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**Generation of a PLLA Scaffold Functionalised with
Mesenchymal Cells and Microvascular Fragments for
Chronic Wound Regeneration**

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ABSTRACT

Chronic wounds, commonly known as ulcers, are non-healing injuries associated with impaired cellular activity, poor vascularization, persistent inflammation, and altered tissue homeostasis. Their persistent nature poses severe challenges for patients, who often experience pain, reduced mobility, and, in extreme cases, lower limb amputation, while also placing substantial burdens on their families and the healthcare system. Since these wounds often fail to respond to conventional medical treatments, there is an urgent need for innovative therapeutic strategies. Tissue-engineered approaches have emerged as a promising solution, as they can produce skin substitutes to provide structural support, stimulate the stroma, and promote rapid angiogenesis, key factors for effective wound healing. In this study, we developed and characterized a multifunctional three-dimensional construct based on porous poly-L-lactic acid (PLLA) scaffolds functionalized with dermal fibroblasts and microvascular fragments (MVs) for chronic wound regeneration.

PLLA scaffolds fabricated by thermally induced phase separation were optimized in terms of pore size, surface biofunctionalization, seeding strategy, and biochemical supplementation. Primary rat and human dermal fibroblasts were isolated, characterized, and seeded onto the scaffolds to evaluate adhesion, proliferation, phenotypic activation, and ECM deposition. MVs were isolated from rat adipose tissue and assessed for angiogenic potential in both two- and three-dimensional environments. The integration of MVs into PLLA scaffolds was investigated under different conditions, and a co-culture system combining rat fibroblasts and MVs was established.

Optimized PLLA scaffolds supported robust fibroblast colonization and multilayered growth. Microvascular fragments retained structural integrity and formed stable, lumenized vascular networks. Co-culture conditions promoted synergistic interactions between fibroblasts and MVs, resulting in dense intraporous vascularization. Human dermal fibroblasts from donors of different ages effectively colonized the scaffolds and remodeled the extracellular matrix, confirming the translational relevance of the approach.

Overall, this study demonstrates that the coordinated integration of structural, biochemical, and cellular cues enables the generation of a functional, pre-vascularized, ECM-rich tissue construct, offering a promising strategy for chronic wound regeneration.

INTRODUCTION

Acute wounds and wound healing

The skin is a highly adaptive and multifunctional organ composed of the epidermis, dermis, and hypodermis (subcutis) (Figure 1). It provides essential protection against ultraviolet radiation, pathogenic invasion, and transepidermal water loss, while also shielding the underlying organs [1, 2]. As a direct consequence of its protective functions, the skin is continuously exposed to a wide range of environmental stressors that may compromise its integrity and lead to wound formation [3].

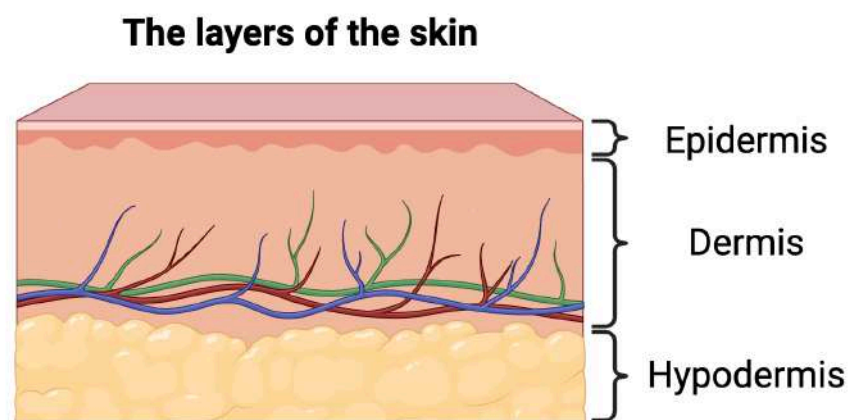


Figure 1. Schematic representation of the skin (BioRender.com).

In response to frequent exposure to injury, the human body has evolved an efficient, tightly regulated process to restore skin integrity following tissue damage, known as wound healing. Cutaneous wound healing is a highly regulated physiological process that restores tissue integrity and barrier function through the coordinated action of multiple cellular and molecular mechanisms. When this process proceeds in an orderly and timely manner, it follows four distinct yet partially overlapping phases: hemostasis, inflammation, proliferation, and remodeling, typically resulting in complete repair within 4 to 12 weeks (Figure 2) [2, 4].

During hemostasis, bleeding is rapidly controlled by vasoconstriction, platelet aggregation, and coagulation. Specifically, platelets form a plug at the injury site and release growth factors that initiate subsequent reparative processes, while fibrinogen is converted into a

fibrin mesh that stabilizes the clot and provides a provisional matrix for cell migration [5]. The following inflammatory phase is characterized by the recruitment of neutrophils and monocytes, which remove pathogens and cellular debris and release cytokines that coordinate the early immune response. Macrophages then support the transition toward repair by clearing apoptotic neutrophils and secreting growth factors essential for tissue regeneration. Dendritic cells and lymphocytes further modulate local immune activity [6].

As inflammation resolves, the wound enters the proliferative phase, characterized by the proliferation of keratinocytes, fibroblasts, and endothelial cells. Re-epithelialization begins within hours after injury. Keratinocytes at the wound edge and from adnexal structures are activated, flatten, and migrate across the provisional matrix to restore epidermal continuity. As these cells proliferate and migrate, they progressively cover the wound bed until contact inhibition halts their movement. Subsequently, they reestablish cell–cell junctions and stratify to rebuild a functional epidermis [7].

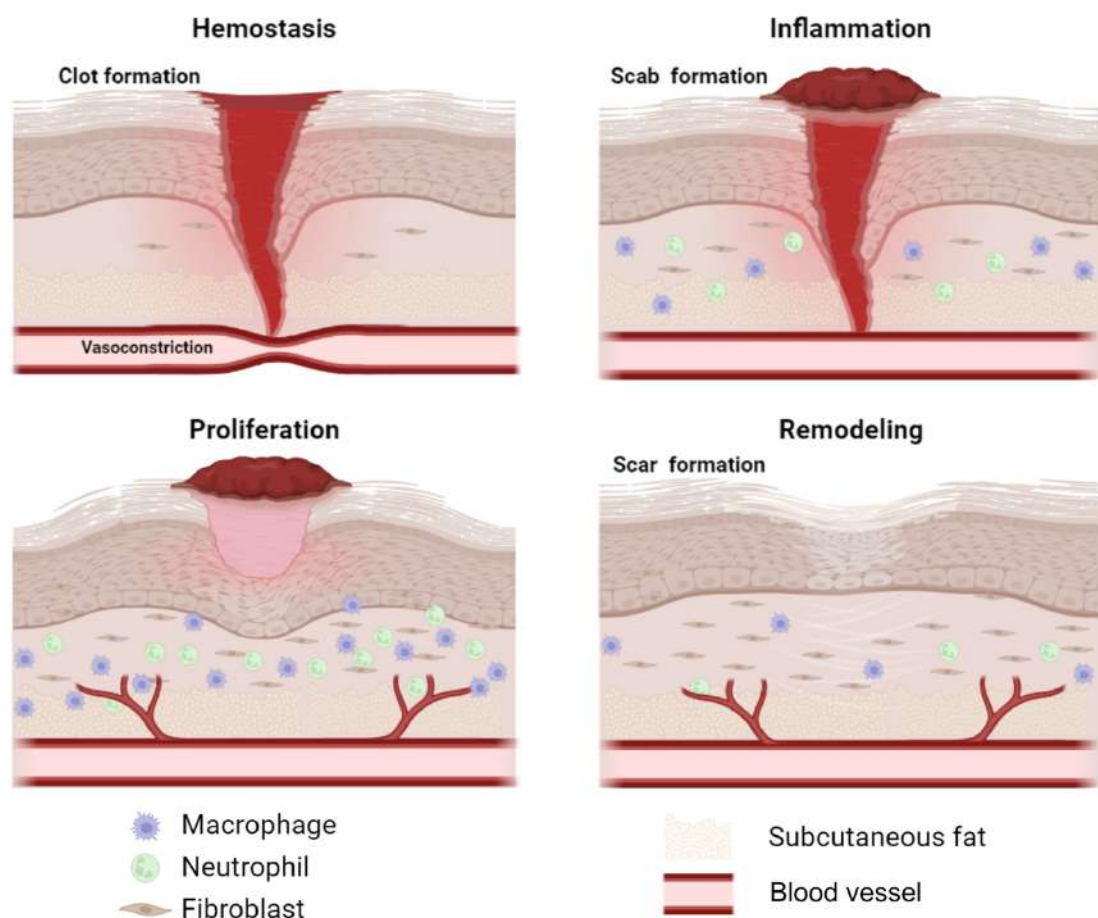


Figure 2. Wound healing phases [49].

Following injury, fibroblasts become activated and acquire a migratory mesenchymal phenotype [8]. Activated fibroblasts migrate into the wound bed, where they proliferate and exert key paracrine functions by releasing pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), thereby promoting angiogenesis and regulating inflammation [8, 9]. Within the wound environment, fibroblasts deposit extracellular matrix components, including fibronectin, hyaluronic acid, and early type III collagen, progressively replacing the provisional fibrin-rich scaffold [8]. In parallel to extracellular matrix (ECM) degradation and synthesis, they coordinate tissue remodeling and wound closure [8, 9]. The resulting ECM supports keratinocyte migration and angiogenesis. Distinct fibroblast subpopulations contribute differently to repair, with some differentiating into α -SMA-expressing myofibroblasts that drive wound contraction and closure, and are essential for regular tissue repair [10, 11].

Concurrently, angiogenesis is driven by endothelial cells (ECs) responding to hypoxia and to pro-angiogenic factors, such as VEGF, FGF, and Platelet-Derived Growth Factor (PDGF), produced by fibroblasts, macrophages, keratinocytes, and platelets. Activated ECs degrade the basement membrane, migrate into the wound, and form capillary sprouts; fusion of tip cells generates lumenized vessels that restore tissue perfusion and support oxygen and nutrient delivery. As tissue oxygenation improves, VEGF levels decline, pericytes are recruited, and vessels mature, strengthened by ECM deposition and hemodynamic forces [8, 12].

Together, fibroblast proliferation, extracellular matrix deposition, and neovascularization lead to the formation of granulation tissue, a highly cellular and vascularized provisional tissue that fills the wound bed and provides structural and biochemical support for re-epithelialization.

Lastly, the remodeling phase can last months to years, during which granulation tissue is gradually replaced by mature scar tissue. Collagen type III is progressively substituted by collagen type I, while myofibroblasts regulate wound contraction and ECM remodeling. Newly formed blood vessels stabilize, and macrophages remove debris, resulting in organized and functional scar tissue [13].

Wounds that successfully undergo this canonical healing trajectory are classified as acute wounds [2, 4]. Acute skin wounds arise from traumatic events that disrupt the continuity of the skin and, in some cases, extend to deeper tissues. They may be caused by physical agents, such as sharp objects, or by environmental factors including chemicals, extreme

temperatures, radiation, or prolonged mechanical pressure [14, 15]. The rate of healing depends on both wound-specific factors, such as size and depth, and patient-related factors, including age and overall health. Actively healing wounds are generally free of infection and are characterized by proliferating fibroblasts, ECM deposition, and the coordinated activity of inflammatory mediators [16].

However, some internal factors, such as pre-existing diseases, and external factors, including bacterial infections, can interfere with the normal progression of wound healing. When these disturbances persist, the healing process may become delayed or fail to progress properly, resulting in wound chronicisation and ultimately in the development of chronic wounds, commonly referred to as ulcers [17, 18].

Chronic wounds

Chronic wounds are a significant global health concern, affecting an estimated 1–2% of the worldwide population at least once in their lifetime [19]. Beyond their physical impact, ulcers profoundly reduce quality of life and impose substantial social and economic burdens on patients and their families [19, 20]. Multiple factors can impair wound healing and are generally classified as local or systemic (Figure 3). Impaired wound healing results from the combined action of local and systemic factors. Local conditions, such as insufficient oxygenation and infection, directly compromise the wound environment, while systemic

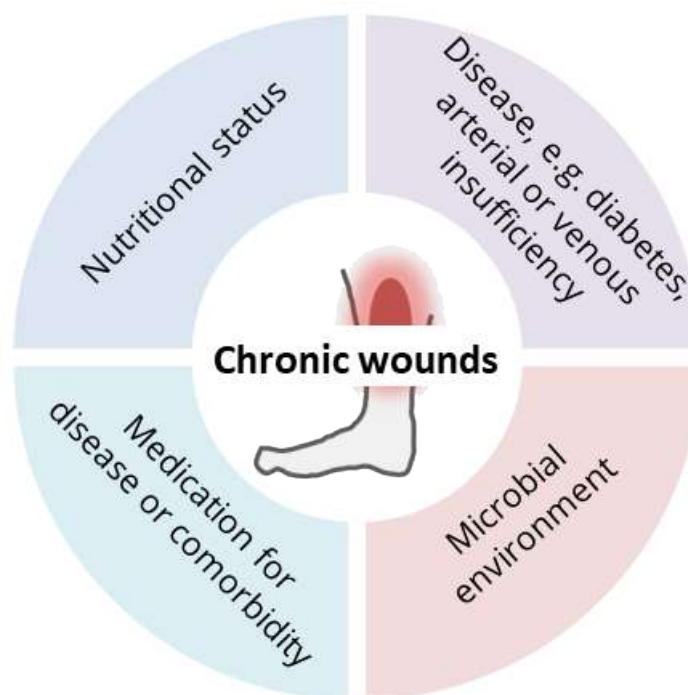


Figure 3. Chronic wound causes.

factors reflect the patient's overall health status and include comorbidities such as diabetes, as well as biological characteristics including age, sex, obesity, and stress. In addition, lifestyle habits such as smoking, poor nutrition, and alcohol consumption can negatively influence healing by altering inflammatory responses, tissue perfusion, and cellular function [21, 22].

The prevalence of chronic wounds is increasing, driven by aging populations and rising risk factors. Age represents a major risk factor, as chronic wounds predominantly affect individuals aged 70–80 years, and their prevalence is projected to increase in parallel with the global aging population [23–25]. Another critical factor is comorbidity, where patients already suffer from one or more chronic diseases, which predispose them to different types of ulcers [24]. Depending on the underlying disease, various ulcer classes can be defined (Figure 3). For example, chronic venous insufficiency, a condition in which the veins in the lower limbs fail to return blood to the heart efficiently, significantly increases the risk of venous ulcer (VU) development. VU typically appear on the lower legs, particularly around the ankles, and are often associated with edema, skin discoloration, and chronic inflammation. In addition, prolonged immobility, common in bedridden or severely debilitated patients, can lead to pressure ulcers (PU), also known as decubitus wounds. These lesions arise in areas subjected to sustained pressure, such as the sacrum, heels, and elbows,

Arterial ulcers	Venous ulcers	Pressure ulcers	Diabetic foot ulcers
Causes: Peripheral artery disease, atherosclerosis/occlusion, critical limb ischemia, dyslipidaemia	Causes: Endothelial dysfunction, ischemia, increase in intraluminal pressure, venous insufficiency	Causes: Deep tissue oedema, traumatic injury, pressure, ischemia-reperfusion injury	Causes: Impaired angiogenesis, neuropathy, endothelial dysfunction, micro-vascular disease, dysregulated cytokines levels
Dysregulation: Predominance of M1 macrophages, inflammation, oxidative stress, necrosis, nutrient deficiency	Dysregulation: Inflammation, leakage of fibrin, elevated MMPs levels, oxidative stress, iron overload, tissue metabolites accumulation	Dysregulation: Inflammation, oxidative stress	Dysregulation: Inflammation, elevated MMPs levels, hyperglycaemia, hyperlipidaemia, advanced glycation end (AGE) products accumulation
Infection risk: High	Infection risk: High	Infection risk: High	Infection risk: High
Standard of care: Restoration of blood flow	Standard of care: Restoration of venous hydrostatic pressure	Standard of care: Relieve of pressure	Standard of care: Multifactorial counteract of pathogenesis for microenvironment restoration
			

Figure 4. Types of ulcers [29].

where reduced blood flow causes tissue ischemia and necrosis [24, 26, 27]. Moreover, Arterial ulcers arise from insufficient blood perfusion and oxygen delivery to the dermal tissue, leading to progressive skin breakdown, particularly in the foot and lower leg [28].

The most severe and clinically challenging cases occur in patients with diabetes and/or obesity, where diabetic foot ulcers (DFU) are prevalent. DFUs typically develop on the plantar surface of the feet, often in areas subjected to repetitive pressure or minor trauma, and are compounded by peripheral neuropathy and impaired vascularization, which reduce pain sensation and delay wound detection. In these patients, even minor injuries can rapidly progress to chronic, non-healing ulcers, increasing the risk of complications such as infection, gangrene, and ultimately, amputation [29, 30]. The annual incidence of DFU among diabetic patients is estimated at 2.5%, while the overall prevalence reaches 6.3%, higher in males (4.5%) than females (3.5%), and higher in type 2 diabetes (6.4%) compared to type 1 (5.5%) [29–31].

Infection can further impair the healing process by inducing excessive and prolonged inflammatory responses. The skin hosts a diverse and dynamic microbiota that interacts closely with the host immune system, contributing to the maintenance of skin homeostasis [32]. When wounds occur, resident skin bacteria initially colonize the wound site, triggering the inflammatory phase, during which immune cells respond to eliminate contamination and recruit signals for tissue repair [33, 34]. In patients with compromised immunity, bacterial colonization can overwhelm host defenses, leading to infection, persistent inflammation, and chronicity. *Staphylococcus aureus* (93.5%) and *Pseudomonas aeruginosa* (52.2%) are particularly notable in ulcers, capable of forming biofilms, bacterial communities encased in extracellular polymeric substances (EPS) that resist antibiotics and antiseptics [33–35].

The microenvironment of chronic wounds is profoundly disrupted, characterized by low mitogenic cell populations, downregulated growth factor receptors, elevated reactive oxygen species (ROS), and high levels of metalloproteases (MMPs), collectively degrading extracellular matrix (ECM) and inhibiting granulation tissue formation (Figure 5) [26]. The inflammatory phase is prolonged due to factors such as hypoxia, persistent trauma, bacterial infections, and impaired host response [36]. Sustained inflammation generates oxidative stress through ROS production while reducing antioxidants like nitric oxide (NO), creating a feedback loop that elevates MMP activity (particularly MMP-2 and MMP-9) and pro-inflammatory cytokines. This cycle hampers ECM deposition, reduces growth factor

availability, and halts tissue regeneration, thereby increasing susceptibility to infection and the potential progression to ulceration and necrosis [36].

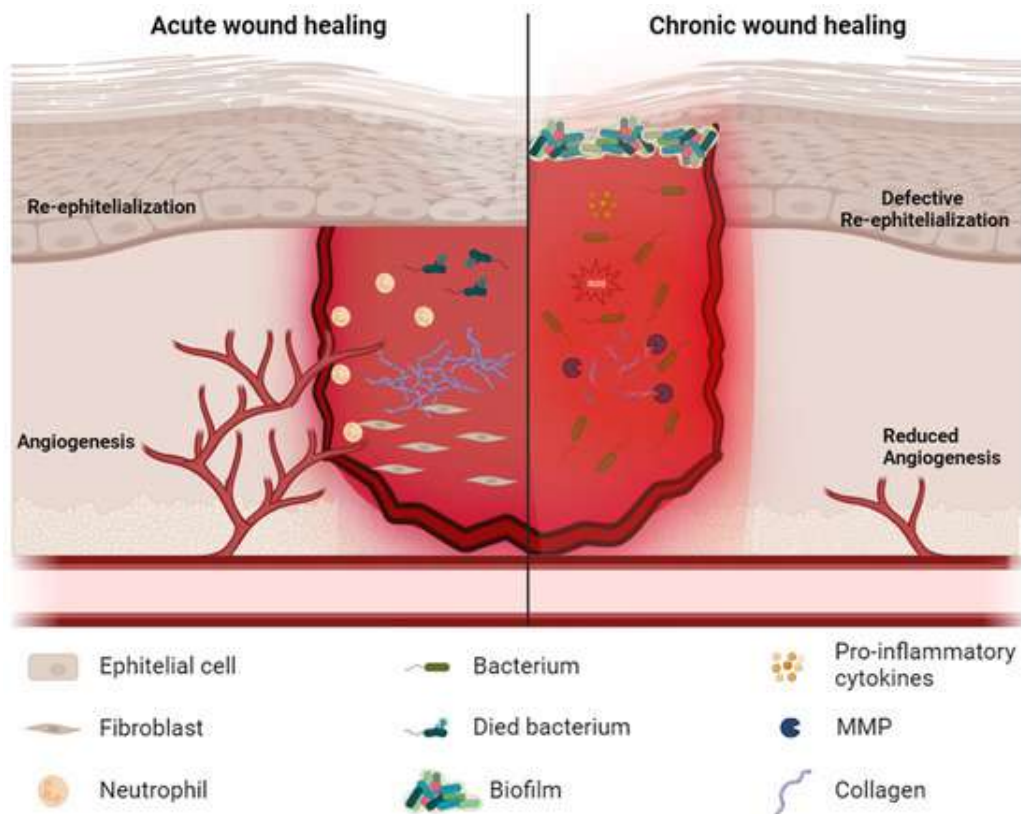


Figure 5. Acute vs chronic wound healing [49].

Chronic wound management imposes a substantial economic burden, accounting for approximately 3% of total healthcare expenditure. Global costs were estimated at USD 28.1 billion in 2014, rising to a maximum of USD 96.8 billion when wounds were considered secondary diagnoses [37, 38]. Diabetic wounds account for the largest share of these costs, with an estimated average annual cost of approximately USD 8,659 per patient [30]. Costs are expected to increase further due to the growing prevalence of diabetes and longer life expectancy among diabetic patients [37].

The clinical management of chronic wounds is often complex, prolonged, and resource-intensive. One key approach to promote wound healing is debridement, which is the removal of necrotic tissue. Necrotic tissue impedes repair and can sustain bacterial persistence, creating an unfavorable wound environment. Debridement exposes viable, well-perfused, and mitotically active cells, thereby facilitating wound closure and tissue regeneration [4, 39]. In cases of infection, topical treatments incorporating antibacterial or antibiofilm agents are commonly employed to control microbial growth and prevent further complications [40].

Additionally, the application of growth factors, often deficient in chronic wounds, is a widely adopted strategy to stimulate cellular activity and tissue regeneration. Adjunctive therapies, such as hyperbaric oxygen therapy to counteract ulcer-associated hypoxia and negative-pressure wound therapy, are also used to enhance healing [4, 39].

Despite these interventions, healing often remains slow and unpredictable. Wound dressings play a central role in standard care by protecting the wound from external contaminants, maintaining optimal moisture levels, and reducing the risk of infection. Advanced dressings can be functionalized with antibiotics, antibiofilm compounds, or growth factors to provide additional therapeutic benefits. Depending on their composition, dressings can serve different purposes; for instance, hydrocolloids and hydrogels maintain moisture and support autolytic debridement, facilitating natural tissue breakdown [41]. However, the increasing prevalence of antibiotic-resistant bacteria, the short half-life of growth factors, and the nonspecific nature of many treatments highlight the need for more innovative approaches that accelerate healing, reduce healthcare costs, and improve patient quality of life.

Tissue engineering

Tissue engineering is among the most promising strategies for enhancing wound repair. By applying engineering principles to biological systems, tissue engineering aims to develop functional skin substitutes that restore damaged tissue. Regenerative medicine (RM) complements tissue engineering by utilizing skin substitutes to repair or replace damaged tissues, restoring anatomical and functional integrity [42]. In clinical practice, RM is increasingly integrated into conventional therapies to reverse tissue or organ damage, potentially reducing the need for transplantation. Its strength lies in its ability to deliver customized treatments by addressing patient-specific concerns (Figure 6) [43].

