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Review

Crosslinking Collagen Constructs: Achieving Cellular Selectivity Through Modifications of Physical and Chemical Properties

Malavika Nair, Serena M. Best * and Ruth E. Cameron *

Cambridge Centre for Medical Materials, Department of Materials Science and Metallurgy, University of Cambridge, 27 Charles Babbage Road, Cambridge CB3 0FS, UK

* Correspondence: smb51@cam.ac.uk (S.M.B.); rec11@cam.ac.uk (R.E.C.)

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Abstract: Collagen-based constructs have emerged in recent years as ideal candidates for tissue engineering implants. For many biomedical applications, collagen is crosslinked in order to improve the strength, stiffness and stability of the construct. However, the crosslinking process may also result in unintended changes to cell viability, adhesion or proliferation on the treated structures. This review provides a brief overview of some of both the most commonly used and novel crosslinkers used with collagen, and suggests a framework by which crosslinking methods can be compared and selected for a given tissue engineering application.

Keywords: collagen; crosslinking; cellular selectivity; materials selection map

1. Introduction

In recent years, there has been considerable interest in the use of collagen as a base biomaterial for tissue engineering applications. Collagen is ubiquitous even in tissues that are biologically and structurally distinct. The natural stratification and localisation of cells achieved within the body is guided by biochemical and biomechanical cues presented by the extracellular matrix and the physiological conditions at the site of the tissue. While several of these cues can be tailored through additive manufacturing in a scaffold, some alterations can arise naturally from the choices made during the core synthesis of a scaffold. As a result, the changes to structure and properties imparted across the length scales by the underlying fabrication processes cannot be overlooked. In particular, the process of crosslinking not only improves the mechanical properties of a collagen scaffold but also results in further modifications to the molecular structure of collagen. In this review, we present an overview of the collagen crosslinking process, with the view to explore the influence of current methods and reagents on both the mechanical and biological properties of the resulting crosslinked constructs.

2. Considerations in Selecting a Chemical Crosslinker

The fundamental aim of chemical crosslinking is to improve the mechanical properties and stability of the final processed collagen product. However, the selection of an appropriate crosslinker may depend on several factors. Since collagen is often used in biomedical applications to replicate the biochemical environment experienced in vivo, amine-based crosslinkers [1–5] are often chosen in order to mimic the lysine-based crosslinks which are naturally present in collagen [6]. The choice and mechanism of crosslinking can also inadvertently modify other structural attributes and the corresponding biological response. For instance, chemical crosslinkers may be broadly classified by their potential to incorporate the crosslinker directly into the protein. This results in the 'zero length' crosslinkers, which do not remain a part of the protein structure post-crosslinking, or 'non-zero length'

crosslinkers, where some or all of the crosslinker is incorporated. While zero-length crosslinkers may modify the local chemical structure, resulting in non-native like cellular interactions [7], there is also some concern with the potential of non-zero length crosslinked collagen films to release cytotoxic products when metabolised [8].

This review is centred around an assessment of some of the most common crosslinking agents which are predominantly amine-based, and cover a range of zero length as well as non-zero length crosslinkers. These include traditional crosslinkers such as glutaraldehyde (GTA), a non-zero length crosslinker, and EDC-NHS, ultraviolet radiation (UV), dehydrothermal treatment (DHT), which are zero-length crosslinkers. Novel and emerging crosslinking methods include the use of genipin as a non-zero length crosslinker, as well as riboflavin and tissue transglutaminase 2 (TG2) as zero-length crosslinkers. This review also focuses on the effect of crosslinking on Type I collagen, and uses a standardised *w/w*% notation to indicate the mass of the crosslinker used per gram of collagen. Following an assessment of these crosslinkers on various physical, chemical and biological properties in collagen-based constructs, an appropriate crosslinker can be therefore be chosen on the basis of the properties demanded by the application of interest.

3. An Overview of Selected Chemical Crosslinkers

3.1. Glutaraldehyde

GTA is a chemical crosslinker widely studied in tissue engineering owing to its excellent ability to increase the elastic modulus of scaffolds [9]. GTA produces imide crosslinked collagen fibres, as illustrated by the crosslinking mechanism in Figure 1. As seen from the reaction mechanism with collagen, GTA is not a zero-length crosslinker and the resulting structure includes parts of the linker molecule in the final crosslinked collagen.

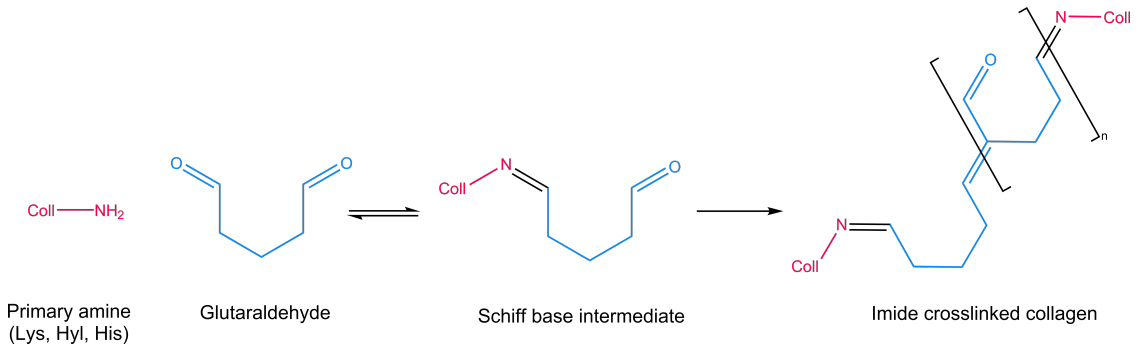

Figure 1. Crosslinking mechanism of glutaraldehyde (GTA). Illustrations redrawn from proposed mechanisms by Olde Damink et al. [1].

Glutaraldehyde has also successfully been shown to achieve an exceptionally high degree of crosslinking, with complete (amine-based) crosslinking achieved above 0.12 *w/w*% on porcine dermal telocollagen-poor collagen gels [10]. This is, however, accompanied by the caveat that crosslinks can be made both within and across collagen fibrils, and that an increase in crosslink density does not always correspond to a similar increase in mechanical properties [1]

One of the earliest systematic studies of the effect of GTA on the mechanical properties of treated collagen was performed by Olde Damink et al. in 1995. GTA crosslinking of dermal ovine collagen was shown to increase the low strain modulus upon crosslinking from 1.7 to 3.5 MPa at 0.5 *w/w*%, although the high-strain modulus was noted to decrease slightly from 32.7 to 21.0 MPa with increasing crosslinking concentration [1].

Furthermore, Chen et al. found that cell seeding and proliferation (by preventing the cell-mediated contraction of a Type I rat tail atelocollagen-derived scaffold) was improved with GTA crosslinking, while differentiation was noted to be significantly hindered in comparison with the non-crosslinked

scaffolds [11]. The by-products of degradation in later metabolic pathways were also observed to result in the cytotoxicity observed, although there is no evidence of carcinogenicity or mutagenicity with the use of GTA [8].

