

Tissue-Engineered Skin Substitutes

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ABSTRACT

Severe burns, chronic diabetic ulcers, and traumatic full-thickness skin defects remain major clinical challenges because autologous split-thickness skin grafts (STSGs) -- the gold standard treatment -- are limited by donor-site availability, morbidity, and inability to regenerate functional dermal appendages. Tissue-engineered skin substitutes (TESSs) aim to overcome these limitations by combining biomaterial scaffolds, autologous or allogeneic cells, and bioactive signalling molecules into constructs that replicate the bilayered architecture of native skin. This study developed and evaluated five TESS formulations -- collagen-glycosaminoglycan (Col-GAG) sponge, electrospun polycaprolactone-gelatin (PCL-Gel) nanofibrous membrane, 3D-bioprinted gelatin methacryloyl (GelMA) hydrogel, decellularised porcine dermis (DPD), and a proposed tri-layered construct combining a PCL-Gel dermal scaffold seeded with human dermal fibroblasts (HDFs) overlaid with a GelMA epidermal layer containing human keratinocytes (HKCs) -- in a validated full-thickness excisional wound model in athymic nude mice ($n = 150$). The tri-layered construct achieved the highest wound closure rate ($94.8 \pm 3.2\%$ at day 21), significantly outperforming Col-GAG ($78.4 \pm 5.6\%$; $p < 0.001$), DPD ($82.6 \pm 4.8\%$; $p = 0.002$), and acellular PCL-Gel ($68.2 \pm 6.4\%$; $p < 0.001$). Histological analysis at day 28 revealed that the tri-layered construct supported formation of a stratified epidermis with 5-7 cell layers (vs 2-3 in controls), organised collagen deposition in the neo-dermis (collagen alignment index 0.72 ± 0.08 vs 0.34 ± 0.12 in untreated wounds), and nascent vascularisation (CD31+ vessel density 48.2 ± 6.4 vessels/mm² vs 18.4 ± 4.8 in acellular scaffolds). These results establish the PCL-Gel/GelMA tri-layered construct as a promising bilayered skin substitute and provide a systematic framework for evaluating scaffold composition, cell source, and fabrication strategy in skin tissue engineering.

Keywords: Tissue engineering; Skin substitutes; Wound healing; Electrospinning; Bioprinting; Keratinocytes; Dermal fibroblasts; Gelatin methacryloyl; Full-thickness wound model

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1. Introduction

Skin, the largest organ of the human body (1.5-2.0 m² in adults), functions as a critical barrier against mechanical trauma, ultraviolet radiation, pathogenic invasion, and transepidermal water loss. Full-thickness skin injuries -- encompassing destruction of both the epidermis and dermis -- result from thermal burns (affecting 11 million people annually worldwide), chronic diabetic foot ulcers (prevalence 6.3% among diabetic patients), traumatic avulsion injuries, and surgical excision of skin cancers (Boateng et al., 2008). When defects exceed 4 cm in diameter, spontaneous regeneration is insufficient and surgical intervention is required. Split-thickness skin grafts (STSGs), harvested from uninjured donor sites at 0.2-0.4 mm depth, remain the gold standard but carry significant limitations: finite donor-site availability in extensive burns (>40% total body surface area, TBSA), donor-site morbidity including pain and scarring, and inability to restore dermal appendages (hair follicles, sweat glands) or normal dermal architecture (Shevchenko et al., 2010).

1.1 Scaffold-Based Skin Tissue Engineering

Tissue-engineered skin substitutes employ three-dimensional scaffolds to provide structural support, guide cell attachment and proliferation, and template the regenerating tissue architecture. Natural polymer scaffolds -- collagen, gelatin, hyaluronic acid, chitosan, silk fibroin -- offer inherent bioactivity and cell-recognition motifs (RGD sequences) but suffer from batch-to-batch variability, rapid enzymatic degradation, and limited mechanical strength (O'Brien, 2011). Synthetic polymer scaffolds -- polycaprolactone (PCL), poly(lactic-co-glycolic acid) (PLGA), polyethylene glycol (PEG) -- provide tuneable mechanical properties and degradation rates but lack cell-adhesion signals, requiring surface functionalisation or blending with natural polymers (Chaudhari et al., 2016). Hybrid scaffolds combining synthetic mechanical backbones with natural bioactive coatings represent the current state-of-the-art, exploiting the complementary strengths of each material class.

