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Evaluation of different chemical methods for cross-linking collagen gel, films and sponges

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Collagen-based films and sponges are widely used as biomaterials. The rate of their biodegradation can be reduced by treating them with different cross-linking agents. The efficiency of different reticulation procedures on thermal stability (measured by differential scanning calorimetry) and susceptibility to bacterial collagenase digestion of the final material (films or sponges) is compared. The chemical agents used on collagen gels or directly on collagen sponges and films were glutaraldehyde (GTA), hexamethylene diisocyanate (HMDC), cyanamide, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and the two acyl azide methods (with hydrazine or diphenylphosphorylazide (DPPA)) developed in the authors' laboratory. Under these experimental conditions, collagen stabilization by the different agents increased in the following order: cyanamide < EDC < HMDC < hydrazine < DPPA < GTA. Sponges with the highest thermal stability were obtained by treating collagen gel with 0.5% GTA for 1 h at pH 5.5 (initial $T_d = 75.8\,^{\circ}C$; after 3 months, $T_d = 75.6\,^{\circ}C$). Direct treatment of collagen films with DPPA gave similar thermal stability ($T_d = 72.6\,^{\circ}C$) and collagenase resistance when compared with treatment with 0.6% GTA for 96 h ($T_d = 74.6\,^{\circ}C$). It is demonstrated that collagen sponges and films can be prepared with a wide range of thermal stability (49–75.8 °C) and collagenase digestion resistance (10–100%). Recent biocompatibility studies show, however, that DPPA and EDC are the best choices for preparing cross-linked collagen sponges and films.

1. Introduction

Collagens are the major structural proteins of connective tissue such as dermis, bone, cartilage, tendons and ligaments [1]. They constitute about one-third of the total body protein in mammals; some 40% of total collagen is found in dermis. Because of its biological properties and easy availability, type I collagen is widely used as a biomaterial. Purification procedures and fabrication processes have made it possible to make collagen products of different textures and shapes including sponges, films, membranes and tubes [2, 3]. As collagen-based biomaterials are rapidly degraded *in vivo*, however, the three-dimensional collagen structure must be reinforced so that it will persist in the body for the required period. The rate of biodegradation of collagen biomaterials can be reduced by treating them with a cross-linking agent. Cross-linking reinforces the collagen structure by introducing intra- and intermolecular cross-links between collagen molecules. Three types of cross-linking procedure have been developed for stabilizing collagen: physical treatments with ultraviolet and gamma irradiation, dehydrothermal treatment and chemical treatments. Chemical cross-linking procedures often involve bifunctional reagents containing reactive groups such as glutaraldehyde (GTA) and diisocyanates, or involve activation of carboxylic acid groups followed by reaction with amino groups (carbodiimide and acyl azide methods).

Glutaraldehyde is now the most widely used reagent; however, as glutaraldehyde cross-linked biomaterials induce local cytotoxicity [4–6], other compounds have been evaluated. We proposed [7–10] use of acyl azide methods, with either hydrazine (referred to here as the hydrazine method [7]) or diphenylphosphorylazide (DPPA) (referred to here as the DPPA method [9]). Comparative analysis of pericardium treated with GTA and with the acyl azide methods demonstrated the excellent cytocompatibility (endothelial cells) of DPPA-treated biomaterials [10]. Other agents used frequently are diisocyanates and carbodiimides [11–18].

In this study, we compared the cross-linking efficiency of glutaraldehyde (GTA), hexamethylene

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diisocyanate (HMDC), two carbodiimides (cyanamide and 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC)) and the two acyl azide methods on type I collagen molecules in the form of gels, sponges and films. The efficiency of the procedures was studied by both measuring thermal stability by differential scanning calorimetry (DSC) and susceptibility to collagenase degradation of the final material.