A key element of this approach is the design of three-dimensional scaffolds that provide structural support for cellular components and enable controlled delivery of bioactive molecules to guide tissue regeneration [44, 45]. Scaffolds in RM serve multiple critical functions: they must be biocompatible, safely biodegradable, and possess pores that allow the transport of nutrients and waste. By providing a structured framework for cell attachment, scaffolds influence key cellular behaviors, including proliferation, differentiation, and tissue formation [45]. Scaffolds for wound dressings can be manufactured in various structural forms, each affecting cell activity and the wound healing process. Fibrous electrospun scaffolds offer tunable fiber diameter and alignment,

supporting cell adhesion and guiding migration. Porous sponges or foams allow control over pore size and interconnectivity, which is essential for nutrient diffusion and cell ingrowth. Hydrogels provide a highly hydrated 3D matrix that mimics soft tissue and maintains a moist environment, although they often require reinforcement to achieve mechanical stability. Finally, thin membranes or meshes, sometimes micro- or nanopatterned, can modulate cell responses through surface topography and chemical functionalization [46].

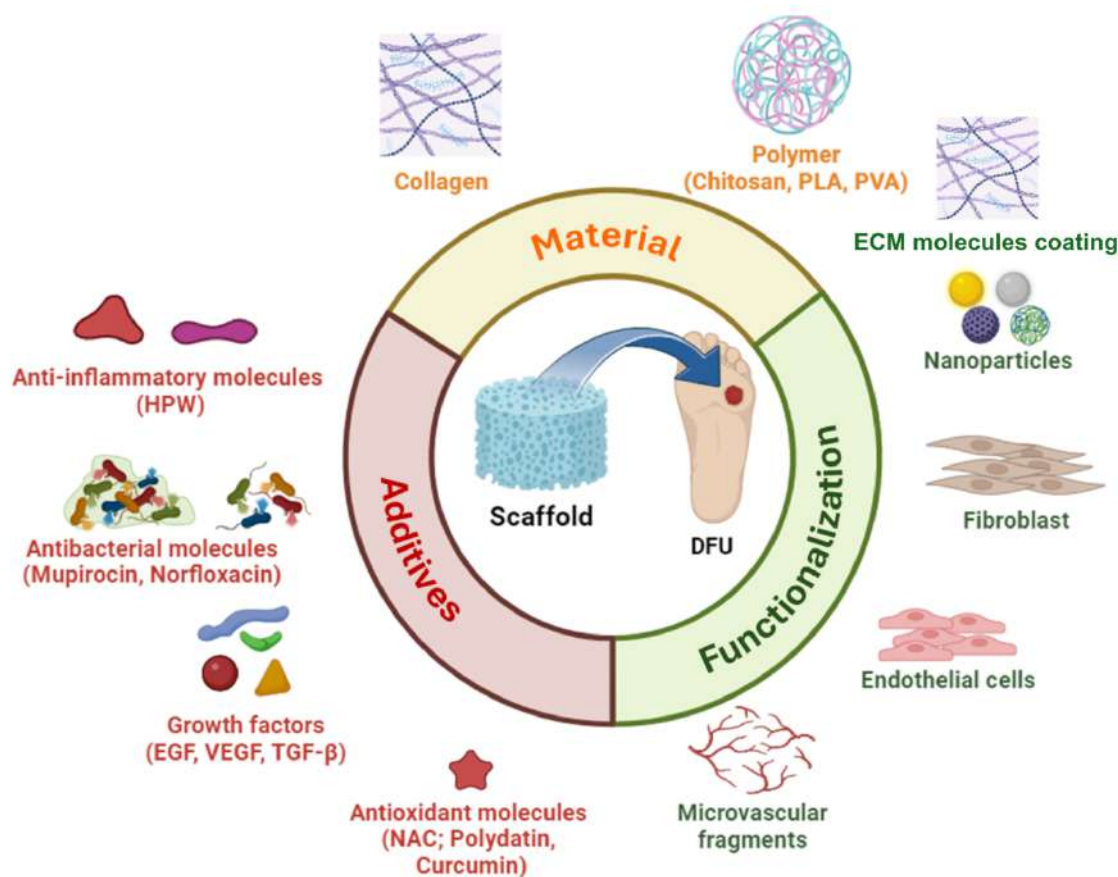


Figure 6. Tissue engineering approaches.

Natural biopolymers are organic molecules produced by living organisms, offering biocompatibility, biodegradability, low antigenicity, and functional effects such as antibacterial or proliferative actions. A wide range of natural and synthetic biopolymers has been employed in tissue engineering to develop skin substitutes [47]. In wound healing, natural biopolymers such as collagen [48], silk fibroin [49], chitosan [50], and hyaluronic acid [51] are among the most extensively investigated. Compared to natural polymers, synthetic polymers offer improved processability and enhanced mechanical properties. Their structure and properties can also be precisely controlled by adjusting material composition

and processing conditions to meet specific therapeutic needs [52]. Common examples include PLA (polylactic acid) [53], PVA (polyvinyl alcohol) [54], and PLGA (poly(lactic-co-glycolic acid)) [52].

Poly-L-Lactic Acid

PLA is an aliphatic polyester derived from renewable resources, such as corn, and produced by fermenting lactic acid. Advances in lactic acid production have reduced manufacturing costs, thereby enhancing PLA's potential for tissue engineering and other medical applications [55]. PLA is widely used in biomedical applications due to its biodegradability, biocompatibility, and thermoplastic processability, allowing its use in surgical implants, drug delivery systems, and porous scaffolds for tissue regeneration. Its versatility is enhanced by the ability to modify its chemical and physical properties, enabling applications ranging from bone fixation devices and ligament reconstruction to injectable microspheres for facial reconstruction and controlled drug release [56].

PLA exists in two chiral crystalline forms: poly-L-lactic acid (PLLA), which exhibits superior mechanical strength and slower degradation, and poly-D-lactic acid (PDLA). In contrast, the racemic mixture (PDLLA) is amorphous and degrades more rapidly [57]. Specifically, PLLA is a semicrystalline polymer composed of crystalline lamellae and interlamellar amorphous regions [58, 59]. Degradation kinetics are strongly influenced by polymer crystallinity, molecular weight, composition, processing-induced morphology, and the presence of additives, with no reported accumulation of degradation products in vital organs [59, 60]. A variety of PLA-based scaffold systems have been successfully applied in tissue engineering approaches for wound healing [53]. Among scaffold fabrication techniques, thermally induced phase separation (TIPS) has been widely employed to produce nanofibrous PLLA scaffolds that mimic the architecture of fibrillar collagen while preserving an interconnected porous network, thereby facilitating nutrient diffusion and cell migration in tissue engineering applications [61].

When implanted *in vivo*, degradation occurs primarily through non-enzymatic hydrolysis of ester bonds and is initiated preferentially within the amorphous domains, which are more accessible to water penetration [58, 59]. Hydrolytic chain scission results in a progressive decrease in molecular weight and mechanical integrity, accompanied by fragmentation of the polymer structure [58, 59]. When PLLA is processed into porous or fibrous scaffolds, including micro- or nanofibrous architectures, the resulting high surface area and

interconnected structure promote cell infiltration and support tissue integration, while also facilitating scaffold degradation over time [58, 60]. The resulting lactic acid-based degradation products are metabolized via the tricarboxylic acid cycle and ultimately eliminated as carbon dioxide and water [59].

PLLA has shown advantages as a biomaterial for wound-healing applications. For instance, porous PLLA nanofiber membranes modified with sulfated chitosan and polydopamine-gentamicin enhanced antibacterial activity, modulated macrophages toward the anti-inflammatory M2 phenotype, and increased VEGF secretion to support vascularisation [62]. Moreover, as mentioned, after implantation of pure PLLA scaffolds, the material undergoes active biodegradation, releasing lactic acid over time. This process is accompanied by increased activity of enzymes such as lactate dehydrogenase (LDH) and NADH-reductase in macrophages and fibroblasts, further supporting tissue integration and remodeling [63]. Moreover, aligned PLLA electrospun nanofibers were able to modulate immune responses and promote healing. They induced macrophages to adopt an anti-inflammatory M2 phenotype, thereby enhancing the activity of fibroblasts and endothelial cells. In vivo, aligned fibers accelerated wound closure, increased collagen deposition, and promoted neovascularisation [64].

From a mechanistic perspective, PLLA promotes wound healing by enhancing collagen production and orchestrating a controlled immune response. In fact, proteins adsorb to the scaffold surface, neutrophils and macrophages infiltrate, and macrophages fuse into foreign body giant cells. These cells recruit fibroblasts, which differentiate into myofibroblasts. The myofibroblasts then synthesize new collagen fibers. Additionally, PLLA gradually degrades into lactate, which further stimulates fibroblast activity and collagen synthesis, reinforcing tissue structure and accelerating repair [65].

Moreover, PLLA mechanical strength [57] might be critical to counteract the contractile forces generated by the panniculus carnosus (PC) in murine skin during in vivo wound-healing experiments. The PC is a thin but highly contractile striated muscle layer that profoundly alters wound-healing dynamics in many animal models, including rodents and rabbits, by driving rapid skin contraction rather than true tissue regeneration. In contrast, human wound healing relies predominantly on cellular proliferation rather than contraction. Consequently, unless this mechanism is carefully controlled, these models fail to accurately recapitulate human healing and therefore have limited translational relevance [66]. Indeed,

it has been demonstrated that wounds heal under the influence of the PC close artificially rapidly, taking only 7–8 days in mice and approximately 22 days in rabbits. By contrast, when PC-mediated contraction is inhibited, closure is significantly delayed, requiring around 13 days in mice and 33 days in rabbits. This delay in wound healing shifts the healing process toward a more human-like pattern, characterized by epithelialization and extracellular matrix deposition [66, 67]. To mitigate this bias, mechanical strategies such as silicone splints, adhesive fixation, elastic bandages, rigid frames, and anatomical models, such as the rabbit ear, have been developed to suppress contraction and force-regeneration-based healing [66, 67]. Nevertheless, the mechanical influence of the panniculus carnosus remains frequently underestimated in scaffold-based wound-healing studies, potentially leading to an overestimation of therapeutic efficacy in animal models. In this context, the use of a scaffold composed of a mechanically stable biomaterial, such as PLLA, may help counteract the contractile forces generated by the PC. By resisting these forces, the scaffold can maintain its position on the wound surface, preventing artificial acceleration of closure and thereby providing a more physiologically relevant model for studying human-like wound healing.

Taken together, these studies demonstrate that PLLA is a highly versatile scaffold material that provides both structural support and biological cues conducive to wound healing.

Scaffold Functionalization

Scaffolds for wound healing can be functionally engineered to address specific pathological features, providing more than mere structural support. Different bioactive molecules can be incorporated to target distinct challenges in the wound healing process, such as poor vascularization, prolonged inflammation, oxidative stress, bacterial infection, or insufficient fibroblast proliferation. These molecules are often designed for sustained or localized release, enhancing therapeutic efficacy while minimizing systemic exposure.

To promote vascularization, scaffolds that deliver sustained VEGF release, thereby promoting angiogenesis, endothelial cell recruitment, cell migration, and wound closure in diabetic models, have been produced [68–70]. The addition of stromal cell-derived factor 1 α (SDF-1 α), which is involved in angiogenesis, to VEGF further stimulated endothelial progenitor cell homing, amplifying angiogenesis, cell proliferation, ECM accumulation, and re-epithelialization, while reducing inflammation [71]. Similarly, in a murine model of diabetic full-thickness skin wounds, scaffolds containing anti-apoptotic factor–modified adipose-derived stem cells (ASCs) increased VEGF expression, enhanced angiogenesis in

wound granulation tissue, and accelerated local tissue revascularization compared to controls [72]. Electrospun collagen/PLGA/chitosan nanofibers loaded with VEGF and bFGF have also been shown to enhance angiogenesis, fibroblast proliferation, collagen deposition, and re-epithelialization in diabetic wound models [73].

To counteract inflammation, anti-inflammatory molecules have been incorporated into scaffolds. Thymosin beta 4 (T β 4), for example, inhibits NF- κ B and cytokines such as TNF- α , IL-1 β , and IL-10, and its gradual release from collagen/chitosan scaffolds promoted angiogenesis and accelerated wound closure in diabetic models [74]. Strategies targeting MMP-9, a key mediator of chronic inflammation, using pioglitazone-loaded scaffolds [75] or gene-silencing tools like MMP-9 siRNA or antisense oligodeoxynucleotides against connexin 43 (Cx43asODN) downregulated pro-inflammatory pathways, enhanced re-epithelialization, and accelerated wound closure [76, 77].

Oxidative stress, caused by excessive reactive oxygen species (ROS), damages cells, affects the ECM, and impairs angiogenesis in chronic wounds. Antioxidant molecules, such as N-acetylcysteine (NAC), reduced ROS by promoting glutathione formation, and their delivery via collagen-based sandwich scaffolds reinforced with polycaprolactone or polyamide improved wound closure, collagen deposition, revascularization, and re-epithelialization [78, 79]. NAC also mitigated oxidative stress induced by graphene oxide in hybrid membranes, thereby promoting angiogenesis, cell migration, and re-epithelialization, and reducing hypertrophic scarring [80]. Moreover, polydatin, a natural compound with antioxidant and anti-inflammatory properties, has been shown to accelerate healing in both diabetic and nondiabetic wounds when conjugated to collagen scaffolds [81].

Chronic wounds are highly susceptible to bacterial colonization and biofilm formation, which protects pathogens from the immune system and antibiotics [82]. For this reason, antibacterial molecules play a critical role in tissue engineering. For instance, silver sulfadiazine incorporated into nanofibrous scaffolds provided antibacterial activity and biocompatibility, and supported cell adhesion and proliferation [83]. Similarly, mupirocin combined with silica microspheres in collagen scaffolds reduced inflammation, enhanced fibroblast proliferation, increased collagen deposition, and accelerated wound closure [84].

Natural extracts and multifunctional bioactive molecules are valuable because they can simultaneously address oxidative stress, inflammation, and infection. Scaffolds

functionalized with plant extracts or resinous compounds accelerated wound closure, reduced inflammation, and promoted fibroblast proliferation, angiogenesis, and ECM formation [85–88].

Overall, these examples illustrate that chronic wounds can be treated with multifunctional tailored scaffolds in which molecules are selected to target specific pathological features. By combining pro-angiogenic, anti-inflammatory, antioxidant, antibacterial, ECM-regulating, and fibroblast-proliferating molecules, scaffolds can provide phase-specific modulation of wound healing, maximizing regenerative outcomes.

Another widely adopted strategy involves incorporating nanoparticles, either as delivery vehicles or as functional components. For instance, metal-based nanoparticles, such as gold [89], copper and cobalt [90], and molybdenum oxide [91], have been shown to impart anti-inflammatory, pro-angiogenic, and antibacterial properties, respectively, thereby improving the overall scaffold potential. Moreover, silver nanoparticles are well known for their antibacterial properties, which effectively inhibit bacterial growth and prevent biofilm formation [92]. Nanoparticles can also be used in combination with drugs or antioxidants (often natural compounds) to achieve controlled release and synergistic biological effects, such as curcumin [93] and pioglitazone [75]. Among these substances, rosmarinic acid is a promising candidate, exhibiting antioxidant, anti-inflammatory, antibacterial, and antiviral activities, with low toxicity and rapid systemic clearance, primarily acting by modulating inflammatory pathways and oxidative stress. [94].

Fibroblasts

In some approaches to wound healing, scaffolds are combined with cellular components, enabling them to serve not only as structural supports but also as reservoirs of growth factors and bioactive molecules that directly stimulate tissue regeneration. Fibroblasts are among the most widely used cell types in scaffold-based wound healing because they are primary dermal components.

The culture of fibroblasts on 3D scaffolds has been shown to enhance the regenerative performance of dermal substitutes. In a rabbit model of full-thickness third-degree burn wounds, a cholecyst-derived scaffold loaded with homologous fibroblasts induced faster re-epithelialization, increased cellular proliferation, more organized collagen deposition, and greater neovascularization compared to the same scaffold without cells.

Immunohistochemical analyses further revealed a more pronounced and temporally regulated myofibroblast response in the cell-loaded group, suggesting that fibroblasts actively modulated the wound-healing process rather than merely providing structural support [95].

Human dermal fibroblasts integrated with platelets within a scaffold, activated by platelet-rich plasma (PRP) and photostimulation, showed increased laminin-5 (LM-5), VEGF, and PDGF expression, promoted cell migration, and reduced reactive oxygen species (ROS) levels, thereby enhancing angiogenesis and tissue regeneration [96]. Oral mucosa-derived fibroblasts seeded onto fibrin scaffolds accelerated wound closure, increased vascular density and cellular proliferation, and reduced scarring [97].

Scaffolds seeded with mouse embryonic fibroblasts (MEFs) have similarly been shown to accelerate wound healing compared to cell-free scaffolds. The presence of MEFs was associated with faster wound closure, improved re-epithelialization, increased collagen deposition, and more prominent capillary formation [98].

Similarly, bi-layer wound dressings containing rat skin fibroblasts (RSFs) seeded onto activated carbon fibers combined with gentamicin-incorporated γ -PGA/gelatin membranes have demonstrated markedly improved healing outcomes compared to acellular dressings, accelerating wound closure, promoting fibroblast migration into the wound site, and enhancing fibronectin and type I collagen expression in vivo [99].

Overall, fibroblasts' ability to attach and penetrate the scaffold, produce extracellular matrix components such as collagens, and secrete factors that stimulate cell proliferation and angiogenesis, highlighting their active role in tissue engineering for promoting both structural repair and vascularization.

Microvascular Fragments

As discussed, a wide range of pro-angiogenic strategies has been explored to stimulate vascularization in skin repair, most notably the delivery of growth factors such as VEGF-A, PDGF, FGF-2, or EGF, as well as non-growth factor mediators, including cytokines, chemokines, and small pro-angiogenic drugs [100]. However, these biologics typically have short half-lives and are rapidly degraded or cleared from the inflamed wound environment, limiting spatial and temporal control of angiogenic signaling. To address these limitations, growth factors and cytokines have been encapsulated or immobilized within biomaterials,

such as hydrogels, polymeric microparticles, and extracellular matrix-based scaffolds, to enhance their stability and local retention [101]. Despite these advances, vascularization in these systems still depends on the patient's own endothelial and stromal cells to migrate, proliferate, and assemble new blood vessels, a process that is often inefficient in chronic wounds or in aged or diseased tissue.

In this context, incorporating cellular components that both release growth factors and contribute to new tissue formation can be particularly beneficial, as they provide a ready source of angiogenic signals and structural building blocks, bypassing reliance on host cell recruitment and accelerating the establishment of functional microvascular networks. Thus, the forces driving healing originate not only from the host cells but also from the introduced cellular components.

For this purpose, stem cells have been used. In diabetic wounds, the use of endothelial cells and their progenitors, mesenchymal stem cells (MSCs), ASCs, and induced pluripotent stem cells, often delivered via scaffolds or hydrogels, promotes vascularization and tissue repair [102]. For example, a fibrin hydrogel scaffold was developed to support the growth and transplantation of human umbilical cord - mesenchymal stem cells (hUC-MSCs) in skin wounds. This scaffold provided a stable 3D microenvironment that enhanced MSC survival and paracrine secretion. In vivo, wounds treated with the hydrogel-hUC-MSCs combination showed higher expression of growth factors (epidermal growth factor (EGF), VEGFA, transforming growth factor (TGF- β 1)), improved re-epithelialization, angiogenesis, and ECM production compared to MSCs alone [103]. Similarly, ASCs enhanced wound healing by differentiating into fibroblasts, keratinocytes, and endothelial cells and by secreting bioactive factors, including VEGF, Hepatocyte Growth Factor (HGF), TGF- β , and PDGF. When combined with hydrogels or scaffolds, ASCs showed improved survival and efficacy in the wound environment, supporting faster healing and increased vascularization [104].

Another alternative is the stromal vascular fraction (SVF), an autologous cell population containing stem cells, fibroblasts, endothelial cells, and immune cells, is well-suited for this purpose. SVF has been shown to enhance wound healing by stimulating angiogenesis, supporting fibroblast proliferation, increasing collagen deposition, remodeling the extracellular matrix, and modulating inflammation both as a standalone therapy and when combined with biomaterial scaffolds, which further improve cell retention and growth factor delivery [105].

Most recently, adipose-derived microvascular fragments (ad-MVF or MVFs) are emerging as a next-generation strategy for prevascularization [106]. MVFs are small, intact segments of functional microvessels derived from adipose tissue via a shorter enzymatic digestion than SVF, which preserves preformed arterioles, capillaries, and venules, including perivascular cells [107]. When implanted, MVFs can sprout, branch, and interconnect to form functional microvascular networks that inosculate with host vessels, rapidly establishing perfusion in the surrounding tissue [108]. This intrinsic microvasculature provides multiple nucleation sites for rapid capillary formation, offering a clear advantage over dispersed single-cell approaches.

The advantages of prevascularizing dermal scaffolds with MVF have been clearly demonstrated using clinically established matrices such as Integra®, a collagen–glycosaminoglycan (CGAG) substitute widely used in burn and reconstructive surgery. When seeded with microvascular fragments (MVFs), Integra® showed improved vascularization, increased granulation tissue formation, better vascular and lymphatic integration, and earlier coverage compared to non-seeded constructs [109]. These results demonstrate that ad-MVF functions as a functional vascular unit, overcoming the limitations of poorly vascularized wound beds and accelerating scaffold incorporation and wound healing.

MVFs have been shown to be more effective vascularization units than other strategies. For instance, direct comparisons of the angiogenic potential of SVF and MVFs demonstrate the superior capacity of MVFs to promote new vessel formation. In fact, SVF spheroids, composed of dispersed single cells, require reassembly into vessel-like structures, whereas MVF spheroids already contain intact microvessel fragments. Consequently, MVFs show faster angiogenic sprouting in vitro, higher densities of endothelial and perivascular cells, and superior vascularization and graft integration in vivo compared to SVF spheroids. [110]. When seeded in 3D collagen–GAG dermal scaffolds, MVF-seeded constructs developed significantly higher vessel density, superior blood perfusion, and better tissue integration than SVF-seeded scaffolds, highlighting the intrinsic advantage of pre-assembled microvessels over single-cell–based approaches [111]. These data indicate that MVF do not merely provide pro-angiogenic cells, but deliver a fully functional microvascular blueprint that enables rapid perfusion and improved survival of engineered tissues.

Importantly, the advantages of MVF are also translationally relevant for human therapy. This has recently been demonstrated using human adipose-derived MVF in a vascularized 3D-bioprinted dermis, which showed rapid perfusion, enhanced wound closure, and improved tissue regeneration in vivo [112]. This finding confirms that MVF-based vascularisation strategies are compatible with human cells and clinically relevant biomaterials, reinforcing their potential for future clinical translation.

Notably, despite this strong biological rationale, only a small number of scaffold-based systems have so far been reported to integrate MVFs as an intrinsic circulatory network. Beyond CGAG matrices such as Integra®, examples include MVFs embedded in collagen hydrogels [108], incorporated into fibrin hydrogels with hydroxyapatite scaffolds for bone tissue engineering [113], prevascularized GelMA hydrogels for osteogenic constructs [114], incorporated within 3D-printed demineralized bone matrix scaffolds for craniofacial grafts [115], and seeded onto synthetic PCL or porous polyethylene scaffolds to enhance vascularization and tissue integration [116]. All these systems, when tested in vivo, demonstrated that MVFs inoscultated with the host circulation, forming functional perfused microvascular networks. Moreover, MVFs delivered in composite or polymeric scaffolds contributed to early vascularization in vivo, inoscultating with host vessels and improving perfusion even in challenging defect sites, such as composite bone–muscle injuries [117, 118].

In skeletal muscle regeneration, MVFs embedded in collagen hydrogels accelerated vascularization in volumetric muscle loss models, increasing microvascular density and ensuring rapid tissue perfusion, which is critical for cell survival and integration of newly formed tissue [119, 120]. MVFs have also been applied in craniofacial and dental tissue engineering. In dental pulp regeneration, adipose-derived MVFs combined with dental pulp stem cell aggregates promoted vascularization within tooth-root–mimicking scaffolds, reduced apoptosis, and supported differentiation of stem cells into odontoblast-like cells [121]. To our knowledge, the interaction of MVFs with certain biomaterials, such as PLLA, has not yet been explored. The closest precedent comes from the use of poly(lactic-co-glycolic acid) (PLGA) microparticles as drug-delivery vehicles in vascular tissue engineering, where biodegradable PLGA microparticles were fabricated, incorporated into hydrogel matrices, and shown to enable controlled release of encapsulated compounds capable of modulating vascular behavior [122]. While this approach addressed drug delivery in vascularized constructs, it did not investigate MVFs as pre-assembled microvascular units.

