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Nail matrix regenerative engineering: in vitro evaluation of poly(lactide-co-glycolide)/gelatin fibrous substrates

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Abstract

Acute traumatic nail injury treatment repair procedures are commonly conducted in emergency departments and primary care offices. Current repair methods use nail splints that are inserted within the nail root to prevent the fusion of the proximal nail fold and the matrix tissue. Splints provide a protective barrier overlying the nail bed soft tissue during recovery periods, but uncertain prognoses (i.e., aesthetic and functional disadvantages) reveal a need for improved nail repair techniques. Nail splints are not specifically designed for nail organ restoration via biological mechanisms, thus, a clinical application that utilizes regenerative engineering techniques can prove useful in improving the nail injury prognoses. Using the coaxial electrospinning method, hybrid poly(lactide-co-glycolide) (PLGA) (85:15) and gelatin fibrous scaffolds (Hybrid₁: PLGA shell, gelatin core and Hybrid₂: gelatin shell, PLGA core) with average fiber diameters of 540 ± 118 and $2,215 \pm 1,135$ nm, respectively, were produced and successful encapsulation of core fibers was observed. Furthermore, nail stem cells expressing stem cell characteristic markers CD90, CD29, and Lgr6 showed preferred attachment to Hybrid₂ scaffolds after 24 hr. Overall, an in vitro regenerative engineered nail matrix may aid to improve the cosmetic appearance and function of injured nail organs post-traumatic injury.

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AUTHOR CONTRIBUTIONS

All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

Keywords

electrospinning; nail matrix; nail stem cells; regenerative engineering

1 | INTRODUCTION

Acute traumatic injuries to the nail may adversely affect the quality of life. While the fingernail contributes to the aesthetic appearance of the hand, the nail also protects the underlying nail bed and can be an indicator of systemic illness (Singal & Arora, 2015). Injuries to the nail may result in uncertain prognoses (Liu, Li, Li, Zhang, & Lu, 2018; Lorea, 2013; Shieh & Cheng, 2015), and injury severity further complicates the scenario. Following injury, the regrown nail may not possess the functionality and aesthetic appearance of the nail pre-injury (Shieh & Cheng, 2015). While there is an option for surgical intervention, corrective surgeries may result in undesirable outcomes including poor cosmesis and impaired sensation at the fingertip (Liu et al., 2018). The recovery process is uncertain and may encompass weeks to months based on injury severity and patient responsibilities often necessitate an expeditious recovery. Thus, an ideal treatment strategy should induce epimorphic regeneration and yield a time considerate recovery process. Currently, there are no stand-alone treatments for nail injuries. Therefore, there is an incentive to explore treatments and procedures that can be utilized in rural and urban settings.

In cases where the nail plate is forcibly removed during injury, nail splints are used to inhibit fusion between the proximal nail fold and nail bed (Tos, Titolo, Chirila, Catalano, & Artiaco, 2012). The splint is inserted into the nail root and anchored with suture. It is removed up to 3 weeks later, and nail regrowth is observed in the subsequent months (Bharathi & Bajantri, 2011). Aside from the native nail plate itself, other materials can be used for a nail splint and include polypropylene prostheses, silicone Inro[®] Nail splints, and sugar cane biopolymer (Figueiredo et al., 2017; Pinto & Oliveira, 2019). There are several limitations to using these synthetic nails splints that include unknown ability to promote regeneration, cost, and excluding Inro[®] splints, they are not specifically designed for assisting nail organ restoration (Figueiredo et al., 2017).