2. Materials and methods

2.1. Materials

Glutaraldehyde, cyanamide and 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) were purchased from Merck (Nogent sur Marne, France). Hexamethylene diisocyanate (HMDC) was obtained from Aldrich (Strasbourg, France). Bacterial collagenase (Clostridium histolyticum type II) was purchased from Sigma (La Verpilliere, France).

Collagen gel was prepared from calf dermis as described previously [16]. The dermis was fragmented, ground, extensively washed in phosphate buffer (pH 7.8) and distilled water, and swollen in dilute acetic acid. Sponges (Haemostagen^{R}) were prepared by freeze-drying the collagen gel (0.7% w/v). Films were prepared by pouring a collagen gel (1.5% w/v) into an aluminium tray and drying them at room temperature for 3 days. The composition and structure of the sponges and films were characterized by amino acid analysis, sodium dodecyl sulfate polyacrylamide gel electrophoresis, high angle X-ray diffraction and differential scanning calorimetry [19, 20]. They were found to be composed of native type I and III collagens (90–95% and 4–9% of dry weight, respectively).

2.2. Cross-linking procedures

Two different cross-linking procedures were used. In procedure 1, cross-linking agents were added to the collagen gel (0.7% w/v) at 20 °C. With this protocol a reaction pH of 6.2 is a maximum in order to avoid fibril precipitation. After reaction, the treated gel was freeze-dried to give sponges. In procedure 2, films and sponges were immersed in a solution of cross-linking agent in water at 20 °C. After reaction, they were rinsed for 1 or 3 days in distilled water. Films were air-dried at 20 °C for 1 day, and sponges were freeze-dried.

The cross-linking treatments were as follows:

Glutaraldehyde treatment. The composition and stability of GTA (absence of aldol condensation products) were characterized as described by McPherson *et al.* [21]. Collagen gels (procedure 1), with pH adjusted to 6.2, were cross-linked with fresh solutions of GTA at 0.0075, 0.01, 0.05 or 1% w/v for 1 and 24 h at 20 °C, extensively washed in glycine 0.1 M pH 7.4 and freeze-dried. Collagen films and sponges (procedure 2) were immersed in 0.6 and 1% w/v GTA solutions for 96 and 24 h, respectively, at 20 °C and pH 6.2.

Carbodiimide treatments. In procedure 1, collagen gels, with pH adjusted and maintained at 5.5, were cross-linked with cyanamide at 1% w/v for 24 h at 20 °C or with EDC at different concentrations (0.1, 0.3 and 1% w/v) for 1 and 24 h at 20 °C. The gels were then freeze-dried. Films and sponges (procedure 2) were treated with cyanamide by immersion in a 1% w/v solution at pH 5.5, 6.2, 7.2 or 10.0 for 1 and 24 h at 24 °C.

Diisocyanate treatment. HMDC was added to a collagen gel adjusted to pH 5.5 (procedure 1) at a concentration of 1% w/v and left at −18 °C for 4 and 96 h. We used this low temperature protocol, previously described by Berg and Eckmayer [12], in order to decrease the HMDC reactivity in aqueous solution but without the use of surfactants [14] or organic solvents [16].

Acyl azide treatments. Films and sponges (procedure 2) were treated with hydrazine as previously described [7]. Briefly, the lateral carboxyl groups of collagen were methylated by immersing the samples in methanol containing 0.2 mol/l hydrochloric acid for 1 week at 20 °C and then washed in 0.1 mol/l NaCl solution. In order to obtain hydrazides from the methyl groups, the tissues were then placed in a 1% hydrazine solution and kept overnight at room temperature. After further washing, acyl azide was formed by dipping the samples into an aqueous solution containing 0.5 mol/l sodium nitrite and 0.3 mol/l hydrochloric acid for 3 min at 4 °C (Step III). After thorough washing the samples were placed in the buffer at pH 8.9 for 4 h at 4 °C to induce collagen cross-linking. With the DPPA method, collagen films and sponges were treated with 0.5% DPPA solution in dimethyl formamide at 4 °C for 24 h [9].

