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Surface tension control of collagen biomaterials by the selective hydrolysis of internal carboxyamides of the protein matrix

Controle da tensão superficial de biomaterias de colágeno por hidrólise seletiva de carboxiamidas internas da matriz protéica.

G. Goissis
Prof. Associado / Depto. Quím. Fís. Molec.
IQSC–USP - São Carlos
Av. Dr. Carlos Botelho, 1465, Cx. P. 780
São Carlos - CEP 13560-970, SP, Brazil
E-mail: ggoissis@iqsc.sc.usp.br

C. Lacerda
Fellowship from CNPq.

M.P. Barbosa
Prof. Dr. / Dept. Hydraulic Eng.
Universidade Federal de Minas Gerais

A. Pinatti
Fellowship from CAPES

Abstract
This work studied the properties of anionic collagen coatings on dacron as a function of pH. Thermal analysis showed that in all the materials studied, transitions were close 50 C°, an indication of the integrity of the protein triple helix structure was maintained. While at pH 7.4 and 3.5 the native collagen showed the expected microfibrillar and the "swollen" amorphous structures respectivelly,the anionic collagen coatings of fully hydrolyzed internal carboxyamides were characterized by an amorphous structure in both pH values. The determined order of decreasing wettability was the following: native (pH 7.4) >> native (pH 3.5) > anionic collagen (pH 7.4) > anionic collagen (pH 3.5) >> dacron. The results show that the controlled hydrolysis of internal carboxyamides of the protein gives rise to collagen biomaterials with variable wettability and microfibrillar content that, besides other applications, its use for the coating of cardiovascular devices may be of extreme interest.
Keywords: Anionic collagen, coating, wettability, microfibril, control.

Resumo
Este trabalho estudou as propriedades de colágeno aniônico sobre superfícies de dacron em função do pH e carga. A análise térmica mostrou em todos os casos, que a estrutura da tripla hélice da proteína foi mantida, apresentando valores para a temperaturas de desnaturação próximas de 50 C°. Enquanto a pH 7,4 e 3,5 o colágeno nativo mostrou estruturas microfibrilar e amorfa, respectivamente, o colágeno aniônico apresentou apenas a estrutura amorfa, nos dois valores de pH. A ordem decrescente de hidrofilidade determinada foi a seguinte: colágeno nativo (pH 7.4) >> nativo (pH 3.5) > aniônico (pH 7.4) > aniônico (pH 3.5) >> dacron. Os resultados mostram que a hidrólise controlada de carboxiamidas internas da proteína originam biomateriais de colágeno com hidrofilidade e intensidade da organização macromolecular variável que, alem de outras aplicações, pode ser de extremo interesse para uso em dispositivos para a área cardiovascular.
***Palavras-chave**: Colágeno aniônico, superfície, ângulo de contato, microfibrila.*

Introduction

The haemocompatibily of cardiovascular devices is a compromise between functionality and biocompatibility closely related to the activation of the clothing system induced by alloplastic materials (Bolz, A. and Schaldach M.,1993). Although the ideal situation is the endothelization seeding of vascular grafts some problems remain such as the lack of efficient and reliable methods of seeding due to inefficient harvesting, poor attachment and low proliferation rates of endothelial cells (Sank et *alii*, 1992). Alternate procedures for the improvements on the haemocompatibility of cardiovascular devices have also been introduced by means of surface coating of synthetic polymers with gelatin (Marois *et alii*, 1995), collagen (Parsson *et alii*, 1994) elastin and elastin peptides (Hess *et alii*, 1995) albumin (Hirt *et alii,* 1993) and fibrinonectine (Hess et alii,1992) to improve endothelial cell adhesion (Dekker *et alii*, 1991; Nojiri *et alii*, 1994). Other procedures include the increase in surface hydrophylicity (Lim *et alii*, 1994), surface tension control of plasma protein adsorption (Vogler *et alii*, 1995) or surface charge density (Bouaziz *et alii*, 1997) and the use of electrically charged surface for the stimulation of endothelial cell adhesion as described for polarized poly(vinyledene difluoride) (Rao *et alius*, 1988).

