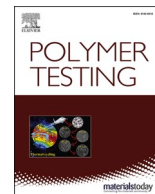




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Exploring the effects of the crosslink density on the physicochemical properties of collagen-based scaffolds

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ABSTRACT

The optimization of collagen-based scaffolds for tissue engineering goes through the careful selection of the crosslinking method(s), which should impart the prerequisite mechanical and degradation properties without impairing the cell/tissue response. Here, we investigated the chemically effective (ρ_{xch}) and the elastically effective (ρ_{xel}) crosslink density of collagen-based scaffolds, induced by various crosslinking methods. The aim was to get a deeper insight into the influence of intramolecular and intermolecular crosslinks on several scaffold properties. Freeze-dried collagen matrices were crosslinked via a dehydrothermal treatment (DHT), and then treated with different chemical agents, including carbodiimide (EDC), glutaraldehyde (GTA), formaldehyde (FA), genipin (GP) and dimethyl suberimidate (DMS). Quantification of primary amines and stress-relaxation compressive tests were performed to evaluate ρ_{xch} and ρ_{xel} , respectively. Scaffolds were then assessed for their water uptake, thermal stability and *in vitro* resistance to enzymatic degradation. Interestingly, for the various crosslinking treatments ρ_{xch} was found to increase in the order DHT < DHT + GP < DHT + DMS < DHT + GTA < DHT + FA < DHT + EDC, while ρ_{xel} increased according to this slightly different trend: DHT < DHT + GP < DHT + DMS < DHT + EDC < DHT + GTA < DHT + FA. Indeed, treatment DHT + EDC induced a higher ρ_{xch} but a lower ρ_{xel} than aldehyde-based ones. This finding, together with the higher denaturation temperature (T_d) of EDC-treated samples compared to others, suggested that zero-length EDC crosslinking promoted intramolecular crosslinks, along with intermolecular ones. Accordingly, the increase of T_d was correlated with the increase of ρ_{xch} rather than ρ_{xel} , whereas the decrease in water uptake was consistent with the increase of ρ_{xel} , as expected. An exponential relationship between ρ_{xel} and the *in vitro* half-life was also determined.

1. Introduction

As a major component of the extracellular matrix (ECM), collagen is an attractive biomaterial for use in tissue engineering and regenerative medicine, due to its excellent biocompatibility, low antigenicity and the presence of cell-instructive cues that elicit cell-material interactions [1–3]. Potential drawbacks of collagen, such as low mechanical stiffness and poor resistance to enzymatic degradation, can be limited or overcome by the use of various crosslinking treatments [4–6]. The establishment of covalent bonds among the collagen molecules, obtained by physical, chemical and/or enzyme-based crosslinking [7–10], is known to yield higher mechanical stiffness and slower degradation in biological fluids. In addition to the indirect effects of these crosslinking-related scaffold properties on the cell behavior [6,11], crosslinking may also directly impair or alter the cellular response, due to the potential

masking of specific collagen sites involved in cell-material interactions (e.g., carboxylate anions typically found on glutamate or aspartate residues that are known to bind to relevant integrin cell surface receptors) [12,13]. As an example, the carbodiimide-based (EDC) crosslinking, which involves the formation of ‘zero-length’ amide bonds between free amine and free carboxylate groups of collagen, has been recently shown to reduce the cellular attachment and spreading to collagen-based films [12,14,15]. In cases where a chemical crosslinker is incorporated within the collagen network, the affinity of the cells to the collagenous substrate may be further reduced or altered by the presence of the crosslinker itself [4,7]. More in general, the *in vivo* host response to collagen-based devices is greatly dependent on the crosslinking treatment used and the extent of achieved crosslinking [16]. In particular, chemical crosslinking methods have been reported to alter the physiological wound healing process, with enhanced pro-inflammatory effects

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associated to heavily crosslinked devices [16,17].

Since no gold standard crosslinking method exists, the quantification of the achieved extent of crosslinking, along with the evaluation of the scaffold properties as well as the assessment of the biological response, appear pivotal to the optimization of collagen-based devices for specific applications. The crosslink density (ρ_x) is generally defined as the number of crosslinked chains per unit volume of the polymer network. According to the classical theory of rubber elasticity [18], the elastic response of the network to deformation is dependent on the elastically effective crosslink density (ρ_{xel}), *i.e.* the number of elastically active crosslinked chains per unit volume. Therefore, ρ_{xel} accounts for the intermolecular crosslinks that are active in deformation and can be estimated from mechanical tests [4,19]. Conversely, the chemically effective crosslink density (ρ_{xch}) takes into account the total number of chains that are engaged in chemical crosslinks, including both of intramolecular and intermolecular crosslinks. As such, ρ_{xch} is generally evaluated by measuring the residual number of free amines [20] or free carboxylic groups [21] achieved upon crosslinking (which may be also an indicator of the potential reduction of cell-binding sites discussed above).

Although ρ_{xel} and ρ_{xch} provide complementary information on the effects of a given crosslinking treatment on the properties of collagen-based scaffolds, the quantification of both parameters is not routinely performed. Most studies on collagen crosslinking assess either ρ_{xch} or ρ_{xel} [4,14,22,23], while others focus on a qualitative assessment of the crosslink density based on some indirect measurements, such as the evaluation of the mechanical stiffness and/or the denaturation temperature [6,16,24].

The aim of this study was to explore the influence of both ρ_{xch} and ρ_{xel} on several physicochemical properties of collagen-based scaffolds. To this purpose, freeze-dried collagen scaffolds were crosslinked via multiple treatments that are commonly used in the literature and involve free amine groups in the formation of crosslinks [4,7,22,25]. These treatments included a dehydrothermal treatment (DHT) and then the further use, right after the DHT, of several chemical crosslinkers in aqueous solutions, for a fixed reaction time: carbodiimide (EDC), glutaraldehyde (GTA), formaldehyde (FA), genipin (GP) and dimethyl suberimidate (DMS).

The scaffolds were first characterized to assess the residual number of free primary amines, as an index of the amount of chemical bonds formed during crosslinking, *i.e.* as an index of ρ_{xch} . The underlying assumption was that all of the crosslinking bonds involved the reaction of free amine groups, thus neglecting the contribution of other potential crosslinks (*e.g.* ester bonds [19]). Then, multiple compressive stress-relaxation tests allowed the determination of ρ_{xel} based on the rubber elasticity theory, together with the evaluation of the scaffold stiffness. Water uptake, thermal stability (denaturation and degradation temperatures) and *in vitro* enzymatic degradation were also investigated, with the aim of finding potential relationships between the crosslink density (either ρ_{xch} or ρ_{xel}) and the assessed properties.

2. Experimental

2.1. Materials

Type I collagen isolated from bovine dermis was supplied by Symatse Biomateriaux (Chaponost, France). All analytical grade chemicals, collagenase and the bicinchoninic acid (BCA) assay kit were purchased from Sigma Aldrich (Milan, Italy) and used as received, unless otherwise noted. Distilled water was obtained from a Millipore Milli-U10 water purification facility.

2.2. Scaffold preparation

An aqueous suspension of 3% w/v collagen was prepared by slowly hydrating dry collagen flakes in distilled water under magnetic stirring,

for 5–6 h. The temperature of the suspension was controlled and kept below 10 °C throughout the stirring, in order to prevent the protein denaturation. At the end, the suspension was degassed via centrifugation (6000 rpm, 5 min, 4 °C) to remove air bubbles. Finally, porous collagen membranes, having a thickness of about 5 mm, were obtained by casting 35 mL of the collagen suspension into a 10 cm Petri dish and further freeze-drying (Virtis Advantage lyophilizer), according to a previously optimized protocol [26].

2.3. Crosslinking treatments

Collagen membranes were subjected to a total of six crosslinking methods to modulate their physicochemical properties, as detailed in the following. Crosslinking methods were selected among those commonly reported in the literature [4,7,22,25], with slight modifications.

Dehydrothermal crosslinking. A dehydrothermal treatment (DHT) was firstly performed, in order to confer the collagen membranes with suitable mechanical and handling properties for further processing. The DHT is a physical zero-length crosslinking method known to induce the formation of amide bonds between carboxyl and amine groups, as well as ester bonds between carboxyl and hydroxyl groups, via condensation reactions [5]. The DHT was here performed by heating the collagen membranes in a vacuum oven ($p < 100$ mTorr) at 120 °C for 72 h, as previously described [19]. Following the treatment, multiple samples were cut from the membranes through a biopsy punch (8 mm) and then used for subsequent crosslinking and/or characterization.