2.3. Evaluation of the cross-linking efficiency

The efficiency of cross-linking was evaluated by differential scanning calorimetry (DSC) and collagenase assay. For the DSC the collagen biomaterials were analysed immediately after cross-linking as well as after 3 months (at room temperature) for the sponges prepared by procedure 1. Calorimetric analysis by DSC allows measurement of the stability of the triple helical structure of collagen molecules [20]. Three temperatures are measured (Fig. 1): the onset temperature, the temperature at the maximal peak (noted denaturation temperature, T_d and chosen here for the comparative analyses) and the recovery temperature, at which the curve returns to baseline. DSC was performed on a Setaram model 111 differential calorimeter (Lyon, France) calibrated using indium standards. Samples weighing approximately 10 mg were immersed in 120 µl distilled water and sealed in cells. Thermal changes were measured against a distilled water sample while the temperature of the samples was raised at a constant rate of 2 °C/min from 0 to 100 °C. For the collagenase assay, the resistance of collagen films and sponges to biodegradation was assessed by immersing them in a bacterial collagenase solution (250 IU/mg collagen in 10.0 mmol/l Tris,

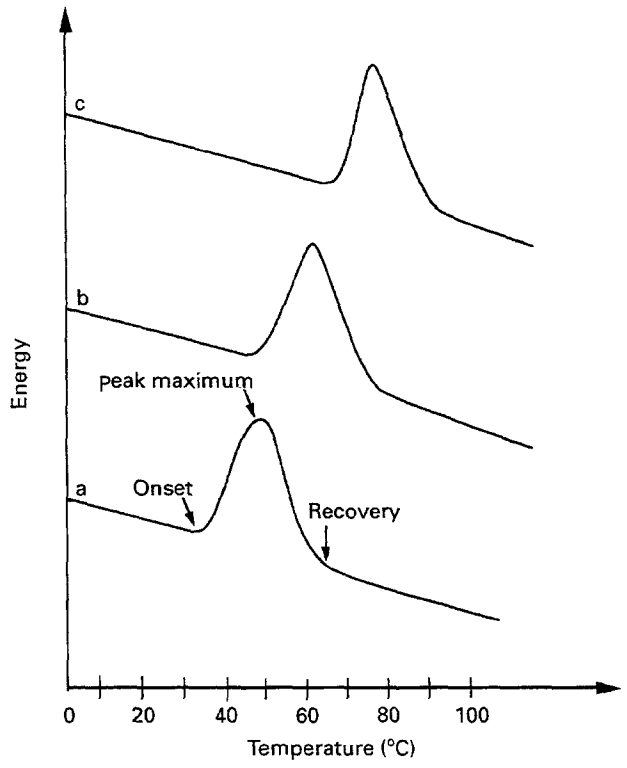

Figure 1 Thermal transition, measured by differential scanning calorimetry (DSC), of (a) control sponges and sponges cross-linked by treatment of collagen gel (procedure 1) with (c) 0.01% glutaraldehyde (GTA) for 24 h or with (b) 1% 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) for 24 h. The temperature of the samples was raised at a constant rate of 2 °C/min from 0 to 100 °C.

25 mmol/l $CaCl_2$, pH 7.4). A 10 mg collagen sample was immersed in 10 ml collagenase solution in a dialysis tube. The amount of degraded collagen present in the external volume, was determined by a hydroxyproline assay. Two independent series of experiment were performed.

3. Results

3.1. Cross-linking of collagen gels (procedure 1)

The thermal stability and collagenase susceptibility of sponges resulting from procedure 1 are shown in Table I and Fig. 2a. A complete statistical analysis is not presented as only three measurements (thermal stability) and two series of experiments (collagenase) were performed.

Treatment with cyanamide (1% pH 5.5) had no effect on collagen stability. The second carbodiimide (EDC) was more effective, and a dose- and time-dependent increase in T_d was observed with a maximal T_d of 61 °C obtained with a concentration of 1% and a treatment duration of 24 h. No variation in sponge stability was observed after 3 months storage.