The purpose of this work was to study the properties of surfaces of anionic collagen coated on dacron. This material was prepared by the selective hydrolysis of internal carboxyamides groups of asparagine and glutamine residues of native collagen, the extent of which may control surface tension and the macromolecular organization of collagen molecules into microfibrils, both phenomena closely related to the haemocompatibility of cardiovascular devices. Beside being biocompatible (Goissis *et alii*, 1997; Cirelli *et alii,* 1997), fully hydrolyzed carboxyamides matrices in the form of reconstituted membranes are characterized by improved dielectric properties as shown by a pyroelectric coefficient of $1.16x10^{-4}$ Cm^{-2} K^{-1}, which is considerably higher than native collagen (Plepis *et alii*, 1996) and P(VDF-TrFE) (Dias, 1994) and of respectively $0.37x10^{-4}$ Cm^{-2} K^{-1} and $17x\ 10^{-6}$ Cm^{-2} K^{-1}, at the same temperature.

Experimental

Anionic Collagen Preparation

Porcine serosa (PS) bovine tendon (BT) and bovine pericardium (BP) were treated at 20 °C for a period of 72 hr, with an alkaline solution containing 6% wt% dimethylsulfoxide and salts and bases of alkaline and alkaline earth metals (Goissis *et alii*, 1994). Excess salts were removed by extensive washes with 3% boric acid solution (3 times, 6 hr), 0.3% EDTA pH 11.0 (3 times, 6 hr) and deionized water (6x, 2 hr). The material was suspended in deionized water and the pH adjusted to 3.5 by the addition of acetic acid for collagen extraction. Collagen gel concentration was adjusted to 0.8 wt% as determined by hydroxyproline assay (Stegemann *et alius* , 1997). One sample of BP was treated for 48 and was not submitted to extraction.

Native Collagen Gel

This was prepared by the treatment of PS and BT at 20 °C with an aqueous 6% (m/m) dimethylsulfoxide solution, made pH 2.5 with acetic acid and, with occasional stirring for a period of 7 days. The suspension was then homogenized and the resulting gel dialyzed against an aqueous acetic acid solution pH 3.5, until complete removal of dimethylsulfoxide. Final gel concentration was adjusted to 0.70 wt% as described for anionic collagen gels.

Collagen Characterization

Besides molecular mass (SDS-polyacrylamide gel electrophoresis) and infrared spectroscopy (IR) collagen materials were characterized by shrinkage temperature (Ts) determinations as follows: Samples of 2.0 x 0.2 cm, previously equilibrated in buffers with the same ionic strength in the pH range from 3.4 to pH 8.0 were introduced in a graduate (mm) Pyrex tubing containing the appropriate buffer. The whole system was immersed in a silicone oil bath of a melting point equipment adapted for Ts determinations and heated from 20 to 100 °C with a rate of 2.0 °C/min. Ts values were determined from the average of 4 determinations.

Potentiometric Titration of BP Matrices

Samples of approximately 600 mg of native and alkali BP treated as described above, for 48 and 72 hr, were swollen with 1M acetic acid solution for 24 hr, frozen in liquid nitrogen and lyophilized. The samples were then equilibrated in 0.5M trifluoracetic acid solution for 24 hr, lyophilized (3 times) and dried to constant weight under vacuum over KOH. 20 mL of deionized water was added and, after submission to vacuum for complete soaking of the matrices, final pH were close to pH 2.0. Titrations were performed with standardized NaOH solution under a stream of nitrogen from pH 2.0 to pH 7.0. Titration results are the average of 3 independent determinations.

