J Mater Sci: Mater Med (2009) 20:1979–1989
DOI 10.1007/s10856-009-3778-1

Collagen- and gelatine-based films sealing vascular prostheses: evaluation of the degree of crosslinking for optimal blood impermeability

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Received: 13 November 2008 / Accepted: 5 May 2009 / Published online: 18 May 2009
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Abstract The stiffness as well as the biodegradation rate of collagen and gelatine products can be modulated by performing a number of crosslinking treatments. In many biomedical applications, an optimal degree of crosslinking seems to exist, depending on the mechanical and/or biosynthesis properties of the host site. The aim of this study was to evaluate the optimal degree of crosslinking of collagen and gelatine films, to be used as sealants for vascular prostheses. Various crosslinking treatments, including exposure to aldehydes, dehydrothemal treatment, carbodiimide crosslinking and combinations of them, were performed on collagen and gelatine films, and the resulting increases in stiffness, degree of crosslinking and denaturation temperature were evaluated. Analogue crosslinking treatments were also performed on sealed prostheses, which were then tested for blood leakage. The experimental results showed that a good blood impermeability of both collagen and gelatine films was obtained for crosslinking density of about 1.2–1.3 × 10^{-5} mol/cm^3, which could be yielded by a dehydrothermal crosslinking treatment (DHT). In particular, dehydrothermally treated gelatine-coated prostheses were found to perform better than analogue collagen-coated ones. The presence of glycerol in crosslinked collagen films was found to have plasticizing effects, which are likely to facilitate blood impermeability, and to increase the thermal stability of collagen.

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1 Introduction

Collagen and gelatine (i.e. denatured collagen) are among the most used sealants for woven polyester vascular prostheses. The use of a sealant clearly obviates the need for preclotting prior to implantation, to avoid leakage of blood during in vivo implantation. Collagen is advantageous as a sealant since it also improves cell adhesion and migration onto the surface of the prosthesis, thus leading to a better integration with the host tissues [1]. However, an optimal degree of crosslinking of the sealing collagen film seems to exist. Indeed, a slight crosslinking of the collagen film deposited onto the prosthesis surface might be required to stabilize the film and make it resistant to the blood flow upon implantation. Conversely, an excessive crosslinking might be detrimental, making the film too stiff and fragile, thus less resistant to traction and shear stresses. The negative effect of a strong crosslinking has been recently observed while storing commercially available collagen-impregnated prostheses (Biomateriali Srl, Italy) in a glutaraldehyde solution in the long term (i.e. 18–24 months). In this case, the collagen film on the prosthesis surface undergoes a chemical crosslinking process activated by glutaraldehyde. The resulting mechanical modifications deeply compromise the prosthesis performance, as failure of the sealant under blood flow occurs during implantation, as documented by in vitro blood leakage tests.

In this study, we quantified the increase in stiffness of the collagen-sealing film caused by the glutaraldehyde solution used for storage (0.6% v/v), and we sought to evaluate the optimal degree of crosslinking for blood impermeability, achievable through alternative crosslinking treatments. The knowledge of the optimal degree of crosslinking might indeed help in choosing or setting up the best crosslinking treatment(s) for the given application.

We also looked at the use of gelatine as alternative sealant, which differs from collagen for its rubber-like mechanical behaviour [2]. Collagen and gelatine sheet-like samples were crosslinked via chemical and/or physical processes, and their shear modulus, degree of crosslinking and denaturation temperature were evaluated, both before and after a further crosslinking treatment with the glutaraldehyde solution. The latter case was considered to verify the effect that some crosslinking treatments, performed before the exposure to glutaraldehyde, might have on the reaction of collagen with glutaraldehyde. At the same time, vascular prostheses impregnated with analogue collagen and gelatine samples were subjected to similar crosslinking treatments and then tested for blood leakage. The role of glycerol, which was present in the starting collagen slurry used to impregnate the prostheses, was also discussed.

2 Materials and methods

2.1 Sample preparation

An aqueous suspension of Type I collagen isolated from calf skin (3 wt%, Semed S collagen, Kensey Nash Corporation) was obtained according to a proprietary process (Biomateriali Srl, Italy). The suspension contained also ethyl alcohol, used as a bacteriostatic agent, and glycerol, used as a plasticizer. Sheet-like or film-like collagen samples ($\sim$2 mm thickness) were obtained by casting 35 ml of the collagen slurry into a 100 mm Petri dish and drying the slurry for at least 48 h at room temperature. Gelatine samples were prepared through the same procedure, but the slurry was kept at 80°C for 2 h before casting, to allow collagen denaturation.

Collagen- and gelatine-coated vascular prostheses (diameter 28 mm) were obtained by impregnating the prostheses with the same suspension described above, according to the production process adopted by Biomateriali Srl, Italy.

2.2 Crosslinking treatments

Collagen and gelatine film-like samples were subjected to several crosslinking treatments, either alone or in combination, as summarized in Table 1. Each crosslinking procedure was performed in triplicate. Although the plasticizer, i.e. glycerol, was present in the starting samples, most of the crosslinking treatments, performed in aqueous solutions, led to the final loss of glycerol, due to its solubility in water. In order to compare the results obtained for different crosslinking treatments, the crosslinked samples were stored in excess phosphate buffered saline (PBS) at 4°C for at least 24 h, before the characterization.

Collagen- and gelatine-impregnated vascular prostheses were also subjected to various crosslinking treatments (Table 1) and then tested for blood leakage.

