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Interaction of anionic collagen with chitosan: Effect on thermal and morphological characteristics

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ABSTRACT

This work describes the freeze-drying technique preparation and characterization of porous scaffolds (sponges) of blends between chitosan and anionic collagen. Chitosan (CHI) was obtained from the partial deacetylation of squid pen chitin and the anionic collagen was prepared by alkaline hydrolysis of porcine serosa at different times (COL24, COL48, COL72 and COL96 h). Separate materials and sponges (1:1) were characterized by thermal analysis (DSC, TG/DTG), FT-IR and SEM. The time course of alkaline hydrolysis in a collagen structure has a remarked influence on sponge properties. In DSC curves, negative net charge increased in the collagen molecules reducing the denaturation temperature in the sponges, 58.4 °C (CHI:COL24) to 49.2 °C (CHI:COL72). In TG/DTG curves the collagen presence influenced the thermal stability, whose tendency for degradation temperature reduced (155, 148.8 °C at CHI:COL24 and CHI:COL96, respectively). Different mean pore sizes were observed for the sponges, where a great reduction of the pore size with alkaline hydrolysis time occurred, which varies from 88.5 ± 7.4 (CHI:COL24) to 59.1 ± 8.6 (CHI:COL96).

1. Introduction

Collagen is the main structural protein accounting for approximately 30% of all vertebrate body protein. Currently, at least 29 types have been isolated that vary in the length of the helix and the nature and size of the nonhelical portions (Shöderhäll et al., 2007). Type I collagen is predominant in higher order animals, especially in the skin, tendon, and bone where extreme forces are transmitted.

Chemical reactions can be used to modify collagen like esterification, acylation, deamination of the ε-amino group of lysine, or blocking of the guanidine groups of arginyl residues or even alkaline hydrolysis, which produces a negatively charged anionic collagen matrix, at pH 7.4. The increase in time for the alkaline hydrolysis of carboxyamide groups of asparagines (Asn) and glutamine (Gln) increase the number of negative charge (Bet, Goissis, & Lacerda, 2001).

Collagen can be processed into a number of forms such as sheets, tubes, sponges, powders, fleeces, injectable solutions and dispersions, all of which have found use in medical systems for drug delivery in a range of applications such as ophthalmology, wound and burn dressing, tumor treatment, and tissue engineering (Lee, Singla, & Lee, 2001).

Chitosan is obtained from *N*-deacetylation of chitin and both polysaccharides are copolymers of β (1 → 4) linked *N*-acetyl-D-glucosamine and D-glucosamine units. The degree of acetylation represents the proportion of *N*-acetyl-D-glucosamine units with respect to the total number of units (Kim et al., 2008).

Chitin has been reported to occur in three crystalline forms, with the main ones designated as α and β-chitin. In the α-crystallographic structure the chain segments are antiparallel inside a polymer sheet while β-chitin chain segments are parallel, and there is no hydrogen bond between two successive chain segments. Consequently, β-chitin exhibits better reactivity, swelling and solubility than α-chitin (Lamarque, Cretenet, Viton, & Domard, 2005).

Chitosan is used in a variety of biomedical fields such as drug delivery carriers, surgical thread, and wound healing materials, due to its hemostasis properties and as an aid to tissue regeneration aid (Ma et al., 2003). One of the most promising features of chitosan is its excellent ability to be processed into porous structures for use in cell transplantation and tissue regeneration (Sionkowska, Wisniewski, Skopinska, Kennedy, & Wess, 2004). Moreover, structural characteristics of chitosan showed to be similar to glycosaminoglycans. In the native extracellular matrix, proteoglycans and glycosaminoglycans have important roles in interlinking with the fibrous structure of collagen in order to obtain mechanical stability and compressive strength (Tan, Krishnaraj, & Desai, 2001).

Arvanitoyannis (1999) discussed, in detail, the preparation and potential properties of totally and partially biodegradable polymer

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blends based on natural and synthetic macromolecules. Due to the properties such as biocompatibility, biodegradability and bioactivity, both collagen and chitosan are considered very interesting materials for applications in tissue engineering. Studies of collagen and chitosan matrices with and without crosslinking are described in literature, all involving β-chitin (Lee, Kim, Chong, & Lee, 2004) or chitosan derivates from α-chitin and collagen (Gingras, Paradis, & Berthod, 2003; Ma et al., 2003; Shanmugasundaram et al., 2001; Tangsadthakum et al., 2007; Sionkowska et al., 2006).

