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EDC cross-linking improves skin substitute strength and stability

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Abstract

Collagen-based scaffolds are extensively utilized as an analog for the extracellular matrix in cultured skin substitutes (CSS). To improve the mechanical properties and degradation rates of collagen scaffolds, chemical cross-linking is commonly employed. In this study, freeze-dried collagen-GAG sponges were crosslinked with increasing concentrations of 1-ethyl-3-3-dimethylaminopropylcarbodiimide hydrochloride (EDC; 0, 1, 5, 10, 50 mM). Cross-linking with EDC at concentrations >1 mM was shown to greatly decrease degradation by collagenase up to 21 days. Ultimate tensile strength (UTS) of acellular collagen sponges scaled positively with EDC concentration up to 10 mM. At 50 mM EDC, the UTS decreased dramatically likely due to the brittle nature of the highly crosslinked material. Co-culture of human fibroblasts (HF) and keratinocytes (HK) on these substrates reveals an apparent cytotoxicty of the EDC at high concentrations with reduced cell viability and poor cellular organization in CSS fabricated with scaffolds crosslinked with 10 or 50 mM EDC. From the data gathered in this study, intermediate concentrations of EDC, specifically 5 mM, increase collagen sponge stability and strength while providing an environment in which HF and HK can attach, proliferate and organize in a manner conducive to dermal and epidermal regeneration.

Keywords: Skin; Tissue engineering; Mechanical properties; Collagen; Wound healing

1. Introduction

Morbidity and mortality of patients with massive burns are closely related to the limited availability of donor sites [1]. Conventional treatments to promote recovery of these patients involve harvesting and grafting split-thickness skin grafts [2–5]. Unfortunately, harvesting donor skin inflicts additional injury and in severely burned patients sufficient donor sites are not available. Thus, alternative means of skin replacement must be utilized. Viable [2,6] allodermis, allodermis with autologous cultured keratinocytes [7–9], and acellular [10,11] or fibroblast populated collagen sponges [12,13] have been utilized to promote wound closure. For burns, however, it is commonly accepted that both the epidermis and dermis are required to achieve functional wound closure [14].

Bioengineered skin substitutes are able to generate greater surface area expansion from donor skin than conventional methods [15]. Collagen is commonly used as the scaffolding material for bioengineered skin [16,17] due to its many advantageous properties including low antigenicity and high growth promotion. Unfortunately, poor mechanical properties and rapid degradation rates of collagen scaffolds can cause graft instability and difficult handling. In addition to suboptimal mechanical properties, native materials have inherent heterogeneity due to variability in source animals and processing conditions, which makes quality control of such scaffolds problematic.

The high rates of degradation and deficient mechanical properties of collagen often fail to meet the requirements of specific applications, consequently limiting the use of collagen-based scaffolds. Cross-linking collagen scaffolds via chemical methods has been widely utilized to slow degradation rates and optimize mechanical properties. Historically, glutaraldehyde (GA) has been the most widely utilized chemical cross-linking reagent [18]. However, GA

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cross-linked biomaterials have been shown to release toxic monomeric GA upon hydrolyzation of the material [19,20]. GA crosslinked biomaterials have been reported to exhibit reduced cellular ingrowth in vitro and in vivo [19,20]; thus alternate reagents have been employed. To overcome problems associated with reagent toxicity, carbodiimides have been used to cross-link collagen because they are members of the zero-length class of cross-linkers. Carbodiimides activate the carboxylic acid groups of glutamic or aspartic acid residues to react with amine groups of another chain, forming amide bonds [18,21–23]. Cross-linking with carbodiimides is especially appealing for biological applications as the carbodiimide does not remain in the chemical bond but is released as a substituted urea molecule [24]. Collagen scaffolds crosslinked with 1-ethyl-3-3-dimethylaminopropylcarbodiimide hydrochloride (EDC) have been shown to possess decreased degradation rates [21–25], increases denaturation temperature [26], improved the mechanical properties of collagen scaffolds [23,27] and maintained porous structure of the matrix [28] while supporting the growth of human keratinocytes (HK) [29], smooth muscle cells [30], and fibroblasts [25,31–33]. EDC cross-linked collagen has been investigated as a scaffold for dermal replacements [16,28,29,33]. However, thus far no research has been reported on the effect of EDC on cultured skin substitutes (CSS) containing both dermal and epidermal components.