Beyond classic tissue-engineering applications, MVFs have shown potential in pathological or ischemic conditions. In murine lymphedema, collagen-embedded MVFs contributed to both angiogenesis and lymphangiogenesis, reducing dermal backflow and swelling [123]. In peripheral ischemia models, transplanted MVFs are directly incorporated into newly formed blood vessels, improving tissue perfusion and reducing necrosis more effectively than single-cell populations [124]. In musculocutaneous flap models, MVF injections enhanced microcirculation, decreased tissue necrosis, and supported survival of distal flap regions [125].

The number of studies in regenerative medicine and tissue engineering involving adipose-derived microvascular fragments (MVFs) remains relatively limited, largely because this approach is still emerging. Nevertheless, their use has been increasing, particularly in recent years. Through a systematic analysis of the literature—focusing exclusively on original research articles and excluding reviews—we identified, to the best of our knowledge, 126 published studies to date about MVFs. As illustrated in Figure 7, scientific interest in MVFs has steadily grown since their initial introduction, with a noticeable acceleration in publications in recent years. Despite this upward trend, the overall body of evidence remains relatively small, indicating that MVFs remain underexplored and that further research is needed to fully understand and exploit their potential for regenerative applications.

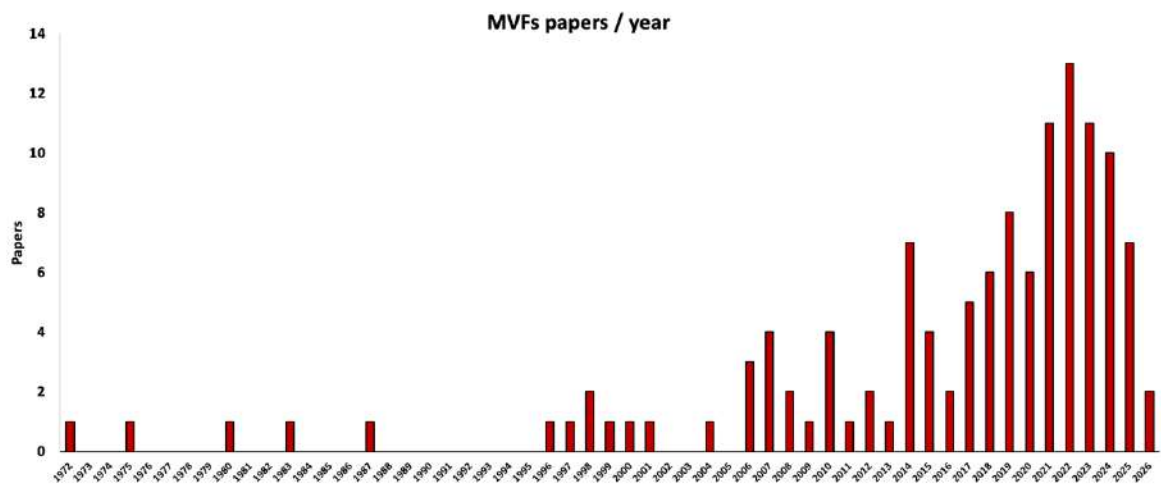


Figure 7. Original studies on microvascular fragments (MVFs) identified in the literature.

The limited number of such systems underscores the infrequent use of microvessels as an intrinsic circulatory architecture in tissue engineering, despite their superior biological performance. As a result, the fundamental mechanisms by which MVFs interact with biomaterial microenvironments, self-organize, and establish functional perfusion within scaffolds remain incompletely understood. Therefore, any additional study in this field contributes not only to expanding the currently limited evidence base but also to improving our understanding of how MVFs interact with biomaterials, self-organize, and establish functional microvascular networks, ultimately enhancing their potential for regenerative medicine applications.

Co-culture of fibroblasts (FBS) and microvascular fragments (MVFs)

The importance of FBS for MVF functionality was first demonstrated in early studies. In 1985, isolated MVFs from rat epididymal fat pads were separated from single myofibroblastic cells by filtration and cultured for the first time to investigate capillary growth in vitro. Microvessels cultured alone did not exhibit sprouting, whereas in the presence of confluent myofibroblasts, they formed vascular-like structures. These structures displayed endothelial lumina, intercellular junctions, and vacuoles, confirming their endothelial identity. Myofibroblasts supported capillary growth through both direct cell–cell interactions and the release of soluble factors, highlighting the cooperative interaction between the two cell types [126].

When implanted in vivo, MVFs together with myofibroblasts formed functional microvascular networks across the peritoneum [127], ischemic random-pattern skin flaps [128], and the superficial myocardium [129], whereas MVFs or myofibroblasts alone were largely ineffective. In all models, the combination promoted vessel sprouting, network integration with host circulation, and improved tissue survival or perfusion, demonstrating that stromal support is crucial for the angiogenic potential of MVFs in vivo.

In another work, 3D bioprinted dermal constructs were generated using human dermal fibroblasts (HNDFs) embedded within a GelMA–fibrinogen hydrogel, either alone or in combination with human adipose-derived microvascular fragments (HaMVFs). Constructs containing fibroblasts alone already showed improved cell proliferation and matrix deposition compared to acellular scaffolds. However, the addition of HaMVFs further enhanced the system's biological performance. In vivo, constructs with both fibroblasts and HaMVFs exhibited a markedly faster wound closure compared to fibroblast-only and

scaffold-only groups. This improvement was associated with significantly increased re-epithelialization and more extensive collagen deposition [112].

Overall, these findings indicate that while fibroblasts contribute to extracellular matrix production and tissue organization, their combination with prevascularizing elements, such as HaMVFs, is crucial for optimal vascular integration and accelerated wound healing.

AIM OF THE STUDY

The current project focuses on developing a multifunctional therapeutic unit to enhance wound healing using a porous poly (L-lactic acid) (PLLA) scaffold functionalized with microvascular fragments (MVF) and dermal fibroblasts, thereby integrating mechanical support, intrinsic vascularization, and matrix-producing cells into a single regenerative platform. Importantly, all cellular components are isolated from clinically accessible autologous tissues: fibroblasts from dermal biopsies and MVF from adipose tissue, both of which can be harvested through minimally invasive procedures such as small skin-punch biopsies and liposuction. This enables the generation of patient-specific, fully autologous skin constructs, eliminating the need for immunosuppression while enabling rapid, scalable clinical translation.

The core of this approach is a PLLA scaffold, which provides a three-dimensional structure that can be customized to match the geometry of experimentally induced full-thickness wounds in future in *in vivo* rat studies. Beyond providing physical support for cell attachment, the scaffold serves as a template for tissue regeneration, facilitating the effective localization and organization of fibroblasts and vascular networks within the wound site. The ability of PLLA scaffolds to mimic the fibrillar architecture of native collagen further enhances their suitability for wound healing applications. Moreover, the predictable degradation of PLLA into non-toxic byproducts allows the scaffold to be progressively replaced by newly formed tissue, a key feature for supporting long-term regeneration. In addition, the scaffold provides anchorage sites that facilitate fibroblast migration from the wound margins, potentially enhancing endogenous cell recruitment and accelerating wound repopulation, while also possessing sufficient mechanical strength to counteract panniculus carnosus (PC)-driven contraction forces.

The incorporation of primary dermal fibroblasts represents a key biological component of the system. Fibroblasts are central regulators of dermal repair, playing pivotal roles in ECM deposition, growth factor secretion, and modulation of inflammation and angiogenesis. Seeding fibroblasts directly onto the scaffold can promote early ECM deposition, thereby forming a biologically active environment that supports subsequent stages of healing. By secreting a broad range of signaling molecules, fibroblasts can further recruit and activate host cells, further amplifying endogenous regenerative responses and contributing to active remodeling of the wound environment.

To support vascularization and counteract hypoxia, a major limiting factor in chronic wound healing, adipose-derived MVFs are incorporated into the scaffold as a prevascularization strategy. MVFs preserve the native microvessel architecture, including endothelial and perivascular cells, and, upon implantation, demonstrated the potential to rapidly form interconnected microvascular networks that anastomose with the host vasculature. This early restoration of perfusion would enable efficient delivery of oxygen and nutrients and removal of metabolic waste products.

The coexistence of fibroblasts and MVFs within the scaffold further enhances the regenerative microenvironment through synergistic interactions. Fibroblasts secrete pro-angiogenic signals that support vessel sprouting and maturation, while newly formed vascular networks sustain fibroblast survival and function.

To assess the translational potential of this strategy, human primary fibroblasts have been used for preclinical evaluation. The use of human cells provides valuable insight into how the scaffold may perform in a clinical setting, particularly regarding ECM deposition and scaffold biofunctionalization.

Overall, this study presents a comprehensive therapeutic strategy for chronic wound healing, integrating structural support, scaffold biofunctionalization, ECM deposition, pre-vascularization, and synergistic cellular signaling to establish a fully regenerative microenvironment. By targeting multiple key factors in the wound microenvironment simultaneously, this multifunctional scaffold system holds the potential to accelerate healing, restore tissue integrity, and overcome the limitations of conventional wound care approaches.

MATERIALS AND METHODS

RDFs isolation

Enzymatic method

The dorsal area of donor Wistar rats was shaved and disinfected. A dermal fragment was harvested, quickly washed in 70% ethanol, and placed in high-glucose Dulbecco's Modified Eagle's Medium (DMEM; SIAL-DMEM-HPA, YourSial) supplemented with 2% Penicillin/Streptomycin (PS; Sigma Aldrich P433) and 1% Antimycotics (AM; Sigma Aldrich A2942). Under a laminar flow hood, the tissue was rinsed three times with sterile phosphate-buffered saline (PBS) lacking Ca^{2+} and Mg^{2+} (Sigma-Aldrich D1408), containing 2% PS and 1% AM, then minced into small fragments using sterile scissors and a scalpel. The fragments were transferred to 15 ml Falcon tubes (1.5 g per tube) and incubated in 10 ml of digestion buffer containing collagenases H (450 U/g) and G (106 U/g; Abiel s.r.l.) and thermolysin (266 $\mu\text{g/g}$; Promega) in DMEM with 2% PS, 1% AM, and 2 mM CaCl_2 (Sigma Aldrich). Digestion was carried out under rotation (power level 5, 1 hour, 30°C, Rotary shaker with carousels RX-RD-Pro, Dutscher) and stopped by adding two volumes (20 ml) of complete fibroblast medium (DMEM with 1% PS/AM, 1% L-glutamine [Sigma Aldrich G7513], and 10% FBS [SIAL-FBS-SA, YourSial]). The suspension was centrifuged, and the pellet was resuspended in fresh complete medium. Larger tissue fragments were removed, and the cells were seeded into Petri dishes. Cultures were maintained at 37°C, 5% CO_2 , and 100% humidity.

Explant method

Primary dermal fibroblasts were isolated from humans (hDFs) and rats (RDFs). Human fibroblasts were obtained with approval from the ethical committee/review board for experiments with human-derived tissues (Bavarian Medical Association, approval No. 16128) in collaboration with Bert Reichert, MD, Professor and Director of the Clinic for Plastic, Reconstructive and Hand Surgery, and Karl Bodenschatz, MD, Director of the Department of Pediatric Surgery and Urology [130]. Rat fibroblasts were isolated from rat skin using the explant technique. The abdominal region of the animal was shaved with an electric razor, and a small incision was made to separate the skin (epidermis, dermis, and hypodermis) from the underlying muscle fascia using sterile scissors. The excised skin fragment was briefly rinsed in 70% ethanol and washed three times in DPBS without Ca^{2+} and Mg^{2+} (Pan Biotech, Germany) supplemented with 2% PS/AM under sterile conditions.

The tissue was cut into approximately 0.5 cm fragments, the hypodermis removed, and fragments placed dermal side down in Petri dishes. Culture medium (DMEM/F12 1:1, Pan Biotech, Germany) supplemented with 10% FBS (Pan Biotech, Germany), 2% PS, 2% A, 23,79 µg/ml ascorbic acid (Merck, Germany), and 0.5% non-essential amino acids (Pan Biotech, Germany) was added just to cover the dermal surface, leaving the epidermal side exposed, and cultures were maintained at 37°C in a humidified atmosphere with 5% CO₂ (HERACELL 150i CO₂ incubator, Thermo Fisher, USA), with fibroblast outgrowth typically beginning after 6–9 days as cells degraded the surrounding matrix and migrated outward from the explant.

PLLA scaffolds production

Scaffolds were fabricated as previously described [131] using Thermally Induced Phase Separation (TIPS). Briefly, a ternary solution was prepared by mixing 1,4-dioxane (Sigma-Aldrich; Merck) and distilled water at an 87/13 wt/wt ratio, followed by the addition of 4% PLLA Resomer® L 209 S (Evonik Industries) to form the precursor solution. This solution was poured into cylindrical molds, and phase separation was induced by incubation in a thermostatic bath, a step crucial for determining the final pore size of the scaffolds, which depends on both temperature and incubation time. Five different incubation conditions were tested: 20°C for 15 minutes, 25°C for 15 minutes, 28°C for 45 minutes, 25°C for 45 minutes, and 32°C for 30 minutes, resulting in scaffolds with distinct pore sizes (~50 µm, 100 µm, 250 µm, 300 µm, and 400 µm, respectively). After incubation, the molds were transferred to denatured ethanol at -20°C for stabilization, then rinsed and washed overnight in distilled water before drying at 60°C. Once dried, the scaffolds were sliced into 2 mm-thick sections and punched with a 6 mm biopsy punch to obtain the final shape. Pore size was subsequently assessed using optical and scanning electron microscopes.

Seeding DFs (murine and human) on PLLA scaffolds

Scaffolds were soaked in 70% ethanol under vacuum and left overnight to ensure complete sterilization. The following day, they were washed three times (5 min each) to remove ethanol residues, twice with PBS and once with base medium (DMEM 4.5 g/L glucose, YourSial), using a vacuum pump to ensure complete solvent exchange into the porous structure. The scaffolds were then divided into two groups: native PLLA scaffolds and

Geltrex™-coated scaffolds. For the coating, Geltrex™ (Thermo Fisher, USA) was diluted to 100 µg/ml in complete medium (DMEM 4.5 g/L glucose, 10% FBS, 1% PS-A). The scaffolds' medium was aspirated, and the Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix solution was applied, with gentle agitation and vacuum to facilitate infiltration into the pores. Scaffolds were then transferred to Petri dishes and allowed to dry under a laminar flow hood. Once dried, they were briefly rehydrated in ethanol under vacuum and then washed twice with PBS and once with complete medium to remove all traces of ethanol. Cells were collected using trypsin/EDTA (0.5%/0.2% W/V, Sigma), counted, and resuspended in complete medium to reach the desired seeding concentration (150.000 and 300.000 cells) in a final volume of 10 µl. Using sterile forceps, each scaffold was placed in a Petri dish, excess medium carefully removed, and 10 µl of cell suspension applied directly onto the scaffold surface. Constructs were incubated at 37°C for one hour to allow cell attachment, then transferred to low-attachment 24-well plates (Corning...) and completely submerged in 700 µl of complete medium containing 10 ng/ml (EGF; Boehringer Mannheim GmbH, Germany). Seeded scaffolds were finally maintained at 37°C in a humidified incubator for subsequent culture and analysis.

Viability assay: Alamar Blue

The working solution was prepared by diluting the stock Alamar Blue reagent (AlamarBlue™ HS Cell Viability Reagents, Thermo Fisher, USA) 1:6 in complete culture medium. Each scaffold, prepared in at least triplicate for every experimental condition, was transferred into an individual well of a 48-well plate, and 500 µL of the Alamar Blue working solution was added. The samples were incubated for 1.5 hours at 37°C under standard cell culture conditions. After incubation, 300 µL of the supernatant from each well was collected and distributed into three wells of a 96-well plate (100 µL/well) to obtain technical triplicates. Fluorescence, directly related to cell viability, was measured using a TECAN Infinite M200Pro microplate reader with excitation and emission wavelengths set at 560 nm and 590 nm, respectively. Fluorescence intensities at different time points were then plotted over time to quantify and compare cell proliferation and metabolic activity. For human experiments, statistical analyses were performed using ANOVA in GraphPad Prism.

Immunostaining (2D and 3D)

2D samples were fixed in 4% paraformaldehyde (PFA, Thermo Fisher, USA) for 10 minutes, while 3D scaffolds were fixed for 30 minutes. Cells (in both 2D and 3D environments) were then permeabilized and blocked for 20 minutes in a solution containing 1% Bovine Serum Albumin (BSA, Merck, Germany), 0.1% Triton X-100, and PBS to minimize nonspecific antibody binding and ensure adequate antibody penetration. Samples were incubated with primary antibodies overnight at 4°C, then washed three times in TBS before incubation with secondary antibodies. When required, 4',6-diamidino-2-phenylindole (DAPI, D9542-1MG, Sigma Aldrich) and Phalloidin-Alexa488 (P5282, Sigma Aldrich) were included. The samples were then incubated for 1 hour at room temperature, protected from light, and washed three additional times with PBS. 2D samples were mounted on glass slides using mounting solution (Fluoromount-G, Invitrogen by Thermo Fisher, USA) and stored until imaging, whereas 3D scaffolds were placed directly on glass coverslips (Cover glasses round Ø 10 mm, thickness 1 (0,13-0,17 mm), Langenbrinck) suitable for confocal laser scanning microscopy and analyzed immediately. Antibodies used are listed below.

- **Primary Antibodies:**

- Collagen Type I (Rabbit anti-Mouse/Rat; Millipore, 234167; 1:20) – Burlington, Massachusetts, US – used for RDFs
- Vimentin (Mouse IgG1 κ anti-Human/Mouse/Rat/Pig; Santa Cruz Biotechnology, sc-6260; 1:20) – Dallas, Texas, US – used for RDFs
- VE-cadherin (Goat anti-Human/Mouse/Rat; Santa Cruz Biotechnology, sc-6458) – Dallas, Texas, US – used for MVFs
- Collagen Type IV (Rabbit IgG anti-Human/Mouse/Rat; Invitrogen, PA5-115036) – Waltham, Massachusetts, US – used for MVFs
- Actin, α -Smooth Muscle (Mouse anti-Human/Sheep/Chicken/Goat/Bovine/Rat /Mouse/Canine/Rabbit; Sigma-Aldrich, A5228; 1:1) – St. Louis, Missouri, US – used for hDFs
- Fibronectin (Mouse anti-Human; Invitrogen, MA5-11981; 1:5) – Waltham, Massachusetts, US – used for hDFs
- Collagen Type I (Goat anti-Human; Southern Biotech, 1310-01; 1:5) – Birmingham, Alabama, US – used for hDFs
- Vimentin (Rabbit anti-Human; Zytomed Systems, 522-3204; 1:5) – Berlin, Germany – used for hDFs

- **Secondary Antibodies:**

- Cy3 Donkey anti-Mouse (Jackson ImmunoResearch, 715-166-150) – Bar Harbor, Maine, US
- Alexa Fluor 488 Goat anti-Mouse (111-095-045; 1.5 ng/ml)
- Alexa Fluor 555 Goat anti-Rabbit (ZYMED Laboratories, Invitrogen, 65-61111) – Carlsbad, California, US

Western Blot

Protein expression was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Western blotting. Proteins were separated on 10% resolving and 4% stacking polyacrylamide mini-gels polymerized with ammonium persulfate and N, N, N', N'-tetramethyl ethylenediamine (TEMED; T9281, Sigma-Aldrich). Samples were prepared in 6× sample buffer containing Tris–HCl, SDS, glycerol, β-mercaptoethanol, and bromophenol blue, denatured at 95 °C for 5 min, and loaded at 17 µg of total protein per lane, together with a molecular weight marker. Electrophoresis was performed in Tris–glycine SDS running buffer at 80 V through the stacking gel and 150 V through the resolving gel. Proteins were transferred onto methanol-activated Polyvinylidene fluoride (PVDF – Merck) membranes using a wet transfer system in Tris–glycine buffer containing 20% methanol at 100 V for 90 minutes at room temperature. Transfer efficiency was verified by reversible Ponceau S staining. Membranes were blocked for 1 hour in 5% non-fat dry milk in PBST, incubated overnight at 4°C with primary antibodies diluted in blocking solution, washed extensively, and incubated for 2 hours at room temperature with secondary antibodies conjugated with horseradish peroxidase (HRP). After further washing, protein bands were visualized with an enhanced chemiluminescence substrate and detected on a ChemiDoc imaging system with automatic exposure settings. Band intensities were quantified by densitometry with ImageJ, and the resulting data were plotted in Microsoft Excel.

Paraffin embedding and histological staining

Tissue samples were fixed overnight at 4°C in 4% PFA, embedded in HistoGel (HistoGel Specimen Processing Kit, Thermo Scientific, HG-4000-012), and placed into standard

histological cassettes (company?). The cassettes were processed for dehydration and clearing using a Shandon Pathcentre (Thermo Electron Corporation) and subsequently embedded in paraffin with the Tissue-Tek TEC embedding station (Sakura). Paraffin blocks were molded to match the dimensions of the scaffold or tissue, trimmed, and sectioned using a MICROM HM 325 microtome (Thermo Scientific) to obtain 5 μ m sections. Multiple sections were placed on each glass slide to improve adherence, and all slides were incubated overnight at 60°C to ensure firm attachment prior to staining.

Hematoxylin / Eosin

For hematoxylin and eosin (H&E) staining, histological sections were placed in a slide holder (up to 19 slides per run) with uniform orientation and deparaffinized by incubation in xylene for 12 minutes in a fume hood. Samples were rehydrated in a graded ethanol series (100%, 96%, 80%, and 70%) for 2 minutes each, followed by a 2-minute rinse in distilled water. Harry's Hematoxylin solution (Carl Roth GmbH) was vacuum-filtered before use, and sections were stained for 4 minutes. Excess stain was removed by rinsing in tap water for 1 minute, followed by a brief dip (~5 seconds) in fresh 96% ethanol. Sections were then counterstained with Eosin Y (Merck) for 1 minute and 30 seconds, dehydrated through an ascending ethanol series (70%, 80%, 96%, and 100%) for 2 minutes each, and cleared in two consecutive xylene baths for 5 minutes each. Finally, sections were mounted using Roti® Histokit Rot mounting medium (Carl Roth, Germany), coverslipped, and allowed to dry overnight at room temperature prior to microscopic analysis.

Sirius red

For Sirius Red histological staining, up to 19 slides were placed in a slide holder and incubated twice in xylene for 10 minutes each, followed by sequential rehydration in descending ethanol concentrations (96%, 80%, 70%, and 60%) for 4 minutes each. Slides were then rinsed in tap water for 4 minutes. Weigert's Hematoxylin was freshly prepared by mixing 55% solution A and 45% solution B, and slides were stained in this mixture for 8 minutes. After a brief 5-second rinse in tap water, slides were washed for 10 minutes under gentle running water until clear, then rinsed in distilled water for 1 minute. Samples were subsequently incubated in Picro Sirius Red solution (MORPHISTO®, 250 ml) for 1 hour at room temperature, washed twice in 30% acetic acid for 1 minute each, and dehydrated in 96% ethanol for 4 minutes. Finally, slides were cleared in two consecutive xylene baths for 5 minutes each, mounted with Roti® Histokit Rot (Carl Roth, Germany) medium, and allowed to dry overnight at room temperature before microscopic analysis.

Alcian blue

Alcian Blue (AB) staining at pH 2.5 was performed to visualize the deposition of sulphated glycosaminoglycans (sGAGs), which appear blue. Briefly, tissue sections were washed in distilled water for 2 minutes and subsequently incubated in 1% acetic acid (Carl Roth GmbH & Co. KG) for 3 minutes. Sections were then stained in 1% Alcian Blue 8GS solution (Carl Roth GmbH & Co. KG, pH 2.5) for 30 minutes. After staining, slides were rinsed in 3% acetic acid, then washed in distilled water for 2 minutes. Counterstaining of cell nuclei was carried out using nuclear fast red aluminium sulphate solution (Carl Roth GmbH & Co. KG) for 5 minutes. Finally, sections were rinsed again in distilled water for 2 minutes before further processing and analysis.