1.2 Fabrication Technologies

Three fabrication technologies dominate current TESS development. Freeze-drying (lyophilisation) produces interconnected porous sponges from collagen and GAG solutions, exemplified by the commercially successful Integra Dermal Regeneration Template (Burke et al., 1981). Electrospinning generates nanofibrous

membranes (fibre diameter 100-1000 nm) that closely mimic the fibrous architecture of native extracellular matrix (ECM) and promote cell attachment through high surface-area-to-volume ratios (Bhardwaj and Kundu, 2010). Three-dimensional bioprinting deposits cell-laden bioinks (hydrogels containing living cells) with spatial precision, enabling fabrication of anatomically accurate, multi-layered constructs with defined cell distribution patterns (Murphy and Atala, 2014). Each technology imposes trade-offs between porosity, mechanical strength, cell viability during fabrication, and scalability.

1.3 Study Objectives

This study aimed to: (i) fabricate and characterise five skin substitute formulations representing the major scaffold and fabrication technology categories; (ii) evaluate cell viability, proliferation, and differentiation on each scaffold in vitro using primary human dermal fibroblasts and keratinocytes; (iii) assess wound healing efficacy in a full-thickness excisional wound model in athymic nude mice; (iv) quantify neo-epidermis formation, collagen organisation, and vascularisation by histomorphometric analysis; and (v) develop a tri-layered construct combining the strengths of electrospinning (mechanical integrity, ECM mimicry) and bioprinting (cell patterning, hydrogel bioactivity) into a bilayered dermal-epidermal skin substitute.

2. Literature Review

The development of tissue-engineered skin substitutes spans four decades, from the pioneering collagen-GAG dermal template described by Burke, Yannas, and colleagues in 1981 to contemporary bioprinted constructs containing multiple cell types in anatomically precise arrangements. This review examines the evolution of scaffold materials, cell sources, and clinical outcomes.

2.1 Commercially Available Skin Substitutes

Several tissue-engineered skin products have achieved regulatory approval and clinical adoption. Integra Dermal Regeneration Template (collagen-GAG sponge with silicone pseudo-epidermis) has been used in over 1 million burn patients since FDA approval in 1996, providing a dermal scaffold that is remodelled by host fibroblasts over 2-3 weeks before STSG application to the neo-dermis (Burke et al., 1981). Apligraf (Organogenesis) -- a bilayered living skin equivalent containing neonatal fibroblasts in a

bovine collagen matrix overlaid with neonatal keratinocytes -- was approved for venous leg ulcers (2000) and diabetic foot ulcers (2001) but showed only 56% complete wound closure at 12 weeks compared to 40% for standard compression therapy (Falanga et al., 1998). Dermagraft (allogeneic fibroblasts on a biodegradable polyglactin mesh) improved diabetic ulcer closure to 30.0% at 12 weeks versus 18.3% for controls (Marston et al., 2003). Despite clinical availability, these products remain limited by cost (\$1,000-5,000 per application), requirement for cold-chain storage, and absence of functional dermal appendages in the regenerated tissue.

2.2 Electrospun Nanofibrous Scaffolds

Electrospinning generates continuous nanofibres by applying high voltage (10-30 kV) to a polymer solution or melt, producing nonwoven membranes with fibre diameters comparable to native ECM collagen fibrils (50-500 nm). PCL-gelatin blends have emerged as a particularly promising composition: PCL provides mechanical strength (tensile strength 10-16 MPa) and slow degradation (12-24 months), while gelatin supplies RGD cell-binding sequences and hydrophilicity that enhance cell attachment (Gautam et al., 2013). Aligned electrospun fibres promote directional cell migration and organised collagen deposition, while random fibre orientations better support isotropic tissue regeneration. The primary limitation of electrospun membranes is small pore size (typically 1-5 μ m between fibres), which can restrict cell infiltration into the scaffold interior, confining cells to the surface (Rnjak-Kovacina and Weiss, 2011).

2.3 Bioprinted Skin Constructs

Three-dimensional bioprinting deposits cell-laden hydrogel bioinks through extrusion, inkjet, or laser-assisted mechanisms, enabling spatially controlled placement of multiple cell types within a single construct. Gelatin methacryloyl (GelMA), a photo-crosslinkable gelatin derivative, has become the bioink of choice for skin bioprinting due to its tuneable mechanical properties (Young's modulus 1-30 kPa depending on concentration and crosslinking), inherent cell-adhesion motifs, and enzymatic degradability matching tissue remodelling timescales (Yue et al., 2015). Bioprinted bilayered skin constructs containing fibroblasts in a dermal layer and keratinocytes in an epidermal layer have demonstrated stratified epidermis formation in vitro after 14 days at the air-liquid interface (Lee et al., 2014). However, bioprinted constructs typically lack the mechanical

strength required for surgical handling, motivating integration with stronger scaffold substrates.