An ideal approach to assist with nail organ healing should include regenerative engineering techniques that harness the potential of combining cell biology and biocompatible materials. Establishing a biomimetic cell-delivery system composed of stem cells and a biodegradable substrate could prove useful in stimulating indigenous regeneration while decreasing recovery time and cost, and improving surgical and aesthetic outcomes. When devising strategies to bioengineer the nail matrix, factors such as biocompatibility, cost, FDA approval, and practical use of the system must be considered. PLGA is a commonly selected synthetic polymer that is known for its biocompatibility, tailorable degradation rates, and mechanical strength. It is important that the biocompatible substrate is sturdy enough to protect the delicate matrix and nail bed, and also has porous hydrophilic properties to attract cell proliferation and accommodate drainage from the wound site. Gelatin, a hydrolyzed form of collagen has been shown to promote cell adhesion, migration, and proliferation due to the presence of the RGD peptide (Norouzi, Shabani, Ahvaz, & Soleimani, 2015). Gelatin

can provide the hydrophilic nature to the scaffold to assist with cell attachment, migration, and proliferation.

In order to construct biomimetic matrices, it is important to consider the native tissue structure. Stem cells proliferate and differentiate into hard-cornified tissue to maintain homeostasis of the nail. The extracellular matrix resembles a fibrous structure and fabricating this environment may assist with basal layer nail stem cell (NSC) transformation into hard-cornified epithelia in the stratum corneum. To replicate this process, coaxial electrospinning was used to combine the physical properties of PLGA and gelatin to fabricate a biocompatible fibrous scaffold for in vitro analysis. This study aims to isolate and characterize NSCs from the nail matrix region and observe their behavior on hybrid fibrous scaffolds.

2 | MATERIALS AND METHODS

PLGA 85:15 was purchased from Lactel Absorbable Polymers (Birmingham, AL). Gelatin (Type A) was obtained from MD Biomedicals, LLC (Solon, OH). 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), methanol, and 4% paraformaldehyde solution were purchased from Sigma-Aldrich (St. Louis, MO). Glutaraldehyde (GA), Alamar Blue Assay, Crystal Violet, Rhodamine Phalloidin stain, EpiLife media, minimum essential media, fetal bovine serum, Penicillin Streptomycin and Gelatin from Pig Skin Fluorescein Conjugate (FITC-gelatin), and Hexamethyldisilazane (HMDS) were purchased from ThermoFisher Scientific (Waltham, MA). Accutase was obtained from MilliporeSigma (Burlington, MA). CD29 (1:100), CD90 (1:100), Lgr6 (1:50), and K10 (1:100) primary antibodies and AlexaFluor[®] 488 and 594 secondary antibodies were purchased from Abcam (Cambridge, MA).

3 | METHODS

3.1 | Coaxial electrospinning

PLGA (shell) 10% w/v and gelatin (core) 4% w/v and 11% w/v gelatin (shell) and 11% w/v PLGA (core) (From this point further denoted as Hybrid₁ and Hybrid₂, respectively) solutions were used to fabricate nanofibers. Briefly, PLGA and gelatin were dissolved overnight in HFIP to use for the coaxial electrospinning method. For Hybrid₁ fibers, the needle-to-tip distance was 22 cm, applied voltage was 16 kV, and flow rate for the shell fibers was set at 1.0 ml/hr and core fibers at 0.5 ml/hr. Hybrid₂ fibers had a needle-to-collector distance of 30 cm, applied voltage was 22 kV, and the flow rate for shell fibers was 1.30 ml/hr and core fibers was 0.850 ml/hr. Fiber mats were collected on aluminum foil (0.3 ± 0.1 mm thick) and then placed under vacuum for 48 hr to rid scaffolds of residual solvent.

3.2 | GA vapor crosslinking

Based on modified methods similar to Nguyen and Lee, Hybrid₂ scaffolds were exposed to GA vapors 25% v/v for 1 hr at 37°C to complete the crosslinking process (Nguyen & Lee, 2012a). Scaffolds were then placed under vacuum for 48 hr to rid scaffolds of residual GA.