2.2.1 *Crosslinking with aldehydes*

Formaldehyde (F) crosslinking was carried out on samples (either films or impregnated prostheses) soon after their preparation, according to a procedure developed by Biomateriali Srl. Briefly, samples were placed in contact with gaseous F for 2 h. Since F reacts in gaseous form, this treatment preserves the presence of glycerol in the samples. F is a monofunctional crosslinking agent, and is known to react with several protein groups, especially lysine residues along the collagen chain and peptide nitrogen atoms, to form a methylene bridge or a crosslink [3, 4].

The crosslinking with glutaraldehyde (GA) was performed by keeping the samples into an excess amount of aqueous solution of GA (0.6% v/v), for a selected time (Table 1). Being bifunctional, GA is much more reactive than F, and is able to react with a number of protein sites, such as ammine, amide and carboxylic groups [3, 4].

The main disadvantage in using aldehydes for collagen crosslinking is related to the fact that these toxic molecules are incorporated into the collagen network, thus they might be released as collagen degrades in vivo, leading to undesired side effects [3]. As alternatives, several crosslinking procedures have been set up, which allow the formation of chemical bonds between collagen macromolecules, without the incorporation of any exogenous molecules. Two of these procedures are the dehydrothermal process (DHT) and the crosslinking with carbodiimide (EDAC), which have been used in this work.

2.2.2 *Dehydrothermal crosslinking (DHT)*

The dehydrothermal (DHT) process is known to induce the formation of both ester bonds between carboxyl and hydroxyl groups, and amide bonds between carboxyl and ammine groups, via condensation reactions. These reversible reactions are activated at high temperatures. The elimination of the resulting water molecules, to allow the crosslinking reaction to take place along the whole process, is achieved working under high vacuum. The vacuum is also necessary to avoid the denaturation of collagen that would otherwise occur in the presence of water [5]. Typical DHT conditions involve temperatures between 105 and 140°C, and vacuum of approximately 30–50 mmHg, for a period of time of approximately 24 h or more. The degree of crosslinking achievable is dependent on the temperature and duration of the process. In this work, the film samples were first washed in distilled water, in order to remove residual glycerol which limits the desiccation of the

Table 1 Summary of the crosslinking treatments performed on collagen and gelatine films

Abbreviation	Description
F	Gaseous formaldehyde
G1	Glutaraldehyde solution, for 1 week
G5	Glutaraldehyde solution, for 5 weeks
G7	Glutaraldehyde solution, for 7 weeks
DHT	Dehydrothermal process
EDAC	Carbodiimide solution
F + EDAC	Gaseous formaldehyde, followed by carbodiimide solution
F + DHT	Gaseous formaldehyde, followed by dehydrothermal process
EDAC + G1	Carbodiimide solution, followed by glutaraldehyde solution for 1 week
F + EDAC + G1	Gaseous formaldehyde, followed by carbodiimide solution and then glutaraldehyde solution for 1 week

Vascular prostheses coated with analogue collagen and gelatine films were subjected to treatments DHT, EDAC and G1

samples, and were then dried under a chemical hood at room temperature for 48–72 h, before running the DHT procedure. The samples were then crosslinked in a vacuum oven at 121°C and 30 mmHg for 48 h.

The vascular prostheses impregnated with either collagen or gelatine were directly subjected to the same DHT treatment, without the need to wash residual glycerol. In particular, in order to ensure an uniform temperature profile and crosslinking throughout the prosthesis surface, the prostheses were mounted onto appropriate rotating mandrels and kept in rotation (10 rpm) during the entire length of the treatment.

2.2.3 Crosslinking with carbodiimide (EDAC)

A chemical crosslinking process through the use of the water soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) involves the formation of a peptide linkage between two collagen macromolecules, without the incorporation of the carbodiimide in the polymer network [6]. The crosslinking reaction was carried out by keeping the samples into an aqueous solution 14 mM EDAC and 5.5 mM NHS (molar ratio EDAC:NHS = 5:2), with 6 mmol EDAC/g collagen, for 2 h [6]. The film samples were then properly washed with distilled water, to remove any unreacted chemicals and the by-products of the reaction, and finally stored in PBS at 4°C until further characterization.

2.3 Calculation of the degree of crosslinking

The degree of crosslinking of a polymeric network is defined as the density of junctions joining the macromolecular chains into a permanent structure, thus is given by:

$$\mathrm{d}c = \frac{v}{2V} \tag{1}$$

where $v/2$ is the total number of chemical crosslinks and V is the total volume of polymer.

In the case of a perfect network with no dangling ends, loops and entanglements, the concentration of the elastically effective chain elements corresponds to the concentration of all chemically crosslinked polymer segments:

$$\rho_x = \frac{v_e}{V} = \frac{v}{V} = \frac{1}{\bar{v} M_c} \tag{2}$$

where $\bar{v}$ is the specific volume of the polymer, M_c is the average molecular weight between crosslinks and v_e/V are the moles of elastically effective chains per unit volume of network.

An approach to directly estimate the elastically effective crosslink density is provided by the theory on the entropic elasticity of rubbers, which relates the degree of crosslinking of a rubber-like crosslinked polymer to its macroscopic mechanical properties.