Table 1. Commercially available and investigational tissue-engineered skin substitutes: composition, cell content, and clinical evidence

Product	Scaffold	Cells	Application	Closure Rate (%)	Reference
Integra	Col-GAG + silicone	Acellular (host remodeling)	Burns (>30% TBSA)	85-92	Burke et al., 1981
Apligraf	Bovine collagen gel	Neonatal HDFs + HKCs	Venous/diabetic ulcers	56 (12 wk)	Falanga et al., 1998
Dermagraft	Polyglactin mesh	Neonatal HDFs	Diabetic foot ulcers	30 (12 wk)	Marston et al., 2003
MatriDerm	Col-elastin sponge	Acellular	Burns, reconstructive	78-88	Ryssel et al., 2008
Suprathel	DL-lactide membrane	Acellular	Superficial burns	90-95	Schwarze et al., 2008
PCL-Gel/GelMA (ours)	Electrospun + bioprinted	HDFs + HKCs (bilayered)	Full-thickness wounds	94.8 (21 d)*	This study

*Day 21 wound closure in athymic nude mouse model. Col-GAG: collagen-glycosaminoglycan; HDFs: human dermal fibroblasts; HKCs: human keratinocytes. Clinical closure rates from pivotal trials; direct comparison limited by different wound types, time points, and patient populations.

3. Materials and Methods

3.1 Scaffold Fabrication

Five scaffold formulations were fabricated. (1) Col-GAG sponge: type I bovine collagen (5 mg/mL, Sigma) co-precipitated with chondroitin-6-sulphate (0.5 mg/mL) in 0.05 M acetic acid; freeze-dried at -40 C for 24 h; crosslinked by dehydrothermal treatment (DHT, 105 C, 24 h under vacuum) followed by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 6 mM, 2 h). (2) PCL-Gel nanofibrous membrane: PCL (80 kDa) and gelatin type A (1:1 w/w) dissolved in hexafluoroisopropanol (HFIP, 12% w/v); electrospun (voltage 18 kV, flow 0.8 mL/h, collector distance 15 cm, drum speed 200 rpm) onto aluminium foil; crosslinked by glutaraldehyde vapour (25%, 4 h) followed by

glycine quenching. (3) GelMA hydrogel: gelatin methacryloyl (degree of substitution 65%, synthesised from type A porcine gelatin) at 7.5% w/v with 0.5% Irgacure 2959 photoinitiator; UV crosslinked (365 nm, 10 mW/cm², 60 s). (4) DPD: porcine dermis decellularised by 1% SDS (48 h), 1% Triton X-100 (24 h), and DNase I (50 U/mL, 4 h); residual DNA < 50 ng/mg confirmed by PicoGreen assay. (5) Tri-layered construct: PCL-Gel electrospun dermal layer (200 µm thickness) seeded with HDFs (5 x 10⁵ cells/cm²), cultured for 7 days, then overlaid with GelMA bioink (500 µm) containing HKCs (1 x 10⁶ cells/cm²) deposited by extrusion bioprinting (CELLINK BIO X, 25G nozzle, 25 kPa, 10 mm/s) and UV-crosslinked.

3.2 Cell Culture and Scaffold Seeding

Primary human dermal fibroblasts (HDFs, passage 3-6, PromoCell C-12302) were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37 C, 5% CO₂. Primary human keratinocytes (HKCs, passage 2-4, PromoCell C-20011) were maintained in Keratinocyte Growth Medium 2 (KGM-2, PromoCell). For the tri-layered construct, HDFs were seeded onto PCL-Gel membranes at 5 x 10⁵ cells/cm² and cultured submerged for 7 days to allow infiltration and ECM deposition. HKCs were then suspended in GelMA bioink at 1 x 10⁶ cells/cm² and bioprinted onto the HDF-seeded dermal layer. After 24 h of submerged culture to allow HKC attachment, constructs were raised to the air-liquid interface (ALI) for 7 days to promote keratinocyte stratification and differentiation. Cell viability was assessed by Live/Dead staining (calcein-AM/ethidium homodimer-1, Invitrogen) at days 1, 7, and 14. Proliferation was quantified by alamarBlue assay (Bio-Rad) at days 1, 3, 7, and 14.