Crosslinking with aldehydes (GTA and FA). Aldehydes such as GTA and FA are well known to react with collagen amino groups [27]. DHT-treated collagen scaffolds, preliminarily hydrated at 4 °C in PBS for 24 h, were immersed either in a 0.25% v/v GTA aqueous solution or in a 0.4% v/v FA solution at room temperature for 2 h, under gentle stirring (300 rpm). Right after chemical crosslinking, extensive washings with distilled water were carried out to remove unreacted chemicals.

Crosslinking with dimethyl suberimidate. DMS, which is reported to be less cytotoxic than aldehydes [28], forms crosslinks between its imidoester groups and the free amino groups of collagen, and precisely with free amino groups separated by a distance equivalent to its molecular length [4,28]. The crosslinking of collagen scaffolds, previously hydrated at 4 °C in PBS for 24 h, was here carried out with 1% w/v DMS in 0.2 M Tris-buffer solution, pH 9.0, at room temperature for 2 h, under gentle stirring (300 rpm). Samples were then extensively washed in distilled water.

Crosslinking with carbodiimide. Chemical crosslinking by means of water soluble EDC was carried out as previously described [7], by soaking hydrated collagen samples in a 14 mM EDC and 5.5 mM N-Hydroxysuccinimide (NHS) aqueous solution, at room temperature for 2 h, under stirring (300 rpm). Whereas NHS is used to enhance the reaction rate and efficiency [29], EDC mediates the condensation reaction between the carboxylate of one amino acid side chain (*i.e.* aspartate, glutamate) with a primary amine on an adjacent amino acid (*i.e.* lysine) [15]. As such, EDC crosslinking is a zero-length crosslinking method.

Crosslinking with genipin. GP is an organic compound derived from the fruit of the gardenia plant (*Gardenia jasminoides*), which is able to react with the primary amino groups of collagen [30], by forming intramolecular and intermolecular crosslinks [22]. GP may bridge peptide chains by introducing dimeric or oligomeric crosslinks [22,31]. In this work, collagen samples were soaked in a 0.03% w/v GP solution buffered with PBS, pH 7.4, for 2 h at room temperature, under stirring (300 rpm).

All of the crosslinked samples were finally stored in PBS at 4 °C for at least 24 h before further characterization or freeze-dried for longer storage.

2.4. Primary amine group content – determination of ρ_{xch}

The concentration of free primary amine groups (-NH₂) in both crosslinked and non-crosslinked collagen matrices was determined using a 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay, as previously described [20]. Briefly, 0.5 mL of a 4% w/v NaHCO₃ solution was added to a dry sample of each scaffold type (about 2 mg). After 30 min, 0.5 mL of a freshly prepared solution of 0.05% w/v TNBS was added. The reaction mixture was heated at 40 °C for 4 h and then 1.5 mL of 6 M HCl solution was added. The samples were hydrolyzed at 60 °C for 90 min. Subsequently, the reaction mixture was diluted with 2.5 mL of distilled water, cooled down to room temperature and the absorbance at 339 nm read using a UV-visible spectrophotometer (Agilent Cary 5000). The absorbance was correlated to the concentration of free amines using a calibration curve obtained with glycine in aqueous NaHCO₃ solution (0.1 mg/mL). Blank samples were prepared with the same procedure described above, except that HCl was added before the addition of the TNBS solution. Assessments of free (-NH₂) were performed in triplicate.

Assuming that non-crosslinked samples contain 100% of the available amine groups of collagen, this value was used as a reference to firstly calculate the percentage of reacted (-NH₂) after a given crosslinking treatment. Since the chemically effective crosslink density (ρ_{xch}) estimates the number of chains engaged in crosslinks, for the subsequent calculation of ρ_{xch} we took into account the stoichiometric number of amines involved in each crosslink. Therefore, for crosslinking treatments that engage only one amine group per crosslink, such as DHT and DHT + EDC crosslinking (1:1 amine:crosslink), ρ_{xch} was calculated according to the following:

$$\rho_{xch} = \frac{\text{Free NH}_{2\text{uncrosslinked}} - \text{Free NH}_{2\text{crosslinked}}}{\text{Free NH}_{2\text{uncrosslinked}}} \times 100 \quad (1)$$

For all the other doubly crosslinked samples, involving both DHT treatment and the use of chemicals (i.e. GP, DMS, GTA and FA) that bind two amine groups per crosslink (2:1 amine:crosslink), the equivalent number of chains engaged in crosslinks was estimated as the sum of: (a) the number of chains/aminos reacted via the preliminary DHT treatment (assumed constant) and (b) the number of amines reacted with the given chemical, divided by the stoichiometric ratio 2. Hence, for DHT + GP, DHT + DMS, DHT + GTA and DHT + FA samples, the equivalent ρ_{xch} was calculated as follows:

$$\rho_{xch} = \left(\frac{\text{Free NH}_{2\text{uncrosslinked}} - \text{Free NH}_{2\text{DHT}}}{\text{Free NH}_{2\text{uncrosslinked}}} + \frac{1}{2} \times \frac{\text{Free NH}_{2\text{DHT}} - \text{Free NH}_{2\text{crosslinked}}}{\text{Free NH}_{2\text{uncrosslinked}}} \right) \times 100 \quad (2)$$

2.5. Mechanical testing – determination of ρ_{xel}

Multiple stress relaxation compressive tests were performed on denatured collagen membranes, in order to evaluate both the stiffness and the elastically effective crosslink density, based on the classical theory of rubber elasticity [18]. Hence, upon hydrothermal denaturation collagen behaves as a rubberlike material, so as the rubber elasticity theory can be applied to estimate the value of ρ_{xel} [32].

Briefly, for an ideal, perfect network, the number of elastically effective chains (ν_e) corresponds to the total number of chemically

crosslinked chains (ν), so that ρ_{xel} can be calculated as:

$$\rho_{xel} = \frac{\nu_e}{V} = \frac{\nu}{V} = \frac{\rho}{M_c} \quad (3)$$

where ρ is the polymer density, M_c is the average molecular weight between two consecutive crosslinks and V is the volume of the polymer network.

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 [18] derived the following relationship between the uniaxial stress and the uniaxial deformation of a swollen crosslinked polymer with a rubberlike behavior:

$$\sigma = RT\rho_{xel}V_2^{1/3} \left(\alpha - \frac{1}{\alpha^2} \right) = G \left(\alpha - \frac{1}{\alpha^2} \right) \quad (4)$$

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, $\alpha = L/L_i$ is the deformation ratio, with L the actual thickness of the deformed sample and L_i the initial thickness of the sample ($\alpha > 1$ for elongation and $\alpha < 1$ for compression, respectively) and G is the shear modulus of the swollen polymer. Therefore, the plot of σ against the quantity $(\alpha - 1/\alpha^2)$ is linear, with a slope that defines the shear modulus G and is directly correlated to the value of ρ_{xel} .

In the case of collagen, the polymer volume fraction V_2 can be calculated as the inverse of the volumetric swelling ratio SR , defined by the following:

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

where 1.32 g/cm³ is the density of anhydrous collagen [33].

According to a previously described protocol [28], mechanical tests were carried out on hydrated collagen matrices using a universal testing machine (LR5K, Lloyd Instruments, Bognor Regis, UK), equipped with a 10 N load cell, custom-made clamping tools and a bath chamber. Cylindrical matrices ($n = 5$ for each type) were firstly denatured by placing them in PBS for 2 min at 80 °C, then mounted on the testing machine and let to equilibrate in PBS at room temperature for 2 h, before starting the measurement. Multiple stress relaxation compressive tests were performed, by subjecting the collagen matrices to 4 steps of loading and

stress relaxation, under displacement control (5% increased elongation and 180 s dwelling time at each step).

At the end of the test, samples were gently blotted with soft paper and weighed, in order to measure their wet mass. After dehydrating in oven (60 °C) for 4 h, the dry mass of the samples was measured and used to calculate the collagen volume fraction, as the inverse of the SR defined in Eq. (5). Finally, ρ_{xel} was estimated from Eq. (4).

2.6. Water uptake

The water uptake of the collagen matrices was measured as follows. Dry matrices ($n = 9$ for each type) were weighed (M_{coll}), and then hydrated in PBS for 3 h at room temperature. After removing excess water

with filter paper, wet matrices were weighed ($M_{\text{coll}} + M_{\text{water}}$). The ratio of the weight of the hydrated sample ($M_{\text{coll}} + M_{\text{water}}$) to that of the dried sample (M_{coll}) was used to calculate the volumetric swelling ratio SR, according to Eq. (5).

