The Stability and Biocompatibility of Electrospun Collagen Using Common Cross-linking Agents

Portocarrero, G., Masih, P., Amara, S., Collins, G., Livingston Arinzeh, T.
Department of Biomedical Engineering, New Jersey Institute of Technology, Newark, NJ 07102, USA.

Statement of Purpose: Electrospinning is a widely used method for fabricating tissue engineering scaffolds. The resulting nonwoven fiber structure mimics the extracellular matrix of natural tissue. Collagen is one of the most routinely used naturally-derived biomaterials. However, electrospinning collagen causes denaturation due to the use of fluorinated solvents and results in complete dissolution of the scaffold in aqueous medium.[1] Thus, various crosslinking techniques are being utilized to improve stability and physiochemical properties of electrospun collagen. This study evaluated the effect of crosslinking using four commonly used crosslinking agents on the dimensional stability, mechanical properties and biocompatibility of electrospun collagen mats. The goal of the study was to fabricate a scaffold which can mimic both the architecture and composition of the natural ECM and retain its fibrous structure in an aqueous, physiological environment for tissue engineering applications that may require long-term structural support for adequate repair.

Methods: *Fabrication of collagen scaffolds:* Type I collagen was purified from bovine tendon, dissolved in trifluoroacetic acid and electrospun using previously reported methods.[2] Collagen mats were crosslinked using genipin, glutaraldehyde, N-(3-Dimethyl aminopropyl)-N'-ethyl carbodiimide hydrochloride (EDC)[3], and EDC with *N*-hydroxysulfosuccinimide (EDC-NHS). *Stability study:* The crosslinked samples were immersed up to 3-months in phosphate buffered saline (PBS) at 37°C. The samples were evaluated for structural changes using differential scanning calorimetry (DSC), degree of cross-linking using free amino acid analysis, changes in overall dimension, fiber dimension and morphology, weight and mechanical properties over time. *Biocompatibility*: Human mesenchymal stem cells (MSCs) derived from the adult bone marrow were seeded at $1.5x10^4$ cells/cm^2 onto cross-linked collagen mats and grown in standard growth media (DMEM, 10% fetal bovine serum, 1% antibiotic) for up to 7 days. Cell proliferation was evaluated by XTT assay (Invitrogen) and cell morphology by confocal microscopy using phalloidin for F-actin and Dapi for the nucleus.

Results: *Stability Study:* The uncrosslinked mats had uniform fiber morphologies. After crosslinking, no fibers could be detected for gluteraldehyde samples. Fibers swelled for 1, 5 and 10% genipin (Figure 1). For the EDC and EDC-NHS crosslinked mats, the fibers were easily distinguishable with interfiber spacing still apparent between the fibers. After 1 month of immersion in PBS, glutaraldehyde and 1% genipin mats completely disintegrated. After three months, fibers for 10% genipin, EDC, and EDC-NHS mats were still present, however, a significant reduction in fiber size occurred for EDC-NHS mats as compared to day 0 ($p<0.05$) (Figure 1). Significant dimensional changes occurred in the overall size of the mats for 5 and 10% genipin, EDC and EDC-NHS crosslinked samples by 3 months as compared to day 0 ($p<0.05$). A significant weight loss was only detectable for 5% genipin at 3 months ($p<0.05$). DSC analysis showed higher glass transition temperatures for all crosslinked mats as compared to uncrosslinked control. Free amino acid absorbance values were significantly lower for all groups except for gluteraldehyde crosslinked mats at day 0 in comparison to uncrosslinked mats ($p<0.05$). The lowest values occurred for EDC and EDC-NHS samples. Free amino acid values increased with increasing incubation period in PBS for all mats. After 3 months of immersion, the highest young's modulus and ultimate tensile stress occurred for EDC-NHS mats wherein average values were 0.26 MPa and 0.22 MPa, respectively. *Biocompatibility:* MSCs grew significantly on EDC and EDC-NHS mats for up to 7 days with the greatest growth on EDC-NHS mats, as analyzed by XTT ($p<0.05$) and had a well-spread cell morphology (Figure 2). Genipin was not supportive of cell growth throughout the culture period as analyzed by XTT and confocal microscopy.

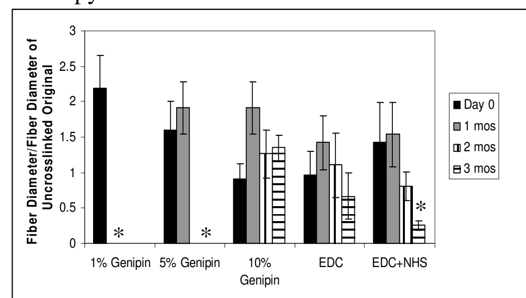

Figure 1: Fiber diameter of cross-linked mats normalized to uncrosslinked mats over time. *$p<0.05$, significantly lower than day 0.

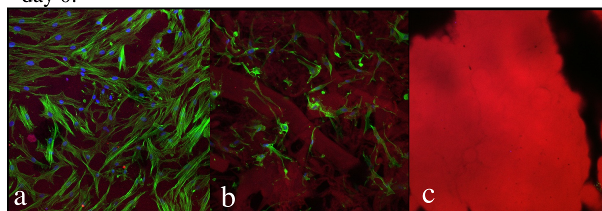

Figure 2: Confocal images of MSCs on a) EDC-NHS, b) EDC and c) genipin crosslinked collagen mats at day 7. Green is F-actin, blue is nucleus and red is the collagen, 20x objective.

Conclusions: This study examined commonly used crosslinking agents for supporting the overall stability of the fibrous structure of electrospun collagen. Mats crosslinked with EDC and EDC with NHS were the most stable, retaining their fiber morphology and mechanical properties over three months. EDC with NHS mats also supported the greatest cell growth. Therefore, EDC and EDC with NHS are suitable crosslinking agents to be pursued for future investigations *in vivo*.

References:[1] Zeugolis DI. Biomat. 2008;29:2293-2305. [2]Patlolla A. Acta Biomat, 2010;6:90-101. [3]Barnes CP. Tiss Eng. 2007;13:1593-1605.

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