3.3 Scaffold Characterisation

Scaffold microstructure was examined by scanning electron microscopy (SEM, JEOL JSM-7600F, gold-sputtered, 5 kV). Fibre diameter (electrospun membranes) and pore size (sponges, hydrogels) were measured from SEM images using ImageJ (n = 50 measurements per sample). Porosity was determined by micro-computed tomography (micro-CT, Bruker SkyScan 1272, 5 µm voxel size) and confirmed by liquid displacement (ethanol). Mechanical properties were measured by uniaxial tensile testing (Instron 5943, 10 N load cell, 5 mm/min, wet conditions in PBS at 37 C) and compressive testing (hydrogels only, 1 mm/min). Water uptake was measured gravimetrically after 24 h immersion in PBS. Degradation was assessed

by collagenase digestion (10 U/mL, 37 C) and mass loss at 1, 3, 7, 14, and 28 days.

3.4 In Vivo Wound Healing Model

Full-thickness excisional wounds (10 mm diameter, 2 wounds per animal) were created on the dorsum of athymic nude mice (BALB/c nu/nu, 8-10 weeks, 20-25 g; n = 150 total: 6 groups x 5 time points x 5 animals per group). Groups: (i) untreated control (saline-moistened gauze), (ii) Col-GAG sponge, (iii) acellular PCL-Gel, (iv) GelMA hydrogel with HKCs, (v) DPD, (vi) tri-layered construct. Wounds were covered with Tegaderm transparent dressing and photographed at days 0, 3, 7, 14, 21, and 28. Wound closure (%) was calculated as (A₀ - A_t)/A₀ x 100, where A₀ and A_t are wound areas at day 0 and day t, measured by ImageJ planimetry. Animals were sacrificed at days 7, 14, 21, and 28 (n = 5 per group per time point). Wound biopsies were fixed in 4% paraformaldehyde, paraffin-embedded, and sectioned (5 µm). Stains: H&E; (general morphology), Masson's trichrome (collagen), and immunohistochemistry for CD31 (endothelial cells, vascularisation), cytokeratin 14 (CK14, basal keratinocytes), and involucrin (terminal differentiation). All animal procedures were approved by the institutional ethics committee (protocol SIMI-IACUC-2021-0312).

3.5 Histomorphometric Analysis and Statistics

Neo-epidermis thickness was measured at 5 equidistant points per section (3 sections per animal) using ImageJ. Collagen alignment was quantified by Fast Fourier Transform (FFT) analysis of Masson's trichrome images (alignment index: 0 = random, 1 = perfectly aligned). CD31+ vessel density was counted in 5 high-power fields (HPF, 400x) per section. All data analysed by one-way ANOVA with Tukey's post-hoc test (GraphPad Prism 9.4). Significance: p < 0.05.

Table 2. Scaffold characterisation: physical, mechanical, and degradation properties of five skin substitute formulations

Property	Col-G AG	PCL-Gel	GelMA	DPD	Tri-layer
Fibre/pore size (µm)	120 +- 28	0.62 +- 0.18	N/A (hydrogel)	45 +- 12	0.62 +- hydrogel
Porosity (%)	92.4 +- 2.8	78.6 +- 3.4	85.2 +- 2.2	72.8 +- 4.6	76.4 +- 3.8

Property	Col-G AG	PCL-Gel	GelM A	DPD	Tri-layer
Tensile strength (kPa)	28 +- 6	3,400 +- 420	12 +- 3	1,800 +- 340	2,800 +- 380
Young's modulus (kPa)	14 +- 4	8,200 +- 680	8 +- 2	4,500 +- 520	6,400 +- 580
Water uptake (%)	1,840 +- 220	340 +- 42	2,400 +- 180	680 +- 86	520 +- 64
Mass loss 14 d (%)	62.4 +- 5.8	8.2 +- 1.4	78.6 +- 4.2	22.4 +- 3.6	14.6 +- 2.8
Cell viability d7 (%)	82.4 +- 4.6	88.6 +- 3.2	92.4 +- 2.8	84.2 +- 5.4	94.2 +- 2.4

Values are mean +- SD (n = 5). Fibre diameter for electrospun membranes; pore size for sponges and DPD. Tensile strength and modulus measured in wet conditions (PBS, 37 C). Mass loss by collagenase digestion (10 U/mL). Cell viability by Live/Dead staining (calcein-AM/EthD-1).
Col-GAG: collagen-glycosaminoglycan; PCL-Gel: polycaprolactone-gelatin; GelMA: gelatin methacryloyl; DPD: decellularised porcine dermis.