2.7. Denaturation and degradation temperatures

Differential scanning calorimetry (DSC Mettler Toledo, DSC1 STARE System) was employed to evaluate the influence of the crosslinking treatments on the thermal stability of the collagen scaffolds. The denaturation temperature (T_d), at which the triple helix is transformed into a random coil structure, is known to be affected by the degree of crosslinking [1,34], similarly to the degradation temperature (T_{II}), at which residual bound water evaporates, other conformational modifications occur and crosslinks are broken [24]. For the determination of T_d , the matrices were hydrated at 4 °C overnight in phosphate buffered saline (PBS), weighed (10–15 mg), introduced into aluminum pans and heated from 5 °C to 80 °C at a constant heating rate of 5 °C/min, in nitrogen atmosphere. In order to assess T_{II} , dry matrices were weighed (10–15 mg), introduced into the aluminum pans and heated from 25 °C to 300 °C at a constant rate of 10 °C/min, in nitrogen atmosphere. The denaturation and degradation temperatures were measured at the mid-point of the corresponding endothermic peak. Different sample types were assessed in triplicate.

2.8. In vitro collagenase degradation

In vitro collagenase digestion experiments were carried out by a procedure reported earlier [7,26]. Briefly, dry collagen scaffolds of about 6 mg were accurately weighed, hydrated in PBS and then incubated in 6 mL of PBS with 0.1 mg/mL collagenase (from *Clostridium histolyticum*) solution, at 37 °C. At fixed time points (0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4 and 6 h), degradation was stopped by freezing the supernatant solution at –40 °C. The weight loss (%) of the matrices at a given time point was evaluated as the amount of collagen solubilized in the incubating solution, measured by means of the BCA assay. Three independent measurements were performed at each time point, for each sample type.

A single exponential decay rule, valid for enzymatic degradation [5], was then used to fit the weight loss data:

$$\frac{M_t}{M_0} = e^{-kt} \quad (6)$$

where M_t is the residual mass of the sample at time t , M_0 is the initial mass and k is the degradation rate constant. The *in vitro* half-life of the device, *i.e.* the time $t_{1/2}$ at which $M_t/M_0 = 0.5$, was thus calculated by substituting the corresponding value of M_t/M_0 in Eq. (6).

2.9. Statistical analysis

Data are expressed as the mean \pm the standard deviation. Analysis of variance (ANOVA) was applied to determine the effect of the crosslinking treatment on the number of moles of free primary amine groups,

Table 1

Number of free primary amine groups, percentage of reacted amine groups and equivalent chemically effective crosslink density (ρ_{xch}) yielded by the different crosslinking treatments, as estimated from the TNBS assay ($n = 3$; mean \pm SD); shear modulus G and elastically effective crosslink density (ρ_{xel}) of the collagen-based scaffolds, as obtained via stress relaxation compressive tests ($n = 5$; mean \pm SD). n.d. = not detected.

Crosslinking treatment	Free -NH ₂ (10 ⁵ mol/g)	Reacted -NH ₂ (%)	ρ_{xch} (%)	G (kPa)	ρ_{xel} (10 ⁻⁵ mol/cm ³)
None	5.7 \pm 0.7	0.0 \pm 0.0	0.0 \pm 0.0	n.d.	n.d.
DHT	5.4 \pm 0.1	4.2 \pm 1.6	4.2 \pm 1.6	8.6 \pm 0.7	1.0 \pm 0.1
DHT + GP	5.3 \pm 0.1	6.7 \pm 1.7	6.2 \pm 1.0	7.2 \pm 0.8	0.9 \pm 0.1
DHT + DMS	3.6 \pm 0.5	36.6 \pm 9.1	20.9 \pm 4.3	16.7 \pm 1.6	2.1 \pm 0.2
DHT + EDC	2.7 \pm 0.1	52.5 \pm 0.3	52.5 \pm 0.3	32.3 \pm 1.6	3.7 \pm 0.2
DHT + GTA	2.4 \pm 0.2	57.5 \pm 0.5	31.4 \pm 1.6	39.8 \pm 4.9	4.3 \pm 0.5
DHT + FA	2.3 \pm 0.1	59.1 \pm 1.2	32.2 \pm 0.8	44.6 \pm 0.4	4.7 \pm 0.1

chemically effective crosslink density, shear modulus, elastically effective crosslink density, water uptake and denaturation/degradation temperatures of the tested samples. Fisher's PLSD tests were also performed to compare individual sets of data. A probability value of 95% ($p < 0.05$) was used as the criterion for significance.

3. Results

3.1. Primary amine group content

The reaction of TNBS with the primary amine groups of proteins was here used to determine the number of free primary amine groups of crosslinked collagen scaffolds with respect to non-crosslinked ones, in order to estimate the chemically effective crosslink density ρ_{xch} achieved by the different crosslinking treatments. Values of ρ_{xch} from Eq. (1) and Eq. (2) accounted for the different stoichiometric amine:crosslink ratios involved in the various crosslinking reactions. In particular, a 1:1 ratio holds for DHT and DHT + EDC crosslinking (Eq. (1)), while a combination of 1:1 (DHT) and 2:1 ratios (GP, DMS, GTA and FA) holds for all the other double crosslinking methods (Eq. (2)). The number of free primary amine groups per gram of material, before and after each crosslinking treatment, is reported in Table 1 together with the corresponding values of reacted amines and ρ_{xch} .

As expected, the crosslinking treatment had a significant impact on the number of free primary amines ($p < 0.0001$, power = 1). Physical DHT treatment was able to induce a moderate decrease (approximately 4%) of the free amine groups of collagen, compared to untreated samples ($p < 0.05$). Then, as expected an additional chemical crosslinking treatment was found to further reduce the number of free primary amine groups, *i.e.* to increase the number of reacted amines according to the following trend: DHT < DHT + GP < DHT + DMS < DHT + EDC < DHT + GTA < DHT + FA. However, the number of free amine groups measured for treatment DHT + GP was not significantly different from that of DHT only ($p = 0.45$), while the number of free amines for treatment DHT + EDC was comparable to treatments DHT + GTA ($p = 0.15$) and DHT + FA ($p = 0.06$). Notably, when calculating the corresponding values of ρ_{xch} , the double treatment DHT + EDC was the one inducing the highest ρ_{xch} , equal to about 52% (*i.e.* about 12-fold with respect to DHT alone). This value was also significantly different from that shown by treatments DHT + GTA (about 31%, $p < 0.0001$) and DHT + FA (about 32%, $p < 0.0001$). In terms of ρ_{xch} , treatment DHT + GP was comparable to DHT alone ($p = 0.26$), while treatment DHT + DMS, which achieved a ρ_{xch} of about 21% (*i.e.* about 5-fold compared to DHT alone), showed an intermediate effectiveness between that of DHT + GP and DHT with aldehydes (either GTA or FA). The overall trend describing the increase of ρ_{xch} was as follows: DHT < DHT + GP < DHT + DMS < DHT + GTA < DHT + FA < DHT + EDC.

3.2. Mechanical testing

Multiple stress-relaxation compressive tests, performed on denatured collagen scaffolds, allowed estimating the elastically effective crosslink density ρ_{xel} , based on Eq. (4). Non-crosslinked scaffolds could not be

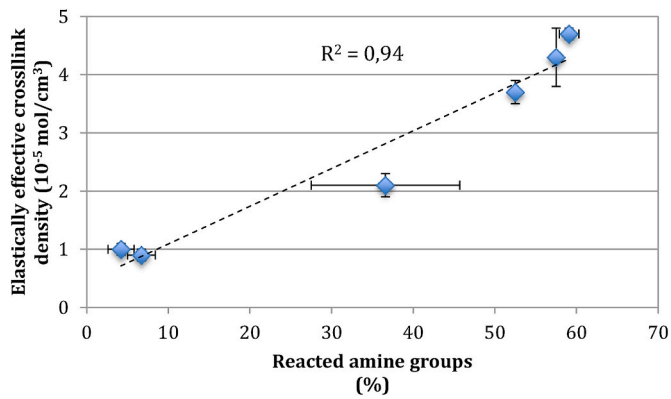


Fig. 1. Linear correlation between ρ_{xel} and the percentage of reacted amine groups measured for the collagen scaffolds ($R^2 = 0.94$).

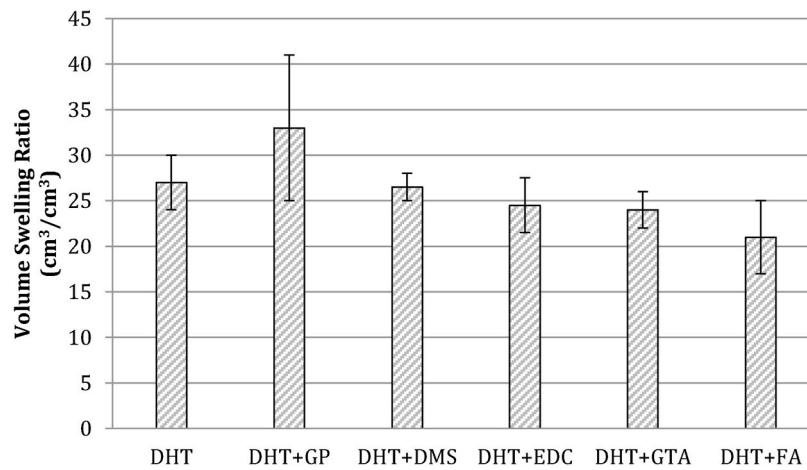
tested, since they rapidly dissolved upon preliminary collagen denaturation.