At the same concentration (1%), HMDC induced a large increase in initial sponge thermal stability (T_d = 70.8 °C after 4 days treatment), which, however, decreased after 3 months (T_d = 63.9 °C). Similar resistance to collagenase was induced in collagen sponges cross-linked by EDC and HMDC (Fig. 2a).

TABLE I Thermal transition temperatures of non cross-linked collagen sponges and of cross-linked sponges prepared by treatment of collagen gel (procedure 1) with cyanamide, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC), hexamethylene diisocyanate (HMDC) and glutaraldehyde (GTA)

Conditions of reticulation				Thermal transition temperature (°C)					
Reticulation agent	Concentration (%, w/v)	pH	Duration of reticulation (h)	Initial stability			Long-term stability (3 months)		
				Onset	Peak	Recovery	Onset	Peak	Recovery
None	–	–	–	36.8 ± 0.7	48.9 ± 0.2	63.1 ± 0.3	35.4 ± 0.4	47.7 ± 0.3	63.0 ± 0.4
Cyanamide	1	5.5	24	43.3 ± 2.9	49.7 ± 2.2	59.7 ± 3.4			
EDC	0.1	5.5	1	40.0 ± 0.8	51.2 ± 3.4	65.6 ± 0.8	41.7 ± 2.6	51.2 ± 0.7	66.6 ± 1.7
	0.1	5.5	24	40.7 ± 3.7	51.1 ± 3.3	71.0 ± 2.9	40.7 ± 1.4	49.9 ± 5.2	66.7 ± 4.0
	0.3	5.5	1	40.7 ± 1.1	54.9 ± 1.2	70.4 ± 3.8	61.7 ± 1.4	65.4 ± 0.5	75.1 ± 0.7
	0.3	5.5	24	54.0 ± 0.9	59.7 ± 0.3	71.0 ± 2.8	53.4 ± 0.9	59.4 ± 0.5	72.6 ± 1.4
	1	5.5	1	49.7 ± 0.3	57.0 ± 0.5	73.6 ± 1.4	56.1 ± 2.1	63.9 ± 0.9	75.0 ± 2.6
	1	5.5	24	50.0 ± 4.0	61.0 ± 0.5	72.3 ± 2.0	51.7 ± 1.6	60.4 ± 0.5	68.6 ± 2.2
HMDC	1	5.5	4	59.0 ± 1.2	66.6 ± 2.1	76.9 ± 2.6	46.0 ± 0.9	53.7 ± 0.8	63.4 ± 0.9
	1	5.5	96	63.6 ± 6.9	70.8 ± 5.3	81.0 ± 3.9	56.7 ± 0.7	63.9 ± 0.4	79.8 ± 4.8
GTA	0.0075	6.2	1	49.2 ± 1.1	61.7 ± 0.7	74.6 ± 0.3	49.4 ± 1.5	65.4 ± 1.4	76.1 ± 1.1
	0.0075	6.2	24	67.6 ± 1.6	75.6 ± 0.5	84.6 ± 0.4	67.6 ± 0.8	73.9 ± 0.5	83.6 ± 1.4
	0.01	6.2	1	57.4 ± 1.9	63.6 ± 0.3	73.6 ± 3.8	51.7 ± 0.7	70.6 ± 0.7	80.5 ± 0.5
	0.01	6.2	24	64.6 ± 1.2	74.6 ± 0.3	84.6 ± 0.3	66.8 ± 0.7	73.4 ± 0.7	83.4 ± 1.3
	0.5	6.2	1	68.6 ± 0.8	75.8 ± 0.5	84.3 ± 1.7	71.6 ± 0.8	75.6 ± 0.7	83.6 ± 2.1
	0.5	6.2	24	64.6 ± 0.3	73.3 ± 0.9	83.9 ± 0.9	65.9 ± 0.9	71.5 ± 0.6	81.9 ± 0.5
	1	6.2	1	62.4 ± 0.5	69.6 ± 0.8	81.6 ± 0.8	63.9 ± 0.8	70.6 ± 2.4	79.3 ± 1.2
	1	6.2	24	63.1 ± 1.5	70.2 ± 0.8	79.5 ± 0.7	62.5 ± 1.4	69.2 ± 0.4	77.4 ± 2.1

Each value is the mean and standard deviation of at least three measurements.