Making the basic assumptions that the deformation of the chains is affine and that the volume of the polymer does not change upon uniaxial deformation (V is constant), Flory [7] derived the following relationship between the uniaxial stress and the uniaxial deformation of a swollen crosslinked polymer with a rubber-like behaviour:

$$\sigma = RT\rho_x V_2^{1/3}\left(\alpha - \frac{1}{\alpha^2}\right) = G\left(\alpha - \frac{1}{\alpha^2}\right) \tag{3}$$

where σ is the stress, R is the universal gas constant, T is the absolute temperature, V_2 is the polymer volume fraction in the swollen state, i.e. the inverse of the volume swelling ratio, $\alpha = L/L_i$ is the deformation ratio, with L the actual thickness of the deformed sample and L_i the initial thickness of the swollen sample ($\alpha > 1$ for elongation and $\alpha < 1$ for compression, respectively) and G is the shear modulus of the swollen polymer. Therefore, for a rubber-like crosslinked polymer the plot of σ against the quantity $(\alpha - 1/\alpha^2)$ is linear, with a slope that defines the shear modulus G.

Crosslinked collagen does not show a rubber-like mechanical behaviour; however, when collagen is denatured, i.e. brought to high temperatures in order to destroy

its triple helical structure, the resulting material, known as gelatine, does show a rubber-like state [2]. Since the denaturation process involves the rupture of only the weak hydrogen bonds that stabilize the triple helix without disrupting the chemical linkages between collagen macromolecules [8], the above theory can be applied to denatured collagen to determine its degree of crosslinking.

It is worth noting that the above equation links the modulus G directly to ρ_x, thus it is possible to argue that, even when measuring G from mechanical tests other than uniaxial elongation or compression, the obtained value for G might be used to calculate ρ_x, once V_2 is known. In the present work the modulus G for several gelatine samples was measured from dynamic mechanical analysis (DMA) [9].

2.4 Dynamic mechanical measurements

For mechanical testing, the sheet-like samples, swollen in PBS at 4°C, were let to equilibrate in the solution at room temperature, and then analyzed by means of a parallel plate rheometer (ARES, Scientific Rheometric). Three disks (25 mm diameter) were cut from each hydrated sheet (with three sheets for each sample type, for a total of nine measurements per sample) and tested at 20°C. The thicknesses of the different samples varied slightly among them, and were usually between two and 3 mm, with the only exception of dehydrothermally treated samples, which had a thickness of about 0.5 mm. The surface of the parallel plates was properly modified to prevent the films from slipping during the measurements. A strain sweep test was first performed to select an appropriate strain amplitude at which the linear viscoelastic behaviour could be observed. The measurements were then carried out in a constant strain (0.001) mode as a function of frequency, in the range 0.1–100 rad/s. The results were recorded in terms of storage and loss moduli, respectively G' and G'', and of loss factor tgδ (= G''/G'). The storage modulus G' is representative of the elastic energy stored by the material, whereas the loss modulus G'' provides information on the energy dissipated via viscous effects. The shear modulus G, used both to evaluate the mechanical properties and to estimate the degree of crosslinking, was calculated at a fixed frequency (10 rad/s) as the value of the complex modulus $|G^*|$:

$$G = |G^*| = \sqrt{G'^2 + G''^2} \tag{4}$$

Collagen samples were tested only to assess their mechanical properties, whereas gelatine samples were tested to evaluate also their degree of crosslinking according to Eq. 3.

Moreover, uncrosslinked collagen films, not swollen in PBS, were analyzed and compared to analogue swollen samples, to assess the effect of glycerol on the mechanical properties of the samples.

2.5 Differential scanning calorimetry (DSC) and swelling ratio

After swelling in PBS at 4°C, hydrated samples ($n = 3$–4 for each sample type) weighing 5–10 mg were sealed in aluminium pans and analyzed by means of differential scanning calorimetry (Mettler-Toledo, DSC 822^e) in the range 5–90°C, with a scanning rate of 5°C/min, to detect the denaturation temperature of collagen. After each DSC run, the sample pan was punched with a pin and the specimens were dehydrated in oven at 105°C overnight. The ratio of the weight of the swollen sample ($M_{coll} + M_{water}$) to that of the dry sample (M_{coll}) was used to calculate the volume swelling ratio as follows:

$$SR = \frac{V_{coll} + V_{water}}{V_{coll}} = 1.32\frac{M_{coll} + M_{water}}{M_{coll}} - 0.32 \tag{5}$$

where 1.32 g/cm^3 is assumed to be the density of anhydrous collagen [10].

The collagen volume fraction in the swollen network, needed to calculate the crosslink density according to Eq. 3, is thus given by:

$$V_2 = \frac{1}{SR} \tag{6}$$

Both uncrosslinked and F-crosslinked samples, not swollen in PBS and thus containing glycerol, were also analysed by means of DSC, to determine the effect of glycerol on the denaturation temperature of the samples.

2.6 Morphological analysis

Scanning electron microscopy (SEM) was used to assess qualitatively the morphology of collagen and gelatine films. Air-dried films were directly positioned onto the sample holder, and observed, both in longitudinal and transverse sections, in a variable pressure mode with a Zeiss EVO scanning electron microscope.

2.7 Blood tests

The so-called blood test is a qualitative experimental procedure used to characterize the blood retention capacity of vascular prostheses in vitro, with a descriptive final outcome that is based on visual inspections by the operator. Briefly, the test consists of pumping bovine blood, heated at 40°C, inside the prosthesis and to observe if great or local leakages occur. The prosthesis is grabbed and closed

on one side and connected, on the other one, to a pump working at a frequency of 37 rpm, in order to simulate the mechanical stresses caused by the blood pressure on the prosthesis upon implantation. During the test, the prosthesis is handled by the operator and subjected to tensile, bending and torque stresses, so that the prosthesis quality, in terms of blood impermeability, can be evaluated in highly critical conditions.