Scanning Electron Microscopy (SEM)

After extensive washes with PBS, samples were fixed in 3.7% formaldehyde at 4°C for 30 minutes, rewashed with PBS, and dehydrated through a graded ethanol series (15%, 30%, 50%, 75%, and 100% v/v), allowing the ethanol to evaporate in air after the final step. The samples were then coated with a thin layer of gold (~5 nm) using a sputter coater to make them conductive for SEM imaging. Scanning electron microscopy was performed using a [model] at an accelerating voltage of [tension kV].

Isolation of Microvascular Fragments (MVs) from Adipose Tissue

Adipose tissue from different regions (intestinal, perirenal, and subcutaneous) was aseptically excised using sterile forceps and scissors, with instruments cleaned between dissection steps in ethanol and sterile PBS. Samples were transferred into 50 ml Falcon tubes containing transport medium (DMEM 4.5 g/L glucose, 10% FBS, 2% PS-A) and kept on ice until processing. Under sterile conditions, tissue samples were washed three times in PBS without Ca^{2+} and Mg^{2+} , supplemented with 2% PS and 2% A. The washed tissues were weighed, finely minced with sterile scissors, and distributed at approximately 1 g per 15 ml tube. Enzymatic digestion was performed by adding 300 U ultrapure recombinant collagenase H (Abiel s.r.l.), 100 μl of 1 mg/ml thermolysin (10 $\mu\text{g}/\text{ml}$, Promega), and 2 mM CaCl_2 (Merck, Germany) in digestion buffer (DMEM 4.5 g/L glucose, 2% PS-A) to a final volume of 10 ml. Tubes were incubated at 37°C under constant agitation for 30 minutes,

followed by mechanical dissociation through repeated pipetting. After an additional 20-minute incubation, a second dissociation step was performed. Digestion was stopped by adding culture medium (DMEM 4.5 g/L glucose, 10% FBS, 2% PS-A). Samples were centrifuged at $200 \times g$ for 10 minutes; the supernatant was discarded, and the pellet was pooled. The pellet was washed twice—first with digestion buffer, then with culture medium—with centrifugation at 200 rcf for 10 minutes after each wash, and finally resuspended in 5 ml of culture medium. For quantification, 50 μ l of the suspension were plated in a 96-well plate, a 1:10 dilution was prepared, and vessels were counted under a microscope as individual microvascular fragments (MVs).

Angiogenic potential evaluation of MVs (2D and 3D)

For 2D assessment, a total of 2,000 MVs were seeded onto 24-well plates, and complete medium (DMEM, 4.5 g/L glucose, 10% FBS, 2% PS-A) was added to a final volume of 500 μ L per well. Samples were maintained under standard culture monolayer conditions and monitored by optical microscopy for up to 7 days. For 3D collagen hydrogel seeding, 2,000 MVs were suspended in 500 μ L of rat tail collagen type I hydrogel at a final concentration of 3 mg/mL. To prepare the gel, a 2 $\times$ PBS solution supplemented with 100 mM HEPES was mixed 1:1 (v/v) with the rat tail collagen stock solution, prepared as described in our previous work [132]. The MVF-containing medium was then added to obtain the desired final collagen concentration. All components were kept sterile and ice-cold throughout preparation to prevent premature polymerization. The suspension was dispensed into 24-well plates and allowed to polymerize at 37°C for 30 minutes. After polymerization, complete medium was added to the gels, which were then incubated under standard culture conditions for 7 days.

Live/Dead assay

The Live/Dead assay is a fluorescence-based method used to assess cell viability by simultaneously detecting live and dead cells within a sample. The assay relies on two dyes with distinct properties: Fluorescein Diacetate (FDA), a non-fluorescent compound converted into green-fluorescent fluorescein by intracellular esterases in viable cells, and Propidium Iodide (PI), a red-fluorescent nucleic acid stain that penetrates only cells with

compromised membranes, thereby labeling dead cells. For the assay, a staining solution was prepared in PBS with final concentrations of 5 $\mu\text{g/ml}$ FDA and 1 $\mu\text{g/ml}$ PI. The solution was mixed thoroughly and stored in amber tubes to protect it from light until use. FDA was excited at approximately 488 nm, with emission collected between 515–530 nm (green channel), while PI was excited at around 535 nm, with emission collected near 617 nm (red channel). Fluorescence images obtained from this procedure were used to evaluate cell viability by distinguishing live (green) and dead (red) cells.

Seeding MVFs on scaffolds

PLLA scaffolds were pre-sterilized as previously described for RDF seeding. When required, scaffolds were coated with Geltrex following the same procedure detailed in that section. Before cell seeding, both sterile uncoated and Geltrex-coated scaffolds were equilibrated in complete medium. For cell seeding, 10 μL of a suspension containing 5,000 MVFs were pipetted onto the surface of each scaffold. MVFs were seeded on uncoated scaffolds, on Geltrex-coated scaffolds, and on Geltrex-coated scaffolds with medium supplemented with 10 ng/mL VEGF. Scaffolds were incubated at 37°C for 1 hour to allow cell attachment. After this initial adhesion period, the scaffolds were transferred to individual wells of 24-well plates, and complete medium was added to each well to cover the scaffolds. The constructs were maintained under standard culture conditions for 14 days, with medium changes performed periodically to provide fresh nutrients and sustain cell viability.

Coculture seeding

PLLA scaffolds were pre-sterilized as previously described for RDF seeding. When required, scaffolds were coated with Geltrex following the same procedure reported in that section. Sterile uncoated or Geltrex-coated scaffolds were equilibrated in complete medium before cell seeding. A total of 10 μL containing 300,000 RDFs and 5,000 MVFs was pipetted onto the surface of each scaffold and incubated at 37°C for 1 hour to allow cell attachment. After this initial adhesion period, the scaffolds were transferred to 24-well plates. Complete medium supplemented with 10 ng/mL EGF and VEGF was gently added to each well to cover the scaffolds. The constructs were maintained under standard culture conditions for 14 days, with medium changes performed as needed to provide fresh nutrients and sustain cell viability.

DNA Quantification by CyQuant Assay

DNA content in cell-seeded scaffolds was quantified using the CyQuant assay. Scaffolds stored at -80°C after 1, 7, and 14 days of culture were thawed at room temperature. Cell lysis was performed by incubating each scaffold with 300 μL of lysis buffer composed of 0.1 M dipotassium hydrogen phosphate (K_2HPO_4), 10 mM ethylenediaminetetraacetic acid (EDTA), and 55 mM sodium citrate, supplemented with Proteinase K (PK) at a 1:40 dilution (7.5 μL PK and 292.5 μL lysis buffer). Samples were vortexed thoroughly and incubated overnight at 56°C , either in a thermomixer or in a drying oven, depending on the number of samples. Following incubation, samples were frozen at -80°C for 2 hours, then thawed and centrifuged at 10,000 rpm to clarify the lysates. Lysates were either processed immediately or stored at -80°C for subsequent analyses. For the CyQuant assay, 10 μL of each supernatant was diluted with 150 μL of TE buffer. Aliquots of 25 μL of each diluted sample were pipetted in triplicate into black 96-well plates. The CyQuant staining solution was prepared immediately before use by mixing molecular biology-grade water, $5\times$ Hank's Balanced Salt Solution (HBSS), and CyQuant dye, and 25 μL of this solution was added to each well in a 1:1 volume ratio. A DNA standard curve was generated by serially diluting a 1 mg/mL DNA stock solution to concentrations ranging from 6400 to 0 ng/mL and was processed in triplicate under identical conditions. Plates were covered with aluminum foil and incubated at 37°C for 1 hour. Fluorescence was measured using a Protean Infinite M200 plate reader with excitation at 485 nm and emission at 530 nm, and DNA content was quantified from the standard curve.

Quantification of Sulfated Glycosaminoglycans (sGAGs)

Sulfated glycosaminoglycan (sGAG) content in seeded scaffolds was quantified using the 1,9-dimethylmethylene blue (DMMB) colorimetric assay. This method relies on the specific binding of the DMMB dye to sulfated groups on GAGs, which can be measured spectrophotometrically. The DMMB stock solution was prepared by dissolving 3.1 mg of DMMB in 1 mL of molecular biology-grade ethanol. A working staining solution was obtained by adding 375 μL of the stock solution to 49.5 mL of DMMB color buffer and stored in amber tubes at 4°C for up to three weeks. A calibration curve was generated using chondroitin sulfate standards prepared from a 10 mg/mL stock solution in 0.9% NaCl at concentrations of 50, 40, 30, 20, 10, 5, and 0 ng/mL. The same lysates used for DNA quantification were employed for sGAG analysis. Specifically, 8 μL of each supernatant

were diluted with 72 μL of PBE buffer (100 mM NaHPO_4 supplemented with 5 mM DTaP , pH 8). Aliquots of 20 μL of each diluted sample were pipetted in triplicate into a white 96-well plate, and 180 μL of DMMB staining solution was added to each well immediately before measurement. Absorbance was measured at 525 nm with a reference wavelength of 595 nm using a Protecan Infinite M200 plate reader. sGAG content was calculated based on the calibration curve. For this experiment, nine samples were analyzed, including coated and uncoated scaffolds seeded with fibroblasts from donors 2, 10, and 11 at days 1, 7, and 14.

RESULTS AND DISCUSSION

This section presents the experimental steps undertaken to develop the proposed 3D tissue-engineered platform. To enable project realization, it was first necessary to obtain and validate three fundamental components: three-dimensional PLLA scaffolds, primary rat dermal fibroblasts (RDFs), and MVFs isolated from adipose tissue. The establishment and optimization of reliable protocols for scaffold fabrication, cell isolation, and culture, therefore, represented an essential preliminary phase. This section will first characterize each component under single-culture conditions to assess cell viability, morphology, and phenotypic stability within the 3D environment. It will then focus on the development and analysis of co-cultures of RDFs and MVFs on PLLA scaffolds, investigating cell–cell interactions, spatial organization, extracellular matrix deposition, and vascular network formation. After demonstrating the feasibility of the system using rat-derived cells, the discussion will shift to human dermal primary fibroblasts to evaluate the platform's translational potential.

Primary Rat Dermal Fibroblasts (RDFs) Isolation

At first, a reliable primary fibroblast isolation protocol was established. Because the construct was designed for skin regeneration, fibroblasts were isolated from rat dermis. Two complementary strategies were evaluated: the explant method and the enzymatic digestion method.

Explant Method

The explant method is a classical approach for isolating primary fibroblasts, which relies on the intrinsic ability of dermal fibroblasts to migrate from small tissue fragments and adhere to the culture dish surface. In our setting (Figure 8), skin from the abdominal region was shaved, collected, cleaned, and trimmed into approximately 0.5-cm pieces. After removal of the hypodermis, skin fragments were positioned with the dermal side down to maximize cell–surface contact, while the epidermal layer was intentionally left exposed. Only a minimal volume of medium was added, allowing the tissue to adhere firmly to the plate before full immersion.

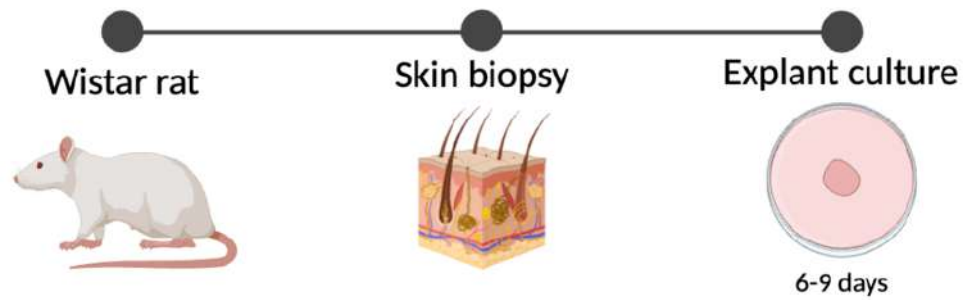


Figure 8. Explant method phases.

Over time, fibroblasts enzymatically remodeled the surrounding ECM and migrated outward, typically becoming detectable after 6 to 9 days. This migration led to the progressive formation of a monolayer of thin, spindle-shaped cells radiating from the explant. The resulting cultures exhibited the expected morphology of dermal fibroblasts, and no other contaminating cell types were observed (Figure 9). We referred to these cells as primary rat dermal fibroblasts (RDFs).

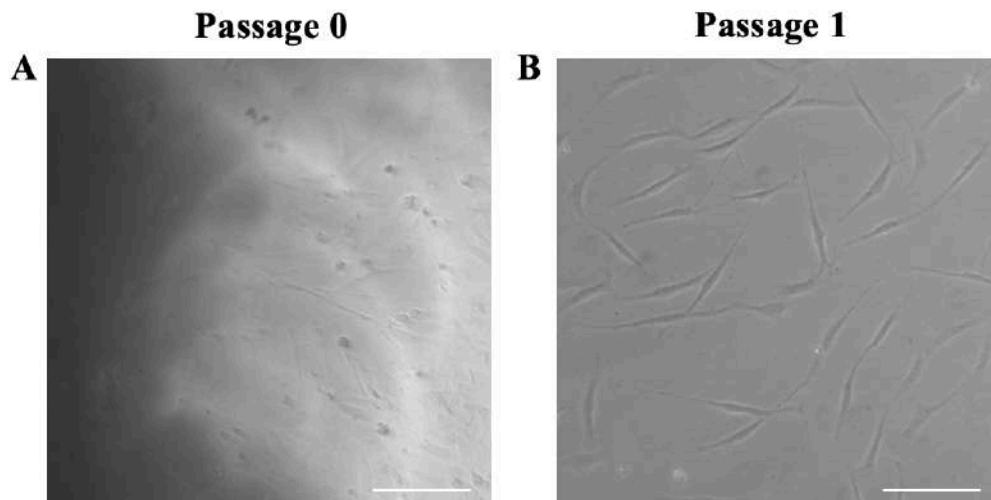


Figure 9. **A.** Primary rat dermal fibroblasts (RDFs) emerging from the skin after explant culture (passage 0). **B.** Primary RDFs monolayer culture in the first passage. Scale bars: 100 μ m.

Enzymatic Method

In parallel, an enzymatic isolation protocol was optimized. While explant culture relies on the spontaneous outward migration of fibroblasts from intact tissue fragments, enzymatic digestion disrupts ECM components, thereby accelerating cell release and reducing the time required to obtain a primary culture. For this purpose, ultrapure recombinant collagenases G and H derived from *Clostridium* species were employed in combination with Thermolysin

to achieve efficient, reproducible dissociation of the dermal ECM. Several experimental conditions were evaluated, including varying enzyme concentrations and digestion times, ultimately yielding an optimized protocol, as described in the Materials and Methods section (Figure 10).

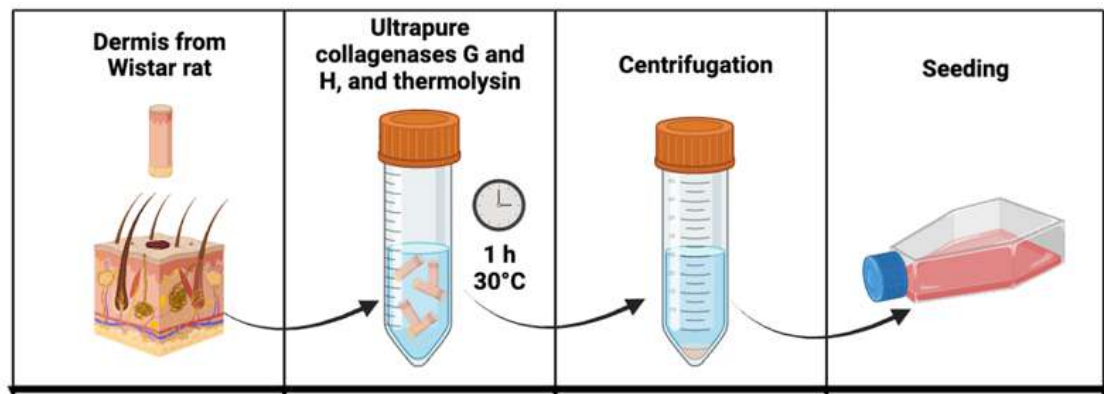


Figure 10. Enzymatic method phases.

Following processing, cells began to attach to the culture plates and migrate out from micro-tissue residues within 1 to 4 days. At this stage, the resulting cell population exhibited marked morphological heterogeneity, as expected given the variety of dermal cell types released during enzymatic digestion. This heterogeneity is evident in Figure 11, where optical microscopy images taken from different regions of the same plate (same donor) reveal cells with diverse morphologies. Specifically, the spindle-shaped cells shown in Figure 11B are consistent with fibroblast-like morphology, whereas the cells observed in Figures 11A, 11C, and 11D appear larger, flattened, and more spread, likely representing other dermal cell types. These latter cells may resemble keratinocytes, which are typically lost during culture because they require specialized growth conditions.

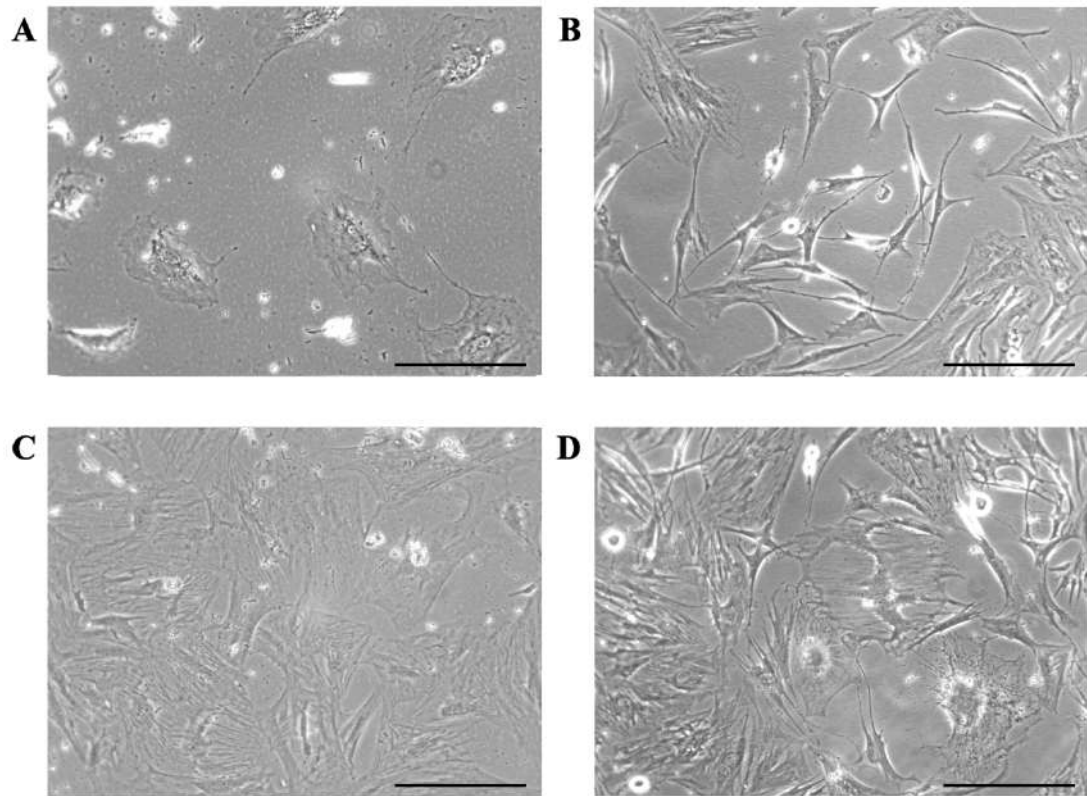


Figure 11. A-B-C-D. Different morphologies of enzymatically isolated rat dermal fibroblasts at passage 0 in monolayer culture. Scale bar: 100 μm .

Nevertheless, progressive passaging selectively favored the expansion of fibroblast-like cells. By the third passage, the culture had become largely enriched in thin, elongated cells exhibiting the characteristic spindle-shaped morphology of dermal fibroblasts, which emerged as the predominant population (Figure 12).

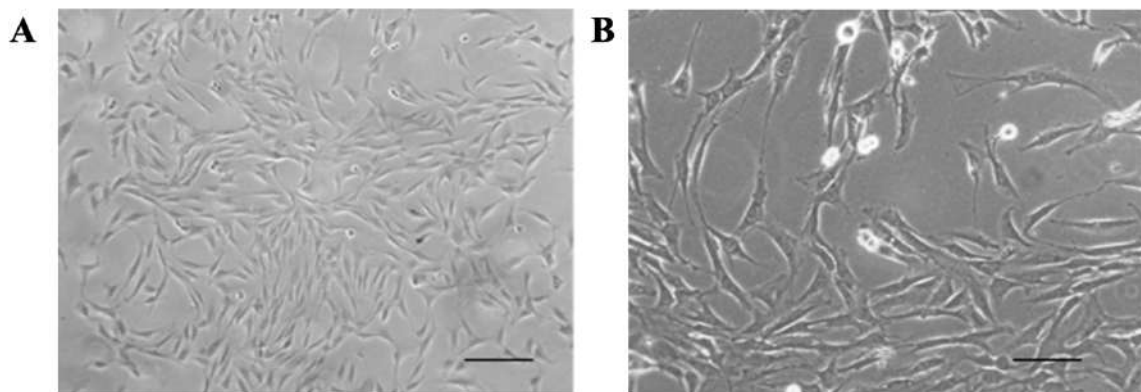


Figure 12. Primary rat dermal fibroblasts (RDFs) at passage 3 at 50 X (A – scale bar: 200 μm) and 100X (B – scale bar: 100 μm) magnification.

Characterization

Immunostaining confirmed the identity of cells obtained using both the explant and enzymatic isolation methods, as cultures derived from either protocol showed positive expression of collagen type I and vimentin. Figure 13 shows fibroblasts isolated by enzymatic digestion and analyzed by immunostaining. Notably, collagen type I showed a pronounced perinuclear signal associated with the rough endoplasmic reticulum region, a pattern often associated with active protein synthesis and intracellular processing.

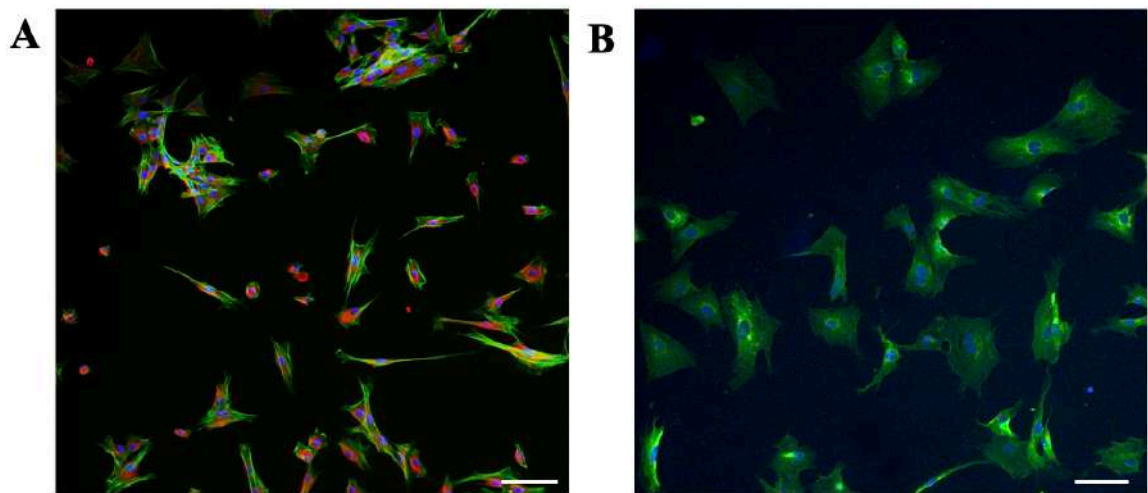


Figure 13. Rat dermal fibroblasts (RDFs) immunostained for (A) **Phalloidin/Vimentin/DAPI** and (B) **Collagen type I and DAPI**. Scale bars: 100 μm .