3.2. EDC-NHS

EDC(1-ethyl-3-(3-dimethylaminopropyl) carbodiimide)-NHS(N-hydroxysuccinimide) is a common collagen crosslinker offering a non-cytotoxic, zero-length crosslinking alternative to GTA. The zero-length crosslinking results in access being limited to 'neighbouring' free amines [12], following a reaction mechanism, as illustrated in Figure 2. These crosslinks occur between glutamates and arginines/lysines, in either case affecting the GXOGER sequence that is recognised by the integrins which mediate cell binding on collagen, where the amino acid X is most commonly phenylalanine (F).

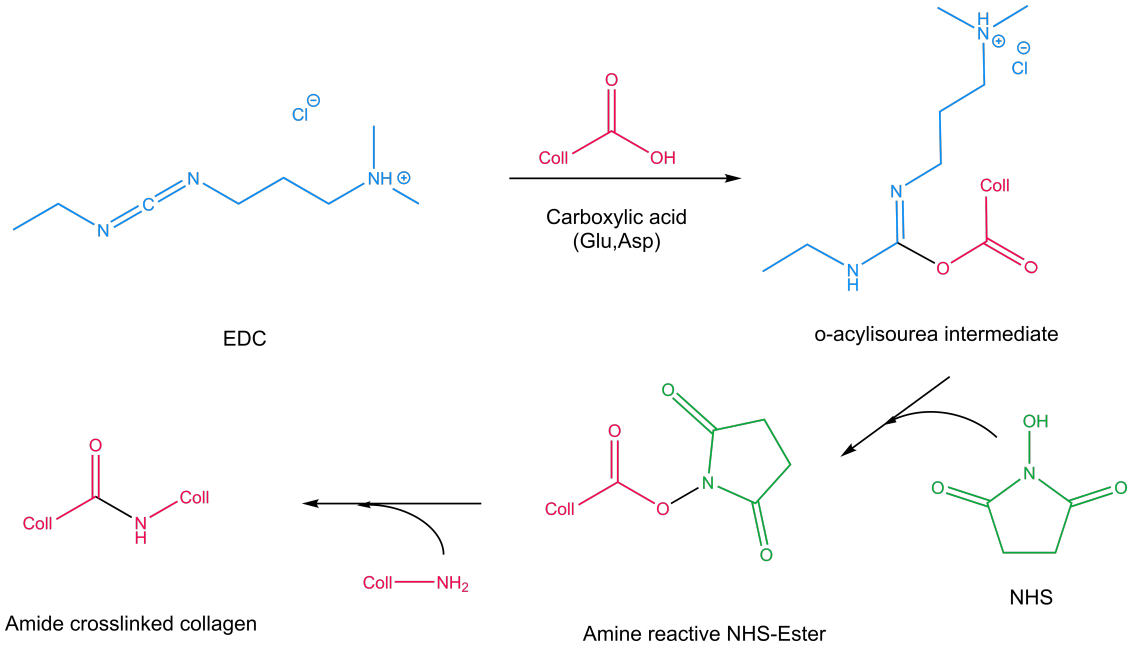

Figure 2. Crosslinking mechanism of EDC-NHS. Illustration redrawn from proposed mechanisms by Haugh et al. [13] Image modified from Nair et al. [14] licensed under CC-BY 4.0.

With EDC-NHS, the standard '100% concentration' [2,15] is often defined as a precise molar ratio of 5:2:1 EDC:NHS:COO^-, where COO^- refers to the carboxylate groups in the protein. Assuming a ratio of 120 COO^- for a collagen chain with a molecular weight of 100,000 [2], this molar ratio can be re-written as a mass ratio corresponding to 1.150:0.276:1 EDC:NHS:Collagen, i.e., 1.150 *w/w*% EDC and 0.276 *w/w*% NHS. A scaling from this standard concentration would then be represented by the proportion of EDC and NHS to collagen. For instance, a 50% standard concentration would refer to a 0.575:0.138:1 mass ratio of EDC:NHS:COO^-. For consistency, concentrations will be expressed in terms of this industry standard. Where modifications are made to this standardised ratio, concentrations will be reported in terms of the mass ratio (*w/w*%) so that comparisons can be made with other chemical crosslinkers.

EDC-NHS crosslinking allows for a lower crosslink density than traditional crosslinkers such as GTA, but provides a more hydrophilic surface, that has been shown to be beneficial for fibronectin activity and a greater degree of swelling compared with traditional crosslinkers on Type I bovine dermal swollen gel-derived collagen fibres (with chondroitin-6-sulfate additives) [12]. Furthermore, EDC-NHS was also shown to induce the self-assembly of collagen bundles roughly 300-nm in width with both acid-soluble and insoluble Type I bovine dermal collagen, suggesting the possibility of both intra-fibril and inter-fibril binding, in spite of being a zero-length crosslinker. The insoluble

collagen fragments revealed an enhancement and localisation of the piezoelectric response along these self-assembled fibre bundles [16].

Historically, up to 200% (i.e., 10:4:1 EDC:NHS:COO^-) of the standard condition has been used in crosslinking collagen, but extensive research on EDC-NHS composition and its effect on both cell migration and mechanics [13,15,17–19] suggest that much lower concentrations (10–100%) can retain the improvements in the mechanical properties imparted by crosslinking. For instance, Ahmad et al. investigated extruded collagen fibres (Type I bovine dermal acid-swollen gel collagen) which were crosslinked at three different EDC-NHS concentrations (0.02 *w/w* EDC%—0.006 *w/w*% NHS, 0.002 *w/w* EDC%—0.0006 *w/w*% NHS and 0.0002 *w/w* EDC%—0.00006 *w/w*% NHS) [18]. Of the three mechanical properties of interest to their study, the ultimate tensile strengths, in particular, were observed to be unaffected by the crosslinking concentrations used.

In the same study by Ahmad et al., human tenocytes derived from anterior cruciate ligaments and seeded on these extruded collagen fibres demonstrated lower levels of attachment on fibres that were heavily crosslinked after 24 h [18]. Analysis of the cell population after three weeks of seeding also indicated lower levels of tenocyte proliferation on the heavily crosslinked fibres. From these results, the authors speculated that the crosslinking conditions could be reduced by nearly two orders of magnitude without affecting the tensile properties desired for tendon repair [18].

Furthermore, a recent patent by Geistlich Pharma AG also supported the used of a low-EDC-NHS crosslinking concentration. Their collagen-based sponges (created from a wide variety of mammalian sources) for tissue regeneration in oral cavities have been shown to allow complete regeneration of gingival cells whilst withstanding the forces accompanied by the movement of the jaw and tongue during mastication and speech. This collagen-based sponge was crosslinked using 0.3 g of EDC per gram of collagen [20], which can be expressed as 26% of the standard crosslinking concentration.

Similar observations of reduced cellular attachment with EDC-NHS crosslinking were reported with C2C12 mouse cardiomyocytes by Grover et al., as well as platelet and HT1080 fibrosarcomas by Davidenko et al., on crosslinked collagen films (Type I microfibrillar bovine dermal and Achilles tendon) but not on crosslinked gelatin films (bovine dermal) [15,17]. The hypothesis emerging from these studies related the observed reduction in the cellular attachment to the ablation of the GXOGER motifs in collagen through the carbodiimide crosslinking process. Due to the availability of RGD motifs for cell binding in gelatin (which is cryptic in collagen), similar decreases in cell binding are not observed with the crosslinking of gelatin.