In this study, the blood retention capacity of collagen and gelatine films deposited onto vascular prostheses was evaluated on three experimental groups (respectively DHT-, EDAC- and G1-treated prostheses). Formaldehyde treated samples had been previously shown to work well in terms of blood impermeability and were thus considered in this study as positive controls. Three samples for each prosthesis type were tested. All the blood tests were carried out at Biomateriali Srl by the same, experienced operator.

2.8 Statistical analyses

Data are expressed as mean ± the standard deviation. Analysis of variance (ANOVA) was applied to determine the effect of the crosslinking treatment on the shear modulus, degree of crosslinking and denaturation temperature of the tested samples. A probability value of 95% ($P < 0.05$) was used as the criterion for significance.

3 Results

3.1 Dynamic mechanical analysis

All film samples displayed a prevalently elastic behaviour, being the storage modulus G' significantly higher (at least an order of magnitude) than the loss modulus G'' (Fig. 1). As expected, the presence of glycerol in uncrosslinked collagen films was found to have a plasticizing effect, as denoted by the corresponding higher values of loss factor tgδ (Fig. 1). The crosslinking treatment was found to affect significantly the shear modulus of collagen and gelatine samples, as well as the degree of crosslinking (Fig. 2 and Table 2; $P < 0.0001$, power = 1 for both dependent variables). Moreover, for a given crosslinking treatment the collagen samples were found to be stiffer than the gelatine ones, although a significant difference could be found only for F and F + EDAC + G1 treatments (Fig. 2; $P = 0.047$ and $P = 0.0002$, respectively). No significant difference in stiffness was detected between samples F and F + DHT for collagen ($P = 0.17$). However, the DHT treatment appeared to be more effective when performed on gelatine samples ($P = 0.035$, samples F and F + DHT).

Treatments F and EDAC were not significantly different in terms of shear modulus ($P = 0.24$ collagen, $P = 0.10$ gelatine). However, the combination of both treatments, F + EDAC, yielded significantly higher values of shear moduli when compared to those obtained with F or EDAC treatments alone (Fig. 2). In particular, the EDAC treatment seemed to be very efficient, since in 2 h it yielded a modulus that was approximately 70% of that obtained by G5 (5 weeks of treatment with glutaraldehyde), and the combination F + EDAC yielded a modulus not significantly different from that yielded by G5 and G7 (Fig. 2; $P = 0.36$ and $P = 0.11$, respectively). The crosslinking reaction activated by glutaraldehyde (GA) was not inhibited when the EDAC treatment was previously performed (samples EDAC + G1 and F + EDAC + G1 in Fig. 2), probably due to the higher number of functional groups of collagen that can react with GA but not with EDAC. It is

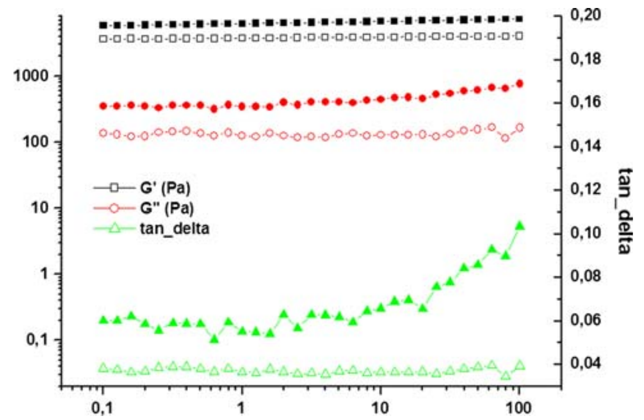

Fig. 1 Effect of glycerol on the dynamic mechanical spectra of uncrosslinked collagen films at 20°C. Samples containing glycerol (*full symbols*) showed a higher loss factor tgδ when compared to the ones without glycerol (*empty symbols*)

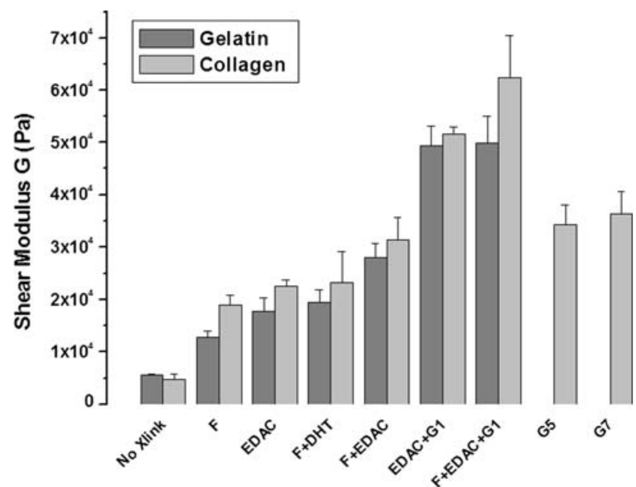

Fig. 2 Comparison between the shear moduli G of gelatine and collagen films as a function of the crosslinking treatment(s). Treatments G5 and G7 were performed only on collagen samples. Error bars represent the standard deviation of the mean (results averaged over 9 measurements)

Table 2 Degree of crosslinking for denatured film samples as a function of the crosslinking treatment

