Crosslinking of Dermis-Derived Hydrogels Increases Stiffness and Alters Resistance to Degradation

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Summary: Hydrogels have been investigated extensively as biomaterials for three-dimensional tissue reconstruction and regeneration. Incorporation of tissue-specific features into synthetic materials is difficult, due to limited ability to recreate the complex multi-component nature of extracellular matrices (ECM). ECM-rich hydrogels extracted and assembled from soft tissues have been shown to stimulate the formation of vascularized tissue *in vitro* and *in vivo*.[1,2,3] Hydrogels derived from dermal tissue using this technique contain basement membrane proteins, including laminin β3, collagen IV, and collagen VII, all of which are essential for proper skin function.[2] While these materials have significant biological activity, their poor mechanical properties and rapid degradation *in vivo* hinder their performance in wound healing applications. This study investigates the effect of covalent crosslinking on the mechanical properties, biological activity, and degradation (*in vitro* and *in vivo*) of dermal-derived hydrogels. Glutaraldehyde (GA) is a well-known crosslinking agent used in clinically approved products to prolong lifetime and increase strength of materials. Compression tests indicated increasing elastic modulus and yield stress of crosslinked hydrogels with crosslinking time ($p<0.05$). The crosslinked ECM were resistant to pepsin degradation *in vitro*. Gels and gel extracts were non toxic, and fibroblasts adhered and spread on gels at all crosslink densities. Crosslinking drastically slowed degradation relative to controls (non-crosslinked gels) *in vivo* in a subcutaneous implant model relative to control. While degradation was slowed, inflammation was low and mature vascularized tissue formed in the gels, suggesting that the materials retained the ability to induce tissue invasion. These results support the potential use of dermis-derived hydrogels as promising constructs for applications in tissue engineering and suggest that covalent crosslinking can be used to enhance mechanical properties and prolong hydrogel lifetime while inducing vascularized tissue formation.

Introduction: Biomaterials for soft tissue reconstruction are often designed to mimic the chemical, physical and mechanical properties of native tissue to induce cellular differentiation, proliferation, and angiogenesis. Patients with soft tissue trauma and pathological conditions could benefit directly from the use of these tissue constructs to provide alternative methods for the treatment of diseased or damaged tissues. A technique was previously developed to create tissue-derived ECM hydrogels using any soft tissue source, including dermis and subcutaneous fat. The end-product of this process is an extract that is rich in basement membrane proteins and growth factors specific to the tissue source. The materials induce vascularized tissue formation *in vitro* and *in vivo*, suggesting their significant potential for tissue engineering. However, the hydrogels assemble through weak secondary interactions and the maximum stiffness achieved is approximately 95 Pa, making them prone to deformation and degradation *in vivo*.[4] Covalent crosslinking of these materials may enhance the strength of the materials and increase resistance to degradation. Given its extensive use as a crosslinking agent for clinically approved ECM-based biomaterials, GA was investigated as a first-pass crosslinking agent for tissue derived hydrogels. The goal is to crosslink the materials to improve their properties without changing biologic function. This design criterion is of particular importance given that the unique properties of this biomaterial lie within the diversity and applicability of its protein components to tissue engineering, with the intent of facilitating cell-protein interactions resulting in the degradation and remodeling of the hydrogel into a mature, vascularized tissue.

Materials and Methods: Hydrogels were extracted from dermal tissue samples as described previously[1]. Briefly, full-thickness skin was harvested from Sprague-Dawley and Long Evans rats and dermis tissue was collected after removing the subcutaneous fat and epidermis. The tissues were decellularized, washed with high salt, and ECM proteins extracted overnight in urea buffer. Extracts were induced to assemble into hydrogels through a pH-induced gelation mechanism[1] and then gels were crosslinked in 0.625% (v/v) GA for 0.5hrs, 1hr, 12hrs, and 24hrs. Mechanical properties of the hydrogels were assessed by compression, allowing determination of the elastic modulus and yield stress for the materials. Resistance of hydrogels to enzymatic degradation was determined by incubation in 0.4% porcine pepsin. The toxicity of residual GA in the hydrogels was determined by exposure of fibroblasts to hydrogel extracts, with viability assessed using an MTS assay. To investigate cell adhesion, gels were seeded with pKH-labeled fibroblasts (5,000 cells) and imaged after 3 days by fluorescence microscopy to examine cell spreading. Following *in vitro* analysis, hydrogels were implanted into the subcutaneous space of Lewis rats and harvested at 1, 3, and 6 weeks. Samples were paraffin embedded and sectioned at 4 μm thickness before staining for Masson's trichrome