3.3 | Physical and chemical characterization of hybrid fibers

Morphology of hybrid scaffolds was observed using scanning electron microscopy (SEM). Samples were sputter-coated with Polaron E5100 then taken for imaging using the FEI Nova NanoSEM 450. Transmission electron microscopy (TEM) was used to confirm the encapsulation of core fibers during the coaxial electrospinning process. To further confirm successful coaxial electrospinning parameters, PLGA and gelatin^{FITC} containing solutions were electrospun using the previously stated parameters to yield Hybrid_{1_FITC} and Hybrid_{2_FITC} fluorescent fiber mats. The scaffolds were imaged using confocal microscopy to observe the intensity of FITC within the samples.

3.4 | Swelling study

To analyze the hydrophilicity characteristics of hybrid scaffolds, 0.3 ± 0.1 mm thick scaffolds (Hybrid₁, Hybrid_{2_UCL}, and Hybrid_{2_CL}) were cut into 1.5×1.5 cm² squares and the dry weight was measured. Samples were then submerged in phosphate buffer solution (PBS) and incubated at 37°C for 1, 3, and 7 days. At each time point of interest, scaffolds were harvest and wet-weight was measured. Samples were dried and taken for SEM analysis.

$$\% \text{percent swelling} = \frac{(\text{wet weight} - \text{dry weight})}{\text{dry weight}} \times 100$$

3.5 | Nail stem cell isolation

Male Sprague–Dawley rats (6–8 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). All animal handling and procedures were carried out in accordance with the Institutional Animal Care and Use Committee at the University of Connecticut Health Center, Farmington, CT. NSCs were isolated from the proximal nail matrix of 6–8 week old Sprague–Dawley rats. Briefly, separation of the hard nail plate and the proximal nail fold revealed the underlying matrix tissue for harvest. The distal matrix was disposed of and proximal nail matrix tissue was isolated for explant culture. Nail matrix samples were washed three times with PBS containing 1% P/S before culturing. Matrices (30 proximal nail matrices) were cultured in EpiLife media supplemented with 10% FBS, 1% P/S, and 0.06 mM CaCl₂ at 37°C containing 5% CO₂. The media was changed every 2–3 days.

3.6 | Flow cytometry

NSCs were removed from tissue culture flasks via accutase and suspended in PBS containing 3% FBS. Cells were separated into Eppendorf tubes at 1.0×10^5 densities and K10, CD29, CD90, and Lrg6 primary antibodies were added and incubated for 1 hr at 4°C. Cells were centrifuged at 4g for 5 min and the pellet was washed with PBS/3% fetal bovine serum (FBS) solutions three times. Cells were then fixed with 4% paraformaldehyde for 20 min, centrifuged, and washed with PBS three times. Secondary antibodies (AlexaFluor 488 and 594) were added to samples and incubated for an additional hour. NSCs were then centrifuged and washed three times, then taken for flow cytometry analysis.

3.7 | NSC attachment

Briefly, scaffolds were cut in $1.5 \times 1.5 \text{ cm}^2$ squares then subjected to UV sterilization for 30 min per side. Scaffolds were then incubated in EpiLife media solutions containing 10% FBS and 5% pen/strep for 24 hr. 1.0×10^5 cells were seeded Hybrid₁ and Hybrid₂ scaffolds then fixed with 4% paraformaldehyde solutions at 0 min, 30 min, 1 hr, 8 hr, and 24 hr. Hybrid scaffolds without cells were used as control. After the final timepoint, samples ($n = 4$) were stained with rhodamine phalloidin and taken for confocal imaging. Samples were incubated with crystal violet reagent for 5 min then rinsed with PBS until the solution remained clear. 100% methanol was then added to dissolve the samples and the absorbance was read at 590 nm.

3.8 | SEM to observe NSC attachment

NSCs (seeding density of 1.0×10^5 cells) were seeded on Hybrid scaffolds and cultured for 24 hr. At each time point, samples were submerged in a 1% GA solution for 1 hr at room temperature then fixed for 24 hr in 3% GA solution at 4°C. Scaffolds were subjected to serial dehydration (30, 50, 70, 90, and 100% ethanol) then incubated with various HDMS/ethanol solutions. Samples were allowed to air dry and desiccated until ready for SEM imaging.