As expected, the crosslinking treatments were found to significantly affect the shear modulus G of the scaffolds, as well as their ρ_{xel} (Table 1; $p < 0.0001$, power = 1). A progressive increase of G and ρ_{xel} was indeed detected when moving from DHT to DHT + FA treatment, with both G and ρ_{xel} showing approximately a 5-fold increase for the double treatment DHT + FA, with respect to DHT alone. Such an increasing trend was similar to the one found for the reacted amine groups (i.e. DHT < DHT + GP < DHT + DMS < DHT + EDC < DHT + GTA < DHT + FA). In this regard, a linear correlation between the values of ρ_{xel} and the percentage of reacted amines was detected (Fig. 1, $R^2 = 0.94$).

In agreement with the number of reacted amines, the DHT + GP crosslinking was comparable to the DHT one, in terms of both G ($p = 0.59$) and ρ_{xel} ($p = 0.69$), while crosslinking with aldehydes (GTA and FA) led to the highest values of G and ρ_{xel} , with no significant difference between the two treatments (DHT + GTA vs. DHT + FA: $p = 0.18$ for G , $p = 0.12$ for ρ_{xel}).

When comparing the values of ρ_{xch} and ρ_{xel} for each crosslinking treatment, it is interesting to observe that both crosslink densities correlated well with each other, with the exception of the values

(A)



(B)

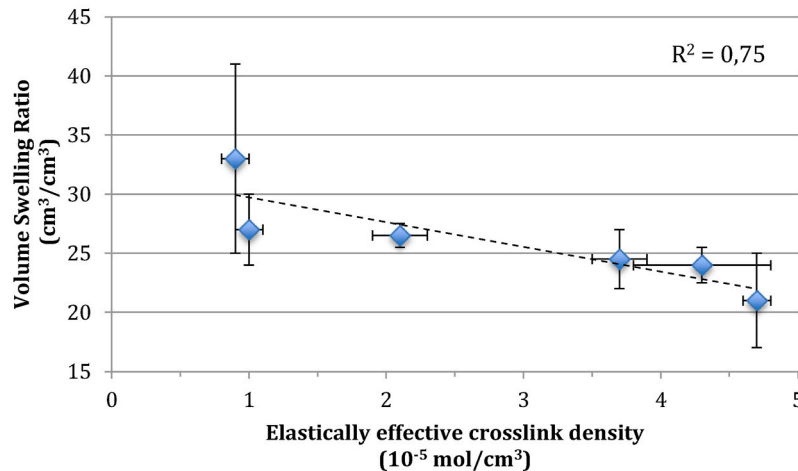


Fig. 2. (A) Volumetric swelling ratio (SR) of the collagen scaffolds upon different crosslinking treatments ($n = 9$; mean \pm SD); (B) linear reduction of the SR as a function of ρ_{xel} ($R^2 = 0.75$).

measured for treatment DHT + EDC. Indeed, this treatment was found to lead to a significantly lower ρ_{xel} than DHT + FA (Table 1; $p < 0.05$), in spite of achieving the highest ρ_{xch} . This suggested that an appreciable amount of zero-length intramolecular crosslinks (which are not elastically effective) formed upon the EDC treatment, along with intermolecular ones.

3.3. Water uptake

The volumetric swelling ratio SR of the crosslinked collagen scaffolds, calculated according to Eq. (5), is represented in Fig. 2A. As expected, the crosslinking treatment was found to significantly affect the SR, with decreasing SR values yielded for increasingly effective crosslinking treatments ($p = 0.0044$, power = 0.943). However, compared to the single DHT treatment, only the DHT + FA one was found to lead to a noticeable reduction of the SR ($p = 0.05$). Moreover, scaffolds crosslinked by the DHT + GP treatment showed an average SR higher than that obtained for the other samples ($p < 0.05$). This is consistent with the swelling increase of GP crosslinked collagen scaffolds reported by Mekhail et al. [35], which may be attributed to the potential formation of GP oligomers upon crosslinking, with the consequent formation of porosity [36].

Linear regression analysis highlighted a linear reduction of the SR with the increase of ρ_{xel} (Fig. 2B, $R^2 = 0.75$), while this trend was not observed when considering ρ_{xch} .

3.4. Calorimetric analysis

Differential scanning calorimetry was employed to analyze the thermal stability of the collagen scaffolds, by detecting their denaturation (T_d) and degradation (T_{II}) temperatures. Collagen denaturation involves the rupture of the hydrogen bonds that stabilize the triple helix, without affecting the crosslinking bonds [25]. Conversely, thermal degradation involves more severe conformational changes, including the rupture of chemical crosslinks [24]. As expected, the crosslinking treatment had a significant effect on the values of both T_d and T_{II} (Table 2; $p < 0.0001$, power = 1), with a general increase of the two temperatures for increasing crosslink densities.

With specific regard to denaturation, DHT and DHT + GP treatments did not result in a significant increase of T_d compared to uncrosslinked samples. This was consistent with their mild increase of ρ_x (both ρ_{xch} and ρ_{xel}), as shown in Table 1. Interestingly, while the T_d measured for the DHT + DMS samples was similar to that of DHT + GTA and DHT + FA ones ($p = 0.12$ and $p = 0.13$, respectively), the average T_d shown by the DHT + EDC scaffolds was the highest one to be detected. This finding, along with the results obtained for ρ_{xch} and ρ_{xel} , suggested that the establishment of intramolecular crosslinks, likely yielded by the zero-length EDC treatment, contributed to enhance the stability of the collagen molecule, thus the collagen resistance to denaturation. In particular, a linear correlation between T_d and ρ_x was found, with a much higher R^2 coefficient obtained for the plot of T_d vs. ρ_{xch} (Fig. 3, $R^2 = 0.94$) than for the plot of T_d vs. ρ_{xel} ($R^2 = 0.65$).

As for the degradation temperature, a certain increase of T_{II} was noted upon crosslinking. However, DHT and DHT + DMS samples

Table 2

Denaturation (T_d) and degradation (T_{II}) temperatures of the collagen scaffolds, measured upon different crosslinking treatments ($n = 3$; mean \pm SD).

Crosslinking treatment	T_d (°C)	T_{II} (°C)
None	36.9 \pm 0.8	205.5 \pm 1.0
DHT	37.4 \pm 0.6	205.7 \pm 0.7
DHT + GP	37.5 \pm 0.1	226.3 \pm 0.3
DHT + DMS	48.7 \pm 2.7	213.0 \pm 11.5
DHT + EDC	58.4 \pm 0.8	223.8 \pm 2.2
DHT + GTA	47.0 \pm 1.2	231.7 \pm 0.1
DHT + FA	50.4 \pm 0.7	232.3 \pm 0.0

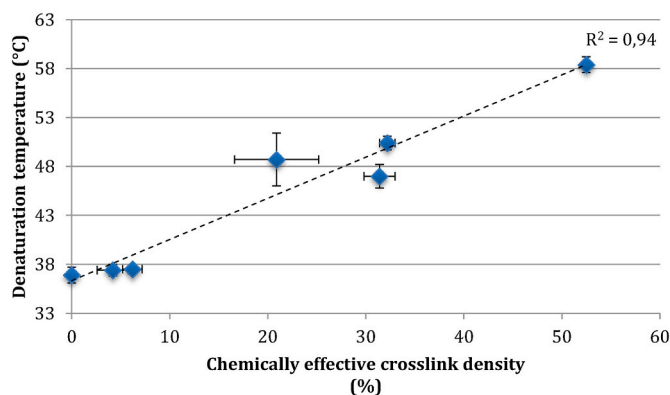


Fig. 3. Linear increase of the denaturation temperature (T_d) of the collagen scaffolds with the increase of ρ_{xch} ($R^2 = 0.94$).

showed a similar T_{II} to uncrosslinked samples ($p = 0.96$ and $p = 0.07$, respectively), in spite of having very different crosslink densities (Table 1). Analogously, DHT + GP samples had T_{II} values very close to those of DHT + EDC samples ($p = 0.5$), regardless of their dissimilar crosslink density. Therefore, differently from what found for T_d , no clear correlation could be detected between the degradation temperature and the crosslink density of the collagen scaffolds. The value of T_{II} seemed to be greatly affected by the specific crosslinking treatment and the incorporation of a given molecule (such as GP) within the collagen network, in addition to the achieved crosslink density.