Crosslinking treatment	Mean collagen volume fraction	Crosslink density (mol/cm^3)
None	0.058	$5.90 \times 10^{-6} \pm 2.89 \times 10^{-7}$
F	0.083	$1.21 \times 10^{-5} \pm 1.04 \times 10^{-6}$
DHT	0.110	$1.30 \times 10^{-5} \pm 2.54 \times 10^{-6}$
F + DHT	0.127	$1.58 \times 10^{-5} \pm 1.95 \times 10^{-6}$
EDAC	0.045	$2.05 \times 10^{-5} \pm 3.01 \times 10^{-6}$
F + EDAC	0.066	$2.84 \times 10^{-5} \pm 2.61 \times 10^{-6}$
EDAC + G1	0.049	$5.51 \times 10^{-5} \pm 4.22 \times 10^{-6}$
F + EDAC + G1	0.051	$5.49 \times 10^{-5} \pm 5.72 \times 10^{-6}$

All samples were swollen in PBS overnight before measurements. Results are expressed as mean ± the standard deviation of the mean and have been averaged over nine measurements. The mean value of the collagen volume fraction used to calculate the crosslink density is also reported ($n = 3$–4)

worth noting that there was no significant difference between treatments G5 and G7, i.e. glutaraldehyde crosslinking for five and 7 weeks, respectively (Fig. 2; $P = 0.49$). This observation suggests that the polymerization of GA that occurs parallel to the crosslinking reaction might impede the further penetration of GA into the sample, thus inhibiting a further crosslinking [3, 4].

The relative efficacy of the various crosslinking treatments was confirmed by the calculation of the degree of crosslinking of gelatine samples (Table 2). On the overall, no significant differences were detected among treatments F, DHT and F + DHT, although DHT and F + DHT treatments were significantly different from the uncrosslinked control ($P = 0.032$ DHT, $P = 0.0016$ F + DHT). The EDAC treatment alone was comparable, in terms of degree of crosslinking, to the combination F + DHT ($P = 0.14$). As expected, the samples crosslinked with glutaraldehyde (EDAC + G1 and F + EDAC + G1, $P = 0.97$) showed the highest degrees of crosslinking.

3.2 Differential scanning calorimetry

Both collagen and gelatine samples were tested by means of DSC to analyse their thermal behaviour and detect their denaturation temperature (some renaturation, i.e. aggregation of collagen random coils to form a triple helix structure, is found in gelatine samples [11]). Depending on the particular crosslinking treatment, some samples showed multiple denaturation peaks in the range of temperatures studied (5–90°C). However, when considering only the first endothermic peak, it could be observed that the corresponding denaturation temperature tended to increase as increasing the degree of crosslinking, as expected (Tables 2 and 3, power = 0.97) [12–14], although there was no significant difference between treatments EDAC and F + EDAC ($P = 0.07$ for collagen, $P = 0.99$ for gelatine). Moreover, in spite of the similar crosslink density yielded, the F treatment and the DHT one had different effects on the thermal stability of the samples. DHT-treated collagen and gelatine samples showed denaturation temperatures lower or close to 37°C, whereas F-treated ones could reach much higher temperatures without denaturing. Interestingly, if comparing a glycerol-containing collagen sample with a PBS-swollen collagen sample, the denaturation temperature of the former was found to be significantly higher than that of the latter (Table 3). This significant difference could not be detected for analogue gelatine samples ($P = 0.14$ and $P = 0.41$ for none and F treatments, respectively).

3.3 Morphological analysis

All the film types, both collagen and gelatine-based ones, were found to possess a quite homogeneous structure, devoid of micropores, which is clearly advantageous in terms of blood impermeability (Fig. 3). It is worth highlighting that it was not possible to detect any significant morphological differences among the different samples prepared, and, for a given sample, no microstructural changes were detected before and after a given crosslinking treatment (Fig. 3).

Table 3 Denaturation temperature as a function of different crosslinking treatments

Crosslinking treatment	Denaturation temperature (°C)	
	Collagen	Gelatin
None (with glycerol)	43.03 ± 0.33	42.64 ± 0.64
None	34.75 ± 0.59	34.53 ± 0.41
F (with glycerol)	N.D.	47.33 ± 6.13
F	43.04 ± 0.65	39.22 ± 0.46
DHT	35.83 ± 0.63	37.61 ± 2.42
F + DHT	39.50 ± 0.95	36.11 ± 2.77
EDAC	49.11 ± 1.76	44.08 ± 5.78
F + EDAC	41.56 ± 4.72	44.04 ± 1.59
G5	42.69 ± 3.06	–
G7	43.50 ± 1.37	–
EDAC + G1	54.54 ± 14.34	50.12 ± 17.61
F + EDAC + G1	N.D.	42.07 ± 9.06

All the samples were tested after swelling in excess PBS at 4°C, except the sample containing glycerol. Results are expressed as mean ± the standard deviation of the mean ($n = 3$–4). Only the first endothermic peaks were considered for the evaluation, in case of multiple peaks. N.D. = not detected in the range 5–90°C. Treatments G5 and G7 were performed only on collagen samples

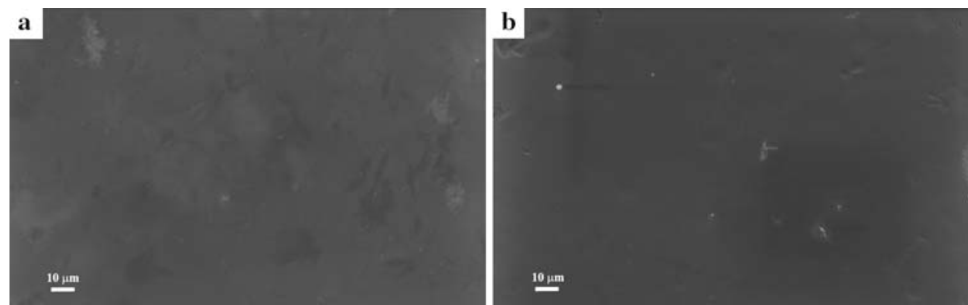

Fig. 3 SEM micrographs (magnification 500×, scale bar 10 µm) showing the microstructure of air-dried gelatine films before **a** and after **b** a crosslinking treatment with gaseous formaldehyde

3.4 Blood tests

The qualitative results of the blood tests performed on DHT-, EDAC- and G1-treated prostheses are summarized in Table 4. Figure 4 also reports representative photographs of the blood tests performed on each type of samples.