Bax et al. further investigated the effect of EDC-NHS crosslinking on integrin binding with the aim to provide a mechanistic understanding of the manner in which the crosslinkers modify the collagen backbone to produce the observed reduction in cell attachment [7]. Bax et al. isolated the biochemical receptors which participate in binding with the crosslinked Type I bovine Achilles tendon collagen substrates by characterising the attachment of two integrin I domains (α_1 and α_2) and four different model cell lines expressing different collagen-binding integrins: platelets, HT1080 human fibrosarcomas, Rugli rat glioma cells and C2C12 mouse fibroblasts with transfected integrin I domains.

Through isolated integrin domain binding and cellular attachment experiments to collagen, the authors were able to conclude that four collagen integrins were affected by the EDC-NHS crosslinking: $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$, and $\alpha_{11}\beta_1$. As a result, the mechanism illustrated in Figure 3 was proposed to described the loss of integrin-binding due to the involvement of glutamic acids in the crosslinks formed by EDC-NHS.

Since the binding of the I-domain in an integrin is dependent on its coordination with a divalent cation supported by MIDAS (metal ion dependent adhesion site) motifs, the loss of glutamates during EDC-NHS crosslinking is suggested to ablate the GXOGER motifs on collagen. At high values of EDC-NHS crosslinking ($\geq$10%), the extent of GXOGER ablation caused by the carbodiimide has been suggested to promote non-native cellular interactions with the substrate [7].

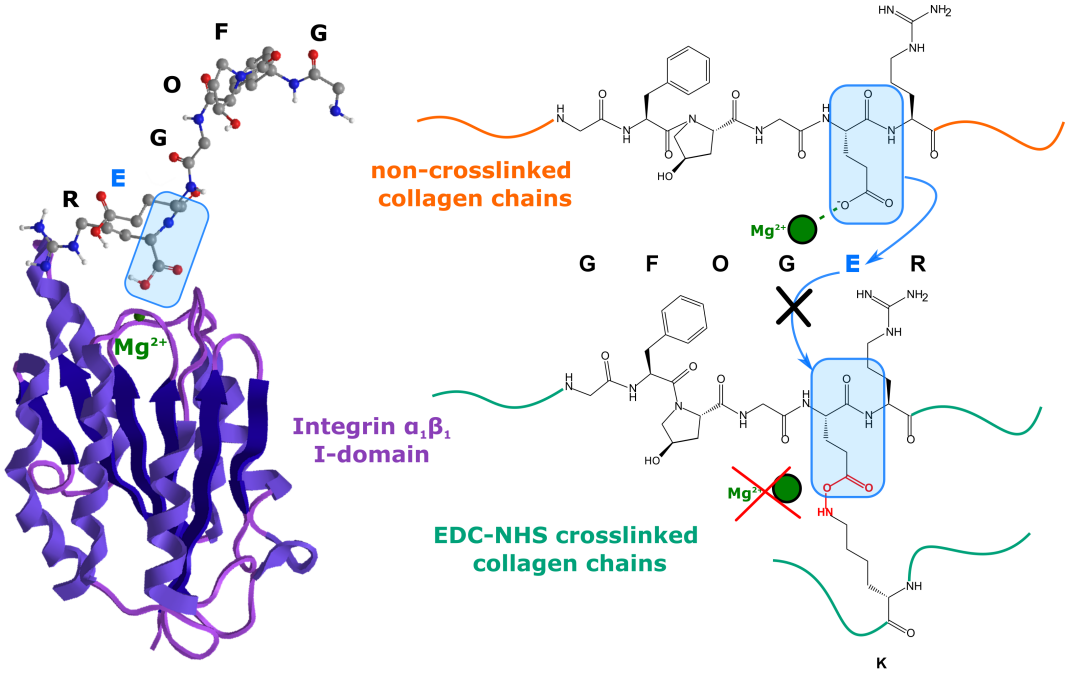

Figure 3. Mechanism of integrin adhesion loss through EDC-NHS crosslinking. The I-domains of integrins coordinate to collagen through a divalent cation (Mg^{2+}), which is supported by the glutamic acid residue in the GXOGER MIDAS motif (GFOGER variant illustrated here). The formation of the peptide bond between glutamic acids (E) and lysines (K) is suggested to be the origin of the observed loss of integrin-specific attachment to EDC-NHS crosslinked collagen. Adapted from Bax et al. [7].

3.3. Dehydrothermal Treatment

DHT exploits LeChatelier's principle of driving a reaction forward by removing the by-product of crosslinking, water, through the application of heat and vacuum, as seen in Figure 4. Madaghiele et al. investigated the effect of DHT held at a range of temperatures (110, 120, 140, 160, 160, 180 °C) at 30–50 Torr for 24 h after ramping for an hour on Type I bovine dermal collagen scaffolds. The compressive elastic modulus of DHT-treated films was shown to increase with the applied DHT temperature, in line with the crosslink density of the films [21]. The treated collagen films were also susceptible to denaturing during the process, with significant denaturing (57.84%), crumpling and embrittlement at higher temperatures (180 °C) [21]. The crosslink density of DHT-treated collagen extracted from steer hide was found to increase with temperature from 105 to 125 °C, but not with time after 3 days [22]. Although some denaturing of collagen is proposed to increase the crosslinking density by allowing access to hidden groups, beyond 145 °C or 5 days, significant denaturing and reduced mechanical properties were observed [22].

Soller et al. conducted an in vivo assessment of DHT-treated Type I microfibrillar collagen–chondrotin-6-sulfate scaffolds in transected rat peripheral nerves [23]. The conditions of study included treatments at 90 °C for both 24 and 48 h, and 120 °C for 48 h under a vacuum of 50 mTorr. In this study, nerve regeneration was supported more successfully by the treatments at higher times and temperatures, providing an optimal degradation time-frame to match the rate of regeneration of the tissue. Additionally, an investigation of the in vitro cell response of DHT-treated Type I collagen–choindroitin-6-sulfate collagen scaffolds was conducted by Haugh et al. using MC3T3-E1 mouse preosteoblasts at higher temperatures. These scaffolds were treated at four temperatures (105, 120, 150 and 180 °C) under a vacuum of 37.5 Torr, although the duration was not specified. DHT-treated scaffolds displayed an extreme seeding and proliferation of the preosteoblasts in comparison with EDC-NHS or GTA-crosslinked scaffolds in the same study, particularly at the higher temperature of 150 °C [13]. While the results of an in vivo and in vitro test cannot be directly compared, it is likely that

the use of DHT-treatment to elicit high cellular activity may be limited to lower treatment temperatures where the risk of denaturing is also low.

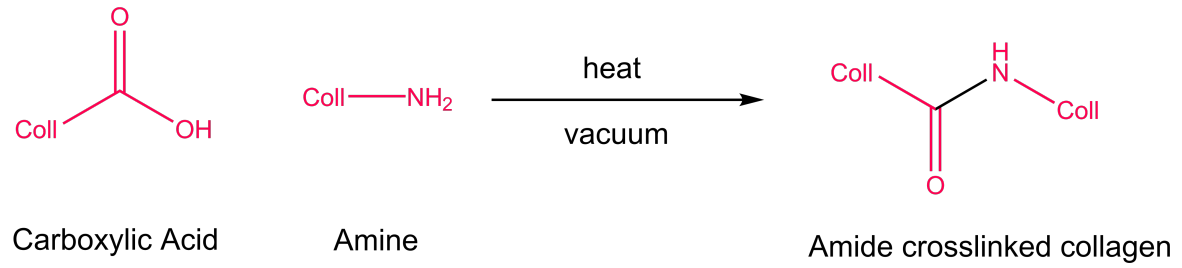

Figure 4. Dehydrothermal mechanism of crosslinking. Illustration redrawn from mechanisms by Haugh et al. [13].