3.5. In vitro collagenase degradation

The susceptibility of the collagen scaffolds to enzymatic degradation was assessed *in vitro* in the presence of bacterial collagenase. While non-crosslinked samples were found to totally disintegrate within 5 min of incubation (data not shown), the complete solubilization of DHT-treated scaffolds occurred within 1 h (Fig. 4). This confirmed that DHT was able to increase the collagen resistance to degradation, although being a mild crosslinking treatment.

Then, the use of further chemical crosslinking treatments, in addition to DHT, influenced the degradation kinetics of the collagen scaffolds in a manner that was proportional to the achieved crosslink density. The GP treatment, which led to ρ_{xch} and ρ_{xel} values similar to DHT (Table 1), did not significantly increase the degradation resistance of the scaffolds ($p = 0.6$), with a complete dissolution occurring after about 2 h. Conversely, the more effective DMS and EDC treatments induced a mass loss of approximately 90% and 50% after 8 h, respectively. It is worth noting that the scaffolds crosslinked with the aldehydes, which exhibited the highest values of ρ_{xel} but lower values of ρ_{xch} compared to the DHT + EDC treated scaffolds, showed the slowest degradation rates, with a mass loss lower than 5% after 7 h of incubation for the DHT + GTA samples and a mass loss lower than 3% after 24 h for the DHT + FA samples (data not shown). The different degradation kinetics of DHT + GTA and DHT + FA scaffolds, with respect to DHT + EDC ones, suggested that ρ_{xel} , rather than ρ_{xch} , is the parameter governing the scaffold degradation rate, *i.e.* intermolecular crosslinks are likely more effective than intramolecular ones to slow down the enzymatic attack.

Weight loss data at the different time points, obtained for DHT, DHT + GP, DHT + DMS and DHT + EDC samples (Fig. 4), were fitted by the single exponential decay rule shown in Eq. (6), in order to estimate the *in vitro* half-life of the scaffolds (Table 3). As expected, increasing values of the *in vitro* half-life, in the approximate range 12–173 min, were obtained for samples having a higher crosslink density.

Considering the calculated values of the half-life of the scaffolds as a function of ρ_{xel} , we found that the half-life increase could be fitted by an exponential relationship of the following type ($R^2 = 0.90$):

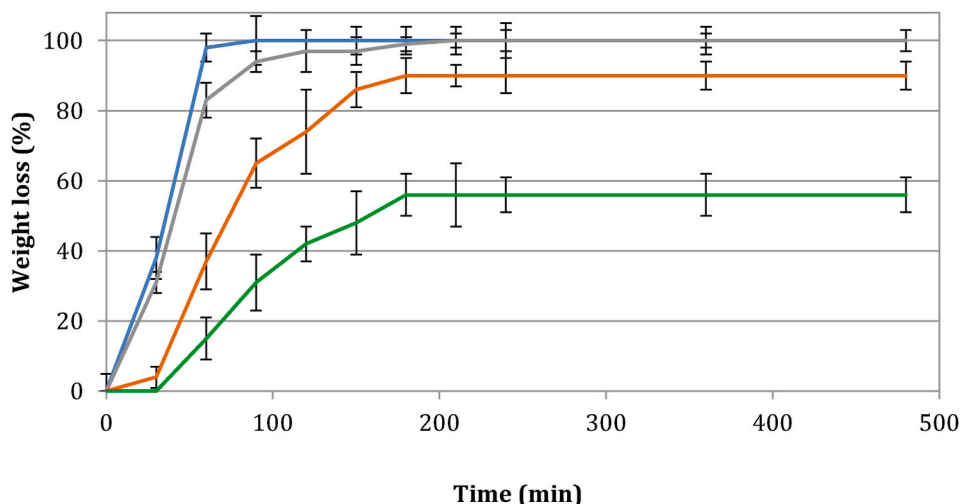


Fig. 4. *In vitro* collagenase degradation kinetics of collagen scaffolds treated by DHT (blue), DHT + GP (grey), DHT + DMS (orange) and DHT + EDC (green), monitored over 6 h ($n = 3$ at each time point; mean \pm SD).

Table 3

Fitting of the degradation data of collagen scaffolds treated by DHT, DHT + GP, DHT + DMS and DHT + EDC with the single exponential decay rule in Eq. (6). Values of R^2 and the degradation rate constant are reported, together with the *in vitro* half-life of the scaffolds.

Crosslinking treatment	R^2	Degradation rate constant k (min^{-1})	Half-life (min)
DHT	0.70	0.055	12.6
DHT + GP	0.93	0.026	26.6
DHT + DMS	0.94	0.012	57.7
DHT + EDC	0.94	0.004	173.2

$$t_{1/2} = Ae^{B/\rho_{\text{set}}} \quad (7)$$

where $t_{1/2}$ is the half-life and A and B are two constants ($A = 8.89$ min and $B = 0.082$ m^3/mol).

This type of relationship was in agreement with the findings of a previous study from our laboratory [7].

4. Discussion

Crosslinking is a fundamental step in the manufacture of collagen-based medical devices, as non-crosslinked collagen would be readily degraded in the physiological environment and unable to sustain mechanical loads [4–9]. While blending with other biomaterials could represent an alternative route to crosslinking [37–39], the formation of crosslinks among the collagen molecules, induced by various physical and/or chemical treatments, is well known to stiffen the collagen-based devices and to increase their *in vivo* residence time, in a manner proportional to the achieved crosslink density ρ_x , *i.e.* the number of crosslinked chains per unit volume [4–10]. Commonly used physical treatments to crosslink collagen include the DHT and the UV crosslinking [9,13,40]. These treatments are intrinsically advantageous over chemical ones, due to the absence of exogenous compounds that may compromise the cytocompatibility of the collagen substrate. However, although the crosslink density can be controlled to a certain extent by varying some crosslinking conditions (*e.g.* temperature, time) [9,19], the crosslinking efficiency of physical treatments is generally limited, thus leading to only mild improvements of the mechanical and degradation properties of the collagen devices [5,7,8]. As such, up to date a plethora of chemical crosslinking agents have been investigated, in order to more effectively modulate the properties of various collagen substrates (*e.g.* films, porous sponges, fibrous mats) as needed. Such agents range from

common and widely investigated ones, such as aldehydes and water-soluble carbodiimide (EDC) [4,7,22,25], to novel and less explored agents, such as shikimic acid [41] and oxidized chitosan oligosaccharide [42]. However, the cytotoxicity that may be induced by the incorporation of certain crosslinkers in the collagen network (*e.g.* aldehydes) should be accounted for, since it may practically limit the applications in the medical field [43,44]. In this regard, crosslinkers of natural origin, such as genipin (GP), or zero-length crosslinkers, such as EDC, may be preferred, since they are generally related to low cytotoxicity and low immunogenicity [23,43,45,46].

With specific regard to the cellular response to crosslinked collagen, it should be noted that crosslinking (achieved through physical, chemical and enzymatic treatments) could alter the exposure of cell-binding domains on the collagen molecules, by masking them or engaging them in crosslinks [12–15]. Upon EDC crosslinking, collagen-based films [13,15] and scaffolds [47] have been found to lead to diminished cellular attachment, with respect to various cell types (*e.g.* platelets, myoblasts, mesenchymal stem cells). Furthermore, the enhanced stiffness and residence time of the scaffold, induced by crosslinking, could additionally affect the cell/tissue response [6,11]. For instance, collagen crosslinking by either EDC or GP has been recently shown to promote the *in vitro* stability of tubular structures, like blood and lymphatic vessels [48]. As for the *in vivo* response, collagen devices that degrade too slowly, due to heavy crosslinking, are generally associated to calcification and foreign body response [16,17]. Hence, both the physicochemical and biological effects of given crosslinking treatments should be accurately evaluated to identify the optimal crosslinking method for the intended application.

In this scenario, the quantification of the crosslink density induced by given crosslinking treatments is fundamental to the understanding of the crosslinking mechanisms, as well as the modulation of the scaffold properties. In this work, we estimated both the chemically effective (ρ_{xch}) and the elastically effective crosslink density (ρ_{xel}) of collagen-based scaffolds achieved upon different crosslinking methods, with the double aim of comparing the efficacy of the different methods and getting more detailed information on the effects of intramolecular and intermolecular crosslinks on the scaffold properties. First of all, in order to finely modulate the crosslink density of freeze-dried collagen scaffolds, we coupled a preliminary DHT treatment with the use of several chemical crosslinkers, including GP, EDC, dimethyl suberimidate (DMS), glutaraldehyde (GTA) and formaldehyde (FA). The combination of two (or more) crosslinking treatments indeed offers a synergistic way to control the scaffold properties [49]. The choice of performing the DHT treatment right before the use of chemical crosslinkers was based

on the findings of previous studies [7,9,19], which showed that: (a) DHT crosslinking is able to stabilize the structure of porous scaffolds for further manipulation as well as for subsequent crosslinking in aqueous solutions, where capillary forces may induce a distortion or collapse of the pore walls [7]; (b) DHT treatment is also able to induce a limited amount of denaturation (in addition to crosslinking) [9,19], which may be useful to increase the number of cell binding sites, by the exposure of cell ligands that are unavailable in the triple helical configuration [14].