Table 1: Elastic modules and yield stress increase with GA exposure time. (* indicates significance from 0.5hr data)

GA Exposure time (hr)	**Elastic modulus, E (Pa)**	**Yield stress, σ (Pa)**
0.5	8876 ± 830	2542 ± 250
1	10887 ± 1020	3174 ± 220
12	14224 ± 1570*	3925 ± 170*
24	16607 ± 2670*	4878 ± 340*

and hematoxylin and eosin (H&E). Quantitative histomorphometric techniques were used to determine amount of gel remaining at each time point. Analysis of variance (ANOVA) with Tukey post hoc test was used for comparison ($p<0.05$ was considered significant).

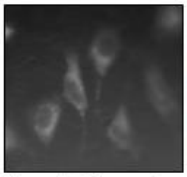
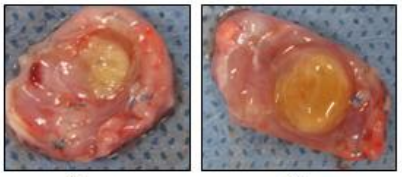


Figure 1. Twenty-four hour crosslinked dermal hydrogel supports fibroblast cell adhesion.



(a) (b)

Figure 2. Non-crosslinked (a) and 24hr crosslinked (b) dermal hydrogel explants from *in vivo* rat subcutaneous model 1 week post implantation.

Results: Following crosslinking, the gels were easier to handle than prior to crosslinking. Compression tests indicated that the elastic moduli and yield stresses of dermal gels increased with GA incubation time (Table 1). Crosslinked gels at all time points were resistant to degradation via 30 minute exposure to pepsin, unlike non-crosslinked gels, which degraded rapidly. After crosslinking, hydrogels contained residual GA, which is toxic to cells, but washing the hydrogels in buffer three times removed excess GA and made the hydrogels suitable for cell attachment and growth. Crosslinked gels also supported cell adhesion regardless of crosslinking time; fibroblasts adhered and spread on the surface of the gels (Figure 1). Currently, work is ongoing in examining how crosslinking may influence cell differentiation *in vitro*.

Images of hydrogels harvested following subcutaneous implantation suggest that control hydrogels (Figure 2a) were nearly completely degraded by three weeks while crosslinked gels were still present at all time points (Figure 2b). Histological analysis of the hydrogel samples stained for H&E and trichrome were used to assess degradation and tissue response. Control gels were rapidly invaded by vascularized tissue by week 1 and had mature vascularized tissues at 3 weeks. However, the control gels had completely degraded by the sixth week with the newly formed tissues also regressing (Figure 4). The crosslinked gels did not degrade by six weeks regardless of conditions. Hydrogel degradation was analyzed by comparing percent of hydrogel remaining at 1, 3, and 6 weeks post-implantation for each crosslinking time (Figure 3). A significant decrease in the percent of hydrogel remaining was noted for all crosslinking time points between weeks 1 and 6 ($p<0.05$), but no statistically significant differences were observed among crosslinking intervals within each group. Little tissue invasion was observed in the gels at 1 week but vascularized tissue could be observed invading the gels at weeks 3 and 6 (Figure 4). Trichrome staining images show a normal wound healing response to hydrogel implantation, with minimal inflammation (Figure 5). Typical acute inflammatory responses were noted, with increased cellular transmigration into the tissue bordering the hydrogel. Current work is investigating whether the ability to induce vascularization is maintained in the crosslinked gels.