Freeze-dried, lyophilized collagen-GAG matrices have been successfully used, both clinically and experimentally, as scaffolds for CSS [34–36]. However, a subset of burn patients have cells which produce elevated levels of matrix metalloproteinases which can cause premature degradation of the collagen in CSS, leading to graft failure in vitro [37]. The goal of this study was to investigate the effect of increasing EDC cross-linking concentrations on the biostability, mechanical properties, and tissue morphogenesis of CSS to determine the optimal processing parameters to obtain stable, reproducible, and well organized skin substitutes.

2. Materials and methods

2.1. Collagen scaffolds

Acellular collagen scaffolds were prepared via freeze-drying and lyophilization as previously described [15] from comminuted bovine hide collagen (Kensey Nash; Exton, PA) and chondroitin-6-sulfate (GAG) (Sigma; St. Louis, MO) except without chemical cross-linking with GA [38]. Briefly, bovine collagen powder was solubilized in 0.5 M acetic acid and co-precipitated with GAG to yield a final concentration of 0.6%wt/vol. The co-precipitate was cast into sheets, frozen, lyophilized and physically cross-linked by vacuum dehydration at 140 °C for 24 h. The sheets were then cut into $9 \times 9\,cm^2$, packaged into peel packs and sterilized by gamma irradiation at ~25 kilo Gray (kGy).

Freeze-dried, irradiated collagen-GAG scaffolds were chemically cross linked for 6 h at room temperature with a sterile filtered solution of 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) hydrate (Sigma, St. Louis, MO) in 40% ethanol–water (pH 5.5) with increasing concentrations (0, 1, 5, 10, or 50 mM) of EDC (Sigma; St. Louis, MO) and *N*-hydroxysuccinimide (NHS; Fluka Chemical Co.; St. Louis, MO) at a molar ratio of 1:1. Following cross-linking, the scaffolds were rinsed twice for 20–30 min with sterile 95% ethanol, twice for 24 h with phosphate buffer solution (PBS, Sigma, St. Louis, MO), four times for 15–20 min with Hepes buffered saline (HBS) solution, and twice for 15–20 min with cell culture media (UCMC 160) [37].

2.2. Scaffold pore size

To determine the effect of cross linking on the resultant collagen-GAG scaffold morphology, cross-linked and control scaffolds which had been processed through the previous rinsing steps were paraffin embedded, sectioned and analyzed via brightfield microscopy. Cross linked and control scaffolds ($n = 3$ per group, randomly selected) which had been fully processed (cross-linked and rinsed) were rinsed, fixed, embedded on face and sectioned ~7 μm thick at 20 μm intervals using standard histological procedures. The sections were stained with aniline blue and imaged with a Nikon FXA photomicroscope (Melville, NY). Image J was used to measure the pore areas from the brightfield images. Distinct sections from each sample were imaged (8 images per sample) and the areas of at least 750 pores per sample were calculated for each sample ($n = 3$). Data are presented as mean pore area + standard error (SEM) rather than pore diameter because the pores are an irregular polygonal shape.

2.3. Matrix degradation studies

The biostability of EDC cross-linked and control scaffolds was evaluated by exposing the collagen-GAG matrices to collagenase of bacterial origin (collagenase type I; Worthington Biochemica Corp., Lakewood, NJ). Dry scaffolds ($n = 12$ per group) were first weighed and placed into a 12-well plate with media containing 25 U/ml collagenase per mg of collagen and incubated at 37 °C and 5% CO_2 for up to 21 days. Collagen content in the media was assessed at 3, 6 h, 1, 2, 3, 5, 7, 14, and 21 days using a hydroxyproline assay [39,40]. Because the matrices contain both type I collagen and chondroitin-6-sulfate, a standard curve was generated by testing the absorbance of known quantities of a collagenase degraded collagen-GAG scaffold was used to extrapolate unknown absorbance values.