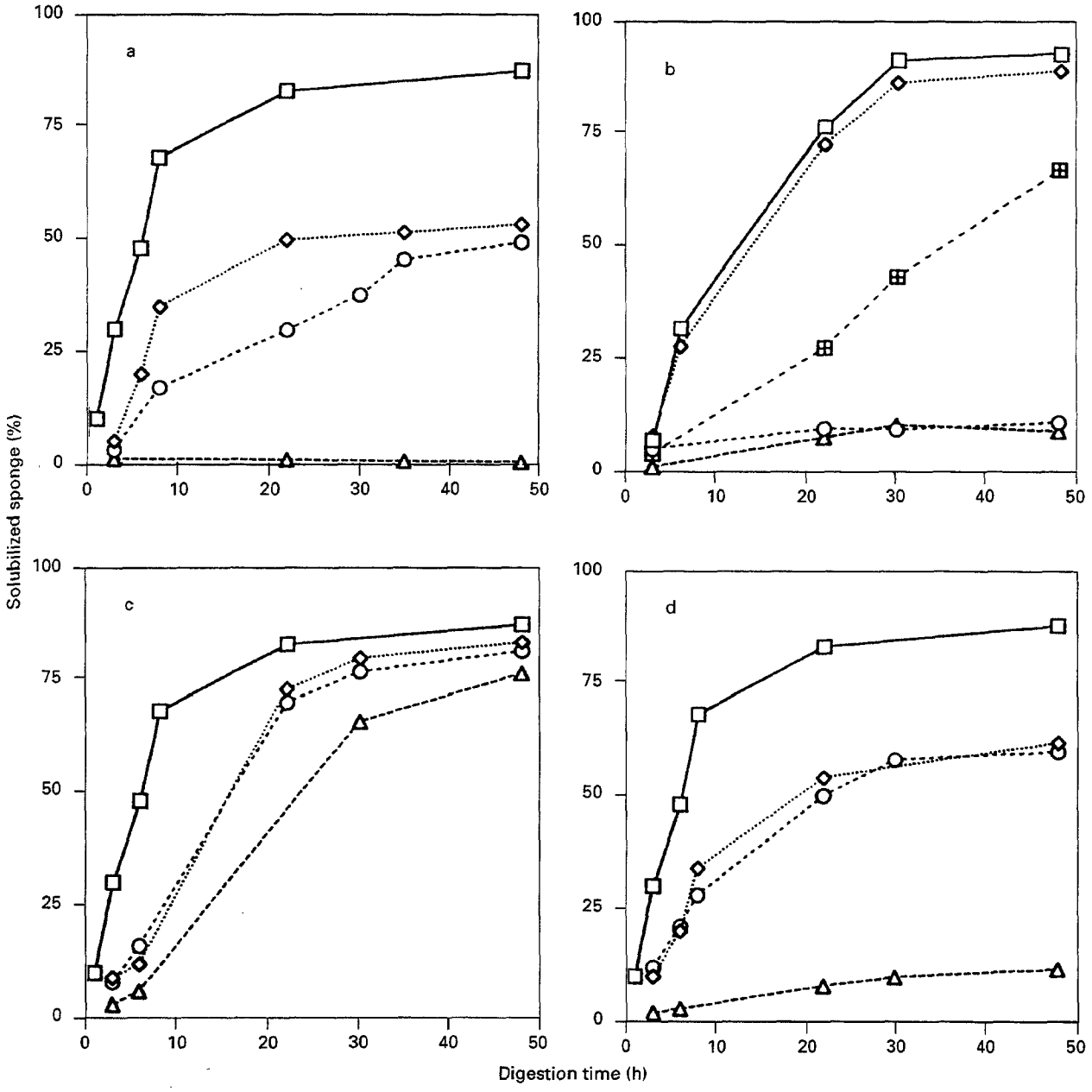

Figure 2 Resistance to collagenase degradation of (a) collagen sponges prepared by procedure 1; (b) collagen films prepared by procedure 2; and (c, d) collagen sponges prepared by procedure 2. (a) –□– control sponge; sponges cross-linked by treatment of collagen gel with ···◇··· 0.3% 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) for 24 h at pH 5.5, - - -○- - - 1% hexamethylene diisocyanate (HMDC) for 96 h at pH 6.2, and - - -△- - - 0.0075, 0.01 or 1% glutaraldehyde (GTA) for 24 h at pH 6.2. (b) –□– control film; films treated with ···◇··· 1% cyanamide for 24 h at pH 7.2, - - - ⊞ - - - 1% cyanamide for 24 h pH 6.2, - - -○- - - 1% hydrazine for 24 h, and - - -△- - - 0.6% GTA for 96 h at pH 6.2. (c) –□– control sponge; sponges treated with 1% cyanamide for 24 h ···◇··· at pH 6.2, - - -○- - - at pH 10 and - - -△- - - at pH 5.5. (d) –□– control sponge; sponges treated with ···◇··· 0.5% diphenylphosphorylazide (DPPA) for 24 h, - - -○- - - 1% hydrazine for 24 h, and - - -△- - - 1% GTA for 24 h at pH 6.2. Similar results were obtained in two independent series of experiment.