Altogether, these results confirmed that both isolation methods ultimately yielded dermal fibroblasts with a stable mesenchymal identity. The explant method, although slower and requiring longer culture times before cells emerged, produced a highly pure fibroblast population with minimal contamination with other dermal cell types. In contrast, enzymatic digestion enabled faster and higher initial cell recovery, but initially yielded a more heterogeneous population. Serial passaging effectively enriched fibroblasts, producing cultures with phenotypes comparable to those obtained via explant outgrowth. Despite these differences, cells isolated with both methods converged toward a fibroblast-dominated population, characterized by a spindle-shaped morphology and robust expression of vimentin and collagen type I, as confirmed by immunofluorescence. The pronounced perinuclear localization of collagen type I indicated active synthesis and intracellular processing, consistent with fibroblasts' role as the primary producers of ECM components in the dermis. Overall, these observations highlighted the functional equivalence of fibroblasts obtained by either approach and indicated that the choice of isolation method

could be guided by experimental priorities, such as purity, speed, or scalability, without compromising cellular identity or matrix-producing capacity. Fibroblasts obtained using both methods were used for subsequent experiments at passages 3–8.

Poly-L-Lactic Acid (PLLA) scaffolds fabrication and characterization

Although human and murine skin share similar healing phases, major structural differences strongly affect wound repair outcomes. Murine skin is thinner, loosely attached to underlying tissues, and contains the panniculus carnosus, a subcutaneous muscle layer virtually absent in humans. This structure confers a high contractile capacity, leading to up to 90% of mouse wounds closing predominantly by contraction. In contrast, human skin is firmly anchored to subcutaneous tissues and heals mainly through granulation tissue formation and re-epithelialization. These biomechanical differences limit the direct translation of murine wound-healing data to humans. Therefore, careful interpretation and adapted experimental models are required in translational studies [133]. Different strategies have been employed in the literature to address this issue, such as dorsal skin chambers that stretch the skin and limit wound contraction [134]. One approach is to use a mechanically resistant scaffold that remains securely in place and withstands contractile forces. Soft or low-resistance materials are prone to displacement, which could compromise the assessment of tissue regeneration. This requirement for structural stability may be equally critical in human applications, particularly for chronic ulcers located in high-pressure areas such as the feet or lower legs, where weak or easily deformable scaffolds risk dislocation or extrusion.

To meet these demands, PLLA was selected as the scaffold material. PLLA combines well-established biocompatibility, biodegradability, and inertness with the mechanical rigidity necessary to counteract contractile forces, maintain scaffold positioning, and provide a stable platform for tissue regeneration. Additionally, the natural contraction of the surrounding skin can help anchor the scaffold in place, ensuring consistent positioning and enabling unbiased evaluation of regenerative performance. Its intrinsic robustness, together with the ability to customize scaffold shape and porosity, ensures both long-term stability and functional integration, making PLLA scaffolds highly suitable for preclinical studies and, potentially, for in vivo therapeutic applications.

Scaffolds were fabricated using the TIPS technique, as detailed in the Materials and Methods section. After polymer dissolution and controlled cooling, the process yielded cylindrical PLLA matrices. By modulating the demixing time and temperature, five distinct scaffold types were generated, each characterized by a specific average pore size: approximately 50 μm (20 $^{\circ}\text{C}$ for 15 min), 100 μm (25 $^{\circ}\text{C}$ for 15 min), 250 μm (28 $^{\circ}\text{C}$ for 30 min), 300 μm (25 $^{\circ}\text{C}$ for 45 min), and 400 μm (32 $^{\circ}\text{C}$ for 30 min). The cylindrical scaffolds were sliced into 2 mm-thick sections to match the average dermal thickness of rats [135], and subsequently shaped using a 6 mm biopsy punch to obtain uniform discs (Figure 14).



Figure 14. Shaping of poly-L-lactic acid scaffolds with 100 μm pore size. From left to right: the initially produced cylindrical scaffold, followed by 2 mm-thick slices. After punching with a 6 mm biopsy punch, the final scaffolds were obtained.

The scaffold groups most extensively used in subsequent experiments (100, 250, and 300 μm) were also examined by scanning electron microscopy (SEM) to characterize their porous structures at high magnification, thereby validating the pore dimensions (Figure 15).

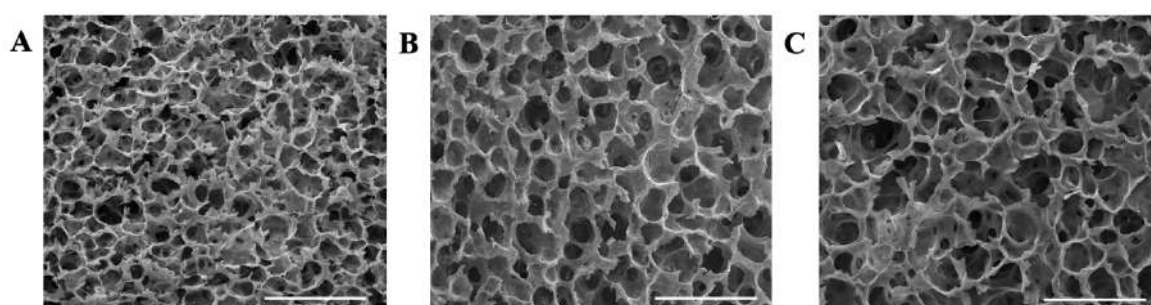


Figure 15. SEM analysis of 100 (A), 250 (B), and 300 (C) μm pore sized poly L lactic acid scaffolds. Scale bar: 300 μm .

In summary, PLLA scaffolds with varying pore sizes were successfully fabricated via TIPS, yielding cylindrical matrices with dimensions compatible with rat dermal thickness. SEM analysis of the most frequently used scaffolds, with pore sizes of 100, 250, and 300 μm , confirmed their porous architecture in greater detail.

Overall, the fabrication process demonstrated good reproducibility and structural uniformity across the entire scaffold. This well-characterized scaffold library provides a reliable platform for the systematic investigation of how microstructural features influence cell behavior and tissue formation in subsequent studies.

Seeding of RDFs on PLLA scaffolds and analyses

Seeding protocol optimization

To determine whether the scaffold architecture supports cell adhesion and proliferation, we first conducted preliminary experiments using the murine NIH/3T3 fibroblast cell line before transitioning to primary dermal fibroblasts. NIH/3T3 cells, an immortalized mouse embryonic fibroblast cell line commonly used as a model system, were seeded onto the scaffolds at a density of 150,000 cells per scaffold (scaffold area: 0.283 cm²), corresponding to a seeding density of 5.3×10^5 cells/cm². The standard seeding protocol, described in the Materials and Methods, involved depositing a defined number of cells in 10 μ l of medium directly onto the scaffold surface, incubating at 37°C to allow cell attachment, then adding additional culture medium for a longer incubation time.

The initial set of assays focused on the 100 μ m and 250 μ m pore-size scaffolds. Cell viability, metabolic activity, and proliferation were evaluated using the Alamar Blue assay. A large number of cells adhered within the first 24 hours. Both scaffold types supported a clear increase in cell number over the 5-day culture period (Figure 16). These results demonstrated that the scaffold material and morphology were compatible with fibroblast growth, providing a foundation for moving toward primary cell experiments. Interestingly, although cells grew on both scaffold types, the 250 μ m pore-size scaffolds supported better cell growth. In fact, on day 1, the number of attached cells was slightly lower than that observed on the 100 μ m pore-sized scaffolds, but after 5 days of culture, the cells reached an higher numbers in the 250 μ m condition.

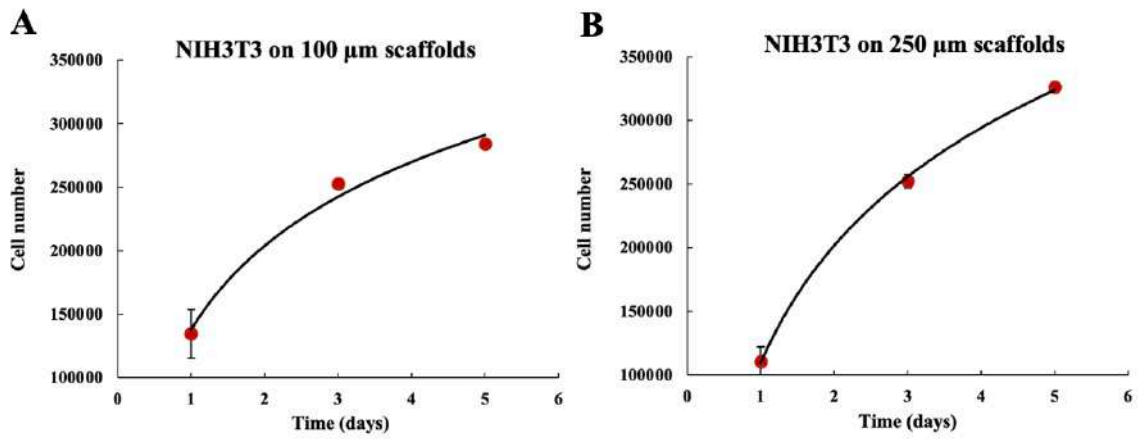


Figure 16. NIH3T3 growth curves (Alamar Blue) onto 100 µm (A) and 250 µm (B) pore sized poly L lactic acid scaffolds over 5 days. 150,000 seeded cells/scaffold. N = 3 replicates.

We then applied the same experimental setup to primary RDFs, seeding 150,000 cells on a single side of the scaffold and assessing proliferation at days 1, 3, and 5 for both 100 µm and 250 µm pore sizes (Figure 17). On day 1, a limited number of cells adhered to the scaffold surface, with a markedly lower abundance than that of NIH/3T3 fibroblasts, indicating reduced initial attachment efficiency. Over the subsequent culture period, the number of adherent cells progressively decreased rather than increasing, regardless of pore size, suggesting impaired cell growth. This trend may reflect reduced cell survival within the scaffold environment and/or a progressive detachment of cells from the scaffold surface over time, ultimately preventing stable colonization of the construct.

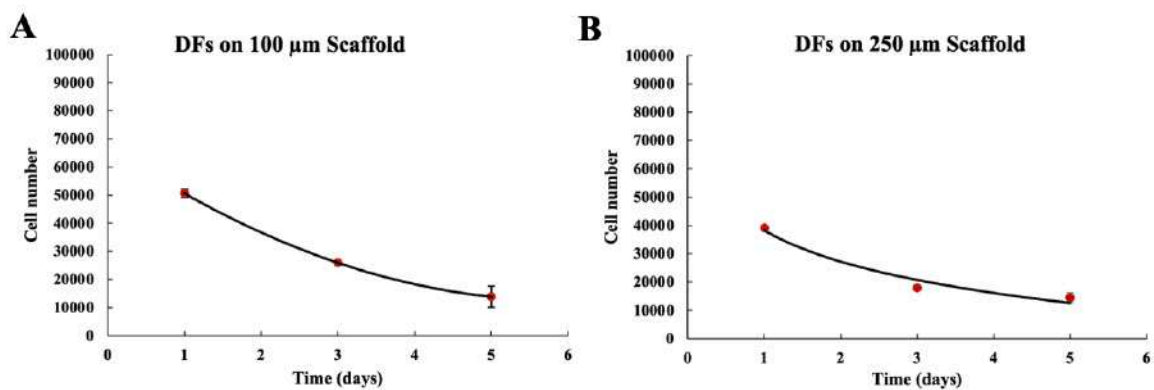


Figure 17. Rat dermal fibroblasts (DF) growth curves (Alamar Blue) onto 100 µm (A) and 250 µm (B) pore sized poly L lactic acid scaffolds over 5 days. 150,000 seeded cells/scaffold. N = 3 replicates.

The contrasts with NIH/3T3 behavior highlight intrinsic differences between immortalized cell lines and primary cells, which often exhibit greater sensitivity to the three-dimensional scaffold environment. For this reason, successful seeding of primary RDFs onto PLLA

scaffolds required careful optimization of both the cell culture method and culture conditions to ensure effective adhesion, proliferation, and long-term colonization.

To investigate whether a longer culture duration could reveal delayed cellular adaptation to the scaffold environment, the subsequent analysis was extended to day 7, using the same 100- and 250- μm pore-size scaffolds and the same seeding density (150,000 cells). In addition, the scaffolds were coated with type I collagen before cell seeding in an attempt to enhance cell–substrate interactions. Collagen coating resulted in improved early cell attachment on both scaffold types. On day 1, the number of adherent cells was higher on the 100 μm pore-sized scaffolds than on the 250 μm ones; however, at subsequent time points, the progressive decrease in cell number followed a comparable trend in both conditions. Overall, despite the initial improvement in adhesion, cell numbers declined over time in all experimental groups, indicating that neither prolonged culture nor collagen coating was sufficient to sustain stable cell growth on these scaffolds (Figure 18).

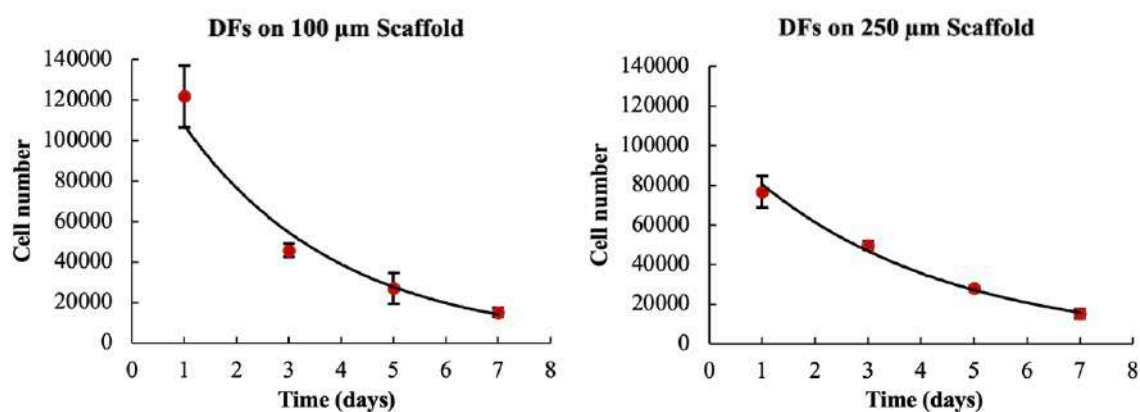


Figure 18. Rat dermal fibroblasts (DF) growth curves (Alamar Blue) onto 100 μm (A) and 250 μm (B) pore sized collagen-coated poly L lactic acid scaffolds over 7 days. 150,000 seeded cells/scaffold. N = 3 replicates.

Since pore size can influence nutrient diffusion, mechanical anchoring, and infiltration capacity, we broadened the analysis to include scaffolds with both smaller (50 μm) and larger (400 μm) pores. However, across all pore sizes (Figure 19) and up to 7 days of culture, primary fibroblasts continued to decrease in number, suggesting that pore size was not the determining factor limiting their proliferation under these conditions.

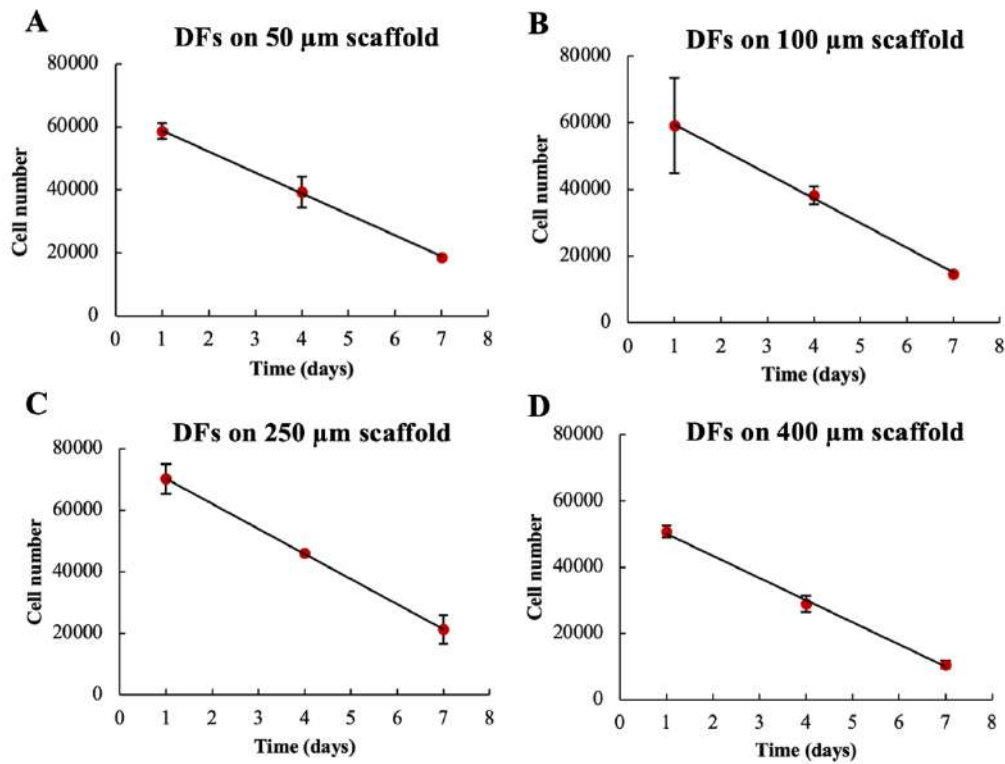


Figure 19. Rat dermal fibroblasts (DFs) growth curves (Alamar Blue) onto 50 (A), 100 (B), 250 (C), and 400 (D) μm pore-sized poly L lactic acid scaffolds over 7 days. 150,000 seeded cells/scaffold. N = 3 replicates.

We then modified the seeding strategy by seeding 150,000 cells on both sides of the scaffold (75,000 per side) to improve access to available attachment surfaces and reduce local cell crowding during seeding. For this test, we used only the 100 μm pore-sized scaffold, which was selected as the model condition for subsequent experiments. Under this configuration, a distinct change in the growth trend was observed for the first time across all tested conditions. Rather than showing a strictly decreasing pattern, cell numbers remained relatively stable from Day 1 to day 4, indicating improved early cell retention and survival. Only between Days 4 and 7 did a decline in cell number become evident (Figure 20). Although this outcome represented a partial improvement over previous seeding strategies, it remained insufficient to support sustained cell growth over longer culture periods.

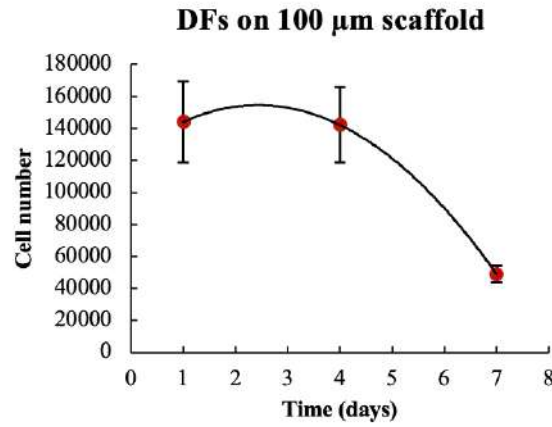


Figure 20. Rat dermal fibroblast (DFs) growth curve (Alamar Blue) onto 100 µm pore-sized poly L lactic acid scaffolds over 7 days (Bilateral seeding). 75.000 seeded cells/scaffold side (150.000 in total). N = 3 replicates.

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Consequently, we investigated whether increasing the initial seeding density could compensate for the early cell losses observed under previous conditions. To this end, 300.000 cells per scaffold (scaffold area: 0.283 cm², corresponding to a seeding density of approximately 1.06×10^6 cells/cm²) were seeded onto one side of the 100 µm pore-sized scaffold. Under these conditions, a clear change in cell behavior was observed. Cell numbers progressively increased between Days 1, 4, and 7, rising from approximately 90,000 to approximately 150,000 cells, indicating that a higher initial cell density supported cell expansion and survival within the scaffold environment (Figure 21). This outcome confirmed that initial cell density is a critical determinant for establishing a viable primary fibroblast population.

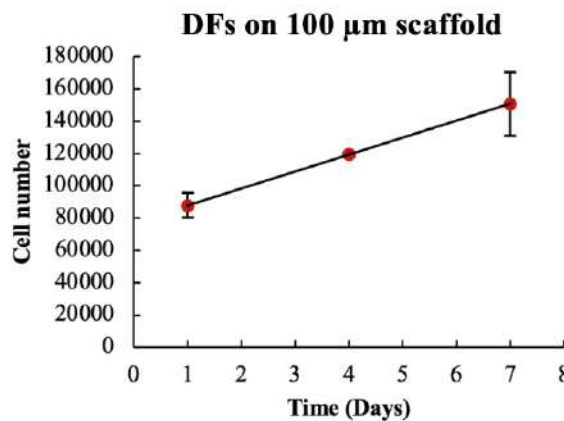


Figure 21. Rat dermal fibroblasts (DFs) growth curve (Alamar Blue) onto 100 µm pore-sized poly L lactic acid scaffolds over 7 days. 300.000 seeded cells/scaffold. N = 3 replicates.

The scaffolds cultured for 7 days were fixed in glutaraldehyde and processed for SEM analysis. The resulting micrographs (Figure 22) revealed extensive cellular coverage across the scaffold surface. At lower magnification, large regions of the matrix appeared completely covered by a continuous layer of cells, which spanned and occluded the underlying porous architecture. This visual evidence confirmed that fibroblasts firmly adhered, spread, and formed multilayered structures on the scaffold surface.

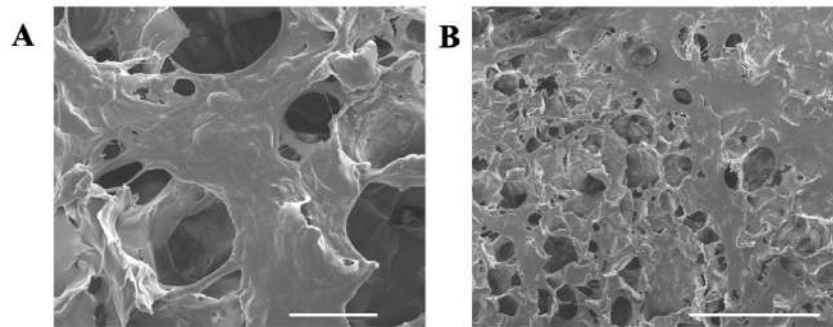


Figure 22. Scanning electron microscopy (SEM) images showing rat dermal fibroblast growth on poly-L-lactic acid (PLLA) scaffolds with a pore size of 100 μm after 7 days of culture, acquired at higher (**A**; scale bar: 50 μm) and lower (**B**; scale bar: 300 μm) magnifications.

However, this outcome was not always reproducible, as most subsequent attempts failed to replicate the same growth trend, suggesting that the improvement was stochastic rather than mechanistically driven.

We subsequently hypothesized that the scaffold environment might require additional biochemical support. Therefore, we supplemented the culture medium with EGF at 10 ng/mL, a mitogen that stimulates fibroblast proliferation, and with ascorbic acid. The supplemented medium was used for a new set of assays performed on scaffolds with pore sizes of 100, 250, and 300 μm , with metabolic activity assessed at days 1, 7, and 14. Under these conditions, primary fibroblasts exhibited sustained, progressive growth across all scaffold types, indicating that supplementation effectively promoted long-term proliferation and enabled the scaffold architecture to support primary cell expansion (Figure 23).

In matrices with 100 μm pores (Figure 23 A), cell proliferation followed a distinctly biphasic trend. During the first seven days, fibroblasts expanded rapidly, with metabolic activity increasing exponentially and cell density reaching the highest value among all tested conditions. After this initial burst, however, the growth curve transitioned into a plateau

phase, with no further substantial increase in cell number between days 7 and 14. This stabilization suggests that the smaller pore network may become saturated over time, effectively limiting nutrient and oxygen diffusion to the underlying layers and restricting further cellular expansion.