In this work blends of chitosan and collagen were prepared in a 1:1 weight ratio. Chitosan was obtained from deacetylation of chitin from squid pens in the β-crystallographic structure that is more reactive and soluble when compared to the α-chitin derivative. Collagen was prepared from porcine serosa at different times of alkaline hydrolysis (24, 48, 72 and 96 h), enabling to obtain collagen samples with controlled net negative charge content.

Thus, a study using chitosan and anionic collagen with controlled increase in negative charge content at pH 7.4 could contribute toward the verification of the possible interactions between these two biopolymers and also to determine if the chitosan presence affects the collagen triple helix structure of the collagen and how collagen hydrolysis time affects the blend characteristics.

2. Experimental

2.1. Blend components

Pens of *Loligo* sp., 40.0 g were treated with HCl 0.55 mol L^{-1} at room temperature for 2 h. The material was then 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 (Kurita et al., 1993). After washing and drying, 11.7 g of chitosan was obtained.

The degree of acetylation of chitosan was determined by nuclear magnetic resonance spectra in hydrogen frequency (^{1}H NMR) (Lavertu et al., 2003) and the obtained value was of 16%, which is less than what is generally obtained for α-chitin deacetylation under the same conditions. This showed the advantages of using β-chitin since only one deacetylation stage is needed and the depigmentation step is eliminated (Kurita et al., 1993).

Anionic collagen was obtained by the treatment of porcine serosa in aqueous alkaline solution (pH 13), for periods ranging from 24 to 96 h. In short, porcine serosa was treated at 20 °C with an alkaline solution (3 mL/g of tissue) containing salts (chlorides and sulfates) of alkaline (K^+ and Na^+) and alkaline earth metals (Ca^{+2}) as described earlier (Lacerda, Plepis, & Goissis, 1998). The material was suspended in deionized water, the pH adjusted with acetic acid (pH 3.5) and stored under refrigeration (4 °C). Gel concentrations were determined by lyophylization ($n = 3$).

2.2. Preparation of blends

Chitosan (16% acetylation degree) 0.5% was prepared by dissolution in 1% acetic acid solution and denominate CHI. Collagen gel concentrations were adjusted to 0.5% with acetic acid pH 3.5 and were labelled COL24, COL48, COL72 and COL96, depending on the collagen alkaline hydrolysis time.

The blended material consisted of 1 part of chitosan and 1 part of collagen (w/w). The Chitosan solution was slowly added to the collagen gel, blended with a mixer and freeze dried to obtain the material in the form of a sponge. They were labelled CHI:COL*x*, where *x* is the collagen alkaline hydrolysis time.

2.3. Characterization

2.3.1. Fourier transform infrared spectroscopy

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

2.3.2. Thermal stability

Differential scanning calorimeter (DSC) data were obtained in nitrogen atmosphere using a DSC-2010 (TA Instruments) with heating rate of 10 °C min^{-1}, between −10 and 120 °C and a sample size of about 10 mg. Thermogravimetric (TG/DTG) curves were obtained with a TGA-2050 module using a heating rate of 10 °C min^{-1} between 25 and 800°C, in synthetic air atmosphere. Sample sizes were about 10 mg.

2.3.3. Scanning electron microscopy (SEM)

The morphology was observed using a Zeiss LEO-440 SEM apparatus. Before the examination, samples were coated with 20 nm of alloy gold-palladium in a Balsers SDC model.

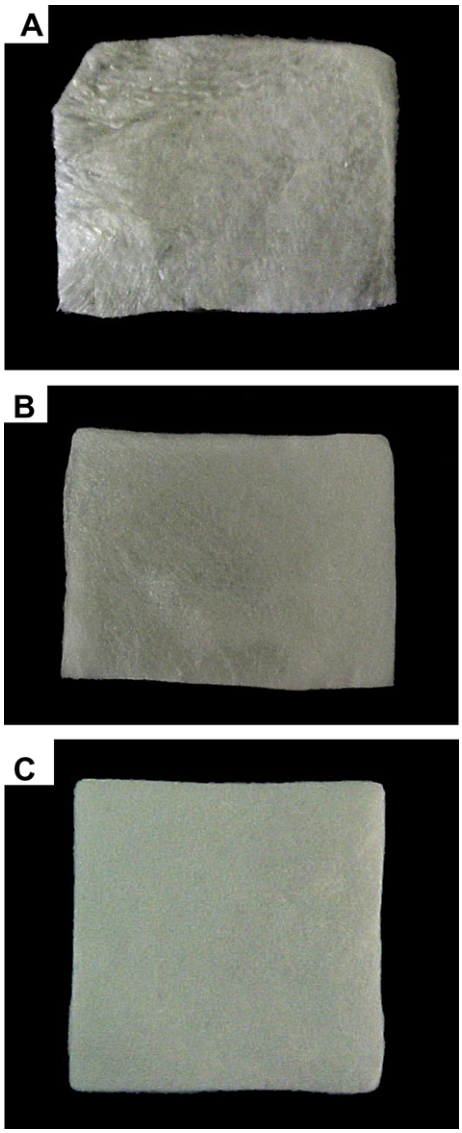

Fig. 1. Sponges obtained by freeze lyophilization (A), CHI; (B), COL24 and (C), CHI:COL24.