The results were consistent within each experimental group. As expected, the G1 crosslinking treatment caused the failure of the treated prostheses under the blood flow, with a diffuse, copious leakage all over the prosthesis length, for both collagen and gelatine samples. The EDAC crosslinked samples displayed many and copious localized leakages, which made the blood impermeability of the prostheses still insufficient. The best results were obtained for the DHT-treated samples, which showed an overall good blood retention capacity, with only poor, localized leakages. In this case, gelatine-coated prostheses performed better than analogue collagen-coated ones, showing a behaviour similar to that of F-treated collagen samples, which were used as positive controls. Such results confirmed those obtained from the evaluation of the degree of crosslinking, suggesting that the DHT treatment is a suitable crosslinking method for yielding blood impermeability of collagen and gelatine films.

4 Discussion

The aim of this work was to quantify the optimal degree of crosslinking of collagen- and gelatine-based films used as sealants for commercial vascular prostheses (Biomateriali Srl, Italy). Indeed the extent of crosslinking affects the mechanical stiffness of the sealant, which plays a fundamental role in ensuring a proper blood impermeability during implantation. A poor crosslinking leads to a low stiffness of the sealing film, which might fail upon implantation, causing undesired blood leakage. Similarly, an excessive crosslinking might make the sealant too stiff and fragile to resist the blood flow. Based on preliminary data obtained by Biomateriali Srl, a crosslinking treatment with gaseous formaldehyde performed on collagen-coated vascular prostheses was already known to yield a sufficient blood retention capability. However, the following long-term storage of such prostheses in a glutaraldehyde solution was found to lead to an excessive increase of stiffness of the sealing films, which caused their failure under the blood flow. In order to quantify the optimal degree of crosslinking for blood impermeability, collagen sheet-like samples were subjected to different crosslinking treatments, respectively based on formaldehyde (F), glutaraldehyde (GA), dehydrothermal process (DHT) and

Table 4 Blood tests results for collagen- and gelatine-coated vascular prostheses subjected to different crosslinking treatments

Crosslinking treatment	Blood impermeability	
	Collagen coating	Gelatine coating
DHT	●●○ Good Minimal, localized leakages	●●● Optimal Minimal leakages, same behaviour as controls
EDAC	●○○ Poor Copious, localized leakages	●○○ Poor Copious, localized leakages
G1	○○○ Insufficient Copious, diffuse leakage	○○○ Insufficient Copious, diffuse leakage

A synthetic, qualitative result is provided. Increasing blood impermeability is expressed on a scale going from 0 (○ ○ ○, insufficient) to 3 (● ● ●, optimal). Collagen-coated vascular grafts crosslinked by formaldehyde had been previously shown to have a good blood impermeability and were used as positive controls. Number of samples tested $n = 3$

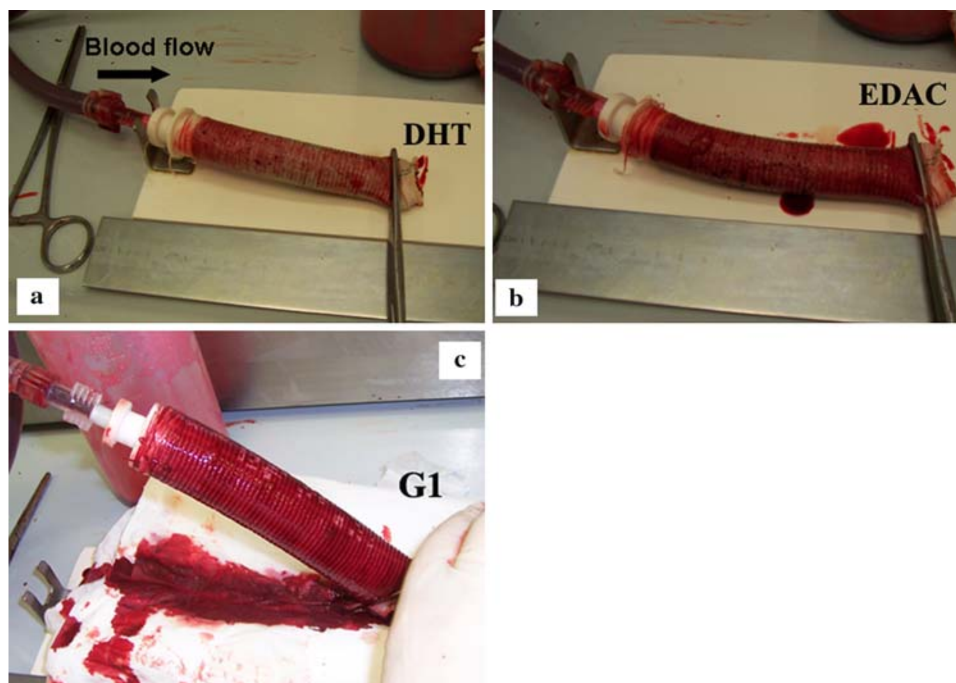

Fig. 4 Blood tests performed on collagen-coated vascular prostheses: **a** dehydrothermally crosslinked (DHT); **b** carbodiimide crosslinked (EDAC); **c** glutaraldehyde crosslinked via immersion in glutaraldehyde solution (0.6% v/v) for 1 week (G1)