2.4. Cell culture

HK and human fibroblasts (HF) were isolated simultaneously from surgical discard tissue (strain #674), grown in selective growth media [37] and cryopreserved at passages 0 and 1 to provide a stock of cells for the CSS. For inoculation, the area of the collagen-GAG scaffold was calculated by measuring the width and length of the rectangular or square sponge. HF (passage 2) were inoculated onto rinsed control and cross linked scaffolds at a density of 0.5×10^6 cells/cm^2 and cultured at 37 °C and 5% CO_2 in CSS medium [37,41]. After 1 day of culture, the area of the HF-collagen substrates was measured again and sponges were inoculated with HK (passage 2) at a density of 1×10^6 cells/cm^2. The following day (CSS incubation day 1) the HK-HF-collagen substrates (i.e. CSS) were placed onto a perforated stainless steel platform covered by a cotton pad to establish an air-liquid interface and cultured up to 21 days with the CSS culture media replaced daily.

2.5. Histology

Biopsies for histology were collected at days 7, 14 and 21 and fixed in formalin for one hour prior to processing and paraffin embedding. Sections were stained with hematoxylin and eosin (H&E) and imaged with light microscopy at 10× and 15× magnifications. Brightfield images were collected with SPOT Advanced imaging software with a total of 8 specimens per condition per time point.

2.6. Surface electrical capacitance (SEC) measurement

A definitive requirement for the closure and healing of full-thickness skin wounds is the restoration of the epidermal barrier which protects the body from microbial infection and loss of endogenous fluids. Barrier properties of human skin have been largely attributed to the presence of the stratum corneum (SC) in the upper epidermis [42–44]. In human skin, a water gradient exists across the SC in which hydration levels are lowest at the surface of the skin and highest within more distal layers. Studies have shown that SEC can be used as direct, convenient and inexpensive method to measure skin surface hydration which is related to barrier function [45,46]. SEC measurements were collected from the CSS grafts in vitro using the NOVA dermal phase meter (DPM 9003; NOVA Technology; Portsmouth, NH). On culture days 7, 14 and 21, measurements were taken from four sites on each CSS (6 grafts per group, 24 measurements total) and the SEC values are expressed in DPM units as mean ± SEM.

2.7. MTT viability assay

On days 7, 14 and 21, 6-mm punch biopsies were collected from the CSS (4 punches/graft, $n = 6$) and each placed into a separate well of a 24-well plate. A standard MTT assay [47] was performed on the punch biopsies. The amount of MTT-formazan product released was measured at 590 nm on a microplate reader with values reported as mean optical density ± SEM.

2.8. Tensile testing

The mechanical properties of cross-linked and control acellular collagen-GAG scaffolds and CSS were assessed via tensile testing ($n = 6$ for each condition). Acellular scaffolds were re-hydrated following the rinsing protocol previously described, placed between two polypropylene sheets (N-interface) for ease of handling and cut into dogbone-shaped specimens with a gauge length of 20 mm and width of 2 mm. After the N-interface was removed from the specimens, they were mounted into the grips of a lever action fiber clamp (Instron; Norwood, MA) of an Instron tensile tester model 4430 (Norwood, MA) with a 5 N load cell and tested to break at a strain rate of 2 mm/min to avoid specimen dehydration during testing. Samples were strained to failure and data from samples which did not break within the gauge length were discarded. CSS were cultured for 14 days and evaluated following the same protocol as the acellular scaffolds with the exception that the test was stopped at the time of break or at the point of delamination of the dermis and epidermis. Peak strain, ultimate tensile strength (UTS) and stiffness values were measured and reported as mean ± SEM.

2.9. Statistical analysis

For all quantitative assays, a one-way analysis of variance (ANOVA), followed by Tukey–Kramer multiple comparison analysis, was performed. The data were presented as mean ± SEM, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of cross-linking on physical and mechanical properties

Histological images reveal that the average pore area in the collagen scaffolds was not dramatically altered by cross-linking. The freeze drying process produced inherently heterogeneous sponges (Fig. 1) thus pore area varied depending on their location within the scaffold. The pore area analysis indicated that the mean pore area tended to increase with increasing EDC concentration (Fig. 2), and the control and 50 mM group were statistically different from each another.