3. Results and discussion

Sponges obtained by chitosan:collagen 1:1 mixture presented a soft aspect and a white coloration (Fig. 1), independent of the collagen alkaline hydrolysis time.

In all cases the FT-IR spectra presented similar behavior to those shown for COL48, CHI and CHI:COL48 (Fig. 2).

The spectrum of collagen (Fig. 2a) depicted characteristic absorption bands (Sionkowska et al., 2004) at 1650 cm^{-1} – amide I; 1557 cm^{-1} – amide II; 1238 cm^{-1} – amide III; 3450 cm^{-1} – stretching O—H. For chitosan (Fig. 2b) the characteristic bands were at: 1654 cm^{-1} – amide I; 1560 cm^{-1} – amide II; 1323 cm^{-1} – group —CO—N—; 1070 cm^{-1} – 1030 cm^{-1} – stretching C—O; 3450 cm^{-1} – stretching O—H.

As shown in the FT-IR spectrum of CHI:COL48 (Fig. 2c) there are no detectable changes, hence indicating that collagen and chitosan interactions are polyelectrolytic with oppositely charged ionic polymers, particularly the cationic group of chitosan ($—NH_3^+$) and negative group in anionic collagen ($—COO^-$) (Chen, Mo, He, & Wang, 2008; Taravel & Domard, 1993).

TG curves which characterize the thermal decomposition in air of COL72, CHI and CHI:COL72 sponges (1:1) are presented in Fig. 3. Other alkaline hydrolysis time present the same characteristics.

Table 1 shows the mass loss obtained by termogravimetry of the chitosan, collagen (with different alkaline hydrolysis times) and the sponges in the mixture 1:1. In the case of the collagen gels the mass loss occurs in three stages: the first one refers to the loss of structural water of the sponges (25–200 °C); the second, to the thermal degradation of the polymeric chains of collagen (200–400 °C) and the third stage, (400–700 °C), to the carbonization of polymeric materials.

For chitosan and sponges the mass loss is observed in four stages: the first one refers to water (25–100 °C), the second one shows more strongly linked structural water (100–200 °C), and the other stages, similar to collagen, are due the degradation of polymeric chains of both polymers and carbonization. With regard to the sponges, peak maximum temperature, observed in DTG curves for the second stage, seems to be related to alkaline hydrolysis, showing a tendency of temperature reduction with higher alkaline hydrolysis time (Table 2).

In the differential termogravimetric curves for CHI, COL72 and CHI:COL72 (Fig. 3) it can be observed that the chitosan degradation shows a maximum at 273 °C (Fig. 3c), while for collagen it is around 327 °C (Fig. 3a). TG/DTG curves for other alkaline hydrolysis time present a similar behavior. Table 2 shows peak maximum temperatures for all studied matrices. With regard to the collagen, there is a decrease of degradation temperature for higher alkaline hydrolysis reaction time. In the prepared 1:1 sponges (CHI:COL72), it was verified that two maximum peaks occur, one in the 244–274 °C range and the other at 300–315 °C, attributed to chitosan and collagen degradation, respectively. In relation to the pure materials

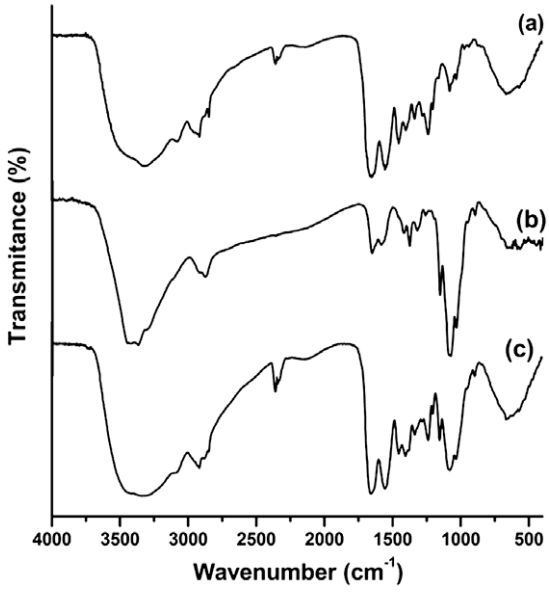

Fig. 2. FT-IR spectra of COL48 (a), CHI (b) and CHI:COL48 (1:1) sponge (c).