carbodiimide (EDAC), and various combinations of them. Gelatine films were also considered as potential sealants and subjected to the same crosslinking procedures. In particular, the F and the GA treatments were considered respectively as positive and negative controls, whereas methods based on EDAC and DHT were considered as valuable alternatives, in view of a better biocompatibility of the resulting devices. Indeed, both treatments do not incorporate exogenous and likely toxic molecules in the collagen network, oppositely to the crosslinking mechanisms activated by F and GA. Various combinations of crosslinking treatments, before and after a further crosslinking with GA, were also taken into account, to assess the effect of those treatments on the chemical reaction initiated by the glutaraldehyde.

The dynamic mechanical properties of both collagen and gelatine films were firstly evaluated, and the degree of crosslinking was estimated from the classical theory on the elasticity of rubbers. The evaluation of the elastically effective degree of crosslinking, instead of the chemically effective one, was preferred in this study, in order to directly correlate the stiffness and the resistance to the blood flow of the samples to their crosslink density. Indeed, although a biochemical assessment of collagen and gelatine crosslinking might have been of interest and complementary to the one performed in this study, it is reasonable to expect different values of the chemical degree of crosslinking compared to the elastically effective one, due to the imperfections of a real polymer network (e.g. intramolecular crosslinking) [7].

The results of the dynamic mechanical analysis (DMA) evidenced a prevalently elastic behaviour, and shear moduli G ranging from about 0.01 to 0.06 MPa, for hydrated collagen and gelatine samples. Such modulus values are much lower than those reported in a recent investigation on the viscoelastic properties of collagen [15]. In that study, a nanoindentation-based technique was used to measure the local viscoelastic properties of aligned collagen scaffolds, at different levels of dehydration, and values of storage modulus E' of approximately 0.70 GPa were recorded. First of all, the striking difference in the measured values of dynamic mechanical moduli may be attributed to the different length scales of the measurements. Indeed, while the nanoindentation method allows to measure the mechanical properties of collagen fibrils, bulk measurements techniques, such as the dynamic mechanical analysis employed in this study, are not sensitive to the microstructural complexity of a given sample and only report average values, which are representative of the mechanical properties of the entire sample. The determination of the viscoelastic properties through the nanoindentation method is thus useful to assess the mechanical behaviour of a given biomaterial or the local variations in an anisotropic sample, whereas bulk DMA provides reliable information on the viscoelastic properties of samples possessing a homogeneous microstructure. Based on the morphological analysis performed by means of scanning electron microscopy, the collagen and gelatine films investigated in this study could be effectively considered as homogeneous. Moreover, the measurement of their bulk

modulus was needed in order to evaluate an average degree of crosslinking. It is worth noting that the values of storage and loss shear moduli reported in this study are consistent with those reported in the literature for crosslinked collagen gels [16]. The extent of crosslinking and the hydration state of the samples are further variables that affect the resulting dynamic mechanical behaviour.

For both collagen and gelatine films, the overall results of DMA highlighted that the GA-mediated crosslinking reaction was not inhibited by previous crosslinking treatments. Moreover, the EDAC treatment was found to lead to a strong increase in stiffness [17], similar to that obtained by glutaraldehyde crosslinking, which suggested that such a treatment is not likely to improve the blood retention capacity of collagen- or gelatine-coated vascular grafts. Conversely, the DHT treatment resulted to be comparable to the F one (i.e. the positive control), in terms of yielded crosslink density (about $1.2–1.3 \times 10^{-5}$ mol/cm^3), thus suggesting that such a treatment might be a more biocompatible option for the crosslinking of collagen-sealed vascular prostheses. As expected, the DMA results highlighted also that gelatine samples were less stiff than collagen ones, which might be advantageous for the envisaged application of the films as sealants for vascular grafts. Moreover, a dynamic mechanical testing performed on uncrosslinked collagen and gelatine films, either containing or not glycerol, confirmed that glycerol is an effective plasticizer.

When analyzing the thermal stability of the samples by means of differential scanning calorimetry, the observed trend was an increase of the denaturation temperature with an increase of the crosslink density of the samples, as expected [12–14]. It is worth noting that the presence of glycerol was found to significantly increase the denaturation temperature of collagen samples. This finding might be ascribed not only to the higher water content of PBS swollen samples (i.e. the ones devoid of glycerol), which decreases the thermal stability of collagen [14], but also to the protective effect exerted by glycerol on collagen, as reported in several studies [18, 19]. Glycerol is supposed to bind to every individual polypeptide chain via hydrogen bonds, thus forming a protective shell around each collagen triple helix. Having three hydroxyl groups, glycerol has indeed a higher ability to form hydrogen bonds, if compared to water. This shielding effect is reported to yield a higher thermal and chemical stability of collagen [18]. Moreover, the presence of glycerol on the surface of the collagen molecules limits the formation of intermolecular interactions, thus preventing fibrillogenesis [19] and probably causing the plasticizing effect, which was detected in this study from the mechanical testing. In a recent work on the fracture of gelatine gels [20], the addition of glycerol to water during the gel preparation has also been found to slow down the propagation of a crack in the resulting gel, probably due to the increased viscosity of the water/glycerol mixture. These observations led us to think that the increased fragility of collagen samples after storage in an aqueous GA solution is related not only to the increased crosslink density, but also to the solubilization of glycerol in water. Therefore, crosslinking methods preserving the presence of glycerol on collagen-coated vascular grafts (e.g. formaldehyde, DHT) should be preferred compared to crosslinking in aqueous solutions. Alternatively, due to the solubility of glycerol in water, crosslinking should be performed in fixed volumes of aqueous solutions, containing a certain amount of glycerol. The desired quantity of glycerol might be directly added to the aqueous solution, or the concentration of glycerol in the starting collagen slurry used to impregnate the vascular grafts might be increased, so that, upon immersion in an aqueous solution, only the amount of glycerol necessary to achieve an uniform concentration profile can be solubilized.