4 | RESULTS

4.1 | Successful fabrication of bead-free coaxial fibrous scaffolds

The coaxial electrospinning method was used to fabricate biomimetic substrates to serve as a potential substrate for NSC delivery purposes (Figure 1). Optimal parameters for the coaxial electrospinning process yielded bead-free uniform Hybrid₁ (10% w/v PLGA-shell, 4% w/v gelatin-core) and Hybrid₂ (11% w/v gelatin-shell, 10% w/v PLGA-core) fibers with average diameters ranging 540 and 2,215 nm, respectively (Figure 1–1).

4.2 | Confirmation of core-shell fiber encapsulation

To confirm the presence of core fibers, TEM imaging was used to view the core of Hybrid₁ and Hybrid₂ scaffolds. Light and dark phases in the TEM images assist us with differentiating the shell fibers from the core. We were able to confirm that our parameters yield encapsulated fibers (Figure 2), and furthermore, we also compared the concentration of gelatin^{FITC} in Hybrid₁ and Hybrid₂ scaffolds to observe encapsulation as well (Figure 2). A noticeable difference in the fluorescent intensity of the gelatin can be attributed to two things, the PLGA shell in Hybrid₁ encapsulates the gelatin, therefore only faint FITC expression was expected and Hybrid₂ has gelatin as the shell and the concentration was increased as well.

4.3 | Crosslinking hybrid₂ fibers using GA vapors

Gelatin has been found to enhance the bioactivity of fibrous scaffolds; however, its disadvantages such as hydrophilicity and low mechanical stability require crosslinking procedures to increase gelatin's stability in solution. One of the most widely used and convenient techniques to crosslink collagen-based materials uses GA vapors to enhance the

properties of the material (Nguyen & Lee, 2012a). To optimize time and GA concentration required to crosslink Hybrid₂ scaffolds, 0, 5, 15, and 25% GA solutions were prepared and fiber mats were exposed to the vapors for 1 hr at 37°C (Figure 3). SEM imaging revealed uniformed fibrous mats (Figure 3), and furthermore, fiber diameter was analyzed using ImageJ and results show that there is an inverse relationship between fiber diameter and GA concentration (Figure 3). At 25% GA, average fiber diameters decreased to 1,599 nm showing that the PLGA and gelatin were successfully crosslinked in some fashion (Figure 3).

4.4 | Measuring the hydrophilic potential of hybrid scaffold

To evaluate the potential of the hybrid scaffolds to retain water, which can have an effect on the behavior of the material in vivo, we submerged Hybrid₁, Hybrid_{2_UCL}, and Hybrid_{2_CL} scaffolds in EpiLife standard media to mock in vitro conditions to observe the swelling of the materials over 1, 3, and 7 days. SEM images reveal that compared to UCL groups, Hybrid_{2_CL} fibers did not hold their morphology due to increased water intake (Figure 4). This is understandable due to the hydrophilic nature of the gelatin shell solution. Hybrid₁ and Hybrid_{2_UCL} scaffolds held fiber morphology for up to 7 days in culture. This can be attributed to the hydrophobicity properties of PLGA and also, when gelatin composes the shell of the fiber and is not crosslinked to the PLGA core fiber, the hydrophilicity of the gelatin will cause it to dissolve in the aqueous solution. The percentage of swelling of Hybrid₁, Hybrid_{2_UCL}, and Hybrid_{2_CL} scaffolds when in aqueous solution further confirmed a significant increase of Hybrid_{2_CL} fibers when compared to hydrophobic UCL groups (Figure 4).