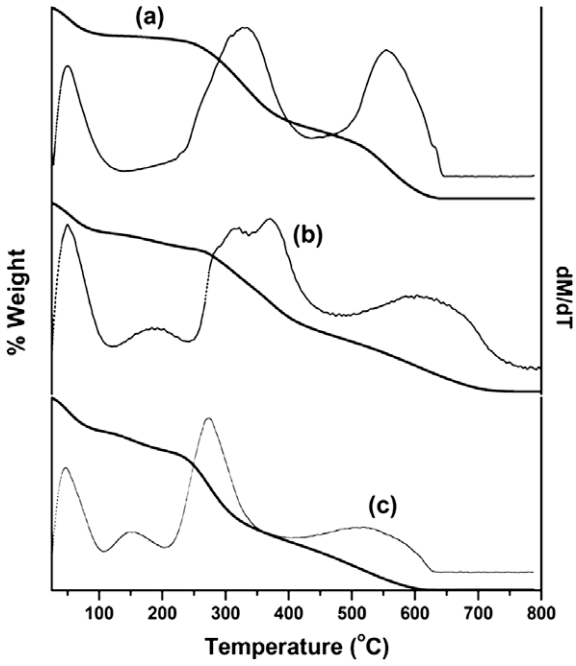

Fig. 3. TG/DTG curves of COL72, (a); CHI:COL72, (b) and CHI, (c) (air, 10 °C min^{-1}).

Table 1
% Weight loss of collagen, chitosan and sponges (1:1).

Sponges	% Weight			
	25–200 °C	200–400 °C	400–700 °C	
COL24	19.9	39.7	38.9	
COL48	17.5	45.2	35.7	
COL72	15.8	43.3	40.4	
COL96	17.8	41.9	38.9	
	25–100 °C	100–200 °C	200–400 °C	400–700°C
CHI	17.1	10.2	47.7	25.4
CHI:COL24	14.5	6.5	48.7	29.8
CHI:COL48	12.5	6.5	51.6	29.6
CHI:COL72	15.7	7.6	46.1	30.5
CHI:COL96	12.3	7.7	46.6	33.5

Table 2
Peak maximum temperature for sponges, as obtained in DTG curves.

Sponges	2° stage (°C)	3° stage (°C)
CHI	156.7	273.2
COL24	–	335.8
CHI:COL24	155.0	244.0 and 310.7
COL48	–	330.8
CHI:COL48	152.6	260.7 and 300.7
COL72	–	327.4
CHI:COL72	151.0	266.9 and 315.7
COL96	–	322.4
CHI:COL96	148.8	274.0 and 315.7

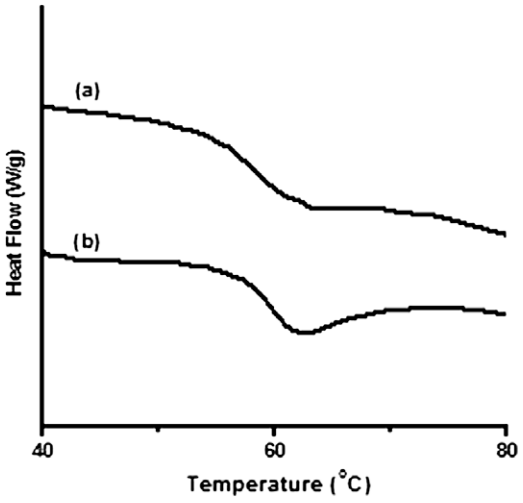

Fig. 4. DSC curves of CHI:COL24, (a) and COL24, (b).

a temperature shift was observed in both cases, suggesting an interaction between the two biopolymers.

TG results showed a possible interaction involving the chitosan groups ($-NH_3^+$) and collagen groups ($-COO^-$) and/or a hydrogen-bonding type as described by Taravel and Domard (1996) that this interaction decreases the thermal stability of both structures, collagen and chitosan.

DSC curves for COL24 and CHI:COL24 (Fig. 4) show characteristic endothermic transitions of structural changes of the collagen triple helix, that is the protein denaturation.

Table 3 shows denaturation temperatures (T_d) of collagenic structure obtained from DSC data. It was not possible to observe the transition for CHI:COL96 due to the low intensity of the signal. Considering that only the sponges contain collagen, it can be verified that the alkaline hydrolysis reduces the denaturation temperature, due to an increase in the amount carboxylic groups in the collagen molecule resulting from the cleavage of Asn and Gln residues (Bet et al., 2001). The integrity of the triple helix structure

Table 3
Denaturation temperature of collagen.