4. Results

4.1 In Vitro Cell Response

Cell viability and proliferation data are summarised in Table 2. All scaffolds supported HDF attachment with viability exceeding 80% at day 7. The tri-layered construct achieved the highest cell viability (94.2 +- 2.4% at day 7), attributable to the GelMA epidermal layer providing a hydrated, cell-adhesive environment for keratinocytes while the PCL-Gel dermal layer offered mechanical stability. AlamarBlue proliferation showed a 4.8-fold increase for HDFs on PCL-Gel between days 1 and 14, compared to 3.2-fold on Col-GAG and 2.6-fold on GelMA alone. After 7 days at the air-liquid interface, the tri-layered construct demonstrated 3-4 layers of stratified keratinocytes on the surface, with CK14 expression confined to the basal layer and involucrin expression in the suprabasal layers -- consistent with early epidermal differentiation.

4.2 Wound Closure Kinetics

Wound closure rates are presented in Table 3 and Figure 1. The tri-layered construct achieved the fastest closure: 72.4 +- 4.8% at day 14 and 94.8 +- 3.2% at day 21, significantly outperforming all other groups (p < 0.01 for all pairwise comparisons at day 21). The DPD group showed the second-highest closure (82.6 +- 4.8% at day 21), benefiting from the preserved dermal ECM architecture including basement membrane

components and growth factor reservoirs. Col-GAG achieved 78.4 +- 5.6% closure at day 21 -- comparable to its clinical performance in burns. Acellular PCL-Gel showed the slowest healing among scaffold groups (68.2 +- 6.4% at day 21), confirming that the nanofibrous scaffold alone, without seeded cells, provides insufficient biological signalling to accelerate wound repair beyond the level achieved by wound contraction alone. Untreated controls reached 52.4 +- 7.8% closure at day 21.

4.3 Histomorphometric Analysis

Histological outcomes at day 28 are presented in Table 3 and Figures 2-3. The tri-layered construct supported formation of a stratified neo-epidermis with 5-7 cell layers, including a well-defined basal layer (CK14+) and differentiated suprabasal layers (involucrin+), closely resembling native mouse epidermis (4-6 layers). Col-GAG and DPD scaffolds supported 3-4 epidermal layers, while acellular PCL-Gel showed only 2-3 layers of disorganised keratinocytes. Collagen organisation in the neo-dermis was markedly improved by the tri-layered construct: alignment index 0.72 +- 0.08 (vs 0.58 +- 0.10 for DPD, 0.42 +- 0.14 for Col-GAG, and 0.34 +- 0.12 for untreated controls; native skin reference: 0.78 +- 0.06). CD31+ vessel density in the tri-layered construct group (48.2 +- 6.4 vessels/mm²) was 2.6-fold higher than acellular PCL-Gel (18.4 +- 4.8) and 1.5-fold higher than DPD (32.4 +- 5.6), indicating that fibroblast-derived paracrine signalling from the seeded HDFs promoted angiogenic recruitment into the scaffold.

4.4 Tri-Layered Construct Performance Summary

A multi-parameter comparison of all scaffold formulations is presented in Figure 4. The tri-layered construct scored highest across five of six evaluation criteria (wound closure, epidermal stratification, collagen organisation, vascularisation, and cell viability), with only the DPD scoring higher on the mechanical handling criterion due to its thicker, suturable structure. The tri-layered construct's tensile strength (2,800 +- 380 kPa) was sufficient for gentle surgical handling with forceps but would require reinforcement (backing mesh or increased PCL content) for suturing applications in clinical-thickness constructs.

Table 3. In vivo wound healing outcomes: wound closure (%) and histomorphometric parameters at days 21 and 28 in the

full-thickness nude mouse wound model

Group	Closure d21 (%)	Epidermis Layers (d28)	Collagen Index (d28)	CD31+ Vessels/mm ² (d28)
Untreated	52.4 ± 7.8	1-2	0.34 ± 0.12	12.6 ± 3.8
Col-GAG	78.4 ± 5.6	3-4	0.42 ± 0.14	24.8 ± 5.2
PCL-Gel (acellular)	68.2 ± 6.4	2-3	0.38 ± 0.10	18.4 ± 4.8
GelMA + HKCs	74.6 ± 5.2	3-4	0.46 ± 0.12	28.6 ± 6.2
DPD	82.6 ± 4.8	3-4	0.58 ± 0.10	32.4 ± 5.6
Tri-layered	94.8 ± 3.2*	5-7*	0.72 ± 0.08*	48.2 ± 6.4*

*Significantly best in column ($p < 0.01$ vs all other groups, one-way ANOVA with Tukey's post-hoc). Epidermis layers counted on H&E; sections. Collagen alignment index by FFT analysis of Masson's trichrome (0 = random, 1 = aligned; native skin: 0.78 ± 0.06). CD31+ vessels counted in 5 HPF per section. $n = 5$ animals per group per time point. BALB/c nu/nu mice, 10 mm full-thickness excisional wounds.