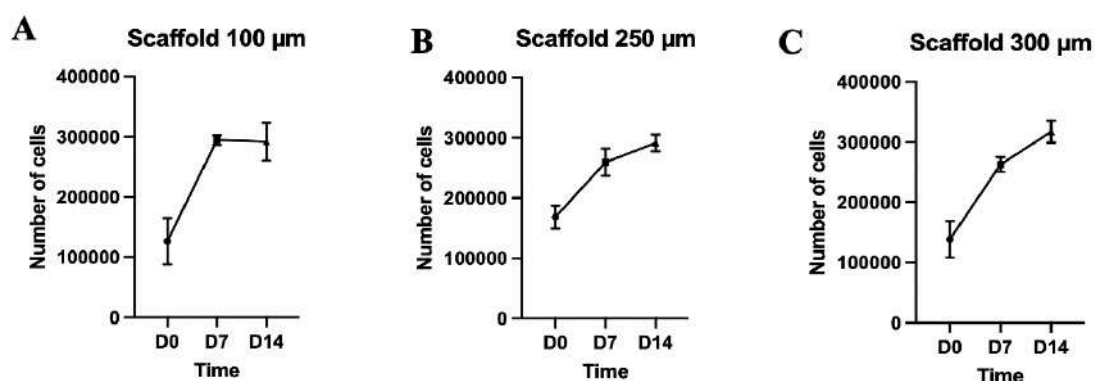


Figure 23. Rat dermal fibroblast growth curve (Alamar Blue) onto 100 μm (A), 250 μm (B), and 300 μm (C) pore-sized poly L lactic acid scaffolds over 14 days. 300,000 seeded cells/scaffold. N = 3 replicates.

In contrast, scaffolds with larger pore sizes (250 and 300 μm – Figures 23 B-C) supported a slower but steadily increasing cell number throughout the 14-day period. Rather than exhibiting an early exponential phase followed by stagnation, these matrices supported a more linear and sustained proliferation profile. Among these conditions, the 300 μm scaffolds consistently yielded the most significant overall increase from day 1 to day 14, indicating that this architecture provides the most favorable environment for long-term fibroblast expansion. Possibly, the largest pores allowed improved nutrient perfusion over time, resulting in more extensive cell growth. Consequently, the 300 μm pore-sized scaffold was selected as the optimal configuration for all subsequent experimental settings. These results underscore the importance of combining structural and biochemical optimization to support primary cell expansion in three-dimensional constructs.

300 μm scaffolds cultured up to 14 days were fixed for confocal immunostaining analysis. A 300- μm -thick scaffold slice was stained with **phalloidin-FITC** to visualize the actin cytoskeleton and with **DAPI** to label nuclei. Confocal imaging revealed predominantly spherical cellular aggregates distributed throughout the scaffold (Figure 24). These aggregates likely represent clusters of fibroblasts occupying and filling individual pores, thereby confirming that cells not only adhered to the scaffold surface but also infiltrated and colonized its internal porous architecture.

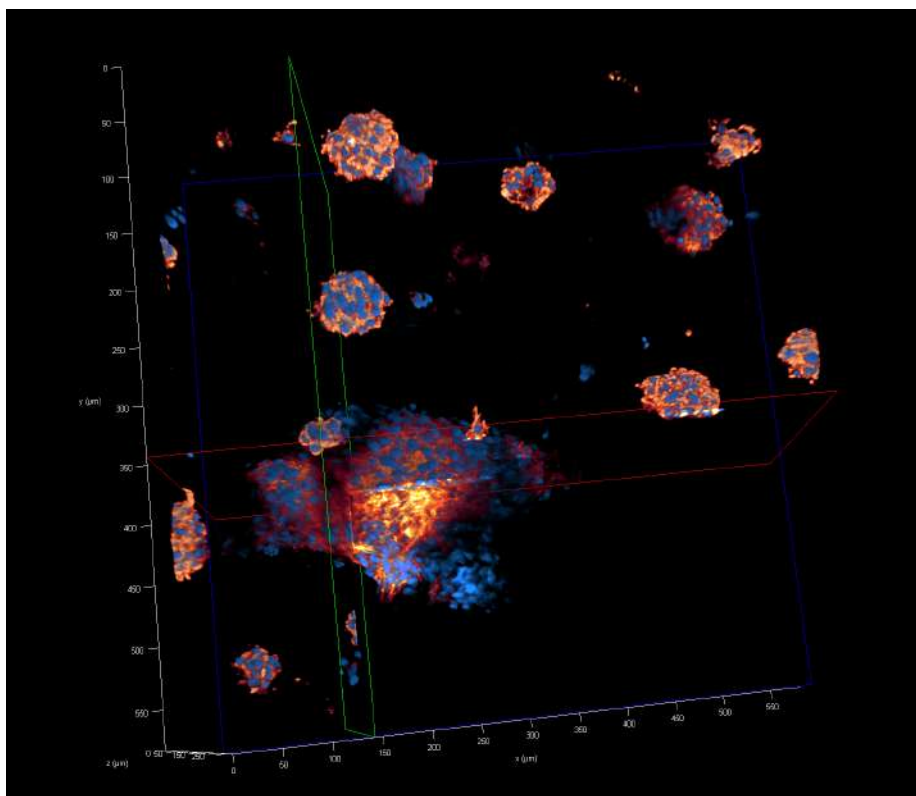


Figure 24. Immunostaining with **Phalloidin-FITC** and **DAPI** on 300 μm pore-sized poly L lactic acid scaffolds seeded with rat dermal fibroblasts.

Medium optimization

To further refine the culture conditions, we evaluated 16 different culture media for fibroblast growth in 2D culture. All formulations shared the same base medium (10% KM), consisting of DMEM/F12 supplemented with 1% antibiotics/antimycotics, 10% FBS, and ascorbic acid. To this baseline medium, EGF, FGF, hydrocortisone, and insulin were added individually or in all possible combinations, yielding 15 supplemented conditions plus one negative control (base medium only). After 4 days of culture, fibroblasts grown in the 16 formulations displayed four distinct phenotypic patterns upon microscopic observation, providing an initial indication of how each factor, alone or in combination, influenced cell morphology and growth behavior. Representative examples of each phenotypic pattern are shown in Figure 25.

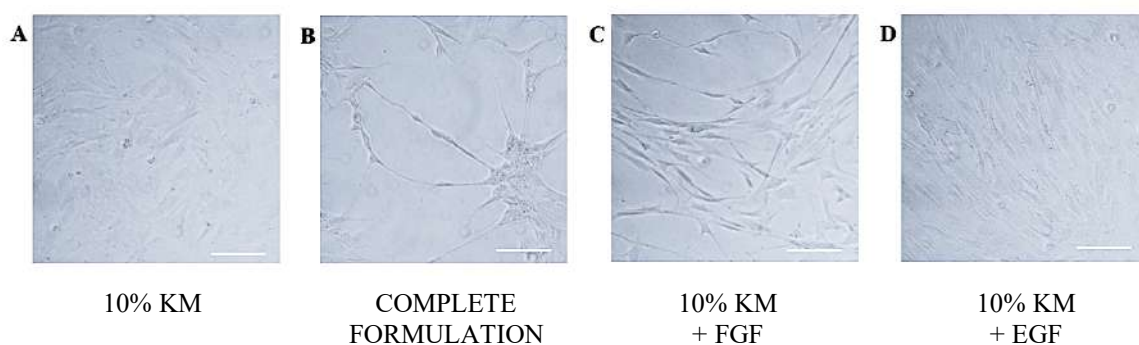


Figure 25. Optical microscopy images of rat dermal fibroblasts cultured in monolayer culture with: **A.** base medium (10% KM); **B.** medium supplemented with all additional components; **C.** base medium + fibroblast growth factor (FGF); and **D.** base medium + epidermal growth factor (EGF). Scale bar: 100 μ m.

Specifically, the base medium, as well as the formulation containing insulin alone, generated cultures in which fibroblasts appeared spread, large, and flattened, with an irregular morphology. When hydrocortisone was included in the medium, either alone or in combination with other additives (including the complete formulation), the cells reduced their adhesion to the substrate and began to contract, reorganizing into compact multicellular aggregates. Thin cytoplasmic extensions connecting the cells persisted, forming delicate intercellular bridges, while empty spaces appeared between the clustered regions.

In media containing FGF, cells assumed a very thin, elongated, and filamentous shape, displaying the classical spindle-like morphology typical of fibroblasts. The culture surface exhibited large empty areas, reflecting the low apparent confluence, possibly due to the extremely slender cytoplasm, even though the total cell number was not dramatically reduced. The nucleus occupied most of the cell body, while the cytoplasmic extensions were extremely delicate and extended across the surface.

In media containing EGF, cells retained an elongated shape and a coherent spatial alignment, but the extreme thinness observed with FGF was markedly reduced. Cells adhered more strongly and formed continuous monolayers, resulting in cultures with higher apparent confluence and increased cell density.

Based on these distinct morphological outcomes, four representative conditions were selected for further analysis on scaffolds: the base medium, the fully supplemented medium, the EGF-supplemented medium, and the FGF-supplemented medium. Cells grown under each of these conditions were seeded onto 300 μ m pore-sized scaffolds and examined after 1 and 14 days of culture using the **Live/Dead** assay, which provided information on both

viability and the spatial pattern of scaffold colonization. The results (Figure 26) showed that fibroblasts responded differently depending on the culture medium, and each condition produced a characteristic pattern of survival and distribution within the scaffold architecture.

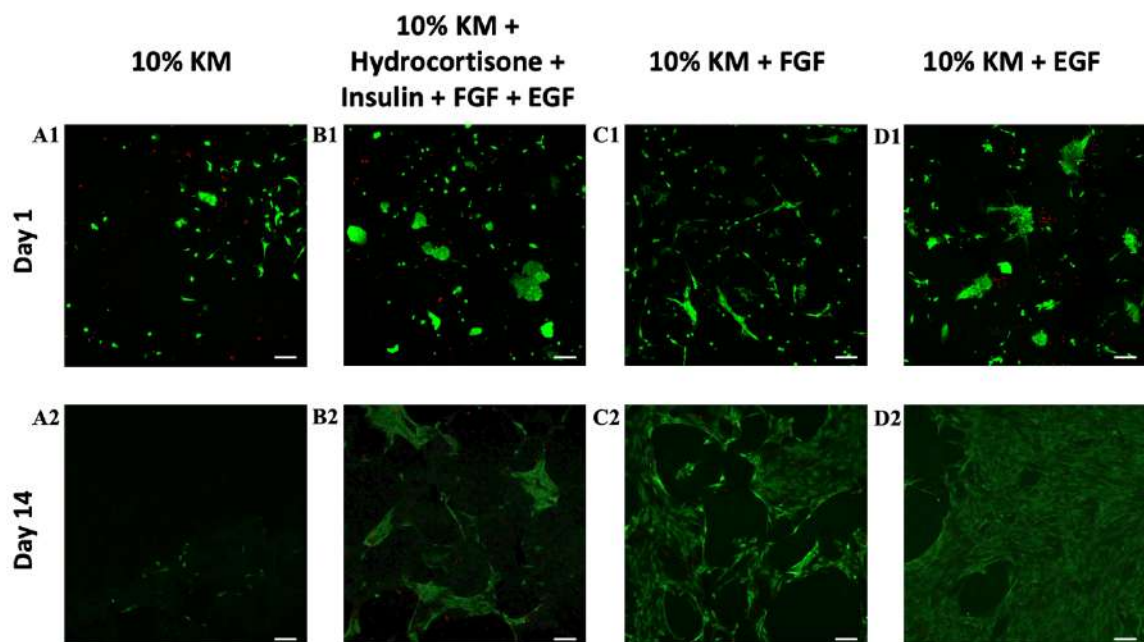


Figure 26. Live/Dead assay of rat dermal fibroblasts cultured in 300 µm pore-sized poly L lactic acid scaffolds treated with **A.** base medium (10% KM); **B.** medium supplemented with all additional components; **C.** base medium + fibroblast growth factor (FGF); and **D.** base medium + epidermal growth factor (EGF) after 1 (A1-D1) and 14 (A2-D2) days. Scale bars: 100 µm.

When the base medium was used, after 24 hours of seeding, only a few cells were observed on the scaffold. In some instances, small aggregates formed along with red-stained cells, indicating cell death. After 14 days, the scaffold surface contained very few cells, considerably fewer than at seeding, indicating a decrease in cell number over time.

In the fully supplemented medium, a substantial number of cells were present on the scaffold after 24 hours, with some dead cells visible. In most cases, the cells formed large spherical aggregates on the scaffold surface. By day 14, aggregates were still present, but they were no longer perfectly spherical. The cells appeared to have started spreading outward, gradually covering a larger surface area of the scaffold, although the increase in coverage was moderate.

In the FGF-supplemented medium, cells were mostly single, elongated, and primarily visible as green-stained cytoplasm surrounding the nuclei, with fine cytoplasmic extensions. After 14 days, the number of cells on the scaffold had increased markedly. Some dead cells

remained, but the scaffold surface was generally well covered, with only a few areas corresponding to pores that were less populated.

In the EGF-containing medium, cell aggregates formed alongside a substantial number of dead cells. Unlike the fully supplemented medium, these aggregates were not spherical; after 24 hours, cells had begun extending outward from the clusters, indicating early spreading and attachment to the scaffold surface, along with single cells and smaller aggregates. By day 14, the scaffold surface was completely covered with cells, and only very few dead cells were visible.

Based on these observations, the EGF-supplemented medium provided the most favorable conditions for cell adhesion, survival, and scaffold colonization; therefore, this medium was used in all subsequent experiments.

Biochemical analyses

To further investigate the functional state of fibroblasts cultured on the 3D scaffolds, Western blot analysis was performed to assess the expression of proteins associated with fibroblast activation and migratory or contractile behavior, specifically vimentin and α -SMA (alpha-smooth muscle actin), using Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) as a housekeeping control (Figure 27). Fibroblasts maintained under standard 2D culture conditions exhibited only low basal levels of vimentin and no detectable α -SMA, consistent with a quiescent phenotype typically observed in monolayer cultures. In contrast, fibroblasts grown on the three-dimensional scaffolds showed a marked upregulation of vimentin, along with a clear induction of α -SMA expression.

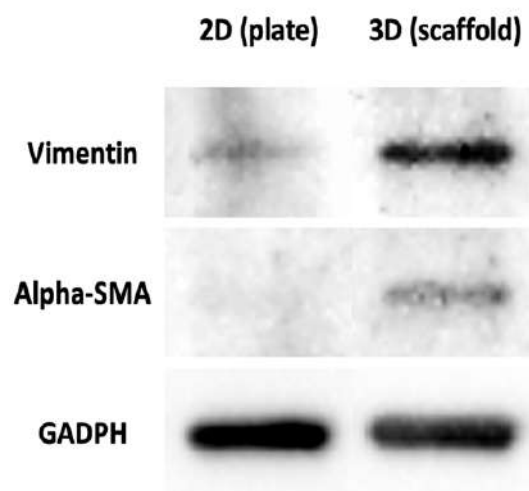


Figure 27. Western blot analysis of Vimentin and α -SMA expression in rat dermal fibroblasts cultured in monolayer [2D (plate)] and on 300 μ m pore-sized poly-L-lactic acid scaffolds [3D (scaffold)]. Housekeeping control: glyceraldehyde-3-phosphate dehydrogenase (GAPDH). N = 1 replicates.

In addition to visualizing the Western blot bands, densitometric analysis was performed to quantitatively evaluate protein expression levels (Figure 28). Specifically, the expression of α -SMA and vimentin in fibroblasts cultured on 3D scaffolds was increased by 2.7- and 2.2-fold, respectively, compared to cells cultured in 2D conditions.

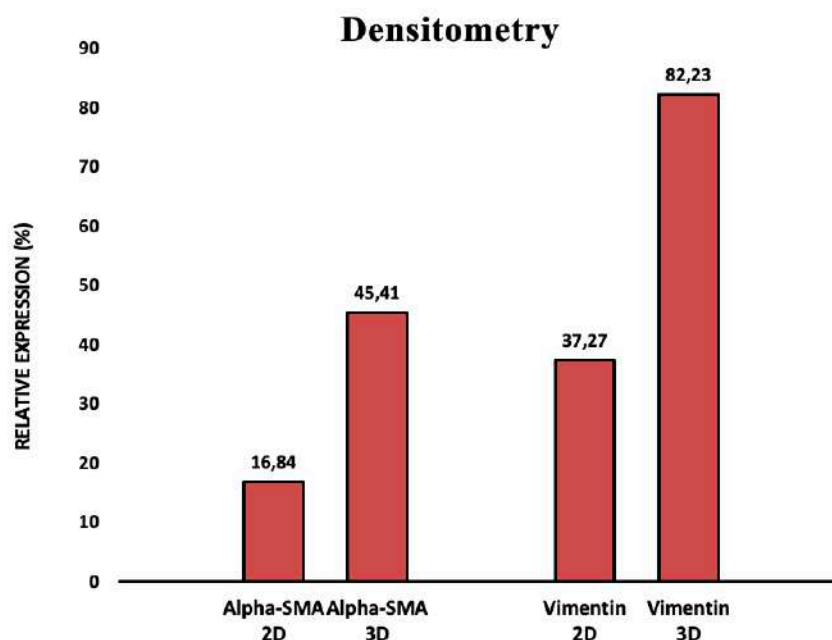


Figure 28. Western blot analysis of Vimentin and α -SMA expression in rat dermal fibroblasts cultured as monolayer (2D) and on 300 μ m pore-sized Poly L lactic acid scaffolds (3D). N = 1 replicates.

Overall, these results demonstrate that both scaffold architecture and culture conditions critically influence primary fibroblast adhesion, proliferation, and long-term colonization. Scaffold pore size was shown to modulate the pattern and rate of cell growth, with larger pores supporting more sustained proliferation over extended culture periods, likely due to improved nutrient diffusion and space for cellular expansion. Structural features alone, however, were insufficient to ensure stable growth, highlighting the importance of biochemical support. Supplementation with growth factors, particularly EGF, proved to be essential for promoting cell survival, attachment, and infiltration throughout the three-dimensional scaffold network. Confocal and SEM analyses confirmed that fibroblasts not only adhered to the scaffold surface but also infiltrated the internal architecture, forming aggregates and multilayered structures within the pores.

Furthermore, Western blot analysis indicated that fibroblasts cultured in 3D scaffolds exhibited pronounced phenotypic activation compared to standard 2D cultures. Scaffold-

grown cells showed increased expression of both α -SMA and vimentin, suggesting a shift toward an activated, myofibroblast-like phenotype. This protein expression pattern is consistent with enhanced cytoskeletal organization, increased motility, and the acquisition of contractile properties, features typically associated with fibroblasts during wound repair. Specifically, elevated vimentin levels support cell elongation and migration, whereas α -SMA upregulation reflects increased contractile capacity and the development of features required for ECM remodeling.

Taken together, these findings indicate that the combination of optimized scaffold design and medium supplementation not only sustains fibroblast viability and proliferation but also actively promotes a wound-relevant, activated phenotype. The integration of structural, mechanical, and biochemical cues within the 3D constructs is therefore essential for effective colonization and functionalization, providing a robust platform for tissue regeneration applications.

Microvascular fragments (MVFs) isolation

MVFs isolation

MVFs were isolated from adipose tissue, which was selected as the source owing to both practical and biological advantages. First, the intrinsic structural characteristics of adipose tissue (soft, loosely organized, and highly compliant) make it particularly amenable to selective enzymatic digestion. The lipid-rich parenchyma can be efficiently dissociated while preserving the architecture of the resident microvascular networks, allowing the retrieval of intact, lumenized microvessel segments rather than dissociated endothelial cells. This property is critical for maintaining the structural and functional integrity of MVFs, which serve as pre-assembled vascular modules capable of rapidly inosculating and forming interconnected vascular networks.

Moreover, adipose tissue is among the most accessible tissues in the body and can be harvested through minimally invasive procedures such as liposuction. This accessibility may enable the isolation of MVFs directly from the same donor, thereby facilitating the generation of autologous vascular networks. In the context of clinical translation, this represents a significant advantage: autologous MVFs inherently improve biocompatibility and reduce the risk of immune rejection. Together, these features indicate that the adipose tissue is an ideal reservoir for MVF isolation, combining ease of processing, preservation of

microvascular integrity, and strong translational potential for personalized, patient-specific *in vivo* applications.

Different adipose sources were used to isolate MVF to identify the most suitable tissue in terms of yield and structural preservation. Subcutaneous, intestinal, and perirenal adipose tissues were harvested from rats and subjected to histological analysis following fixation, paraffin embedding, sectioning, and Sirius Red staining (Figure 29). Subcutaneous adipose tissue appeared denser and more fibrous, characterized by collagen-rich connective layers tightly surrounding the resident vasculature, whose walls appeared notably thicker than those in the other tissue types. In contrast, intestinal and perirenal adipose tissues consisted predominantly of loosely packed adipocytes with minimal fibrous encapsulation and contained abundant, readily accessible vascular structures. Although all depots displayed clearly identifiable vessels, these architectural differences strongly influenced the efficiency of MVF isolation.

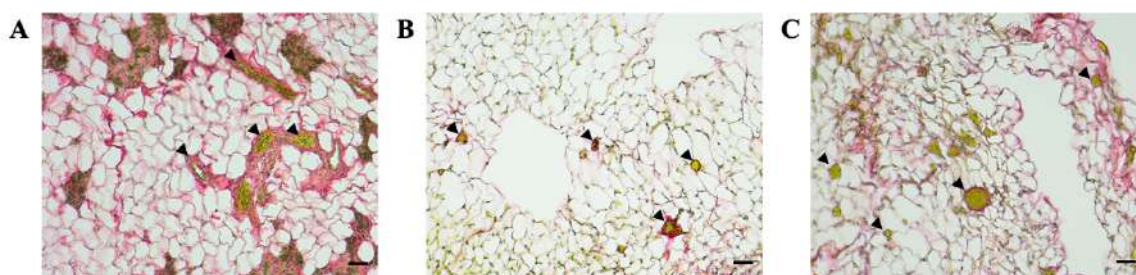


Figure 29. Sirius red histological staining of **A.** subcutaneous, **B.** intestinal, and **C.** perirenal adipose tissues. Blood vessels are indicated by black arrows. Scale bar: 100 μ m. N = 1 replicates.

As observed, adipocytes were surrounded by a thin collagen layer that acted as a structural connection among different cell types. In contrast, blood vessels were enveloped by a similar but generally thicker collagenous layer. For this reason, tissue digestion was performed using ultrapure recombinant collagenase H, supplemented with small amounts of thermolysin, to target additional extracellular matrix components. An isolation protocol for MVFs was established (Figure 30). Under controlled digestion times, the thinner collagen layer surrounding adipocytes was efficiently degraded, while the thicker collagen layer surrounding the vessels was only partially affected, thereby preserving vascular integrity. In parallel, mild mechanical disruption via pipetting was applied to disrupt loosely packed adipocytes. MVFs were subsequently separated by centrifugation, exploiting the lower

density of adipocytes, which floated to the surface, while MVFs sedimented at the bottom as a pellet.

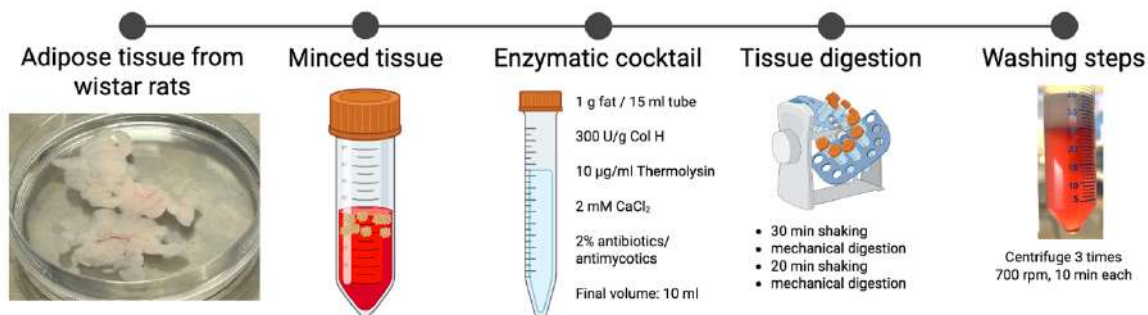


Figure 30. MVFs isolation protocol.

In a representative experiment (Figure 31), MVFs were isolated from each adipose depot. Four grams of each tissue were harvested and digested separately (gram by gram). The digests were then pooled, and the total number of counted vessels was divided by four to estimate the number of vessels per gram of tissue. Subcutaneous adipose tissue yielded only ~700 fragments per gram, likely due to the difficulty of releasing intact microvascular segments from its dense ECM. Conversely, intestinal and perirenal depots produced markedly higher numbers of MVFs, approximately 16,000 and 19,000 fragments per gram, respectively, reflecting their more compliant tissue architecture and reduced connective tissue constraints. These findings are consistent with the notion that tissue compliance and ECM density (higher in subcutaneous vessels) critically influence the efficiency of isolating structurally intact microvessels, and they confirm intestinal and perirenal adipose tissues as the optimal sources of high-quality MVFs.