Treatment with low concentrations of GTA (0.0075 and 0.01% w/v) for only 1 h resulted in an initially low thermal stabilization of collagen (T_d = 61.7 and 64.0 °C), followed during the 3 months storage time by a significant increase (T_d = 65.4 and 70.6 °C, respectively). At these low concentrations, 24 h of treatment was necessary in order to obtain a high initial thermal stability that persisted over time (T_d = 75.6 and 74.6 °C). The best results were obtained after a 1 h treatment with a concentration of 0.05% (initial T_d = 75.8 °C; after 3 months, T_d = 75.6 °C). Increasing the GTA concentration to 1% decreased the thermal stability (T_d = 69.6 °C after 1 h). Resistance to collagenase digestion was optimal after GTA treatment (24 h at 0.0075, 0.01 or 1%), since no degradation was seen after 50 h digestion (Fig. 2a).

3.2. Cross-linking of collagen sponges (procedure 2)

Collagen sponges were treated with cyanamide solution (1%) at pHs ranging from 5.5 to 10.0 for 1 and 24 h. Small increases in thermal stability (Table II) and resistance to collagenase (Fig. 2c) were observed, with a maximum at 1% cyanamide, pH 5.5 after 1 h.

As with procedure 1, the best results were obtained by treatment with GTA. A T_d of 75.3 °C and high resistance to collagenase degradation were observed

TABLE II Thermal transition temperatures of non-cross-linked collagen sponges and of sponges cross-linked (procedure 2) with cyanamide, hydrazine, diphenylphosphorylazide (DPPA) or glutaraldehyde, (GTA)