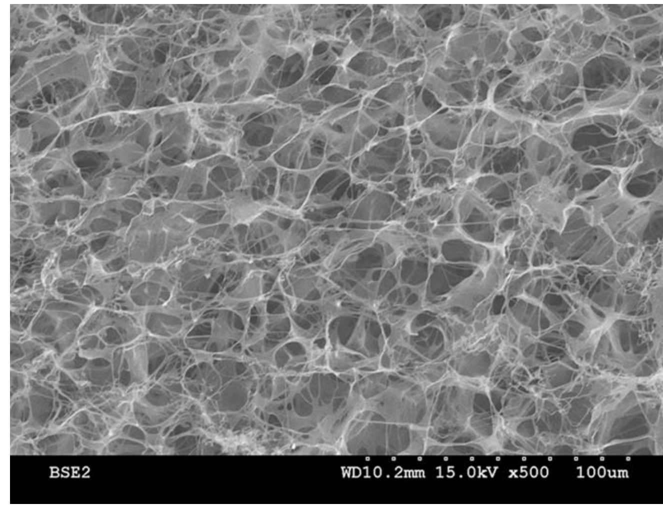

Fig. 1. Scanning electron micrograph of a dry non-crosslinked collagen-GAG sponge. Scale bar = 100 μm.

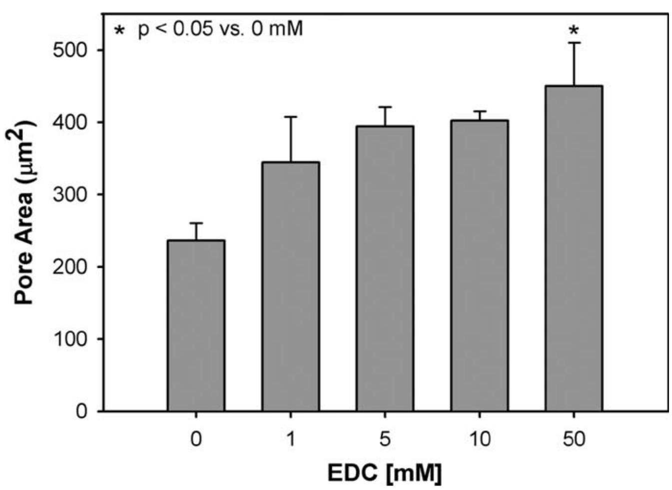

Fig. 2. Mean pore area versus EDC concentration.

In addition to preserving the original structure, a marked increase in scaffold biostability with increasing EDC concentration was found with a ~30-fold reduction in degraded collagen concentration in the 10 mM EDC samples compared to the control samples after 1 day in collagenase (Fig. 3). Increasing EDC concentration to 50 mM significantly reduced the degradation process with only 7.9 ± 1.2% of the scaffold degraded after 30 days of incubation ($p < 0.001$ vs. all other conditions; Fig. 3).

The mechanical properties of the collagen scaffolds were also enhanced by EDC cross-linking. As the concentration of EDC increased to 10 mM, the UTS increased by more than 50% over the control collagen (Table 1). At concentrations above 10 mM the UTS decreased dramatically to less than 70% of the mean value of the control collagen (Table 1). A similar trend in stiffness data was found with the modulus of the scaffolds increasing with the concentration of EDC up to 10 mM after which the stiffness of the materials falls to a mean value significantly lower than that of the 5 and 10 mM samples (Table 1; $p<0.001$). In contrast, strain at break scaled inversely with EDC concentration (Table 1). A maximum percent elongation of occurred in the control samples while the 50 mM EDC samples could be strained the least (Table 1).