3.4. Ultraviolet Radiation

The use of UV to crosslink collagen is a relatively novel technique that relies on the formation of highly reactive radicals to facilitate the formation of crosslinks within the structure. There is no strict chemical mechanism due to the non-specificity of radical based reactions, but amino acid analyses give evidence of crosslinks through aromatic residues, like phenylalanine and tyrosine [24,25] that are able to stabilise the radicals within their de-localised π-systems.

An investigation by Sionkowska on UV-irradiated films made from acid-soluble rat tail tendon collagen revealed a reduction in thermal stability and the creation of surface flaws such as wrinkles and micro-cracks after UV crosslinking at $\lambda = 254$ nm, 0.196 J cm^{-2}min^{-1}, for 2, 4 and 8 h [26]. The UV radiation applied was suggested to disrupt the hydrogen bonds within and across the collagen in these samples, thus initiating the release of water and the production of collagen fragments [26]. This fragmentation of collagen has been shown to be limited through the use of glucose by inhibiting the unwinding of the triple helices in bovine insoluble dermal and Achilles tendon collagen constructs [24,27].

Davidenko et al. conducted an assessment of the physical properties of UV-irradiated collagen films and scaffolds both with and without the presence of glucose. Type I bovine dermal scaffolds and bovine Achilles tendon collagen scaffolds and films were produced, and subsequently treated at a range of UV conditions ($\lambda = 254$ nm for 30 min). Scaffolds were irradiated at a range of intensities (0.06–0.96 J cm^{-2}), whereas films were only irradiated at either 0.42 or 0.96 J cm^{-2}. The crosslink densities achieved were very low despite long exposure times, giving rise to a maximum Young's modulus under 2 kPa for tendon collagen treated with glucose, and 0.5 kPa without any additives [24]. Moreover, the influence of UV-crosslinking on degradation stability in water was found to be dependent on the collagen source. UV crosslinking improved the stability of tendon collagen, displaying no dependence on irradiation intensities whereas the stability of dermal collagen was improved at the lowest intensity and decreased at higher values. This was reconciled by the competing effects of collagen crosslinking and collagen denaturation that occur during UV-treatment [24].

In the same study, the authors also investigated the in vitro cellular integrin-mediated cellular response to UV crosslinking. $\alpha_2\beta_1$ integrin mediated binding of HT1080s and platelets, as well as spreading and proliferation of HT1080s was found to be unaffected by UV crosslinking, suggesting that the GXOGER sequences were unaffected by the UV-treatment [24]. Building upon these results, the synergistic effects of UV crosslinking ($\lambda = 254$ nm, 0.42 or 0.96 J cm^{-2} for 30 min) and EDC-NHS crosslinking (0–200% of the standard condition as defined in Section 3.2) was investigated by Bax et al. on Type I insoluble bovine Achilles tendon collagen. UV-irradiation was found to inhibit the adhesion of α_2 I-domains above the expected effect from EDC-NHS alone, with a EDC-NHS concentration-dependent inhibition of HT1080 cell adhesion and cell coverage). This was proposed to occur due to the involvement of phenylalanine (F) in UV crosslinking, resulting in the loss of GFOGER crosslinking motifs, the most strongly binding of the GXOGER motifs. As a result, binding is

compensated through the GLOGER motifs, where L represents Leucine, which is compensatory for α_1-I domain binding, but a weak association for α_2-I domains [28].

3.5. Genipin

Genipin, a compound extracted from the fruits of the *Gardenia jasminoides Ellis*, emerged as an alternative crosslinker for crosslinking biomaterials in light of the cytotoxicity of crosslinkers such as glutaraldehyde and formaldehyde [29]. Due to the established food safety of genipin, the crosslinking method has been suggested for use in collagen, gelatin and chitosan-based scaffolds and drug-delivery systems [29]. Genipin has been used as a crosslinker in chitosan-based scaffolds and is proposed to follow the same two-step mechanistic pathway in both chitosan and collagen. One of the crosslinking steps has been reported in earlier studies to involve a secondary amide linkage of a free amine to the genipin via an S_N2 nucleophilic substitution [30,31], and, more recently, the second crosslink has been described to form via two further pathways to complete the crosslinking to collagen, through either two imide crosslinks, or two amide crosslinks, as shown in Figure 5 [31]. Genipin crosslinks gelatin through lysine and arginine groups and is expected to follow a similar mechanistic pathway in collagen [32]. The crosslinks formed with genipin offer collagen scaffolds increased elastic modulus by nearly an order of magnitude, as shown by Zhang et al. The authors found that treating Type I rat tail tendon derived collagen scaffolds of 92% porosity was most effective at high crosslinking concentrations (0.7812 *w/w*%) and temperatures (20–37 °C), producing compressive elastic modulus of 30 kPa when compared against a non-crosslinked control at 5 kPa [33].