Conditions of cross-linking				Thermal transition temperature (°C)		
Reticulation agent	Concentration (%, w/v)	pH	Duration of reticulation (h)	Onset	Peak	Recovery
None	–	–	–	36.8 ± 0.7	48.9 ± 0.2	63.1 ± 0.3
Cyanamide	1	5.5	1	58.7 ± 1.6	65.9 ± 0.9	75.9 ± 0.9
		5.5	24	47.7 ± 5.6	57.0 ± 4.7	67.9 ± 4.7
		6.2	1	49.8 ± 6.2	59.5 ± 7.2	71.3 ± 4.7
		6.2	24	51.9 ± 6.8	60.5 ± 0.6	71.0 ± 3.6
		7.2	1	45.4 ± 0.9	54.4 ± 0.5	64.7 ± 0.1
		7.2	24	42.6 ± 1.6	51.4 ± 1.8	65.7 ± 1.6
		10	1	41.7 ± 0.8	53.0 ± 1.9	70.3 ± 3.1
		10	24	42.5 ± 0.3	54.2 ± 0.6	73.5 ± 1.5
Hydrazine	1	–	24	53.4 ± 2.8	64.3 ± 4.0	72.1 ± 1.2
DPPA	0.5	a	24	64.1 ± 2.0	69.2 ± 2.5	86.1 ± 3.0
GTA	1	6.2	24	66.6 ± 1.7	75.3 ± 0.6	84.6 ± 1.2

Each value is the mean and standard deviation of at least three measurements.
a Dimethyl formamide

TABLE III Thermal transition temperatures of non-cross-linked collagen films and of films cross-linked (procedure 2) with cyanamide, hydrazine, diphenylphosphorylazide (DPPA) or glutaraldehyde (GTA)

Conditions of cross-linking				Thermal transition temperature (°C)		
Reticulation agent	Concentration (%, w/v)	pH	Duration of reticulation (h)	Onset	Peak	Recovery
None	–	–	–	44.4 ± 2.9	52.0 ± 1.7	61.3 ± 1.2
Cyanamide	1	5.5	1	46.4 ± 4.5	54.1 ± 3.3	64.8 ± 1.0
		5.5	24	48.3 ± 3.7	55.0 ± 3.2	64.2 ± 2.9
		6.2	1	47.0 ± 4.0	54.8 ± 3.6	69.4 ± 5.0
		6.2	24	49.7 ± 4.1	58.5 ± 5.2	68.3 ± 4.1
		7.2	1	47.4 ± 4.5	55.5 ± 4.4	71.9 ± 2.0
		7.2	24	49.1 ± 1.9	56.7 ± 3.6	67.3 ± 5.5
		10	1	47.4 ± 5.7	55.4 ± 5.4	68.6 ± 2.8
		10	24	52.0 ± 5.2	58.2 ± 3.6	68.1 ± 0.4
Hydrazine	1	–	24	65.3 ± 5.0	69.9 ± 0.6	77.6 ± 3.0
DPPA	0.5	a	24	70.3 ± 5.0	72.6 ± 1.0	79.2 ± 0.5
GTA	0.6	6.2	96	69.6 ± 1.7	74.6 ± 1.5	84.6 ± 1.0

Each value is the mean and standard deviation of at least three measurements.
a Dimethyl formamide

after 24 h treatment with 1% GTA. Treatment of sponges with the acyl azides gave intermediary results for both thermal stability (T_d = 64.3 °C with hydrazine and 69.2 °C with DPPA) and collagenase susceptibility.

3.3. Cross-linking of collagen films (procedure 2)

Collagen films were treated with the same reagents as the collagen sponges (Table III and Fig. 2b). Cyanamide had low cross-linking efficiency (maximal T_d = 58.5 °C). Two films treated at pH 7.2 and 6.2 had similar thermal stability (T_d = 56.7 and 58.5 °C) but different collagenase susceptibilities: the sponge treated at pH 7.2 was similar to the control, but that treated at pH 6.2 had significant resistance. Treatment of the collagen films with 0.6% GTA for 96 h induced high thermal stability (T_d = 74.6 °C) and collagenase resistance, similar to that obtained in sponges (Fig. 2b, d).

Films treated with the acyl azides had high thermal stability, particularly with DPPA (T_d = 72.6 °C). The

resistance to collagenase degradation was similar to that of GTA-treated films (Fig. 2b).