In vitro blood tests performed on collagen- and gelatine-coated vascular prostheses (28 mm diameter) confirmed the results of the previous analyses, as an optimal blood impermeability was detected for gelatine-coated prostheses, crosslinked by means of DHT. Although in this study the thrombogenic response of the sealants was not investigated, it is worth highlighting that, in case of collagen-based biomaterials, such a response is inhibited or limited by disrupting the quaternary structure of collagen, which is known to mediate platelet adhesion [21, 22]. Gelatine films, which have been found to display an optimal blood impermeability in case of DHT crosslinking, are thus potentially non-thrombogenic. In particular, further investigations are needed to assess the thrombogenic behaviour of glycerol-containing collagen sealants, as glycerol might prevent fibrillogenesis [19] thus inducing a non-thrombogenic response.

Considerations about potential thrombosis are particularly relevant for vascular grafts of small diameter. Indeed, it is well-known that the use of vascular prostheses is practically limited to the substitution of large-diameter blood vessels, since small diameter prostheses (<6 mm) are likely to fail due to occlusion of the lumen. Two mechanisms may contribute to occlusion, respectively thrombosis and neointimal hyperplasia. In order to avoid thrombosis, adherence and activation of platelets onto the surface of the prosthesis should be inhibited by either coating with non-thrombogenic materials or inducing the in vitro formation of a confluent and non-thrombogenic endothelium [23]. With regard to intimal thickening, this undesired tissue synthesis might be related to cellular mechanisms promoted by in vivo endothelialization [24], as well as to a compliance mismatch at the junction between the prosthesis and the blood vessel [23]. With the

aim of producing effective alternatives to permanent prostheses, a number of investigations currently focus on the development of tissue-engineered small diameter blood vessels, starting from tubular scaffolds made of extracellular matrix (ECM) component biomaterials. Remarkably, the collagen and gelatine-based sealants produced in this study might find application in the engineering of blood vessels as well as other types of tissues. In an interesting study by Kasyanov and coworkers [24], it is suggested that hydrogels based on hyaluronic acid, coating the internal surface of tubular micro-perforated scaffolds, might be useful both to produce cellularized vascular substitutes and to deliver in vivo specific biomolecules (e.g. growth factors) potentially able to enhance endothelialization while preventing intimal thickening. In a similar way, it can be argued that collagen and gelatine sealants described in this work (which are in a gel form when hydrated) might be employed for the local delivery and sustained release of therapeutic agents, both in tissue engineering and in the pharmacological field [25]. Furthermore, it is worth underscoring that collagen and gelatine gels can be produced in the form of porous three-dimensional scaffolds, rather than films (e.g. by means of freeze-drying). Both the stiffness and the biodegradation rate of the scaffolds [26] can be modulated by performing specific crosslinking treatments. Therefore, collagen and gelatine gels show promise for the design of novel scaffolds and novel composite biomaterials (e.g. mixtures collagen/chitosan [27], collagen/fibroin [28]) for tissue engineering.

5 Conclusions

Collagen and gelatine films, used to seal polyester vascular prostheses, were subjected to various crosslinking treatments, both to verify if such treatments may affect the crosslinking of collagen that takes place during long-term storage in glutaraldehyde, and to quantify the degree of crosslinking that ensures an optimal blood impermeability.

The results evidenced that glutaraldehyde-mediated reaction is not inhibited by previous crosslinking treatments, and that a suitable blood impermeability is obtained for collagen- and gelatine-coated vascular grafts treated either with gaseous formaldehyde or dehydrothermally crosslinked. Both treatments yielded a similar average crosslinking density ($\sim$1.2–1.3 $\times$ 10^{-5} mol/cm^3), which thus might be considered as the optimal one. In particular, the dehydrothermal treatment (DHT) might be preferred compared to the formaldehyde one, in order to avoid concerns about the use of toxic crosslinking agents and their possible release upon degradation in vivo. Both DHT and formaldehyde crosslinking preserved the presence of glycerol in the sealing films, unlike crosslinking reactions performed in aqueous solutions. Since glycerol works as a plasticizer for collagen, we suggest that the blood impermeability of collagen-coated vascular prostheses is dependent not only on the crosslink density of the collagen films, but also on the glycerol content found in the crosslinked collagen films. The plasticizing effect exerted by glycerol on collagen was also related to an increase of the thermal stability of the collagen itself, as demonstrated by DSC analysis. However, such a protective effect of glycerol was not detected for gelatine coatings, which, however, displayed an optimal blood retention capacity when DHT-treated.

Acknowledgements The authors acknowledge the Italian Ministry of Productive Activities (MAP)-'MIS.2.1a PIA Innovazione' for partial financial support.

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