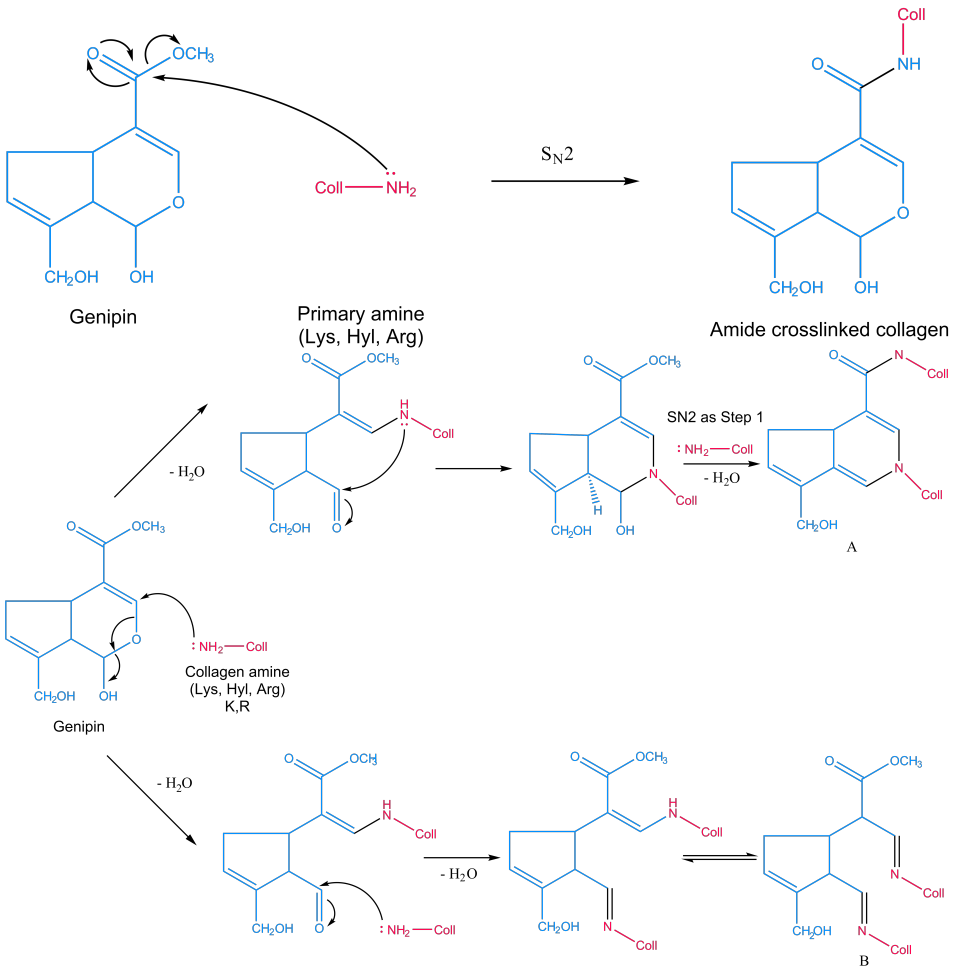

Figure 5. Crosslinking mechanism of genipin. First mechanism proposed in Butler et al. [30] Second proposed in Tambe et al. [31]. Image modified from Nair et al. [14] licensed under CC-BY 4.0.

There is some evidence to support the cytotoxicity of genipin at very large concentrations (>5 mM), according to a study by Sundararaghavan et al. which evaluated the effect of genipin-crosslinked Type I collagen gels without washing post-treatment with genipin [32]. However, the effect on washed scaffolds was found to be minimal. In addition, following neurocompatibility and long-term large animal studies, Singh et al. investigated the use of genipin which was directly injected into the spine of five patients to alleviate their chronic lower back pain by improving the mechanical properties of the annulus [34]. At the end of their six month study, the authors observed an improvement in the flexion-extension instability reduction and patient satisfaction in 75% of their patients. Patients only experienced temporary central pain associated with the procedure, and the leg pain experienced by a patient was treated and successfully resolved by the ninth week of the experiment, supporting the safety of genipin for use in vivo.

Furthermore, an investigation of the mechanical properties of genipin by Nair et al. using Type I bovine dermal insoluble collagen, revealed that genipin at the highest crosslinking concentrations (1.5624 *w/w* % at room temperature) can act as an alternative for the intermediate crosslinking conditions of EDC-NHS, improving both the Young's modulus and the stress to failure [14]. Integrin-specific binding was also observed to be unaffected by the genipin crosslinking, resulting in high human dermal fibroblast proliferation rates and low cellular toxicity [14].

3.6. Riboflavin

Riboflavin, or vitamin B2, has also arisen as a biocompatible means to achieve command-set crosslinking of collagen constructs with blue light. Riboflavin crosslinking of collagen is of particular interest due to the short processing times (15 min) needed to observe significant improvements in mechanical properties (2.5-fold increase in elastic modulus) [35]. Barrett et al. established that riboflavin can create crosslinks within a collagen construct (Type I insoluble, bovine Achilles tendon membranes) specifically through the arginine, histidine and lysine amino acids. Although the loss of arginines can result hinder integrin-mediated adhesion, Barrett et al. hypothesised that arginines are not as critical in stabilising a divalent cation in the GFOGER motifs as the glutamines which are lost during EDC-NHS crossliniing [36]. This was confirmed with cell attachment assays where only the $\alpha_2\beta_1$ integrin-mediated binding was affected, as evaluated with HT1080 fibrosarcomas, whereas the integrin binding in human dermal fibroblasts expressing a range of integrins was unaffected. Unlike EDC-NHS crosslinked films however, riboflavin-crosslinked films did not result in an increase in non-specific binding, whilst possessing comparable ultimate tensile strengths to EDC-NHS crosslinked collagen membranes [36]. Additionally, an investigation of plastically compressed collagen scaffolds by Cheema et al. observed a decrease in oxygen diffusivity and in human dermal neonatal fibroblast viability after crosslinking a compressed collagen (Type I, rat tail, single molecule) scaffold [37]. However, this cytotoxicity may arise from an interaction between the compression and crosslinking, since greater degrees of plastic compression resulted in a more significant decrease in cell viability (67%) than the riboflavin crosslinking process (79%).

Transglutaminase

Transglutaminases refer to a family of transferase enzymes that crosslink proteins through the formation of a bond between an ε-amine (lysine) and γ-carboxyl in glutamines, as shown in Figure 6. There are many types of transglutaminases, including microbial transglutaminase, factor XII, epidermal, keratinocyte and tissue transglutaminases, which are commonly found as crosslinking agents in skin, hair and blood clots in vivo. Of these enzymes, tissue transglutaminase 2 (TG2), is a calcium-dependent enzyme that has shown an exceptionally high cellular response, including an increase in the number of osteoblast adhesion after TG2-crosslinking of freeze-dried Type I calf-skin-derived collagen scaffolds [38].

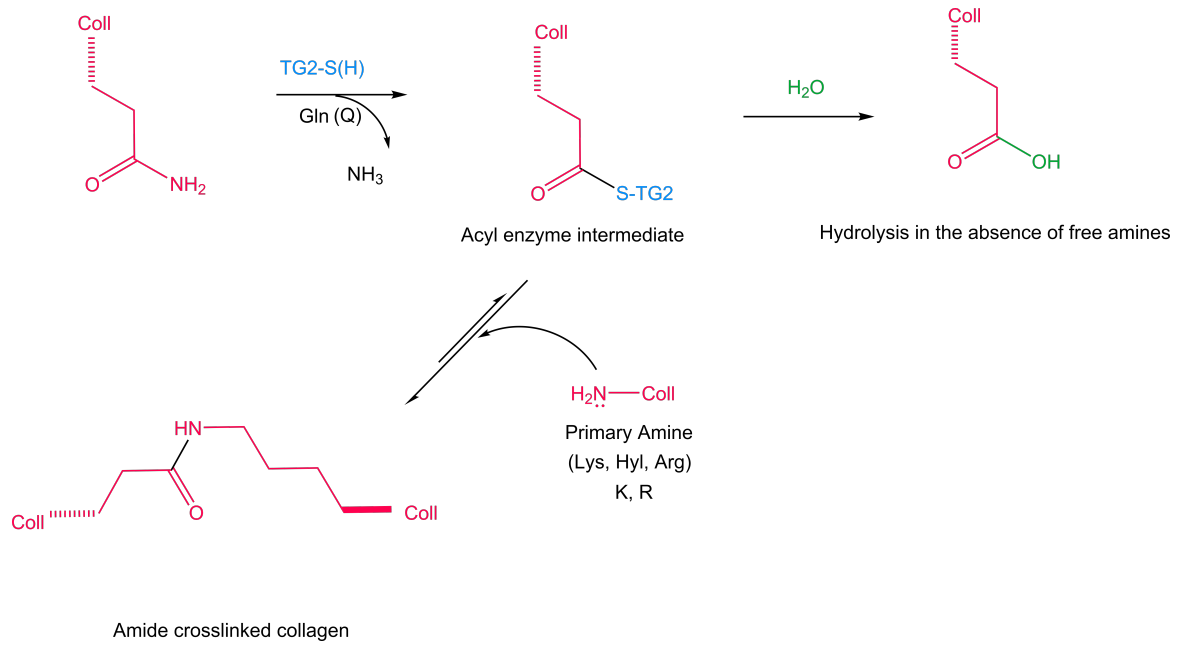

Figure 6. Crosslinking mechanism of tissue transglutaminase. Illustration of proposed mechanisms by Keillor et al. [39]. Image modified from Nair et al. [14] licensed under CC-BY 4.0.