Interestingly, by estimating the values of ρ_{xch} and ρ_{xel} induced by the various crosslinking treatments (Table 1), we observed that: (a) based on ρ_{xch} , which accounts for both intramolecular and intermolecular crosslinks, the efficacy of the treatments varied according to the following order: DHT < DHT + GP < DHT + DMS < DHT + GTA < DHT + FA < DHT + EDC, with values of ρ_{xch} in the approximate range 4%–52%; (b) based on ρ_{xel} , which accounts for intermolecular crosslinks only, the efficacy of the treatments was consistent with the following trend: DHT < DHT + GP < DHT + DMS < DHT + EDC < DHT + GTA < DHT + FA, with values of ρ_{xel} directly proportional to the number of reacted amine groups (Fig. 1) and in the range $1 \times 10^{-5} - 4.7 \times 10^{-5} \text{ mol/cm}^3$. When comparing the values of ρ_{xch} and ρ_{xel} yielded by the different treatments, the crosslink densities were thus found to correlate quite well with each other, with the exception of those obtained for the DHT + EDC treatment. Indeed, the DHT + EDC crosslinking led to a higher ρ_{xch} but a lower ρ_{xel} than the DHT + GTA and DHT + FA ones. This seemed to suggest that, being a zero-length treatment (differently from the aldehydes ones), the EDC crosslinking elicited the formation of a considerable amount of elastically ineffective, intramolecular crosslinks.

After the evaluation of ρ_{xch} and ρ_{xel} , crosslinked scaffolds were assessed for their water uptake, thermal stability and *in vitro* resistance to enzymatic degradation. With regard to the water uptake, expressed in terms of volumetric swelling ratio (SR), we did not find significant differences among the distinctly crosslinked scaffolds (Fig. 2A), with the exception of GP-crosslinked samples, which showed a higher swelling compared to others. However, a linear trend describing a slight reduction of the SR with the increase of ρ_{xel} was found (Fig. 2B), while no clear dependence of the SR on ρ_{xch} was observed. These findings suggested that swelling measurements, performed on porous scaffolds, are not accurate enough to provide a proper comparison of different crosslinking treatments, likely due to the fact that the water uptake is also affected by additional structural parameters, such as hydrophilicity and porosity. However, a certain correlation exists between the SR and ρ_{xel} , in agreement with the rubber elasticity theory.

Notably, the results of calorimetric analyses, which highlighted a higher denaturation temperature (T_d) for the EDC-treated samples compared to others (Table 2), were consistent with the formation of intramolecular crosslinks upon EDC treatment, as suggested by the comparison of ρ_{xch} and ρ_{xel} . Indeed, in addition to intermolecular crosslinks, intramolecular ones are expected to contribute to the stability of the collagen triple helix, thus increasing the resistance to molecular unfolding. Accordingly, we found that the values of T_d are well correlated with the values of ρ_{xch} (Fig. 3), whereas the interdependence with ρ_{xel} is less pronounced. Therefore, the evaluation of T_d by means of calorimetric analyses seems to provide a good qualitative comparison of the ρ_{xch} yielded by different crosslinking treatments. On the contrary, no clear correlation between the crosslink density (either ρ_{xch} or ρ_{xel}) and the degradation temperature (T_{II}) of the scaffolds could be detected, thus suggesting that T_{II} is not a useful parameter to qualitatively assess the efficacy of different crosslinking methods.

Furthermore, collagenase-induced degradation tests showed that the degradation rate of the scaffolds was significantly slowed down by an increase of the crosslink density, especially ρ_{xel} (Fig. 4). Indeed, DHT-crosslinked scaffolds completely degraded within 1 h of enzymatic exposure, GTA- and FA-treated ones showed only a negligible weight loss along the experimental time window, while EDC-treated specimens (that exhibited a lower ρ_{xel} but a higher ρ_{xch} than aldehyde-treated ones) showed a degradation of about 50% after 8 h. The comparison between

aldehyde-treated and EDC-treated samples thus suggested that the degradation rate of collagen-based scaffolds is mostly dependent on intermolecular crosslinks, rather than intramolecular ones. Interestingly, degradation data for samples crosslinked by DHT + GP, DHT + DMS and DHT + EDC (Table 3) were in very good agreement with the exponential decay rule reported in the literature for enzymatic degradation [5]. Moreover, we found an exponential dependence of the *in vitro* half-life on the values of ρ_{xel} (Eq. (7)). These results indicated that enzymatic degradation tests are very useful to compare the efficacy of multiple crosslinking treatments, with the scaffold half-life providing an indirect, quantitative estimation of ρ_{xel} .

Overall, this study provided evidence that the single evaluation of either ρ_{xch} or ρ_{xel} might be a useful method to compare the effectiveness of multiple crosslinking treatments, in cases where the intramolecular crosslinks formed upon crosslinking are negligible. Conversely, when intramolecular crosslinks are substantial, as here found for treatment DHT + EDC, ρ_{xch} and ρ_{xel} values diverge, so that the single determination of either ρ_{xch} or ρ_{xel} may be misleading or provide only a partial understanding of the effects of given crosslinking treatments on the scaffold properties. Therefore, the determination of both ρ_{xch} and ρ_{xel} , together with a comprehensive scaffold characterization (including the evaluation of physicochemical, mechanical and biological properties), appear as the safest way to tailor the design of collagen-based scaffolds to selected applications.

As a final note, it is important to remind that the crosslink density ρ_{xch} evaluated in this work specifically accounts for the reacted amine groups. An alternative route to estimate ρ_{xch} might be also based on the quantification of residual carboxylic groups [21]. Although these approaches to estimate ρ_{xch} can be applied to most of the collagen crosslinking methods, which indeed utilize amine and/or carboxylate groups for crosslinking, there might be other methods of interest that do not involve those functional moieties for bonding. UV crosslinking, for example, is known to induce bonds between free radicals that are generated on aromatic collagen residues, like tyrosine and phenylalanine [8,40]. Due to the small amount of aromatic amino acids (less than 2%) in collagen [13], the efficacy of this method is modest, especially with respect to amine-based crosslinking treatments [13,50]. However, it represents an attractive option for crosslinking, while remarkably preserving the cell binding sites of collagen [13,50]. For UV crosslinking (or any other method that does not utilize amines), the direct evaluation of ρ_{xch} , as here described, does not seem straightforward. Nonetheless, our findings suggest that the denaturation temperature might be a reliable indicator of the achieved ρ_{xch} . Therefore, we expect that the assessment of the denaturation temperature, along with the evaluation of ρ_{xel} , would ideally allow for a proper comparison of the efficacy of different crosslinking treatments, regardless of the specific chemical linkages formed upon crosslinking.

5. Conclusions

In this work, we investigated the use of various crosslinking treatments to modulate the mechanical and degradation properties of collagen-based scaffolds. In order to get an accurate estimation of the extent of crosslinking achieved by the different treatments, we evaluated both the chemically effective (ρ_{xch}) and the elastically effective (ρ_{xel}) crosslink density, and then analyzed several scaffold properties that are commonly assessed as qualitative indicators of the crosslink density (e.g. water uptake, denaturation temperature and degradation rate). Notably, ρ_{xch} and ρ_{xel} correlated well with each other for all the crosslinking treatments except the DHT + EDC one. For this treatment, the dual estimation of ρ_{xch} and ρ_{xel} allowed us to appreciate the formation of a considerable amount of intramolecular crosslinks, compared to the other crosslinking methods.

Moreover, we found that the denaturation temperature was linearly correlated with ρ_{xch} rather than ρ_{xel} , while the water uptake was affected by ρ_{xel} , as expected. The *in vitro* half-life of the scaffolds showed an

exponential dependence on ρ_{xel} . Excluding the use of aldehydes (which are known to induce cytotoxicity), among the tested treatments EDC crosslinking was the most effective one, as it ensured the highest increase (about 4-fold) of the stiffness and the highest increase of the half-life (about 14-fold), compared to single DHT treatment. DMS crosslinking also appeared as an attractive method to enhance the mechanical and degradation properties of the scaffolds. However, the choice of the optimal crosslinking for a given application will require an extensive biological evaluation to analyze the effects of crosslinking on the cell/tissue response.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon request.