Collagen Coating of Dacron

4 pieces of Dacron cloth were immobilized between two plates of acrylic provided with circular openings of 4.5 cm in diameter followed by the addition of native and anionic collagen gels at pH 3.5 prepared from PS. One native and one anionic collagen coatings were covered with a 0.14 M pH 7.4 phosphate buffer solution and the system allowed to stand for 24 hr, with two changes of buffer solution within this period. All systems were submitted to vacuum for the removal of entrapped air and than allowed to dry under laminar flow. Reconstituted collagen membranes were prepared in a similar way. All materials were characterized as follows:

Denaturation Temperature (Td): These were determined in dacron (polytherephtalate, Biochrom, Surgical Fabrics, Mesh E, Pledget OS Biochrom Materials) coated materials in a Du Pont DSC-910 equipment, tha4t was previously calibrated with indium standard. Heating rate was 5 ^{0}C/min from 20 to 120 ^{o}C.

Scanning Electron Microscopy (MEV): Photomicrographs were obtained from dacron coated native and 72 hr alkali treated PS collagen gels and BP materials that were previously equilibrated with pH 3.5 acetic acid or phosphate 0.14 M buffer, pH 7.4, in a Zeiss DSM 960 equipment operating with primary electron beans of 20 keV, with samples previously coated with gold.

Contact Angles

These were determined by the photograph method after digitalization. Contact angles was calculated by the expression q = 2. arctg(2h/d), where q is the contact angle, h, the height of the drop and d, its length (Oshidashida *et alii*, 1993). Photograph were taken with a Cannon camera adapted with a 50mm 1:3.5, lens adapter coupled to an extensor tube FD 50 mm and a 100mm, 1:40 Cannon objective. The dacron coated collagen samples were supported in a Mirage tripod adapted with a mechanical device for movement of the sample in the dimensions. The volume of the drop was 10 µL of saline physiological solution dispensed by means of a Hamilton microserynge. Photographs were taken at 0, 5 and 10 min.

Results and Discussion

The time course for the selective hydrolysis of carboxyamide groups of asparagine (Asn) and glutamine (Gln) of BP, determined within the pH interval from 2.0 to 7.0 (Table 1), showed a progressive increase in the number of titrable groups with increasing time of alkaline treatment that varied from 0.85±0.3 mEq/g for native, to 1.24±0.03 and 1.30±0.01 mEq/g after 48 and 72 hr of alkaline treatment respectively. Calculated values for native and alkali treated BP, assuming complete hydrolysis of Asn and Gln residues and based on molecular mass for tropocollagen of 280.000 Da (Eastoe, 1967), were respectively 0.82 mEq/g and 1.28 mEq/g.

These figures for the experimental values correspond respectively to 235±8 and 364±4 titrable groups/tropocollagen for native and 72 hr alkali treated BP, compared to calculated values of 231 and 361 (Ramirez, 1988). Correction for titrable a carboxyl (3 residues) and imidazole groups from histidine residues (pK_a = 6.5), within the pH range studied, showed 224.5±8 and 353.±4 titrable groups/mole of tropocollagen for native and 72 hr alkali treated BP. These values correspond to a net increase of 129 titrable in treated material, compared to an expected values of 120 for native (Eastoe, 1967; Ramirez, 1988), showing that after 72 hr, all Asn and Gln carboxyamide groups present in BP were completely hydrolyzed to

Table 1. Calculated and experimental values for the number of carboxyl groups present as aspartic and glutamic acid residues for native and alkali treated collagen matrices.

	Tropocollagen		
Time of Alkaline Treatment	*N° mEq/g* [a]	*Total Number of Residues* [b]	*Total Carboxyl Groups* [c]
0	0.84 ± 0,03	235 ± 8	224 ± 8
48	1.24 ± 0,03	347 ± 7	336 ± 7
72	1.30 ± 0,01	364 ± 4	353 ± 4

a - Determined from three independent titration experiments determinations; **b** - Calculated with contributions from N^{im}-histidine and α-carboxyl groups residues included; **c** – Corrected for the contributions N^{im}-histidine and α-carboxyl groups.

carboxyl groups, with the extent of hydrolysis controlled by the time of reaction as shown by the result of 48 hr hydrolysis (Table 1).