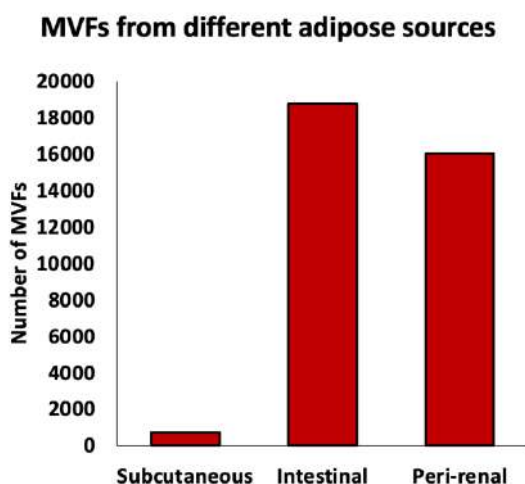


Figure 31. MVFs yields from different adipose sources. N = 1 replicates.

MVFs characterization

Microscopic examination revealed that the isolated MVFs formed a heterogeneous population of microvessel segments ranging from $\sim 30\ \mu\text{m}$ to $500\ \mu\text{m}$ in length, with approximately 75% between 30 and $200\ \mu\text{m}$, and diameters spanning $10\text{--}30\ \mu\text{m}$ (Figure 32). Many fragments exhibited branching morphologies and, in some cases, contained luminal erythrocytes, indicating that the vascular structures remained intact throughout the isolation process.

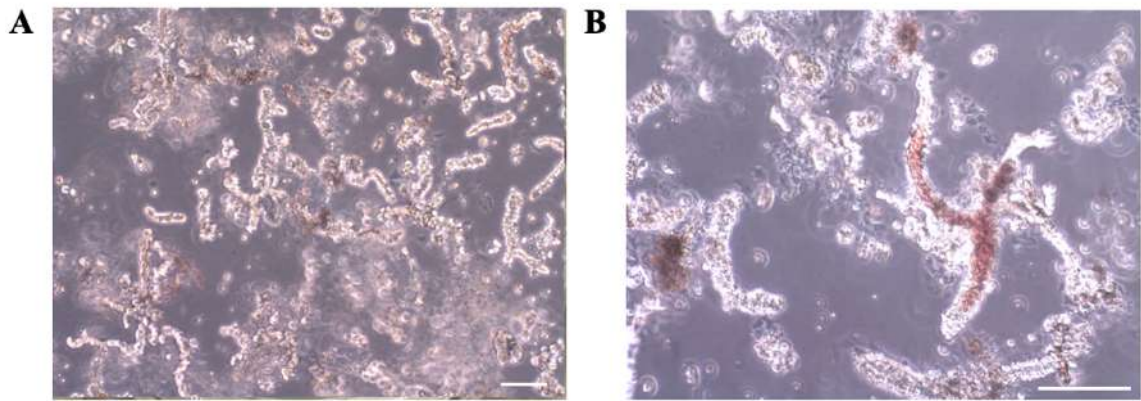


Figure 32. Freshly isolated MVFs from intestinal and peri-renal adipose tissues observed under a light microscope at two different magnifications (**A–B**). Scale bars: $100\ \mu\text{m}$.

SEM imaging further confirmed the elongated, cylindrical morphology typical of microvascular structures (Figure 33). Although SEM images show only the external surface of the fragments, their consistent tubular shape suggests an internal lumen, as expected for microvessel segments. Interestingly, erythrocytes were occasionally detected on the vessel surface, consistent with their vascular origin and possibly reflecting residual blood-associated components retained during isolation.

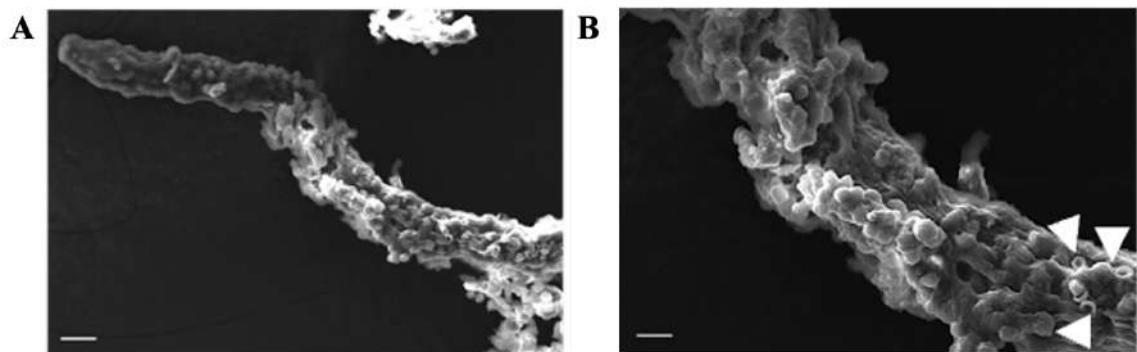


Figure 33. SEM analyses of freshly isolated MVFs from intestinal adipose tissue. Scale bars: $20\ \mu\text{m}$ (**A**) and $10\ \mu\text{m}$ (**B**). White arrows in **B** indicate erythrocytes.

MVFs angiogenic potential evaluation

Angiogenic potential of MVFs: 2D culture

To assess the viability and angiogenic potential of MVFs, they were seeded onto poly-L-lysine-coated coverslips in 24-well plates and cultured for up to 7 days (Figure 34). In 2D culture, the temporal evolution of MVF behavior was monitored at days 1, 4, and 7. After 1 day in culture (Figure 34 A), most microvessel fragments lost their original tubular organization and disintegrated into single cells, leaving only a small number of structurally intact vessel segments still recognizable. Over the following days, single cells reorganized,

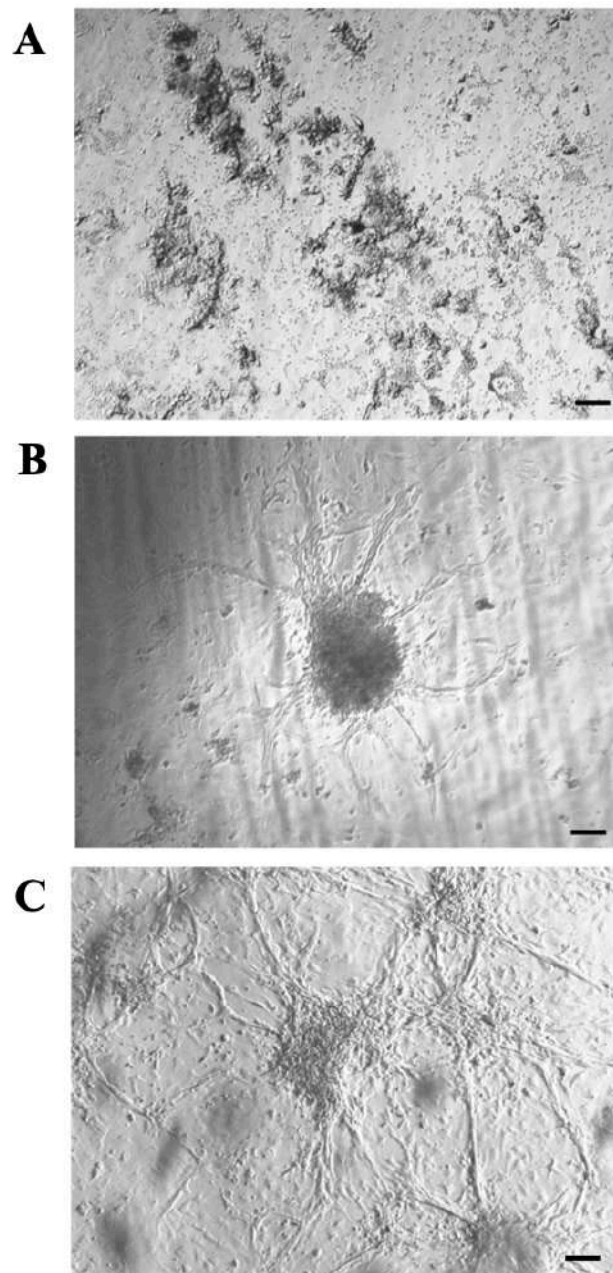


Figure 34. Microvascular fragments seeded in a 2D plate for 1 (A), 4 (B), and 7 (C) days. Scale bars: 100 μm . N = 3 replicates.

progressively aggregating into small multicellular clusters that subsequently adhered firmly to the well surface. By day 4 (Figure 34 B), aggregates actively sprouted, giving rise to early vascular-like extensions that elongated and began to branch. Over the subsequent days, the sprouts continued to extend, interconnect, and mature, gradually forming increasingly complex structures. By day 7 (Figure 34 C), this dynamic remodeling resulted in an extensive, highly interconnected vascular-like network covering the entire well surface, demonstrating the intrinsic angiogenic capacity of the MVFs.

To confirm the endothelial nature of the newly formed structures, day 7 samples were characterized using collagen type IV and VE-cadherin as specific endothelial markers. As shown in Figure 35, the networks were positive for both markers. Collagen type IV was prominently expressed along the developing vascular structures (Figure 35 B), consistent with basement membrane deposition, while VE-cadherin delineated endothelial cell–cell junctions with sharp and continuous staining (Figure 35 A). These results confirmed the endothelial identity of the vessel-like networks and demonstrated the intrinsic angiogenic capacity of MVFs to self-organize and form interconnected vascular structures in a 2D system.

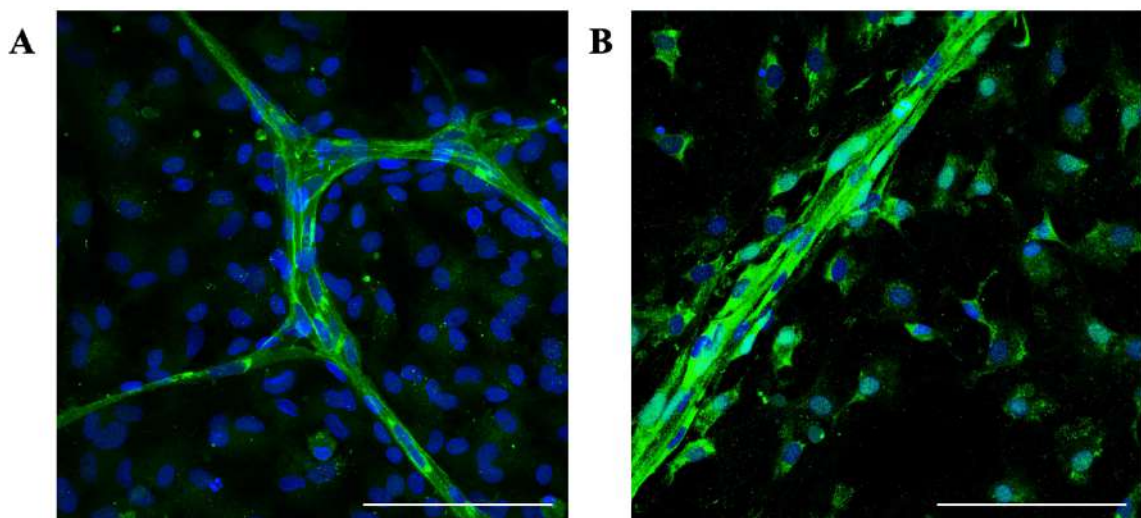


Figure 35. Immunostaining against **VE-Cadherin** and **DAPI** (A) and **Collagen type IV** and **DAPI** (B) of microvascular fragments cultured in a 2D plate for 7 days. Scale bars: 100 μ m.

Angiogenic potential of MVFs: 3D culture

The angiogenic potential of MVFs was further evaluated by embedding the fragments in a 3D collagen hydrogel. Throughout a 7-day observation period using optical microscopy, we monitored the dynamic remodeling of the embedded microvessels. After 24 hours (Figure

36 A), the MVFs preserved their original tubular morphology, remaining structurally intact and clearly identifiable within the collagen matrix, unlike in 2D cultures, where most structures rapidly disintegrated into single cells. Over the following three to four days (Figure 36 B), the preserved microvessels generated sprouts that elongated into the surrounding hydrogel and gradually increased in number. As the culture progressed, the sprouts continued to extend, branch, and interconnect, giving rise to an increasingly complex vascular-like network. By day 7 (Figure 36 C), the constructs exhibited a dense, organized 3D architecture composed of elongated, vessel-like segments linked by multiple anastomoses.

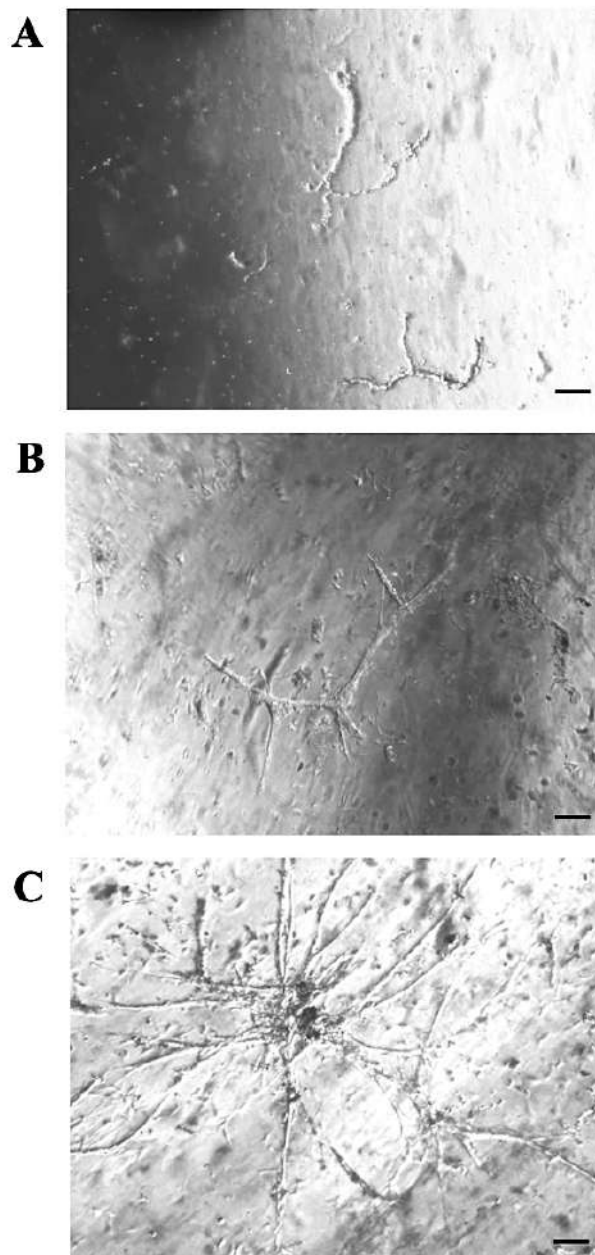


Figure 36. Microvascular fragments seeded in a 3D collagen hydrogel for 1 (A), 4 (B), and 7 (C) days. Scale bars: 100 μm. N = 3 replicates.

Cell viability and overall network maturation were further assessed by Live/Dead assays (Figure 37). On day 1 (Figure 37 A), individual vessel fragments were clearly visible within the hydrogel, maintaining their structural integrity and demonstrating high initial cell viability. By day 7 (Figure 37 B), the cell population remained highly viable, and extensive sprouting and interconnection between vessel fragments were observed, indicating significant maturation of the vascular structures within the hydrogel.

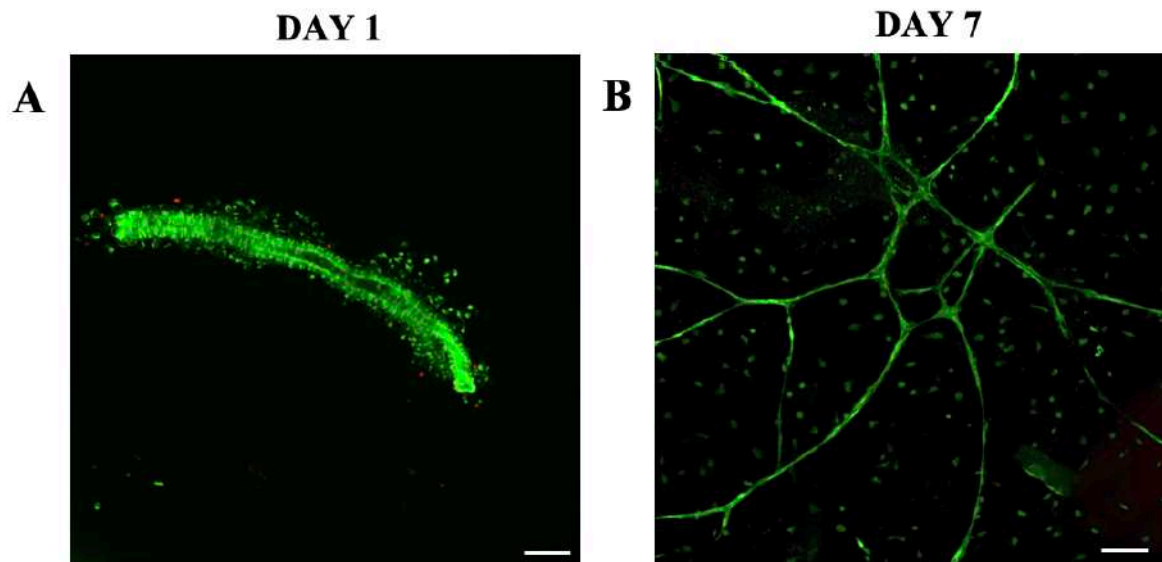


Figure 37. Live/Dead assay of microvascular fragments seeded in a 3D collagen hydrogel after 1 (A) and 7 (B) days. Scale bars: 50 μm (A) and 100 μm (B).

Confocal microscopy of FITC–phalloidin–stained samples revealed extensive elongation, branching, and proliferation of MVFs across the entire gel volume (Figure 38). Three-dimensional reconstruction of the hydrogel further demonstrated that the vessels extended and interconnected across multiple depths (z-planes), highlighting the formation of a complex, spatially organized vascular network that ultimately resulted in a mature, interconnected three-dimensional vascular structure.

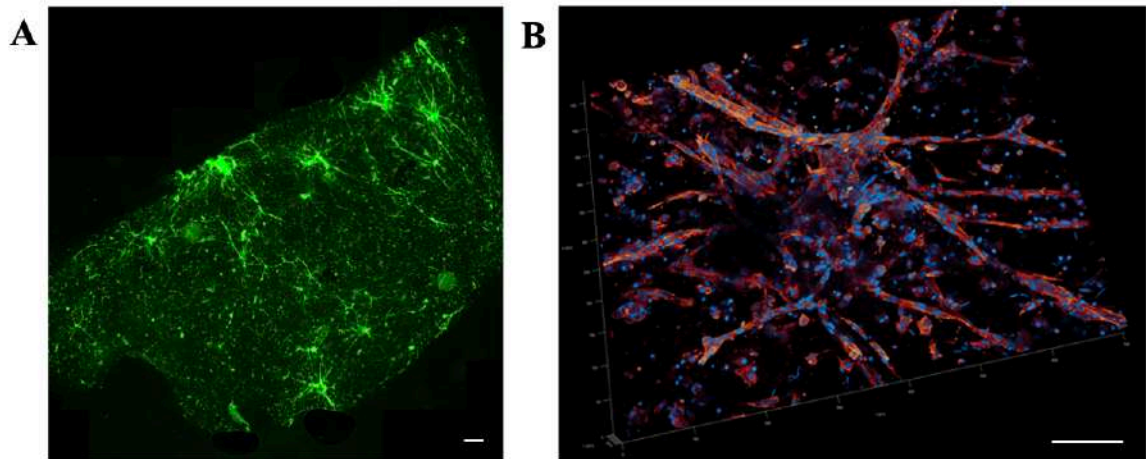


Figure 38. **A.** Confocal microscope analysis of microvascular fragments immunostained against **phalloidin-FITC**. Scale bar: 100 μm . **B.** 3D image reconstruction of MVFs seeded onto 3D collagen hydrogels immunostained with **Phalloidin-FITC** and **DAPI**. Scale bar: 100 μm .

These observations highlight the remarkable adaptability and angiogenic capacity of MVFs, reinforcing their suitability for incorporation into scaffold-based therapies that require rapid vascular integration. Collectively, these results demonstrate that visceral and perirenal adipose tissues provide not only the highest yields but also the highest-quality MVFs, combining structural integrity and compatibility with downstream regenerative applications.

Overall, these findings demonstrate that the isolated microvascular fragments are structurally intact and heterogeneous microvessel units that preserve key morphological features of native microvasculature, including branching architectures, tubular organization, and luminal erythrocytes, confirming the maintenance of vascular integrity during isolation. Both 2D and 3D culture systems revealed a strong intrinsic angiogenic potential of MVFs, characterized by dynamic remodeling, sprouting, and the formation of interconnected vascular-like networks.

In 2D culture, MVFs underwent rapid reorganization, transitioning from fragmented structures to multicellular aggregates that actively sprouted and self-assembled into extensive endothelial networks. The expression of collagen type IV and VE-cadherin confirmed the endothelial identity and functional maturation of these structures, indicating basement membrane deposition and the establishment of stable cell–cell junctions. In contrast, 3D collagen hydrogels provided a more supportive microenvironment, allowing MVFs to preserve their native tubular morphology, sustain high viability, and progressively develop a dense, spatially organized vascular network through sprouting and anastomosis.

Taken together, these results highlight the remarkable adaptability and robust angiogenic capacity of MVFs across different culture contexts. Their ability to maintain structural integrity, remain viable, and form complex vascular networks underscores their suitability for incorporation into three-dimensional scaffold-based regenerative strategies, where rapid and functional vascularization is essential for effective tissue integration and long-term construct viability.

Seeding of MVFs onto PLLA scaffolds

MVFs were seeded onto PLLA scaffolds for 14 days to evaluate their capacity to form vascular networks within the porous 3D matrix, using the standard protocol detailed in the Materials and Methods section. The **Live/Dead** assay (Figure 39) revealed that vessel-like structures were detectable on the scaffold surface as early as day 1 (Figure 39 A). However, by days 7 and 14 (Figures 39 B-C), no organized vascular formations were observed, indicating limited network maturation under these conditions.

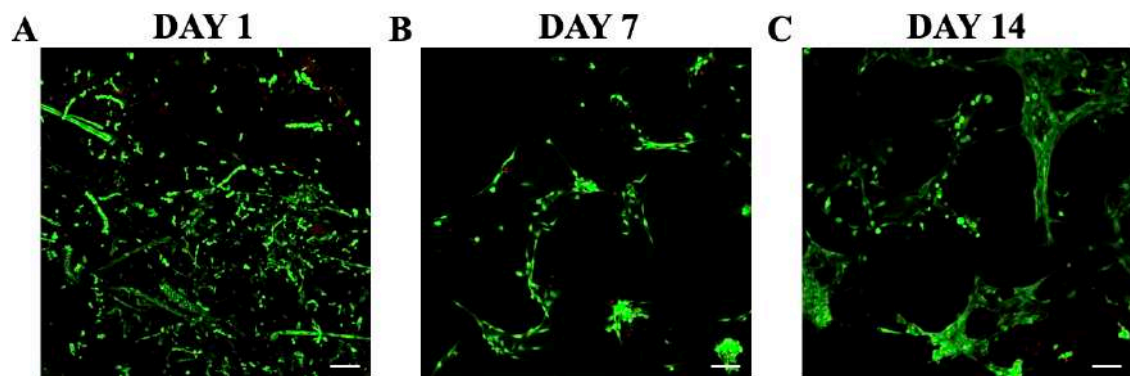


Figure 39. **Live/Dead** assay of microvascular fragments seeded in poly L lactic acid on 300 μm pore-sized scaffolds for 1 (A), 7 (B), and 14 (C) days. Scale bars: 100 μm . N = 2 replicates.