CRediT authorship contribution statement

Luca Salvatore: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing. **Emanuela Calò:** Investigation, Formal analysis. **Valentina Bonfrate:** Investigation, Formal analysis. **Deborah Pedone:** Investigation, Formal analysis. **Nunzia Gallo:** Investigation, Writing - original draft. **Maria Lucia Natali:** Investigation. **Alessandro Sannino:** Resources, Supervision. **Marta Madaghiale:** Conceptualization, Methodology, Supervision, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

- [1] W. Friess, Collagen-biomaterial for drug delivery, *Eur. J. Pharm. Biopharm.* 45 (2) (1998) 113–136, [https://doi.org/10.1016/S0939-6411\(98\)00017-4](https://doi.org/10.1016/S0939-6411(98)00017-4).
- [2] A.K. Lynn, I.V. Yannas, W. Bonfield, Antigenicity and immunogenicity of collagen, *J. Biomed. Mater. Res. B Appl. Biomater.* 71 (2004) 343–354, <https://doi.org/10.1002/jbm.b.30096>.
- [3] S. Gorgieva, V. Kokol, Collagen- vs. Gelatine-based biomaterials and their biocompatibility: review and perspectives, in: Rosario Pignatello (Ed.), *Biomaterials Applications for Nanomedicine*, IntechOpen, 2011, <https://doi.org/10.5772/24118>.
- [4] V. Charulatha, A. Rajaram, Influence of different crosslinking treatments on the physical properties of collagen membranes, *Biomaterials* 24 (5) (2003) 759–767, [https://doi.org/10.1016/S0142-9612\(02\)00412-x](https://doi.org/10.1016/S0142-9612(02)00412-x).
- [5] B.A. Harley, M.H. Spilker, J.W. Wu, K. Asano, H.P. Hsu, M. Spector, I.V. Yannas, Optimal degradation rate for collagen chambers used for regeneration of peripheral nerves over long gaps, *Cells Tissues Organs* 176 (1–3) (2004) 153–165, <https://doi.org/10.1159/000075035>.
- [6] M.G. Haugh, C.M. Murphy, R.C. McKiernan, C. Altenbuchner, F.J. O'Brien, Crosslinking and mechanical properties significantly influence cell attachment, proliferation, and migration within collagen glycosaminoglycan scaffolds, *Tissue Eng.* 17 (9–10) (2011) 1201–1208, <https://doi.org/10.1089/ten.TEA.2010.0590>.
- [7] L. Salvatore, M. Madaghiale, C. Parisi, F. Gatti, A. Sannino, Crosslinking of micropatterned collagen-based nerve guides to modulate the expected half-life, *J. Biomed. Mater. Res.* 102 (12) (2014) 4406–4414, <https://doi.org/10.1002/jbm.a.35124>.
- [8] D.H. Lew, P.H.T. Liu, D.P. Orgill, Optimization of UV cross-linking density for durable and nontoxic collagen GAG dermal substitute, *J. Biomed. Mater. Res. B Appl. Biomater.* 82B (1) (2007) 51–56, <https://doi.org/10.1002/jbm.b.30704>.
- [9] M.G. Haugh, M.J. Jaasma, F.J. O'Brien, The effect of dehydrothermal treatment on the mechanical and structural properties of collagen-GAG scaffolds, *J. Biomed. Mater. Res.* 89 (2) (2009) 363–369, <https://doi.org/10.1002/jbm.a.31955>.
- [10] I. Stachel, U. Schwarzenbolz, T. Henle, M. Meyer, Cross-Linking of type I collagen with microbial transglutaminase: identification of cross-linking sites, *Biomacromolecules* 11 (3) (2010) 698–705, <https://doi.org/10.1021/bm901284x>.
- [11] C.R. Lee, A.J. Grodzinsky, M. Spector, The effects of cross-linking of collagen-glycosaminoglycan scaffolds on compressive stiffness, chondrocyte-mediated contraction, proliferation and biosynthesis, *Biomaterials* 22 (2001) 3145–3154, [https://doi.org/10.1016/S0142-9612\(01\)00067-9](https://doi.org/10.1016/S0142-9612(01)00067-9).
- [12] C.N. Grover, J.H. Gwynne, N. Pugh, S. Hamaia, R.W. Farndale, S.M. Best, R. E. Cameron, Crosslinking and composition influence the surface properties, mechanical stiffness and cell reactivity of collagen-based films, *Acta Biomater.* 8 (8) (2012) 3080–3090, <https://doi.org/10.1016/j.actbio.2012.05.006>.
- [13] N. Davidenko, D.V. Bax, C.F. Schuster, R.W. Farndale, S.W. Hamaia, S.M. Best, R. E. Cameron, Optimisation of UV irradiation as a binding site conserving method for crosslinking collagen-based scaffolds, *J. Mater. Sci. Mater. Med.* 27 (2016) 14, <https://doi.org/10.1007/s10856-015-5627-8>.
- [14] C.N. Grover, R.W. Farndale, S.M. Best, R.E. Cameron, The interplay between physical and chemical properties of protein films affects their bioactivity, *J. Biomed. Mater. Res.* 100A (9) (2012) 2401–2411, <https://doi.org/10.1002/jbm.a.34187>.
- [15] D.V. Bax, N. Davidenko, D. Gullberg, S.W. Hamaia, R.W. Farndale, S.M. Best, R. E. Cameron, Fundamental insight into the effect of carbodiimide crosslinking on cellular recognition of collagen-based scaffolds, *Acta Biomater.* 49 (2017) 218–234, <https://doi.org/10.1016/j.actbio.2016.11.059>.
- [16] L.M. Delgado, Y. Bayon, A. Pandit, D.I. Zeugolis, To cross-link or not to cross-link? Cross-linking associated foreign body response of collagen-based devices, *Tissue Eng. Part B* 21 (2015) 298–313, <https://doi.org/10.1089/ten.TEB.2014.0290>.
- [17] Q. Ye, M.C. Harmsen, M.J. van Luyn, R.A. Bank, The relationship between collagen scaffold cross-linking agents and neutrophils in the foreign body reaction, *Biomaterials* 9192 (2010) 31, <https://doi.org/10.1016/j.biomaterials.2010.08.049>.
- [18] P.J. Flory, *Principles of Polymer Chemistry*, Cornell University Press, Ithaca, NY, 1953.
- [19] M. Madaghiale, E. Calò, L. Salvatore, V. Bonfrate, D. Pedone, M.E. Frigione, A. Sannino, Assessment of collagen crosslinking and denaturation for the design of regenerative scaffolds, *J. Biomed. Mater. Res.* 104 (2016) 186–194, <https://doi.org/10.1002/jbm.a.35554>.
- [20] C.M. Ofner, W.A. Bubnis, Chemical and swelling evaluations of amino group crosslinking in gelatin and modified gelatin matrices, *Pharm. Res. (N. Y.)* 13 (12) (1996) 1821–1827, <https://doi.org/10.1023/A:1016029023910>.
- [21] F. Everaerts, M. Torrianni, M. Hendriks, J. Feijen, Quantification of carboxyl groups in carbodiimide cross-linked collagen sponges, *J. Biomed. Mater. Res.* 83A (4) (2007) 1176–1183, <https://doi.org/10.1002/jbm.a.31398>.
- [22] H.-C. Liang, W.-H. Chang, H.-F. Liang, M.-H. Lee, H.-W. Sung, Crosslinking structures of gelatin hydrogels crosslinked with genipin or a water-soluble carbodiimide, *J. Appl. Polym. Sci.* 91 (2004) 4017–4026, <https://doi.org/10.1002/app.13563>.
- [23] K.G. Shankar, N. Gostynska, M. Montesi, S. Panseri, S. Sprio, E. Kon, M. Marcacci, A. Tampieri, M. Sandri, Investigation on different cross-linking approaches on 3D gelatin scaffolds for tissue engineering application: a comparative analysis, *Int. J. Biol. Macromol.* 95 (2017) 1199–1209, <https://doi.org/10.1016/j.ijbiomac.2016.11.010>.
- [24] K. Pietrucha, Changes in denaturation and rheological properties of collagen-hyaluronic acid scaffolds as a result of temperature dependencies, *Int. J. Biol. Macromol.* 36 (2005) 299–304, <https://doi.org/10.1016/j.ijbiomac.2005.07.004>.
- [25] M. Madaghiale, A. Piccinno, M. Saponaro, A. Maffezzoli, A. Sannino, Collagen- and gelatine-based films sealing vascular prostheses: evaluation of the degree of crosslinking for optimal blood impermeability, *J. Mater. Sci. Mater. Med.* 20 (10) (2009) 1979–1989, <https://doi.org/10.1007/s10856-009-3778-1>.
- [26] G. Monaco, R. Cholas, L. Salvatore, M. Madaghiale, A. Sannino, Sterilization of collagen scaffolds designed for peripheral nerve regeneration: effect on microstructure, degradation and cellular colonization, *Mater. Sci. Eng. C* 71 (2017) 335–344, <https://doi.org/10.1016/j.msec.2016.10.030>.
- [27] J.A. Kiernan, Formaldehyde, formalin, paraformaldehyde and glutaraldehyde: what they are and what they do, *Microsc. Today* 1 (2000) 8–12, <https://doi.org/10.1017/S1551929500057060>.
- [28] V. Charulatha, A. Rajaram, Crosslinking density and resorption of dimethyl suberimidate-treated collagen, *J. Biomed. Mater. Res.* 36 (1997) 478–486, [https://doi.org/10.1002/\(SICI\)1097-4636\(19970915\)36:4<478::AID-JBMS3.0.CO;2-B](https://doi.org/10.1002/(SICI)1097-4636(19970915)36:4<478::AID-JBMS3.0.CO;2-B).
- [29] L.H. Olde Damink, P.J. Dijkstra, M.J. van Luyn, P.B. van Wachem, P. Nieuwenhuis, J. Feijen, Cross-linking of dermal sheep collagen using a water-soluble carbodiimide, *Biomaterials* 17 (1996) 765–773, [https://doi.org/10.1016/0142-9612\(96\)81413-x](https://doi.org/10.1016/0142-9612(96)81413-x).
- [30] H.G. Sundararaghavan, G.A. Monteiro, N.A. Lapin, Y.J. Chabal, J.R. Miksán, D. I. Shreiber, Genipin-induced changes in collagen gels: correlation of mechanical properties to fluorescence, *J. Biomed. Mater. Res.* 87 (2008) 308–320, <https://doi.org/10.1002/jbm.a.31715>.
- [31] M.F. Butler, Y.F. Ng, P.D.A. Pudney, Mechanism and kinetics of the crosslinking reaction between biopolymers containing primary amine groups and genipin, *J. Polym. Sci., Part A: Polym. Chem.* 41 (2003) 3941–3953, <https://doi.org/10.1002/pola.10960>.