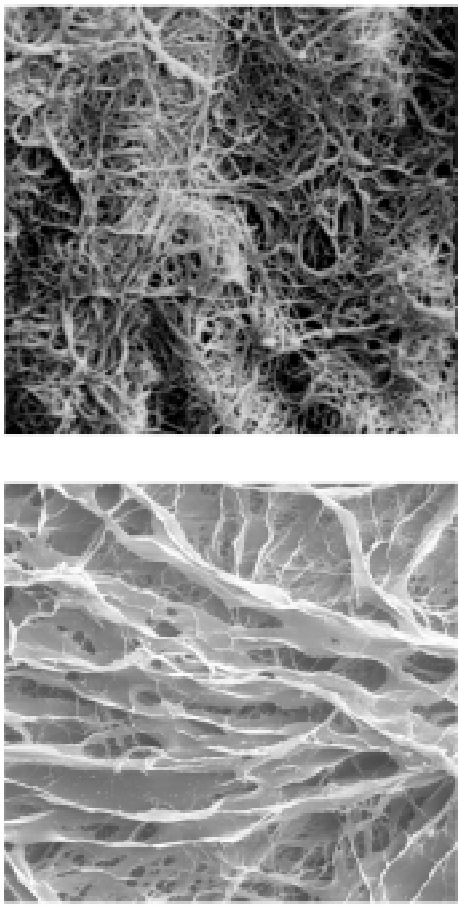
Figure 1 are the SEM micrographs for native (Figure 1a) and anionic BP collagen matrix (Figure 1b) obtained from BP, both equilibrated at pH 7.4.

While in native BP the main structural feature was the presence of a well organized microfibrillar assembly (Figure1a), in the anionic collagen BP matrix treated for complete hydrolysis of carboxyamide groups of asparagine and glutamine residues, the material was completely devoided of such a structure. In its place, a lamellar type of structure was observed (Figure 1b) and only a small number of microfibrils can be observed even at a higher amplification (Figure 2).

These results suggest that the extra negative charges introduced within the BP matrix prevent collagen molecules from organizing into the microfibril assembly and probably, as a result of a new pattern of electrostatic interaction within the matrix. This behavior is similar to that described for succinilated collagen with 85% excess of negative charge with respect to native (Shenoy *et alius*, 1995). This new pattern of electrostatic interaction is supported by the thermal stability pH dependence study of native and 72 hr alkali treated collagen materials determined within the pH range from pH 3.4 to 8.0 (Table 2).

As expected native materials showed minimum Ts values at pH 3.4 with maximum thermal stability observed at pH 7.4, in agreement with a transition from a "swollen" figure to the microfibril structure (Silvester *et alii,* 1989), associated with an increase in electrostatic interactions due to an increase in the number dissociated carboxylic groups. For native BP, Ts increased from 48.0±0.5 °C, for materials equilibrated at pH 3.4, to a maximum value of 62.1±0.3 °C for materials equilibrated at pH 7.4. These values for 72 hr alkali treated PB (anionic collagen BP matrix) were respectively 44.3±0.6 °C and 51.0±0.5 °C, but maximum thermal stability was observed at pH 4.6 with a Ts value of 53.1±0.6°C. Similar behavior was observed for reconstituted native and anionic collagen matrices prepared from BT and BS (Table 1).

Photomicrograph of dacron coated with native (Figure 3a) and anionic collagen gels (Figure 3b) prepared from PS and equilibrated at pH 7.4 were similar to those observed for BP under the same conditions of pH and treatment. While with native, collagen coating provided a surface characterized by



(a)

(b)

Figure 1. Scanning electron microscopy micrographs for native collagen matrices before (**a**) and after 72h alkaline treatment for the selective and complete hydrolysis of the carboxyamide groups of bovine pericardium matrix (**b**), both , equilibrated in pH 7.4, phosphate buffer (5000x).