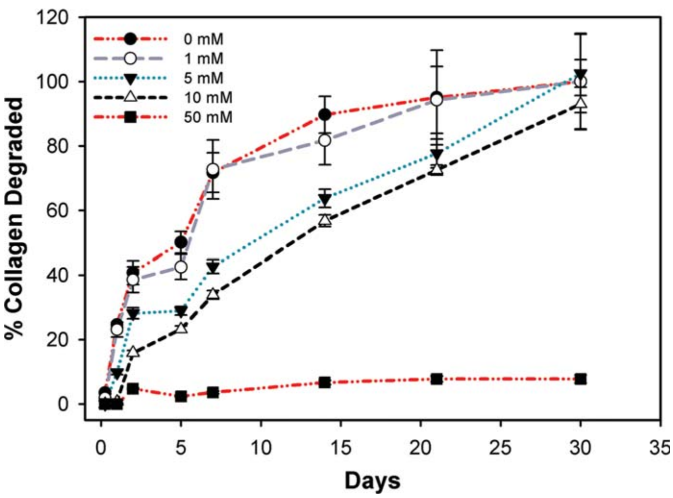

Fig. 3. Percent of collagen degraded by collagenase type I over a 30-day period.

3.2. Co-culture of HK and HF on control and cross-linked collagen

Histological samples showed dense populations of fibroblasts were present in the control, 1 and 5 mM EDC groups at all time points (day 14, Fig. 6). The cell layers were well stratified in the groups with a continuous basal cell layer present in all (arrows, Fig. 4). However, in the 10 and 50 mM EDC groups the density of cells appeared much less with no clear, well-defined stratification of the cells (Fig. 4D and E).

Analysis of cell viability via MTT revealed no statistically significant difference in cell viability among any groups at day 7 (Fig. 5). However at day 14, control, 1 and 5 mM EDC groups had significantly higher MTT values than the 10 and 50 mM groups ($p<0.05$). These results corresponded to the lower densities of cells seen in the histological images taken of the 10 and 50 mM samples.

As the control CSS matured at the air-liquid interface, the epithelium keratinized (Fig. 4), began to form an epidermal barrier and caused the surface of the CSS to dry. SEC measurements on all groups at day 7 revealed the surface of the CSS was still moist and had not yet fully matured. However, at days 14 and 21, significant reductions in surface hydration were seen in the control, 1 and 5 mM conditions, whereas the 10 and 50 mM groups had not begun to dry appreciably and were well above values for normal human skin (Fig. 6).

Table 1
Mechanical properties of acellular control and cross-linked scaffolds and cultured skin substitutes prepared with cross-linked and control sponges

EDC/NHS (mM)	Acellular			After 14 days in culture		
	Percent elongation	Stiffness (mN/mm)	UTS (kPa)	Percent elongation	Stiffness (mN/mm)	UTS (kPa)
0	61.9±2.3	15.6±1.0	49.0±3.0	60.6±2.9	16.1±2.0	86.7±8.4
1	39.1±5.2	17.0±1.0	50.1±3.5	70.0±5.6	16.3±2.0	77.3±15.6
5	28.8±3.6	35.8±3.0	65.8±7.4	52.4±3.1	19.3±2.0	105.3±7.1
10	27.3±3.6	47.5±4.0	74.8±7.0	68.7±4.8	11.2±1.0	62.2±1.4
50	22.9±3.0	23.6±2.0	33.6±19.4	48.8±1.4	18.7±2.0	64.0±4.0