4.5 | NSCs express nail matrix-related cell surface markers

Passage 2 (P2) NSCs expressed Lgr6 (90.9%), which has been found to mark NSCs, and CD90 (54.8%) and CD29 (68.5%), markers for epidermal stem cells (Figure 6a–d) (De Berker et al., 2000; Lehoczy & Tabin, 2015; Nakamura et al., 2006; Westgate & Leigh, 1996). Flow analysis of double-stained Lgr6/K10 NSCs shows limited expression (4.7%) of the K10, a suprabasal cornified epidermis marker (Figure 5) (De Berker et al., 2000). The presence of NSC characteristic markers confirm successful isolation of stem cells from the nail matrix region and are further used to evaluate the behavior of these stem cells on hybrid fibrous matrices.

4.6 | NSC attachment and viability on hybrid scaffolds

To confirm if gelatin shell fibers induced cell attachment better than PLGA shell scaffolds, NSCs were seeded on the fibrous substrates and cell attachment was observed using fluorescent and crystal violet staining mechanisms. Between Hybrid₁ and Hybrid₂ scaffolds, we observed low cell attachment at early time points, however, we start to see NSCs migrate and spread on fibrous substrates at 12 hr and by 24 hr, a noticeable increase in actin staining suggesting an increase in NSC attachment to both substrates (Figure 6). Moreover, crystal violet results suggest that Hybrid₂ fibers induced cell attachment better than Hybrid₁ scaffolds over a 24-hr period (Figure 7). By 24 hr, there is a significant difference between NSC attachment to Hybrid₁ and Hybrid₂ scaffolds (**** $p < .0001$) which is expected due to interaction with favored hydrophilic interactions.

5 | DISCUSSION

Acute traumatic nail injuries are often coupled with an uncertain prognosis and it is unclear if the regenerated tissue will fully resemble the pre-injury nail function and aesthetic appearance. Injuries vary in severity and meticulous and expeditious initial repair strategies are crucial in supporting downstream prognoses that are favorable. Currently, available materials used for nail splints lack bioactivity properties, a possible contributor to unfavorable outcomes in nail plate regeneration post repair. Clinical methods addressing traumatic nail injuries should adopt strategies that can be consistently used in a broad spectrum of injury types and severities.

Nail injury repair strategies can be improved with the implementation of stem cell science and advanced material science, in which the nail extracellular matrix can be simulated with the use of biomimetic substrates and functionalizing the matrix with stem cells. An important strategy is to develop a system where NSCs are delivered to the nail matrix to ultimately produce cornified keratinocytes to generate a hard nail plate. NSCs give rise to the nail plate and maintain an important role in hard nail plate regeneration and digit tip regeneration (Lehoczy, 2016; Takeo, Chou, Sun, et al., 2013). The first step in this process is to demonstrate that NSCs can be isolated and maintain their stem cell potential. In this study, we isolated and characterized NSCs, fabricated hybrid scaffolds, and determined NSC behavior on a hybrid scaffold in vitro. Important findings of our study include that NSCs express stem cell-related markers CD90, CD29, and Lgr6 at passage 2 and do not express differentiation marker K10 at passage 2. The expression of stem cell markers decreases with passage number. Furthermore, increasing GA vapor concentration decreases Hybrid₂ fiber diameter. Ultimately, Hybrid₂ scaffolds promote better NSC attachment at 24 hr.

Some limitations of the study include the use of randomly aligned fibers, an orientation that does not accurately represent the native tissue. Yu et al. reported SEM images of decellularized nail ECM that consisted of aligned fibers. When the fibers are seeded with bone mesenchymal stem cells, they can survive in vivo and stimulate nail regeneration. However, the group demonstrated that decellularized matrices are unable to survive for long periods in vivo and suggests that a tissue engineering approach with the use of biomaterials is needed to further elucidate this disadvantage (Yu et al., 2019). In our study, random electrospun fibers were used, however, to mimic the native ECM we will use aligned fibers in future work. Furthermore, we will need to further determine the benefits of surface modification of the scaffolds. Future studies will also focus on identifying other cell sources, such as adipose-derived stem cells, to decrease donor site morbidity.