Sponges	T_d (°C)
CHI:COL24	58.4
COL24	59.9
CHI:COL48	50.8
COL48	51.8
CHI:COL72	49.2
COL72	51.6
CHI:COL96	–[a]
COL96	47.8

[a] Not observed due to low intensity of the sign.

was preserved in all cases, since denatured collagen does not present any thermal transition in the temperature intervals studied (Flandin, Buffevant, & Herbage, 1984). Chitosan addition reduces denaturation temperature of collagen molecules. This reduction probably occurs due to an influence of chitosan in the electrostatic interactions inside the collagen structure.

SEM was used to examine the microstructure of chitosan:collagen sponges at a magnification of 200× (Fig. 5). Collagen sponges show a porous structure (Fig. 5a and c), with an average pore size decreasing with alkaline hydrolysis time, which varies from 163.9 ± 12.4 (COL24) to 117.7 ± 9.4 (COL96) (Table 4). The presence of chitosan (Fig. 5b and d) induces a great pore size reduction, 88.5 ± 7.4 (CHI:COL24) to 59.1 ± 8.6 (CHI:COL96) (Table 4),

Table 4
Pores size average for the sponges.

Sponges	Pores size average
CHI:COL24	88.5 ± 7.4
COL24	163.9 ± 12.4
CHI:COL48	73.7 ± 4.2
COL48	123.1 ± 10.9
CHI:COL72	67.1 ± 4.7
COL72	119.3 ± 5.1
CHI:COL96	59.1 ± 8.6
COL96	117.7 ± 9.4

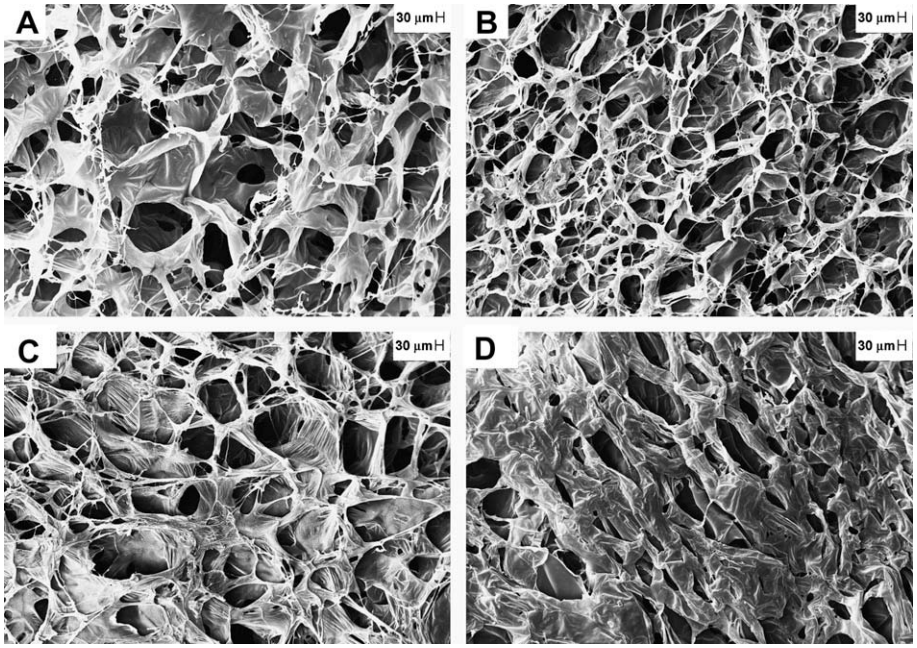

Fig. 5. SEM images of COL48, (A); CHI:COL48, (B), COL96, (C) and CHI:COL96, (D). Magnification: 200×.

showing sponges with a more compact morphology. Pore size is suitable for fibroblast infiltration (O'Brien et al., 2005).

4. Conclusion

Chitosan addition results in interactions involving the chitosan groups ($-NH_3^+$) and collagen groups ($-COO^-$) and/or a hydrogen-bonding type detectable by FT-IR, DSC and TG/DTG. This interaction reduces thermal stability of both structures. Negative net charge increase in the collagen hydrolysis reduces denaturation temperature in collagen sponges, 59.9–47.8 °C at COL24 and COL96, respectively and when chitosan is added a small thermal instability is observed (about 1 °C or 2 °C). Collagen alkaline hydrolysis time influences the mean pore size and the chitosan presence induces a great reduction in pore size with values that ranged from 88.5 ± 7.4 (CHI:COL24) to 59.1 ± 8.6 (CHI:COL96).

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