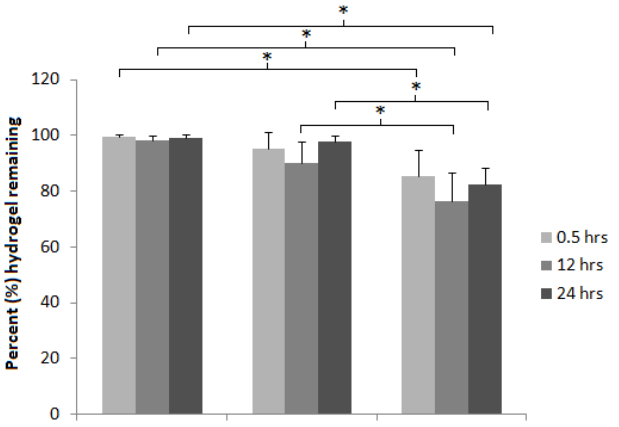


Figure 3. Percent of dermal GA-crosslinked hydrogels remaining at 1, 3, and 6 week post-implantation time points (* indicates statistical significance, $p<0.05$).

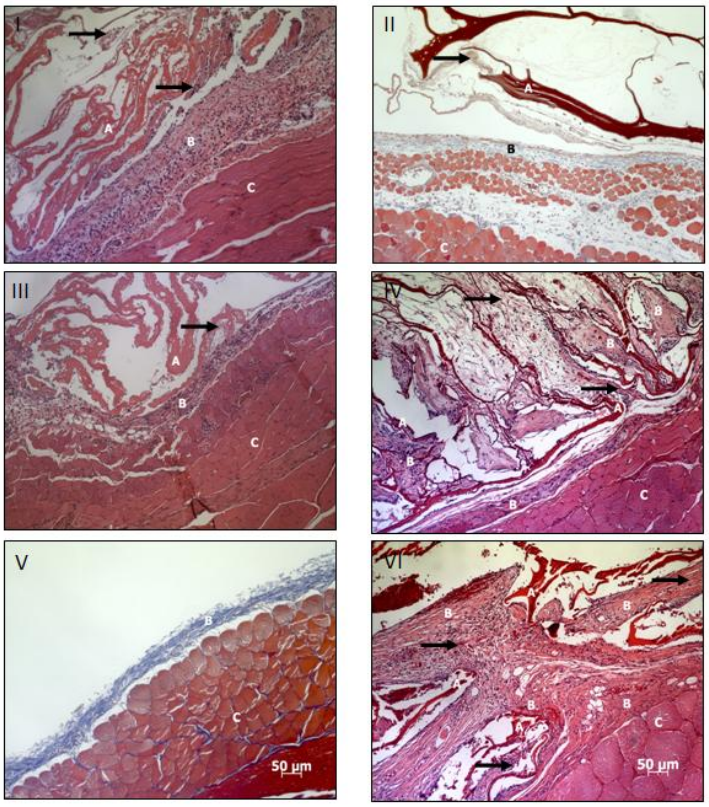


Figure 4. H&E images of non-crosslinked (I, III, V) and 12-hr GA-crosslinked (II, IV, VI) dermal hydrogels implanted subcutaneously into rats at 1, 3, and 6 week time points, respectively. Complete degradation of non-crosslinked gels was observed by the sixth week (V). Areas of tissue invasion (arrows) increased over time for crosslinked hydrogels. Hydrogels, fibrovascular tissue, and muscle tissue are denoted by A, B, and C, respectively.