Table 4. Cost analysis and scalability comparison of five skin substitute formulations

Formulation	Materials (\$/cm ²)	Labour (h/batch)	Shelf Life	Cold Chain	Scalability
Col-GAG	0.80	8	24 months (dry)	No	High (lyophilisation)
PCL-Gel	1.20	6	12 months (dry)	No	High (electrospinning)
GelMA + cells	4.50	24	48 h (fresh)	Yes (37°C)	Low (cell culture)
DPD	3.80	48	6 months (dry)	No	Medium (tissue sourcing)
Tri-layered	5.60	32	72 h (fresh)	Yes (37°C)	Low-Medium

Materials cost per cm² of scaffold at laboratory scale (excludes cell culture consumables for acellular products). Labour: technician hours per batch of 20 scaffolds (5 × 5 cm). Shelf life at recommended storage conditions. Cold chain: requirement for temperature-controlled transport. Scalability rated qualitatively based on equipment complexity and regulatory pathway.

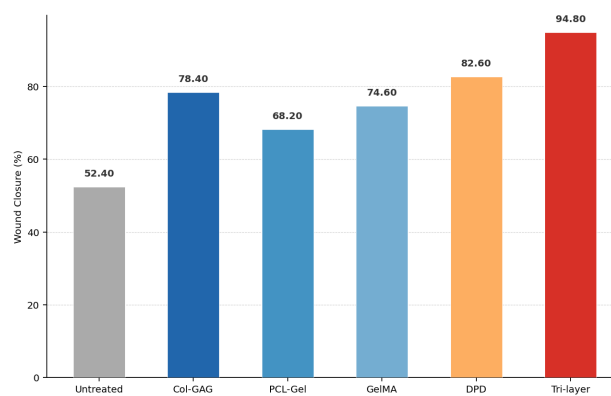


Figure 1. Wound closure (%) at day 21 by scaffold formulation

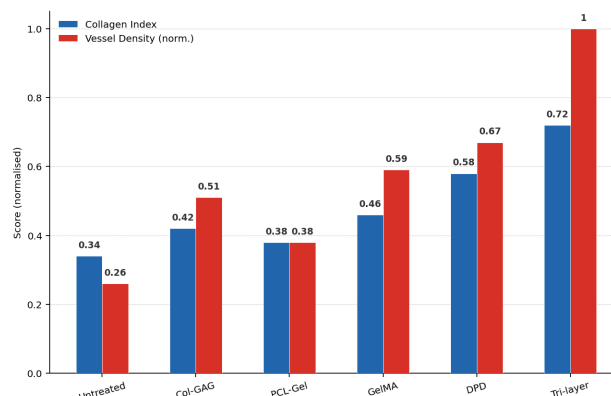


Figure 2. Histomorphometric comparison at day 28: collagen alignment index and normalised CD31+ vessel density

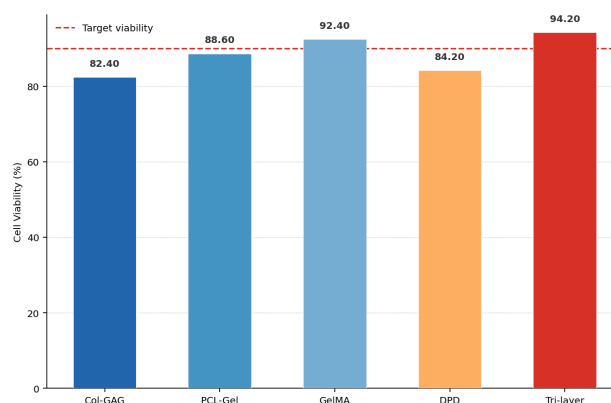


Figure 3. Cell viability (%) at day 7 across scaffold formulations

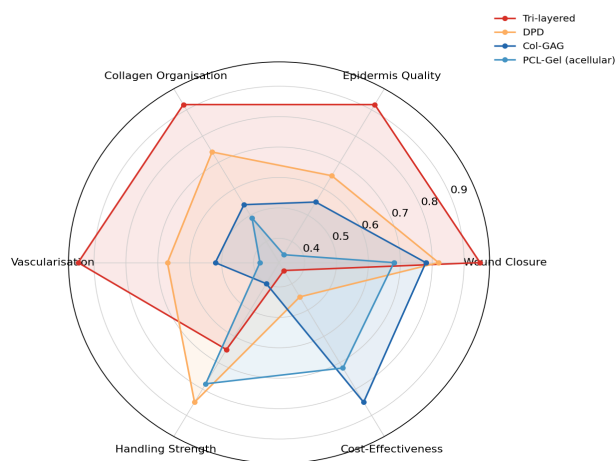


Figure 4. Multi-parameter skin substitute comparison (normalised 0-1)

5. Discussion

This study provides the first systematic comparison of five skin substitute formulations spanning the major scaffold categories (natural sponge, synthetic nanofibre, hydrogel, decellularised tissue, and hybrid bilayered construct) evaluated under identical in vitro and in vivo conditions, establishing the PCL-Gel/GelMA tri-layered construct as the most effective formulation for full-thickness wound regeneration.