4. Discussion

We have shown that collagen-based biomaterials can be prepared with a wide range of thermal stabilities and collagenase susceptibilities by varying the procedures and reaction conditions. The cross-linking agents studied increased collagen stability in the following order: cyanamide < EDC < HMDC < hydrazine < DPPA < GTA. As expected from the numerous studies on this reagent [4–6, 21, 22], GTA was the most effective agent. In order to prepare collagen sponges with the highest thermal stability (75.6 °C), using the lowest GTA concentration, we treated a collagen gel (0.7%) with 0.0075% GTA at pH 6.2 for 24 h and then freeze-dried it. Several results are worth noting: at a low GTA concentration (0.0075 and 0.01%) after only 1 h treatment, stability increased after a 3-month storage period. A similar "after-hardening" phenomenon was observed with gelatin treated by formaldehyde [23]. At a high concentration (1%), treatment of collagen gel for 1 or 24 h produced sponges with lower thermal stability (69.6 and 70.2 °C). The well-known polymerization action of GTA probably competes with its cross-linking efficiency at this concentration; however, under the same conditions (1%, 24 h), direct treatment of a collagen sponge gave rise to materials with highest thermal stability (75.3 °C). This result confirms the data of the comparative study of Cheung and Nimni [22] with collagen molecules and fibres, which demonstrated the effect on cross-linking of the distance between two reactive sites on the collagen molecule. Intermolecular cross-links, responsible for increased thermal stability and resistance to collagenase digestion, would form more easily in collagen fibres (or sponges and films) than between dispersed molecules (gels).

Carbodiimides are used to couple compounds with several functional groups, such as carboxylic, amino and phenolic groups. For example, Lloyd and Burns [24] and Raghunath *et al.* [25] used water-soluble carbodiimide to couple acrylic polymers and heparin, respectively, to collagen. In our study cyanamide treatment of collagen gel was ineffective in cross-linking collagen molecules. However Weadock *et al.* [17] used dehydrothermal and cyanamide treatment of collagen films for optimal cross-linking. In agreement with their results, and as already observed with GTA, direct treatment with cyanamide of collagen in a solid form (sponge or film) is more effective, with an increase in thermal stability of 12 °C under optimal conditions (e.g. collagen sponge treated with 1% cyanamide for 24 h at pH 6.2). As observed by different authors [14, 18], we obtained a significant effect with EDC (T_d = 61.0 °C after treatment with 1% EDC for 24 h). Recently, it was shown [14, 15] that addition of N-hydroxysuccinimide to an EDC-containing cross-linking solution not only increased the rate of cross-linking of dermal sheep collagen but also resulted in materials with very high increase in thermal stability (+ 30 °C).

Diisocyanates react primarily with amino groups and have been used to cross-link collagen [11–16]. Treatment of collagen gel with 1% HMDC, for 4 days at low temperature and without surfactants or organic solvents, led to collagen sponges with high initial thermal stability (T_d = 70.8 °C), which, however, decreased significantly during the 3-month storage period (T_d = 63.9 °C).

We showed previously [7–10] that treatment of collagen-rich tissue such as pericardium with acyl azides results in a material with thermal stability and resistance to collagenase digestion very similar to those obtained after a GTA treatment. We have shown here that collagen films treated by this method have the same resistance to enzymatic digestion by collagenase as GTA-treated films and a thermal stability as high as 70 °C (74.6 °C with GTA). The structure of the biomaterial seems to be important, since collagen sponges treated similarly to collagen films had significantly lower thermal stability and resistance to collagenase.

Our recent results with an organotypic culture model [10] and those of van Wachem *et al.* [15] with a methyl cellulose cell culture system and after subcutaneous implantation in rats, demonstrate that collagen biomaterials treated with acyl azide methods or with carbodiimide (EDC) have better biocompatibility than the same biomaterials treated with GTA or diisocyanate. As acyl azide and carbodiimide methods do not result in the presence of unreacted functional groups in the material and can be used to couple other molecules containing carboxylic groups (such as glycosaminoglycans), they appear to be the best choices for preparing stable, biocompatible collagen-based biomaterials.

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