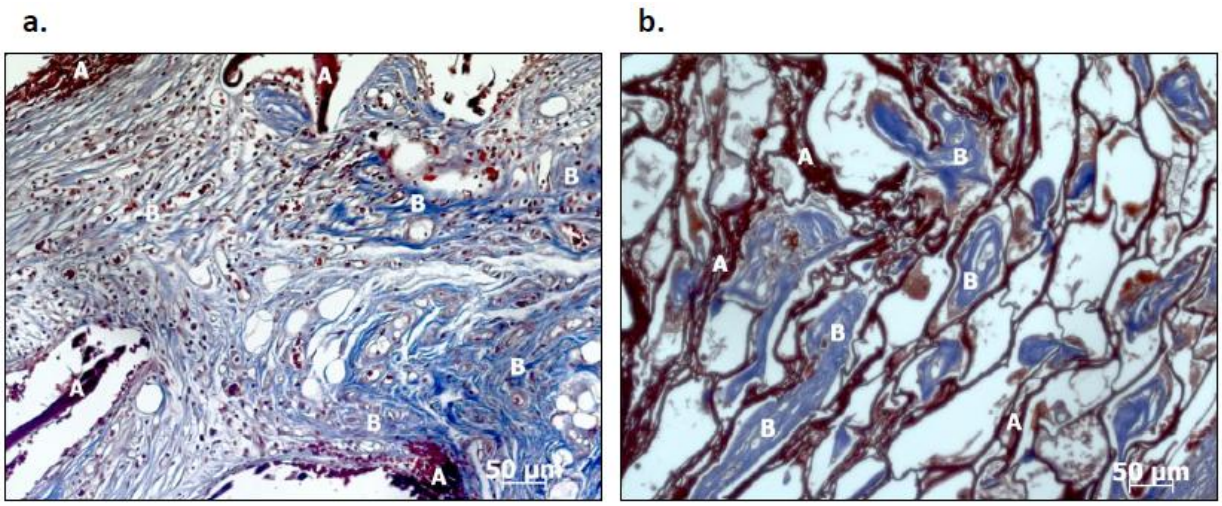


Figure 5. Masson's trichrome stain images of 12hr crosslinked (a.) and 24hr crosslinked (b.) dermal hydrogels at 6 weeks post implantation, showing presence of mature collagen surrounding hydrogel components. Hydrogel and mature collagen are denoted by A and B, respectively.

Discussions: GA-induced crosslinking of dermal hydrogels shows a significant increase in the elastic modulus and yield stress of dermis derived hydrogels with exposure time, suggesting that crosslinking can be used to successfully improve the mechanical properties of tissue-derived hydrogels. The maximum mean elastic modulus was obtained with 24hr crosslinking—up to double the mean value obtained with crosslinking for 0.5hrs. Similarly, results obtained for yield stress indicate a significant increase in the amount of stress needed to deform the hydrogel permanently, corresponding to an increase in crosslinking duration. Resistance of GA-crosslinked hydrogels to degradation was observed both *in vitro* and *in vivo*. This is applicable for *in vivo* studies, given that proper tissue remodeling and regeneration should occur in conjunction with the degradation of the implanted biomaterial. Ideally, the hydrogel matrix should foster cell attachment that allows subsequent synthesis and secretion of new ECM proteins designed to replace those of the biomaterial construct. While results indicate that mechanical properties are improved with GA-induced crosslinking, it is necessary that these hydrogels do not lose their unique biological, tissue-specific properties. The hydrogels supported cell adhesion and spreading. Furthermore, subcutaneous implantation has allowed for an analysis of (1) tissue invasion into crosslinked versus non-crosslinked hydrogels as a determinant of biodegradation characteristics, (2) the effects of crosslinking duration on percent of hydrogel remaining over a period of six weeks, and (3) inflammatory responses of GA-crosslinked hydrogels. Crosslinking drastically slowed tissue invasion and hydrogel degradation relative to controls. However, the results did not indicate a difference between crosslinking conditions. The presence of only a mild inflammatory response, the absence of multinucleated giant cells, and no noticeable fibrous encapsulation of the biomaterial are indicative of hydrogel biocompatibility. Further histological evaluation may be necessary to better characterize the inflammatory response by staining for mononuclear cells such as lymphocytes and macrophages. Overall, crosslinking of dermal derived hydrogels increased hydrogel stiffness and delayed degradation *in vitro* and *in vivo*. The crosslinked gels support cell adhesion and appear well-tolerated *in vivo*. The crosslinking of tissue derived hydrogels may improve their potential for future applications in wound-healing models and for other applications in three-dimensional tissue reconstruction and regeneration.

References:

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