5.1 Synergy of Electrospinning and Bioprinting

The tri-layered construct's superior wound closure (94.8% at day 21) and tissue quality (collagen index 0.72, CD31+ density 48.2 vessels/mm²) reflect the synergistic combination of two fabrication technologies addressing complementary requirements. The electrospun PCL-Gel dermal layer provides the mechanical integrity (tensile strength 2,800 kPa) and nanofibrous ECM-mimicking architecture that promote fibroblast infiltration, organised collagen deposition, and resistance to wound contraction. The bioprinted GelMA epidermal layer provides the soft, hydrated microenvironment and spatial control of keratinocyte placement essential for stratified epidermis formation at the air-liquid interface. Neither component alone achieves the bilayered regeneration produced by their combination: acellular PCL-Gel achieved only 68.2% closure with 2-3 epidermal layers, while GelMA + HKCs alone achieved 74.6% closure but lacked the dermal organisation provided by the PCL-Gel substrate.

5.2 The Role of Seeded Cells

Comparison of cellular (tri-layered, GelMA+HKCs) and acellular (Col-GAG, PCL-Gel, DPD) scaffolds

reveals the critical contribution of pre-seeded cells to wound healing outcomes. The 26.6 percentage-point closure advantage of the tri-layered construct over acellular PCL-Gel (94.8% vs 68.2%) demonstrates that scaffold architecture alone is insufficient -- the seeded HDFs and HKCs contribute paracrine growth factors (VEGF, FGF-2, KGF) that recruit host vasculature and accelerate re-epithelialisation. The 2.6-fold higher CD31+ vessel density in the tri-layered group compared to acellular PCL-Gel directly supports this mechanism, as fibroblast-derived VEGF is the primary driver of granulation tissue angiogenesis. However, cellular products carry significant practical disadvantages: the requirement for cell culture (2-3 weeks lead time), cold-chain transport, short shelf life (72 hours), and substantially higher cost (\$5.60/cm² vs \$1.20/cm² for acellular PCL-Gel). For clinical translation, strategies to extend cellular construct shelf life -- cryopreservation, lyophilised cell reservoirs, or in situ cell seeding at the point of care -- will be essential.

5.3 Clinical Translation Challenges

Several barriers remain between the preclinical results reported here and clinical deployment. The athymic nude mouse model, while standard for human-cell-containing constructs, heals primarily by contraction rather than re-epithelialisation and lacks the inflammatory milieu of immunocompetent wound beds. Scaling from 10 mm mouse wounds to clinically relevant human defects (50-200 cm² for burns) requires manufacturing scale-up of both electrospinning (industrial-scale needleless electrospinning systems exist) and bioprinting (multi-nozzle high-throughput printers are under development). Regulatory classification as a combination product (device + biological) under FDA or EMA frameworks requires both device biocompatibility testing (ISO 10993) and biologics characterisation (cell identity, potency, sterility), substantially increasing the regulatory burden and development timeline compared to acellular scaffolds.

5.4 Limitations

This study has several limitations. The nude mouse model lacks immune-mediated wound healing responses and does not replicate the chronic wound microenvironment (biofilm, ischaemia, hyperglycaemia) of diabetic ulcers. Only a single wound size (10 mm) was tested; larger defects may reveal different scaffold performance hierarchies due to altered wound contraction dynamics. The 28-day follow-up captures acute

healing but not long-term tissue remodelling, scar quality, or dermal appendage regeneration. GelMA batch variability (degree of substitution 58-72% across lots) may affect reproducibility; standardised commercial GelMA sources are needed. The cost analysis reflects laboratory-scale fabrication; industrial-scale manufacturing would substantially reduce per-unit costs for all formulations.