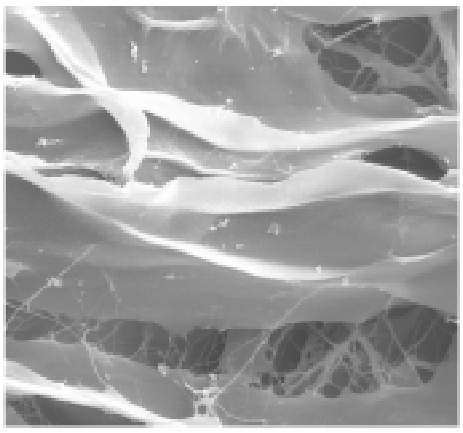


Figure 2. Scanning electron microscopy micrographs collagen matrices after 72h alkaline treatment for the selective and complete hydrolysis of the carboxyamide groups, equilibrated in pH 7.4, phosphate buffer(15000x).

Table 2. Thermal stabilities[a] determined as Ts for native and 72 hours alkali treated bovine pericardium[b] porcine serosa[c] and bovine tendon[c] as a function of pH.

	pH				
Material	**3.4**	**4.0**	**4.7**	**7.4**	**8.0**
Bovine Pericardium					
Native	*48.0±0.5*	*52.5±0*	*57.8±0.3*	*62.1±0.3*	*61.8±0.3*
Alkali treated	*44.5±0.8*	*51.5±0*	*53.1±0.6*	*51.0±0.5*	*51.2±0.6*
Tendon					
Native	*55.5±0.5*	*57.1±0.3*	*61.3±0.3*	*64.8±0.3*	*63.3±0.3*
Alkali treated	*44.3±0.6*	*50.5±0.8*	*55.0±0.5*	*52.8±0.3*	*53.5±0.3*
Serosa					
Native	*44.8±0.4*	*51.7±0.2*	*55.0±0.7*	*58.2±0.05*	*58.5±0.02*
Alkali treated	*41.9±0.1*	*47.8±0.6*	*48.8±0.3*	*48.9±0.2*	*47.6±1.3*

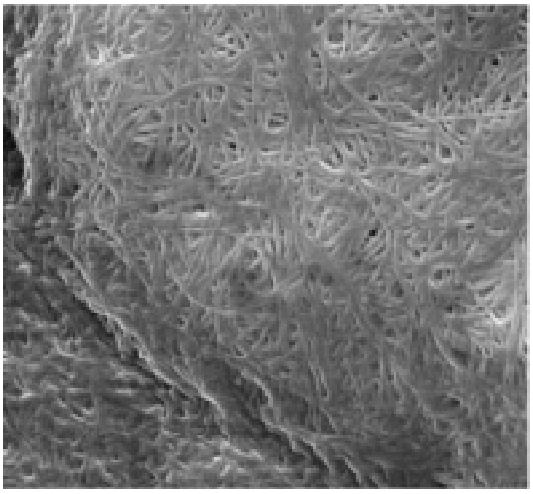
a - in ºC and are the average of at least 4 determinations; **b** - whole tissue; **c** - determined on reconstituted collagen membranes equilibrated in the appropriate pH.

the presence of the microfibrillar structure (Figure 3a), with anionic collagen coating this structure was not observed (Figure 3b).

DSC results for collagen membranes and those dacron coated with similar collagen materials equilibrated at pH 7.4 (Figure 4) showed that, independently from the nature of the collagen material (membrane, coating or state of hydrolysis) thermal transitions were quite similar and close to 50.0 °C, and is indication for the presence of the protein with the intact secondary triple helix structure. Independent from coating, thermal transition for dacron was observed only at 259.9 °C.