Transglutaminases bind to glutamines in the polypeptide chain, activating them for further reactions, as depicted in Figure 6 [39]. In the presence of water, this results in the conversion of the glutamine to a glutamate residue, whereas, in the presence of a suitable amine, an amide bond is formed at the site of the activated glutamine [39]. Thus, TG2 can either serve to act as an amide crosslinker that does not utilise pre-existing aspartic or glutamic acids (E and D) in the formation of crosslinks, or, conversely, could help to re-introduce glutamates (E) in the substrate, which may increase the number of MIDAS motifs available on the substrate for integrin recognition.

In an investigation of the mechanical properties of TG2 by Nair et al., the global mechanical properties such as tensile modulus and failure strength of TG2-treated films were lower than non-crosslinked Type I bovine dermal insoluble collagen films, whereas the strain to failure and plasticity was observed to increase [14]. Regardless, the human dermal fibroblasts seeded on these substrates were well-spread and attached to the substrate, with low cytotoxicity and high cell proliferation.

4. Summary

An ideal tissue engineering construct will possess the mechanical properties required to withstand the global forces, and biochemical cues to promote controlled cell invasion and proliferation at the site of injury. Due to the mechanisms employed by chemical crosslinkers, it is impossible to modify the mechanical properties without altering the chemical structure of collagen. The biological and chemical behaviour exhibited by crosslinked collagen is summarised in Table 1.

Table 1. Summary of the advantages and disadvantages of current crosslinking systems used in collagen-based constructs.

Crosslinker	Advantages	Disadvantages
GTA	• Increased elastic modulus [1,13]	• Non-zero length crosslinker [1] • Cytotoxic [8]
EDC-NHS	• Increased elastic modulus [13,17] • Zero length crosslinker [2,12]	• Loss of some integrin-specific binding [7]
UV	• Integrin binding unaffected [24] • Zero length crosslinking [24,25]	• Non-specific reaction sites [24,25] • Low crosslink densities [24] • Collagen fragmentation [26] • Some denaturing [26] without use of glucose [24,27]
DHT	• Integrin binding unaffected [13] • Zero length crosslinking	• Denaturation of collagen [21]
Genipin	• Non-cytotoxic [34] • Increases in elastic modulus [33] • Mechanism should not affect GXOGER motifs in collagen [31]	• Not a zero-length crosslinker [31] • Imparts a blue colour [33]
Riboflavin	• Mechanism should not affect GXOGER motifs in collagen [36] • Comparable ultimate tensile strength to EDC-NHS [36]	• Some, albeit limited, loss of $\alpha_2\beta_1$ mediated binding
TG2	• Mechanism do not affect GXOGER motifs in collagen [39]	• Can cause morphological changes to collagen ultrastructure [38] • Can result in a reduction of Young's modulus

As the number of crosslinking methods and cell types evaluated in the literature continues to grow, determining the ideal crosslinker choice for a given application has become a non-trivial task. One approach to addressing these issues draws from established representations of materials properties through Ashby Materials Selection plots. In such plots, several of the underlying interactions are abstracted, allowing distinct classes of materials to be compared along the same property space. An attempt to create a materials selection map is illustrated in Figure 7, representing schematic trends for the 'bioactivity' (through attachment and cytotoxicity) and 'mechanics' based on the literature reviewed in this chapter. Within these observations, there is significant complexity and diversity in both mechanical properties (tensile, compressive, shear as well as strength, strain, viscoelasticity, etc.) and biological responses (cell lines, monoculture vs. co-cultures, attachment vs. proliferation vs. migration). As a result, this materials selection map is intentionally left without exact values since the trends were limited to observations made in systematic and comparative studies of crosslinked collagen. Regardless, the schematic demonstrates the potential to extract and compare multiple properties upon crosslinking, with protocol standardisation and systematic comparisons of crosslinkers in future studies.