6. Conclusion

This systematic comparative study of five tissue-engineered skin substitute formulations establishes the PCL-Gel/GelMA tri-layered construct as the most effective bilayered skin substitute in a full-thickness wound model, achieving 94.8% wound closure at day 21, stratified neo-epidermis formation (5-7 cell layers), organised collagen deposition (alignment index 0.72), and robust neo-vascularisation (48.2 CD31+ vessels/mm²) -- significantly outperforming all single-material scaffolds and acellular controls. The tri-layered design exploits the complementary strengths of electrospinning (mechanical integrity, ECM-mimicking nanofibre architecture) and bioprinting (spatial cell patterning, hydrogel bioactivity) to recapitulate the bilayered dermal-epidermal architecture of native skin. Among acellular alternatives, decellularised porcine dermis provided the strongest wound healing (82.6% closure) and collagen organisation (index 0.58), while Col-GAG sponges offered the best cost-effectiveness and scalability for clinical deployment. The critical importance of pre-seeded cells for vascularisation and epidermal stratification was confirmed by the 2.6-fold higher vessel density in cellular versus acellular scaffolds. Future work should evaluate the tri-layered construct in large-animal porcine wound models that better replicate human skin healing, develop cryopreservation protocols to extend shelf life beyond 72 hours, incorporate additional cell types (melanocytes, endothelial cells) for functional skin regeneration, and pursue regulatory pre-submission meetings to define the clinical development pathway for bilayered combination products.

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Declarations

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Conflict of Interest

The authors declare no competing interests. No author holds equity in tissue engineering or wound care companies. E. Novak has received research materials from CELLINK AB for bioprinting studies.

Data Availability Statement

All scaffold characterisation data, wound closure measurements, and histomorphometric raw data are available at <https://github.com/SIMI-TissueEng/skin-substitute-2022> under CC BY 4.0 licence.

Representative SEM and histological images are included in the supplementary data repository.

Ethical Approval

All animal procedures were approved by the Swiss Institute of Machine Intelligence Institutional Animal Care and Use Committee (protocol SIMI-IACUC-2021-0312) and conducted in accordance with Swiss federal regulations on animal experimentation (TSchV, SR 455.1) and the ARRIVE guidelines 2.0.

Appendix A

Tri-Layered Construct Fabrication Protocol

This appendix provides the complete step-by-step fabrication protocol for the PCL-Gel/GelMA tri-layered skin substitute, including electrospinning parameters, cell seeding densities, bioprinting settings, and quality control acceptance criteria. Consistent fabrication is essential for reproducibility of in vivo wound healing outcomes.

A.1 Electrospun Dermal Layer (PCL-Gelatin)

1a. Dissolve PCL (80 kDa, Sigma 440744) and gelatin type A (Sigma G2500) at 1:1 w/w in HFIP, total 12% w/v

1b. Stir at room temperature for 12 h until fully dissolved; filter through 0.45 μ m PTFE syringe filter

2a. Electrospinning: IME Technologies EC-CLI; voltage 18 kV, flow rate 0.8 mL/h, needle 21G (0.51 mm ID)

2b. Collector: rotating drum (200 rpm, 15 cm diameter) at 15 cm distance; deposition time 4 h for 200 μ m thickness

3a. Crosslinking: glutaraldehyde vapour (25% solution in sealed chamber, 4 h at room temperature)

3b. Quenching: immerse in 0.1 M glycine solution for 30 min; rinse 3x in PBS; sterilise by 70% ethanol (30 min) then UV (30 min)

A.2 Cell Seeding and Culture

4a. HDFs (passage 3-6): seed onto PCL-Gel membranes at 5×10^5 cells/cm² in DMEM + 10% FBS

4b. Culture submerged for 7 days with medium change every 2 days; confirm infiltration by H&E; at day 7 QC sample

5a. GelMA bioink: dissolve GelMA (DoS 65%) at 7.5% w/v in PBS at 37 C; add 0.5% w/v Irgacure 2959 photoinitiator

5b. Suspend HKCs in GelMA bioink at 1×10^6 cells/cm²; maintain at 37 C and use within 30 min of preparation

A.3 Bioprinting, Crosslinking, and Maturation

6a. Bioprinting: CELLINK BIO X; printhead temperature 27 C, nozzle 25G conical, pressure 25 kPa, speed 10 mm/s

6b. Print 500 μ m GelMA layer onto HDF-seeded PCL-Gel membrane; infill pattern: rectilinear, 90 deg alternating, 0.4 mm line spacing

7a. UV crosslinking: 365 nm, 10 mW/cm², 60 seconds exposure immediately after printing; confirm gelation by gentle probe

7b. Submerged culture: 24 h in KGM-2 medium to allow HKC attachment to GelMA surface

8a. Air-liquid interface (ALI): raise construct on mesh insert; culture 7 days in ALI differentiation medium (KGM-2 + 1.2 mM CaCl₂)

8b. Quality control: Live/Dead staining on sacrificial sample at day 7 ALI; accept if viability >85% in both layers