- [32] I.V. Yannas, C. Huang, Viscoelastic distinction between helical and coiled macromolecules, *Macromolecules* 5 (1) (1972) 99–100, <https://doi.org/10.1021/ma60025a024>.
- [33] F.J. O'Brien, B.A. Harley, I.V. Yannas, L.J. Gibson, The effect of pore size on cell adhesion in collagen-GAG scaffolds, *Biomaterials* 26 (4) (2005) 433–441, <https://doi.org/10.1016/j.biomaterials.2004.02.052>.
- [34] C.A. Miles, N.C. Avery, V.V. Rodin, A.J. Baley, The increase of denaturation temperature following cross-linking of collagen is caused by dehydration of the fibres, *J. Mol. Biol.* 346 (2005) 551–556, <https://doi.org/10.1016/j.jmb.2004.12.001>.
- [35] M. Mekhail, K.K.H. Wong, D.T. Padavan, Y. Wu, D.B. O'Gorman, W. Wan, Genipin-Cross-linked electrospun collagen fibers, *J. Biomater. Sci. Polym. Ed.* 22 (2011) 2241–2259, <https://doi.org/10.1163/092050610X538209>.
- [36] V. Chiono, E. Pulieri, G. Vozzi, G. Ciardelli, A. Ahluwalia, P. Giusti, Genipin-crosslinked chitosan/gelatin blends for biomedical applications, *J. Mater. Sci. Mater. Med.* 19 (2008) 889–898, <https://doi.org/10.1007/s10856-007-3212-5>.
- [37] L. Salvatore, V.E. Carofiglio, P. Stufano, V. Bonfrate, E. Calò, S. Scarlino, P. Nitti, D. Centrone, M. Cascione, S. Leporatti, A. Sannino, C. Demitri, M. Madaghiele, Potential of electrospun poly(3-hydroxybutyrate)/collagen blends for tissue engineering applications, *J. Health C Eng.* 2018 (2018), 6573947, <https://doi.org/10.1155/2018/6573947>.
- [38] X. Qiao, S.J. Russell, X. Yang, G. Tronci, D.J. Wood, Compositional and in vitro evaluation of nonwoven type I collagen/poly-dl-lactic acid scaffolds for bone regeneration, *J. Funct. Biomater.* 6 (2015) 667–686, <https://doi.org/10.3390/jfb6030667>.
- [39] J. Zhou, C. Cao, X. Ma, J. Lin, Electrospinning of silk fibroin and collagen for vascular tissue engineering, *Int. J. Biol. Macromol.* 47 (2010) 514–519, <https://doi.org/10.1016/j.ijbiomac.2010.07.010>.
- [40] K.S. Weadock, E.J. Miller, L.D. Bellincampi, J.P. Zawadsky, M.G. Dunn, Physical crosslinking of collagen fibers: comparison of ultraviolet irradiation and dehydrothermal treatment, *J. Biomed. Mater. Res.* 29 (2004) 1373–1379, <https://doi.org/10.1002/jbm.820291108>.
- [41] K.V. Srivatsan, N. Durairandy, R. Lakra, K. Sandhiya, U. Ramamurthy, P. S. Korrapati, M.S. Kiran, Nano-caged shikimate as a multi-site cross-linker of collagen for biomedical applications, *RSC Adv.* 5 (2015), 22106, <https://doi.org/10.1039/C5RA02278A>.
- [42] Y. Chen, N. Dan, Y. Huang, C. Yang, W. Dan, Y. Liang, Insights into the interactions between collagen and a naturally derived crosslinker, oxidized chitosan oligosaccharide, *J. Appl. Polym. Sci.* 136 (2019), 48489, <https://doi.org/10.1002/app.48489>.
- [43] H.W. Sung, D.M. Huang, W.H. Chang, R.N. Huang, J.C. Hsu, Evaluation of gelatin hydrogel crosslinked with various crosslinking agents as bioadhesives: in vitro study, *J. Biomed. Mater. Res.* 46 (1999) 520–530, [https://doi.org/10.1002/\(SICI\)1097-4636\(19990915\)46:4<520::AID-JBM10>3.0.CO;2-9](https://doi.org/10.1002/(SICI)1097-4636(19990915)46:4<520::AID-JBM10>3.0.CO;2-9).
- [44] J.E. Gough, C.A. Scotchford, S. Downes, Cytotoxicity of glutaraldehyde crosslinked collagen/poly(vinyl alcohol) films is by the mechanism of apoptosis, *J. Biomed. Mater. Res.* 61 (2002) 121–130, <https://doi.org/10.1002/jbm.10145>.
- [45] Y. Wang, J. Bao, X. Wu, Q. Wu, Y. Li, Y. Zhou, L. Li, H. Bu, Genipin crosslinking reduced the immunogenicity of xenogeneic decellularized porcine whole-liver matrices through regulation of immune cell proliferation and polarization, *Sci. Rep.* 6 (2016), 24779, <https://doi.org/10.1038/srep24779>.
- [46] K. Výborný, J. Vallová, Z. Kočí, K. Kekulová, K. Jiráková, P. Jendelová, J. Hodan, S. Kubínová, Genipin and EDC crosslinking of extracellular matrix hydrogel derived from human umbilical cord for neural tissue repair, *Sci. Rep.* 9 (2019), 10674, <https://doi.org/10.1038/s41598-019-47059-x>.
- [47] C.R. Rowland, D.P. Lennon, A.I. Caplan, F. Guilak, The effects of crosslinking of scaffolds engineered from cartilage ECM on the chondrogenic differentiation of MSCs, *Biomaterials* 34 (2013) 5802–5812, <https://doi.org/10.1016/j.biomaterials.2013.04.027>.
- [48] K.L.S. Chan, A.H. Khankhel, R.L. Thompson, B.J. Coisman, K.H.K. Wong, J. G. Truslow, J. Tien, Crosslinking of collagen scaffolds promotes blood and lymphatic vascular stability, *J. Biomed. Mater. Res.* 102A (2014) 3186–3195, <https://doi.org/10.1002/jbm.a.34990>, 2014.
- [49] Y. Hu, L. Liu, W. Dan, N. Dan, Z. Gu, X. Yu, Synergistic effect of carbodiimide and dehydrothermal crosslinking on acellular dermal matrix, *Int. J. Biol. Macromol.* 55 (2013) 221–230, <https://doi.org/10.1016/j.ijbiomac.2013.01.009>.
- [50] D.V. Bax, N. Davidenko, S.W. Hamaia, R.W. Farndale, S.M. Best, R.E. Cameron, Impact of UV- and carbodiimide-based crosslinking on the integrin-binding properties of collagen-based materials, *Acta Biomater.* 100 (2019) 280–291, <https://doi.org/10.1016/j.actbio.2019.09.046>.