Results on Table 3 are those for the contact angle for dacron surfaces coated with native and anionic collagen coated surfaces after equilibration at pH 7.4 and 3.5 and measured after 5 and 10 min. of contact. At pH 7.4 contact angles for native (Table 3) were respectively 26.2 and 22.6° compared to 81.4 and 79.0° for anionic collagen, suggesting that the lack of the ordered microfibrilar structure and/or the increase in negative charge produces, on a comparative basis, produces more hydrophobic surface. At pH 3.5 the differences in contact angles of dacron surface coated with native and anionic collagen were smaller.

After 5 and 10 min contact angle for native were respectively 84.4 and 75.9° compared to 93.5 and 93.5° observed for anionic collagen (Table 3). The observed smaller difference in relation to that found for coatings equilibrated at pH 7.4 suggest that, the microfibril structure plays an important role in the determination of wettability of collagen biomaterials and probably


(a)


(b)

Figure 3. Illustration of contact angles for saline physiological solution drops over Dacron coated with native **(a)** and anionic collagen **(b)** after equilibration in pH 7.4, phosphate buffer (5000x).

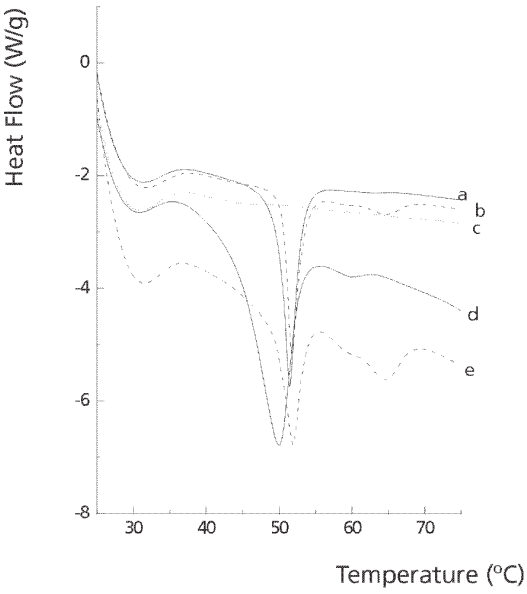


Figure 4. Differential scanning calorimetry profiles for reconstituted collagen membranes and dacron coated with native (——) and anionic collagen (- - - -) at pH 7.4: **a** - dacron coated, native; **b** - dacron coated, anionic; **c** - dacron; **d** - native, membrane; **e** - anionic, membrane.

the slightly higher value determined for the anionic collagen surface due to differences in the content protonated carboxyl groups and the fact that the carboxyamide side chains of asparagine and glutamine residues are more hydrophilic when compared to carboxyl groups (Eastoe, 1967).

Conclusion

The results above showed that anionic collagen materials may be conveniently prepared by selective hydrolysis of internal carboxyamide groups of asparagine and glutamine of the collagen matrix and when completely hydrolyzed is unable to form microfibrils at physiological pH a natural characteristic of native collagen materials. This inhibition is probably associated to the strong electrostatic repulsion effect due to the introduction of 120 extra negative charge at physiological pH. On the other hand the significant changes observed in wettability, particularly at pH 7.4, associated with improved dielectric properties, suggest that the control of the hydrolytic process may provide collagen biomaterials with controlled wettability and microfibrillar content. Besides other applications, this type of collagen biomaterials may be of potential use for the coating of cardiovascular devices.

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Table 3. Contact angles[a] determined for native and anionic collagen coatings[b] over dacron as a function of pH.

	Contact Angle (°)				
	Native		Anionic		
t (min)	3.5	7.4	3.5	7.4	Dacron
0	*91.6*	*48.8*	*93.5*	*83.1*	*135.1*
5	*84.4*	*26.2*	*93.5*	*81.4*	*132.5*
10	*75.9*	*22.6*	*93.5*	*79.0*	*129.8*

a – Average of two independent determinations. **b –** Gel obtained from 72 hours alkali treated porcine serosa.

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