear, for crosslinked film, that the peak frequency was shifted to higher frequencies with an increase in crosslink. The loss peaks and their shifts with crosslink suggest a dielectric relaxation process. The effect of crosslink on the conductivity of collagen–sulphated polysaccharide films were also studied. Fig. 5 shows the logarithmic plots of the conductivity as a function of frequency for unmodified film (CG00) and crosslinked films (CG01 and CG50). The curves present a nonlinear increase of the conductivity with frequency. For low frequencies, in the range below 10 MHz, one can see that the values of σ_{ac} are significantly higher for CG00 film than crosslinked films CG01 and CG50, suggesting a relaxation process.

4. Conclusions

The effect of crosslink sulphated polysaccharide on the physico-chemical properties of collagen was studied using FT-infrared spectroscopy, differential scanning calorimetry (DSC) and dielectric spectroscopy. The resulting films crosslinked with glutaraldehyde (GA) in concentrations of 0.001% and 0.05% when analysed by DSC, showed that the GA treatment does not affect the thermal stability of the collagen and it also increased the thermal transition energy. Dielectric spectroscopy showed that the effect of the crosslink on the blend film was to decrease and stabilize the dielectric permittivity and conductivity at low frequencies. The frequency-dependent conductivity and dielectric relaxation are both sensitive to the motion of charged species and dipoles of the macromolecules in the films.

Acknowledgments

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Fundação Cearense de Amparo à Pesquisa (FUNCAP).

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