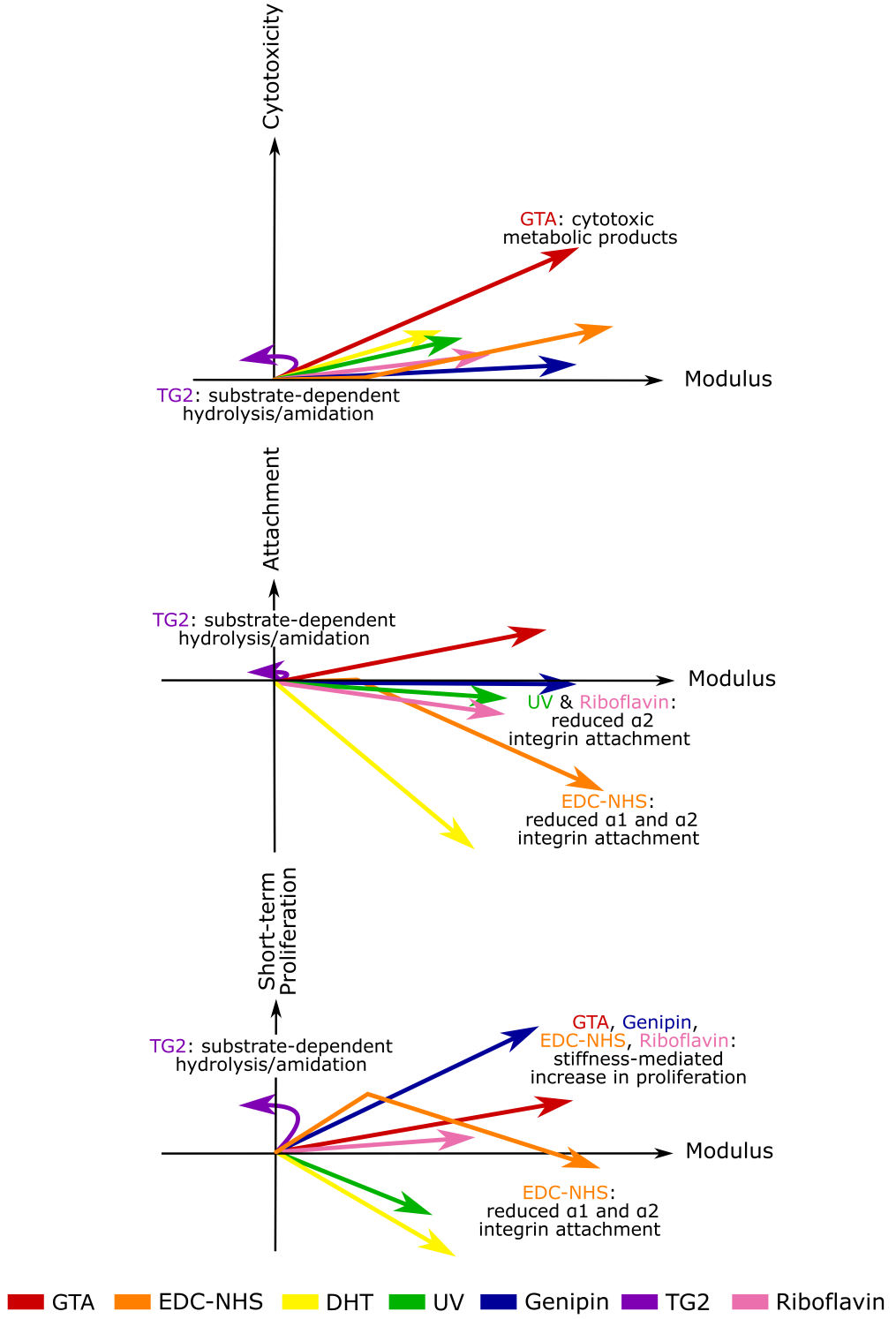

Figure 7. A schematic bioactivity-mechanics materials selection map illustrating a representation of crosslinked or treated collagen substrates in terms of two commonly desired characteristics. The origin represents the behaviour of native uncrosslinked collagen constructs, and the behaviour from the origin following the arrows indicate trends observed with increasing crosslinking concentration. These trends are illustrative of the behaviours observed in studies containing systematic comparisons of collagen crosslinkers, but do not contain exact values and scales since there is significant variability in the types of constructs, cell lines and measurement methods used.

In order to produce a version of Figure 7 that allows for direct comparison of crosslinkers across a range of physiologically relevant systems, we suggest that future experimental methods should, therefore, not only include the global data that are relevant for the application at hand, but also a few additional key datasets. Firstly, as a method to standardise the effect of crosslinkers on a common substrate such as collagen, the 'degree of crosslinking' should be included. Based on the technique of choice, this may involve an amino acid analysis [28,36], or colorimetric amine-based assays such as the TNBS [17,21,24] or Ninhydrin assays [14,16,33] to assess the presence of free amines. Secondly, an evaluation of the mechanical properties should include access to the data for the representative stress–strain curves in addition to the authors' chosen mechanical metric of interest. Secondly, we recommend the inclusion of two biological datasets: (1) cytotoxicity and (2) cell adhesion molecule (CAM) attachment studies. While the complexity of a tissue cannot be recreated by a single cell type, a high cytotoxicity for any cell line is often undesirable. Thus, preliminary cytotoxicity studies on the cells of interest is a valuable inclusion for the purposes of tissue engineering. Moreover, cellular attachment is a precursor to several complex cellular behaviour including migration and proliferation. Although the cells of interest do not remain the same across several tissue engineering applications, the cells studied often share several common characteristics, including the presence of CAMs. By performing CAM adhesion assays in place of, or in addition to cellular assays, a materials selection map can include the attachment properties of CAMs such as integrins, allowing the behaviour of similar classes of cells (fibroblasts, osteoblasts and endothelial cells) to be obtained. The reduction in cellular behaviour to the initial attachment of CAMs to the crosslinked substrates therefore enables the separation of the biochemical and mechanical effects of crosslinking from the cell–cell and chemokine-driven responses in more complex cellular phenomena such as cell migration.

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References

1. Olde Damink, L.; Dijkstra, P.; Van Luyn, M.; Van Wachem, P.; Nieuwenhuis, P.; Feijen, J. Glutaraldehyde as a crosslinking agent for collagen-based biomaterials. *J. Mater. Sci. Mater. Med.* **1995**, *6*, 460–472. [CrossRef]
2. Olde Damink, L.; Dijkstra, P.; Van Luyn, M.; Van Wachem, P.; Nieuwenhuis, P.; Feijen, J. Cross-linking of dermal sheep collagen using a water-soluble carbodiimide. *Biomaterials* **1996**, *17*, 765–773. [CrossRef]
3. Mekhail, M.; Wong, K.K.H.; Padavan, D.T.; Wu, Y.; O'Gorman, D.B.; Wan, W. Genipin-cross-linked electrospun collagen fibers. *J. Biomater. Sci. Polym. Ed.* **2011**, *22*, 2241–2259. [CrossRef] [PubMed]
4. Fortunati, D.; San Chau, D.Y.; Wang, Z.; Collighan, R.J.; Griffin, M. Cross-linking of collagen I by tissue transglutaminase provides a promising biomaterial for promoting bone healing. *Amino Acids* **2014**, *46*, 1751–1761. [CrossRef]
5. Smith-Mungo, L.I.; Kagan, H.M. Lysyl oxidase: Properties, regulation and multiple functions in biology. *Matrix Biol.* **1998**, *16*, 387–398. [CrossRef]
6. Mouw, J.K.; Ou, G.; Weaver, V.M. Extracellular matrix assembly: A multiscale deconstruction. *Nat. Rev. Mol. Cell Biol.* **2014**, *15*, 771. [CrossRef]
7. Bax, D.V.; Davidenko, N.; Gullberg, D.; Hamaia, S.W.; Farndale, R.W.; Best, S.M.; Cameron, R.E. Fundamental insight into the effect of carbodiimide crosslinking on cellular recognition of collagen-based scaffolds. *Acta Biomater.* **2017**, *49*, 218–234. [CrossRef]