Overall, we were able to further elucidate the behavior of NSCs in vitro and their behavior on electrospun matrices, which currently has not been defined in the literature. Furthermore, we fabricated a proof-of-concept biomimetic system for application in traumatic nail injuries. The next phase of the study will focus on assessing the efficacy of this novel strategy in an in vivo animal model. Evaluating the effect of NSCs in the repair and rescue of nails following traumatic injury can aid in the development of clinical interventions. Moreover, such efforts can be applied in future initiatives toward regenerative engineering methods focused on mammalian digit regeneration.

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DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article.

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FIGURE 1.

Fabrication of Hybrid₁ and Hybrid₂ coaxial electrospun scaffolds. (a). Schematic of coaxial electrospinning setup used to create Hybrid₁ and Hybrid₂ fibrous scaffolds. Illustrated by Aundrya B. Montgomery. (b,c) SEM image of Hybrid₁ and Hybrid₂ optimized coaxial electrospun fibrous scaffolds, respectively. (d) Hybrid₁ diameter distribution histogram displaying fiber diameters ranging between 227 and 792 nm. (e). Fiber diameters ranging from 205 to 4,938 nm are observed in Hybrid₂ coaxial scaffolds

FIGURE 2.

Visualizing encapsulation of Hybrid₁ and Hybrid₂ core fibers. (a). TEM images of Hybrid₁ fiber shows core fiber (light contrast, 116 nm) and shell fiber (dark contrast, 383 nm). (b) Faint FITC signal is consistent with a small concentration of gelatin (4% w/v) encapsulated by a shell fiber of 10% w/v PLGA in Hybrid₁ scaffolds. (c) PLGA (dark contrast, 121 nm) is seen within the shell gelatin^{FITC} fibers (light contrast, 436 nm). (d) Gelatin^{FITC} shell fibers show greater intensity of FITC in Hybrid₂ fibers

FIGURE 3. GA vapor crosslinking Hybrid₂ fibrous scaffolds. (a). Process of crosslinking 11% gelatin and 10% PLGA Hybrid₂ coaxial fibrous scaffolds. (b). SEM images showing the morphology of Hybrid₂_{UCL} scaffolds and Hybrid₂ scaffolds crosslinked with different GA concentrations. (c) Fiber diameter distribution of fibers crosslinked with 0, 5, 15, and 25% GA concentrations. (d) Average diameters of Hybrid₂ scaffolds show a decrease as the concentration of GA increases

FIGURE 4. Swelling properties of Hybrid₁, Hybrid_{2_UCL}, and Hybrid_{2_CL} scaffolds. (a). SEM images of Hybrid₁, Hybrid_{2_UCL}, and Hybrid_{2_CL} scaffolds after 1, 3, and 7 days of exposure to EpiLife media. (b). Hybrid_{2_CL} shows a significant increase in swelling over the course of 7 days when compared to Hybrid₁ and Hybrid_{2_UCL} groups

FIGURE 5. NSC expressing stem cell-associated markers. Flow cytometry analysis of NSC cultures show positive staining for characteristic markers CD90, CD29, and Lgr6. NSCs were negative for epidermal differentiation marker K10

FIGURE 6.

Observing NSC attachment on Hybrid₁ and Hybrid₂ scaffolds. (a) Phalloidin (actin = red) staining of NSCs seeded on Hybrid₁ and Hybrid₂ scaffolds at 30 min, 1 hr, 12 hr, and 24 hr. (b, c) SEM images of NSCs seeded on Hybrid₁ and Hybrid₂ scaffolds, respectively, after 24 hr

FIGURE 7.

Quantification of NSC attachment via crystal violet assay. NSCs show significant increase (**** $p < .001$) in Hybrid₂ attachment between 0.5 and 24 hr time points when compared to NSCs that attached to Hybrid₁ scaffolds