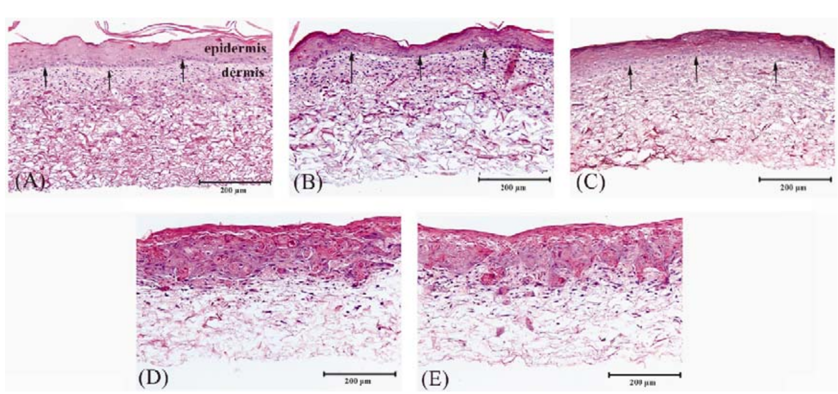

Fig. 4. H&E stained histological sections of of (A) 0 mM (control), (B) 1 mM, (C) 5 mM, (D) 10 mM, and (E) 50 mM EDC cross linked skin substitutes after 14 days in culture. Note continuous layer of basal layer keratinocytes present in control, 1 and 5 mM EDC CSS (arrows). Scale bars = 200 μm.

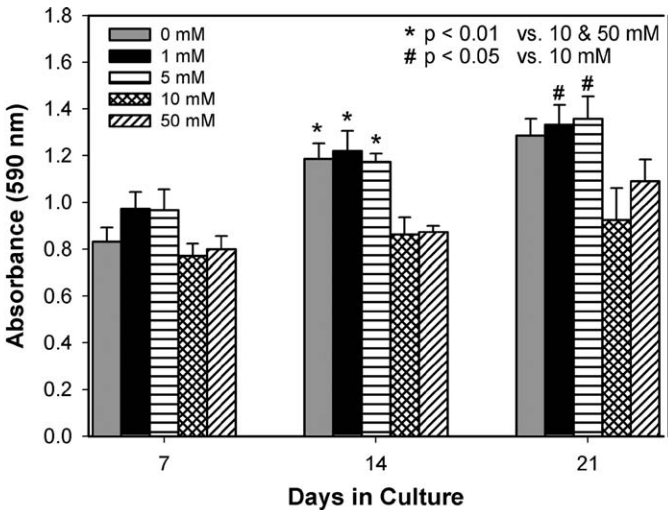

Fig. 5. Assessment of cell viability via MTT assay at days 7, 14 and 21.

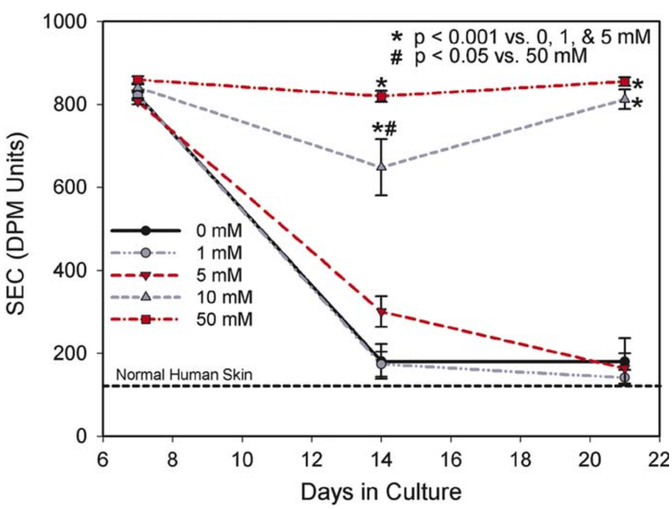

Fig. 6. Surface hydration (SEC) measurements at days 7, 14 and 21 for control and experimental groups. By day 21, control, 1 and 5 mM EDC groups have reached normal human skin levels of hydration.

After 14 days in culture, control and cross linked grafts were tensile tested. Although increasing cross-linking concentrations to 10 mM significantly increased the stiffness of the acellular collagen scaffolds, the same trend was not seen in cellular grafts. The stiffness of cellular grafts, regardless of cross-linking concentration, were not statistically different, though mean stiffness values tend to positively scale with cross-linking concentration (Table 1). UTS of the cellular grafts reached a maximum within the 5 mM group (Table 1) and significantly decreased with higher EDC concentration ($p<0.05$). Maximum percent elongation did not follow any trend, however, the 50 mM group had the lowest percent elongation (Table 1).