8. Takigawa, T.; Endo, Y. Effects of glutaraldehyde exposure on human health. *J. Occup. Health* **2006**, *48*, 75–87. [CrossRef]
9. Schmidt, C.E.; Baier, J.M. Acellular vascular tissues: Natural biomaterials for tissue repair and tissue engineering. *Biomaterials* **2000**, *21*, 2215–2231. [CrossRef]
10. Sheu, M.T.; Huang, J.C.; Yeh, G.C.; Ho, H.O. Characterization of collagen gel solutions and collagen matrices for cell culture. *Biomaterials* **2001**, *22*, 1713–1719. [CrossRef]
11. Chen, D.C.; Lai, Y.L.; Lee, S.Y.; Hung, S.L.; Chen, H.L. Osteoblastic response to collagen scaffolds varied in freezing temperature and glutaraldehyde crosslinking. *J. Biomed. Mater. Res. Part A* **2007**, *80*, 399–409. [CrossRef] [PubMed]
12. Shepherd, J.; Ghose, S.; Kew, S.; Moavenian, A.; Best, S.M.; Cameron, R.E. Effect of fiber crosslinking on collagen-fiber reinforced collagen–chondroitin-6-sulfate materials for regenerating load-bearing soft tissues. *J. Biomed. Mater. Res. Part A* **2013**, *101*, 176–184. [CrossRef] [PubMed]
13. Haugh, M.G.; Murphy, C.M.; McKiernan, R.C.; Altenbuchner, C.; O'Brien, F.J. Crosslinking and mechanical properties significantly influence cell attachment, proliferation, and migration within collagen glycosaminoglycan scaffolds. *Tissue Eng. Part A* **2011**, *17*, 1201–1208. [CrossRef] [PubMed]
14. Nair, M.; Johal, R.K.; Hamaia, S.W.; Best, S.M.; Cameron, R.E. Tunable bioactivity and mechanics of collagen-based tissue engineering constructs: A comparison of EDC-NHS, genipin and TG2 crosslinkers. *Biomaterials* **2020**, *254*, 120109. [CrossRef]
15. Davidenko, N.; Schuster, C.; Bax, D.; Raynal, N.; Farndale, R.; Best, S.; Cameron, R. Control of crosslinking for tailoring collagen-based scaffolds stability and mechanics. *Acta Biomater.* **2015**, *25*, 131–142. [CrossRef]
16. Nair, M.; Calahorra, Y.; Kar-Narayan, S.; Best, S.M.; Cameron, R.E. Self-assembly of collagen bundles and enhanced piezoelectricity induced by chemical crosslinking. *Nanoscale* **2019**, *11*, 15120–15130. [CrossRef]
17. Grover, C.N.; Gwynne, J.H.; Pugh, N.; Hamaia, S.; Farndale, R.W.; Best, S.M.; Cameron, R.E. Crosslinking and composition influence the surface properties, mechanical stiffness and cell reactivity of collagen-based films. *Acta Biomater.* **2012**, *8*, 3080–3090. [CrossRef]
18. Ahmad, Z.; Shepherd, J.H.; Shepherd, D.V.; Ghose, S.; Kew, S.J.; Cameron, R.E.; Best, S.M.; Brooks, R.A.; Wardale, J.; Rushton, N. Effect of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide concentrations on the mechanical and biological characteristics of cross-linked collagen fibres for tendon repair. *Regen. Biomater.* **2015**, *2*, 77–85. [CrossRef]
19. Shepherd, D.; Shepherd, J.; Ghose, S.; Kew, S.; Cameron, R.; Best, S. The process of EDC-NHS cross-linking of reconstituted collagen fibres increases collagen fibrillar order and alignment. *APL Mater.* **2015**, *3*, 014902. [CrossRef]
20. Imhof, C.; Schloesser, L.; Stiefel, N.; Wüest, M. Collagen Sponge. EP Patent 3,055,000, 21 December 2016.
21. Madaghiele, M.; Calò, E.; Salvatore, L.; Bonfrate, V.; Pedone, D.; Frigione, M.; Sannino, A. Assessment of collagen crosslinking and denaturation for the design of regenerative scaffolds. *J. Biomed. Mater. Res. Part A* **2016**, *104*, 186–194. [CrossRef]
22. Chen, X.; Zhou, L.; Xu, H.; Yamamoto, M.; Shinoda, M.; Kishimoto, M.; Tanaka, T.; Yamane, H. Effect of the Application of a Dehydrothermal Treatment on the Structure and the Mechanical Properties of Collagen Film. *Materials* **2020**, *13*, 377. [CrossRef] [PubMed]
23. Soller, E.C.; Tzeranis, D.S.; Miu, K.; So, P.T.; Yannas, I.V. Common features of optimal collagen scaffolds that disrupt wound contraction and enhance regeneration both in peripheral nerves and in skin. *Biomaterials* **2012**, *33*, 4783–4791. [CrossRef] [PubMed]
24. Davidenko, N.; Bax, D.V.; Schuster, C.F.; Farndale, R.W.; Hamaia, S.W.; Best, S.M.; Cameron, R.E. Optimisation of UV irradiation as a binding site conserving method for crosslinking collagen-based scaffolds. *J. Mater. Sci. Mater. Med.* **2016**, *27*, 14. [CrossRef] [PubMed]
25. Kato, Y.; Nishikawa, T.; Kawakishi, S. Formation of protein-bound 3,4-dihydroxyphenylalanine in collagen types i and iv exposed to ultraviolet light. *Photochem. Photobiol.* **1995**, *61*, 367–372. [CrossRef] [PubMed]
26. Sionkowska, A. Modification of collagen films by ultraviolet irradiation. *Polym. Degrad. Stab.* **2000**, *68*, 147–151. [CrossRef]
27. Ohan, M.P.; Weadock, K.S.; Dunn, M.G. Synergistic effects of glucose and ultraviolet irradiation on the physical properties of collagen. *J. Biomed. Mater. Res.* **2002**, *60*, 384–391. [CrossRef]

28. Bax, D.V.; Davidenko, N.; Hamaia, S.W.; Farndale, R.W.; Best, S.M.; Cameron, R.E. Impact of UV-and carbodiimide-based crosslinking on the integrin-binding properties of collagen-based materials. *Acta Biomater.* **2019**, *100*, 280–291. [CrossRef]
29. Manickam, B.; Sreedharan, R.; Elumalai, M. 'Genipin'–the natural water soluble cross-linking agent and its importance in the modified drug delivery systems: An overview. *Curr. Drug Deliv.* **2014**, *11*, 139–145. [CrossRef]
30. Butler, M.F.; Ng, Y.F.; Pudney, P.D. Mechanism and kinetics of the crosslinking reaction between biopolymers containing primary amine groups and genipin. *J. Polym. Sci. Part A Polym. Chem.* **2003**, *41*, 3941–3953. [CrossRef]
31. Tambe, N.; Di, J.; Zhang, Z.; Bernacki, S.; El-Shafei, A.; King, M.W. Novel genipin-collagen immobilization of polylactic acid (PLA) fibers for use as tissue engineering scaffolds. *J. Biomed. Mater. Res. Part B Appl. Biomater.* **2015**, *103*, 1188–1197. [CrossRef]
32. Sundararaghavan, H.G.; Monteiro, G.A.; Lapin, N.A.; Chabal, Y.J.; Miksan, J.R.; Shreiber, D.I. Genipin-induced changes in collagen gels: Correlation of mechanical properties to fluorescence. *J. Biomed. Mater. Res. Part A* **2008**, *87*, 308–320. [CrossRef] [PubMed]
33. Zhang, X.; Chen, X.; Yang, T.; Zhang, N.; Dong, L.; Ma, S.; Liu, X.; Zhou, M.; Li, B. The effects of different crossing-linking conditions of genipin on type I collagen scaffolds: An in vitro evaluation. *Cell Tissue Bank.* **2014**, *15*, 531–541. [CrossRef] [PubMed]
34. Singh, H.; Racadio, J.; Brown, M.; Hedman, T. Six-Month Safety and Efficacy of Genipin Crosslinking Treatment for Chronic Low Back Pain. *Spine J.* **2017**, *17*, S206. [CrossRef]
35. Rich, H.; Odlyha, M.; Cheema, U.; Mudera, V.; Bozec, L. Effects of photochemical riboflavin-mediated crosslinks on the physical properties of collagen constructs and fibrils. *J. Mater. Sci. Mater. Med.* **2014**, *25*, 11–21. [CrossRef] [PubMed]
36. Barrett, D.J.; Bax, D.V.; Best, S.M.; Cameron, R.E. Blue light activated crosslinking of collagen. **2020**, pending publishing.
37. Cheema, U.; Rong, Z.; Kirresh, O.; MacRobert, A.J.; Vadgama, P.; Brown, R.A. Oxygen diffusion through collagen scaffolds at defined densities: implications for cell survival in tissue models. *J. Tissue Eng. Regen. Med.* **2012**, *6*, 77–84. [CrossRef] [PubMed]
38. Chau, D.Y.; Collighan, R.J.; Verderio, E.A.; Addy, V.L.; Griffin, M. The cellular response to transglutaminase-cross-linked collagen. *Biomaterials* **2005**, *26*, 6518–6529. [CrossRef]
39. Keillor, J.W.; Clouthier, C.M.; Apperley, K.Y.; Akbar, A.; Mulani, A. Acyl transfer mechanisms of tissue transglutaminase. *Bioorg. Chem.* **2014**, *57*, 186–197. [CrossRef]