4. Discussion

Physical characterization of control and cross-linked collagen sponges reveals that cross-linking produces no considerable alterations to scaffold morphology but distinct changes to scaffold stability, mechanical properties and cellular organization. Chemical cross-linking of collagen has been used for several years to improve scaffold stability [28,31–33,48] and the data collected here support these findings. Increases in EDC concentration associated with greater resistance to degradation. At concentrations of 50 mM EDC, the collagen sponge almost entirely resists degradation for 30 days (the duration of the study) while control samples were fully degraded within 3 days (Fig. 3). However, these results do not predict degradation rates in vivo which must be confirmed in preclinical transplantation studies. Although these sponges have the highest resistance to degradation in vitro, their inability to be cleared from the wound site in a timely manner may interfere with proper wound healing or delay vascularization. Other studies have demonstrated that GA cross-linked collagen scaffolds grafted to athymic mice are not cleared from the wound after 6 weeks of implantation and this persistence in the wound with may lead to a delayed development of connective tissue [38].

Generally, positive correlations between cross-link density and mechanical properties have been reported [18,23,27]. Although many have seen increases in tensile strength and modulus with higher EDC concentrations, others report decreases in these parameters with increases in EDC [23]. These data indicate the UTS and stiffness of acellular scaffolds scale positively with EDC concentration up to 10 mM at which point the stiffness and strength of the material dramatically declines. Researchers have postulated that at high concentrations, the diffusion of the cross-linker into the collagen is slowed by the rapid initial cross-linking of the surface, limiting the efficacy of the cross linking agent [49–51]. Another possible mechanism for this phenomenon is the formation of local stress concentrations due to the early failure of brittle collagen fibers within the collagen sponge [23]. Because the collagen sponges used for these experiments have an open pore structure and maintain that pore structure after cross-linking, we hypothesize it is unlikely that the diffusion of the crosslinker into the sponge was a limiting factor. It is more probable that local stress concentrators within the highly cross-linked scaffolds caused these grafts to fail with minimal elongation.

The application of cultured HF and keratinocytes to control and cross-linked scaffolds reveals a somewhat unexpected outcome. Despite the fact that carbodiimides are cross-linkers which do not stay in the bond and have been reported to cause no significant cytotoxicity [25,30,32,33,52], elevated concentrations of EDC (10 and 50 mM) result in low cell densities and poor cellular organization in these studies, suggesting that EDC is cytotoxic in this model. The anatomical disorganization of the cells coincides with a failure to form an epidermal barrier as can be seen in the high SEC values in Fig. 6. Hanthamrongwit et al. observed EDC to be cytotoxic when scaffolds were not washed thoroughly [29]. Similar results were detected in preliminary experiments for this study which caused the scaffold rinsing process to be greatly enhanced to the three day procedure used presently. It is possible that at high cross-linker concentrations, the unreacted EDC and reaction by-products cannot be fully released via simple rinsing leaving behind residual chemical species which can be cytotoxic.

Mechanical properties of cell-polymer constructs depend on many factors including the initial strength of the material, population of cells within the matrix and the balance of matrix degradation and synthesis. Control and cross-linked CSS were evaluated at culture day 14 and showed a significant increase in tensile strength in the control, 1 and 5 mM groups versus the 10 and 50 mM groups. Poor cellularity in the high concentration groups likely leads to lower levels of endogenous matrix formation by the fibroblasts. We suggest low matrix production and cell density are the two major contributing factors to the poor mechanical properties of these grafts. These results emphasize the importance of the proper balance between the mechanical properties of a scaffold and its biocompatibility and ability to support tissue morphogenesis.

5. Conclusion

The stability and strength of collagen scaffolds used for tissue engineering must satisfy their intended biomedical needs. Cross-linking of collagen sponges with EDC provides a means of easily tailoring a scaffolds biostability and mechanical properties. These techniques may be used to increase CSS quality and ease of handling in general and more specifically in cases where patient cells produce elevated levels of matrix metalloproteinases [37]. Based on these results, intermediate concentrations of EDC, specifically 5 mM, increases collagen sponge stability and strength while providing an environment in which fibroblasts and keratinocytes can attach, proliferate and organize in a manner conducive to dermal and epidermal regeneration. These advantages may contribute to improved performance and clinical outcome in the treatment of full thickness skin wounds.

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