To enhance vascularization, the scaffolds were subsequently coated with Geltrex, a basement membrane extract predominantly composed of extracellular matrix (ECM) proteins, mainly laminin, collagen IV, entactin, and heparan sulfate proteoglycans. When MVFs were seeded onto Geltrex-coated scaffolds (Figure 40), they displayed improved initial adhesion and angiogenic behavior. On day 1 (Figure 40 A1-B1), MVFs were clearly visible on the scaffold surface, as observed on uncoated scaffolds. After 7 days of culture (Figure 40 A2-B2), a markedly different pattern emerged. Unlike uncoated scaffolds, Geltrex-coated ones supported the formation of elongated, branched, and interconnected structures distributed

across the entire scaffold surface. These networks closely resembled the angiogenic patterns observed in 2D cultures, characterized by multicellular aggregates interconnected by vascular-like sprouts. At this time point, a substantial number of dead cells was also detectable. By day 14 (Figure 40 A3-B3), only limited vessel-like remnants were visible, together with amorphous cell aggregates and isolated single cells. Organized branching structures and interconnected networks were no longer observed.

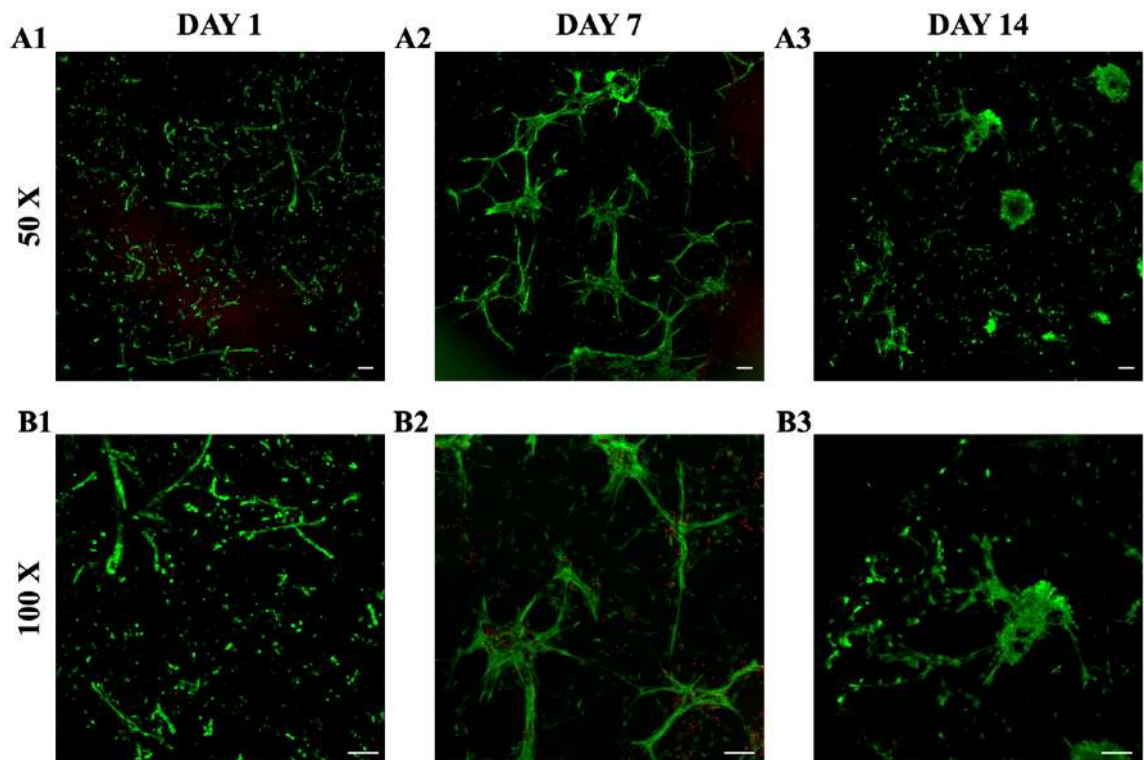


Figure 40. Live/Dead assay of microvascular fragments seeded on Geltrex-coated PLLA scaffolds with 300 μm pores. Images are shown at 50 $\times$ (A series) and 100 $\times$ (B series) magnifications after 1, 7, and 14 days (panels 1–3 for each time point). Scale bars: 100 μm . N = 1 replicates.

The transient presence of angiogenic-like structures at day 7, combined with poorly defined vessel boundaries and their absence at day 14, indicates that although Geltrex coating promoted early angiogenic activity, the seeding and culture protocol was not sufficient to sustain stable and long-term vascular organization. Overall, these observations suggest that further optimization of the seeding strategy and culture conditions is required to stabilize MVF-derived vascular structures within the scaffold over prolonged culture periods.

In light of these observations, the protocol was further refined by supplementing the culture medium with 10 ng/mL VEGF to enhance angiogenic activity, and MVFs were subsequently cultured on Geltrex-coated scaffolds under the same conditions (Figure 41). In contrast to

the previous setup, VEGF supplementation resulted in a rapid and robust angiogenic response. By day 7 (Figure 41 A2–B2), MVFs exhibited pronounced sprouting activity, forming elongated and interconnected projections that progressively organized into a well-defined vascular network across the scaffold surface. This structural organization indicates active endothelial remodeling and suggests the establishment of stable cell–cell interactions, a prerequisite for the formation of functional microvascular structures. Importantly, this vascular network was maintained over time and further evolved by day 14 (Figure 41 A3–B3), becoming denser and more morphologically complex. The appearance of compact cellular aggregates and increased network complexity may reflect ongoing vascular maturation and early stages of tissue organization within the scaffold. In addition, small cells

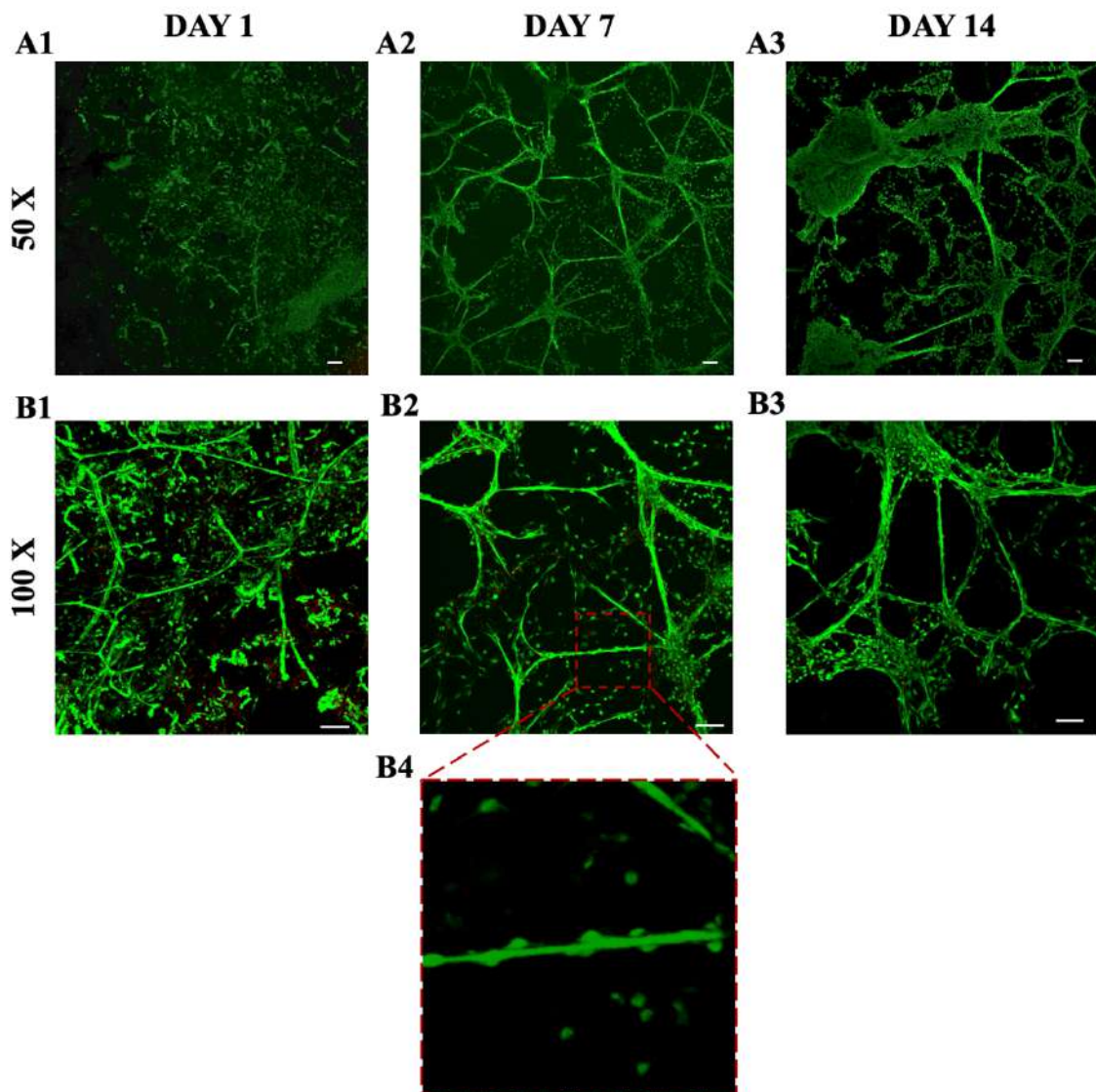


Figure 41. Live/Dead assay of microvascular fragments seeded on Geltrex-coated poly (L-lactic acid) scaffolds with 300 μm pores. Images are shown at 50 $\times$ (**A** series) and 100 $\times$ (**B** series) magnifications after 1, 7, and 14 days (panels **1–3** for each time point). Panel B4 shows a higher magnification of B2 to visualize pericytes surrounding the vessels. Scale bars: 100 μm . N = 3 replicates.

adhering along the vessel-like structures were observed, likely corresponding to perivascular cells, suggesting progressive network maturation and stabilization (Figure 41 B4).

Overlaying fluorescence and brightfield images (Figure 42) confirmed that the vascular structures formed directly on the scaffold surface, demonstrating genuine vessel growth rather than the mere alignment of single cells that mimic network patterns. Thanks to the improved image contrast, high-magnification imaging revealed clearly defined lumens within multiple vessel-like structures, suggesting ongoing vascular maturation and the development of functional microvessels.

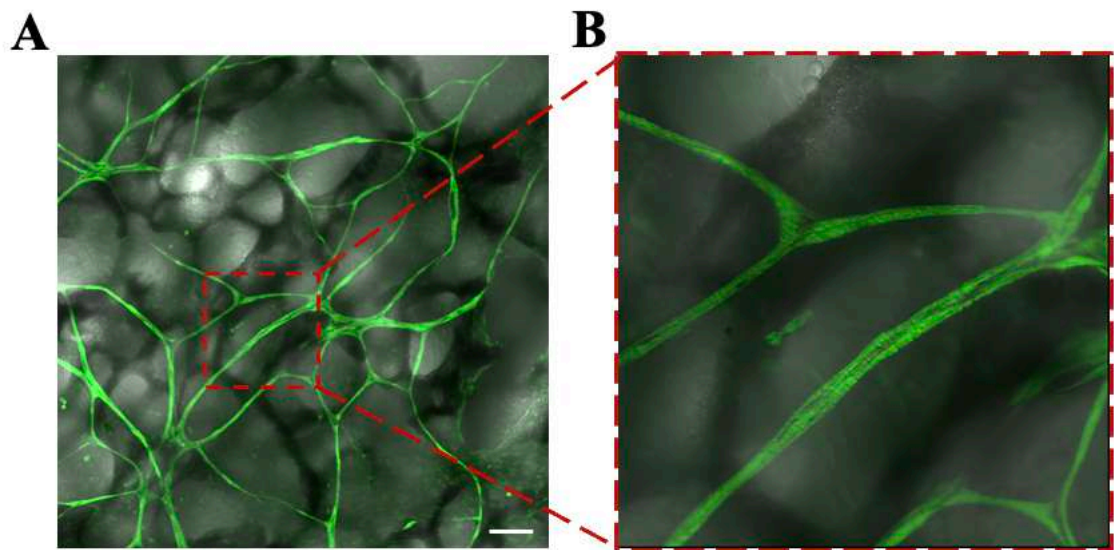


Figure 42. Live/Dead assay of MVFs seeded on GelTrex-coated PLLA scaffolds with 300 μm pores after 7 days of culture, shown with brightfield overlay (A) and a magnified view of a lumenized vessel (B). Scale bar: 100 μm .

Scanning electron microscopy (SEM) analysis corroborated these observations, illustrating a continuous vascular network distributed across the scaffold surface and confirming the successful integration and functional angiogenic activity of MVFs within the 3D matrix (Figure 43).

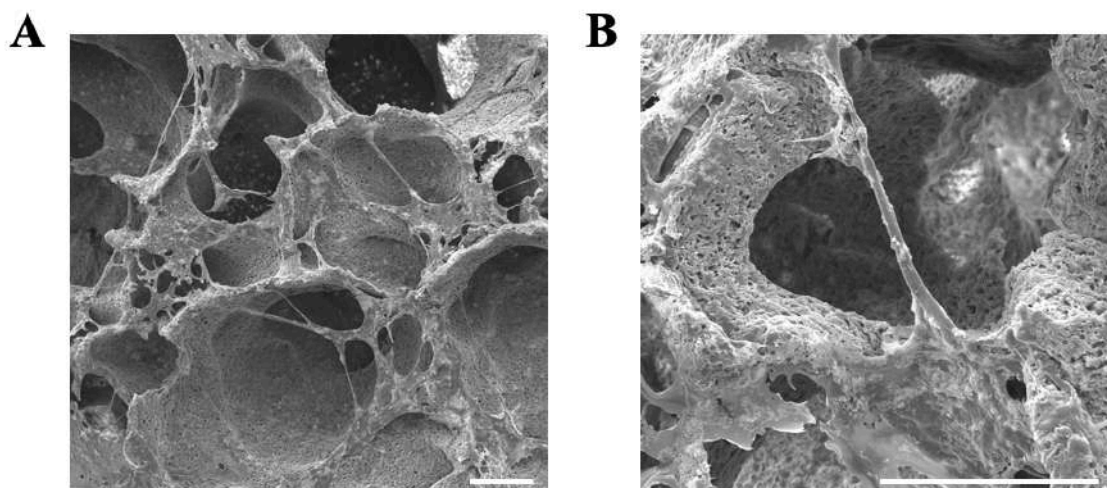


Figure 43. Scanning electron microscopy (SEM) images showing microvascular fragments cultured on Geltrex-coated poly L-lactic acid (PLLA) scaffolds with 300 μm pores after 7 days, acquired at lower (A) and higher (B) magnifications. Scale bars: 100 μm .

Overall, these findings indicate that MVF-driven vascularization within PLLA scaffolds is not an intrinsic outcome of seeding but requires a supportive microenvironment to be sustained over time. While uncoated scaffolds did not permit organized vessel formation and Geltrex coating alone induced only a transient angiogenic response, the addition of VEGF proved essential to stabilize and maintain vascular structures. The resulting networks exhibited lumen-containing morphology and were accompanied by small cells adhering along the vessels, likely representing perivascular cells, highlighting ongoing maturation and stabilization. These observations demonstrate that MVFs retain strong angiogenic potential. Still, their practical expression within synthetic 3D scaffolds depends on the integration of biochemical and matrix-derived signals rather than scaffold architecture alone.

Seeding RDFs on Geltrex-coated scaffolds

Because the successful growth of MVFs required a Geltrex coating, we next examined whether primary RDFs could also adhere to and proliferate efficiently on this substrate. RDFs were seeded onto Geltrex-coated PLLA scaffolds, and their metabolic activity was quantified using the Alamar Blue assay (Figure 44). After 1 day, cells successfully adhered to the scaffold. Over the 14-day culture period, the Alamar Blue signal increased steadily and significantly, ultimately doubling the initial value, indicating enhanced metabolic activity and a corresponding increase in cell number. This trend demonstrates not only

sustained fibroblast proliferation but also the absence of metabolic impairment or cytotoxic effects associated with the Geltrex coating or the scaffold itself.

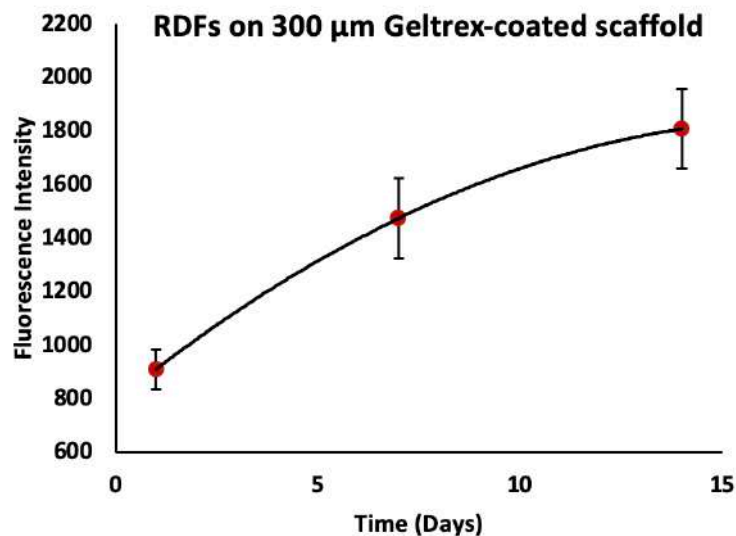


Figure 44. Growth curve (Alamar blue) of rat dermal fibroblasts seeded onto GelTrex-coated 300 µm scaffolds. 300,000 seeded cells/scaffold. N = 3 replicates.

To confirm the identity and to analyze the spatial distribution of the fibroblast population, immunostaining for vimentin and DAPI was performed (Figure 45). Vimentin staining verified the mesenchymal/fibroblastic nature of the adherent cells. The scaffolds appeared red due to their intrinsic autofluorescence, which, rather than interfering with analysis, facilitated the visualization of cell localization relative to the scaffold structure. The uniform distribution of vimentin-positive cells throughout the structure further confirmed effective colonization of the porous architecture. Specifically, on day 1 (Figure 45A), fibroblasts were observed in close proximity to the scaffold surface, displaying an elongated and well-spread morphology, indicative of strong adhesion to the scaffold. Over time, the cells extended across the scaffold, covering smaller pores and progressively creating a continuous cellular layer. Notably, by day 14 (Figure 45 B), the surface was characterized by localized multilayered regions and thickened cell aggregates, suggesting early tissue-like organization and potential matrix deposition, possibly indicating an active and dynamic interaction between fibroblasts and the 3D scaffold environment.

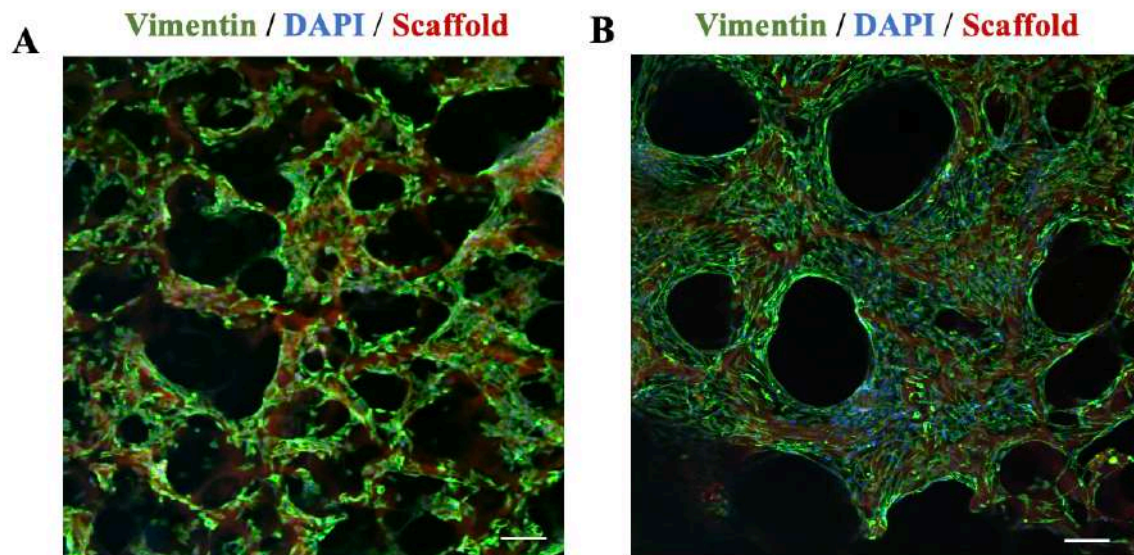


Figure 45. Vimentin/Scaffold/DAPI immunostaining of rat dermal fibroblasts seeded onto GelTrex-coated 300 µm scaffolds at 1 (A) and 14 (B) days after seeding. Scale bars: 100 µm.

SEM analysis (Figure 46) provided complementary morphological information, highlighting regions fully covered by a cell layer, confirming the superficial colonization of the coated scaffolds.

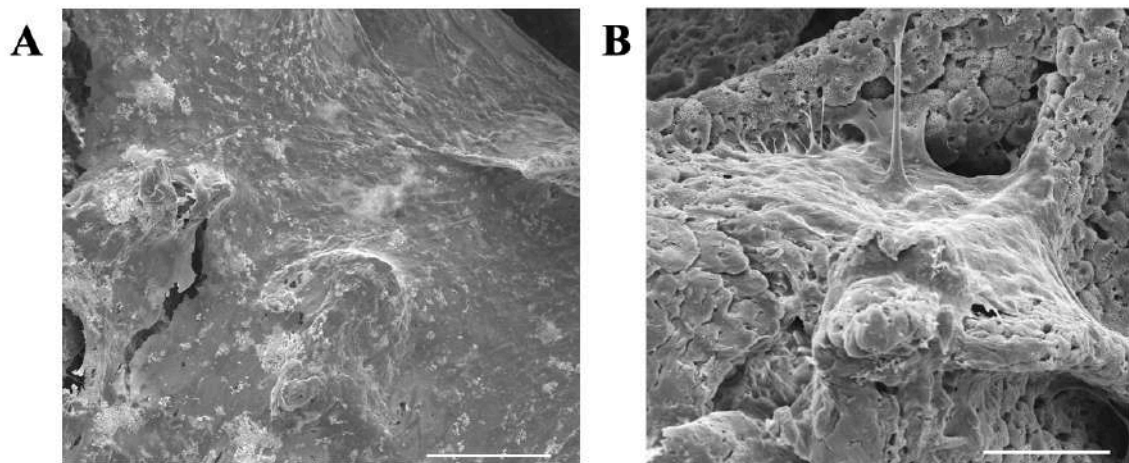


Figure 46. Scanning electron microscopy (SEM) images of rat dermal fibroblasts cultured on Geltrex-coated scaffolds with 300 µm pore size, 14 days after seeding. Images are shown at low (A; scale bar: 300 µm) and high (B; scale bar: 50 µm) magnifications.

Taken together, the Alamar Blue, SEM, and immunostaining data demonstrate that Geltrex-coated PLLA scaffolds provide a highly supportive environment for fibroblast adhesion, survival, proliferation, and spatial organization. These findings confirm the suitability of this

scaffold configuration for subsequent co-culture experiments with MVFs, ensuring that both cell types can thrive under the same biomaterial and culture conditions.

Co-cultures

Following the successful seeding of primary RDFs and MVFs individually on Geltrex-coated PLLA scaffolds, we next investigated their behavior in co-culture to generate a functional, vascularized construct suitable for regenerative applications such as chronic wound repair. Since both RDFs and MVFs proliferated and matured on Geltrex-coated scaffolds and required supplementation with EGF and VEGF, respectively, co-cultures were established using Geltrex-coated scaffolds and a culture medium supplemented with both growth factors.

Live/Dead staining at Day 1 (Figure 47 A1-B1) demonstrated efficient adhesion of both cell types, with MVFs retaining their native vascular architecture, including perivascular matrix components, and fibroblasts spreading along the scaffold surface. This early observation confirmed that the scaffold microenvironment supports the simultaneous survival and initial organization of both cell populations. Interestingly, some MVFs displayed aligned cellular extensions, suggesting that interactions with fibroblasts, which secrete pro-angiogenic growth factors, may further enhance angiogenesis. Over the subsequent culture period, MVFs sprouted and branched, progressively forming interconnected vascular networks. By Day 7 (Figure 47 A2-B2), the networks were clearly established, and they continued to mature through Day 14, maintaining structural stability and high viability (Figure 47 A3-B3). Notably, some vessels appeared to temporarily disappear and then re-emerge at their expected locations, possibly reflecting coverage by fibroblasts and ongoing tissue organization within the scaffold.

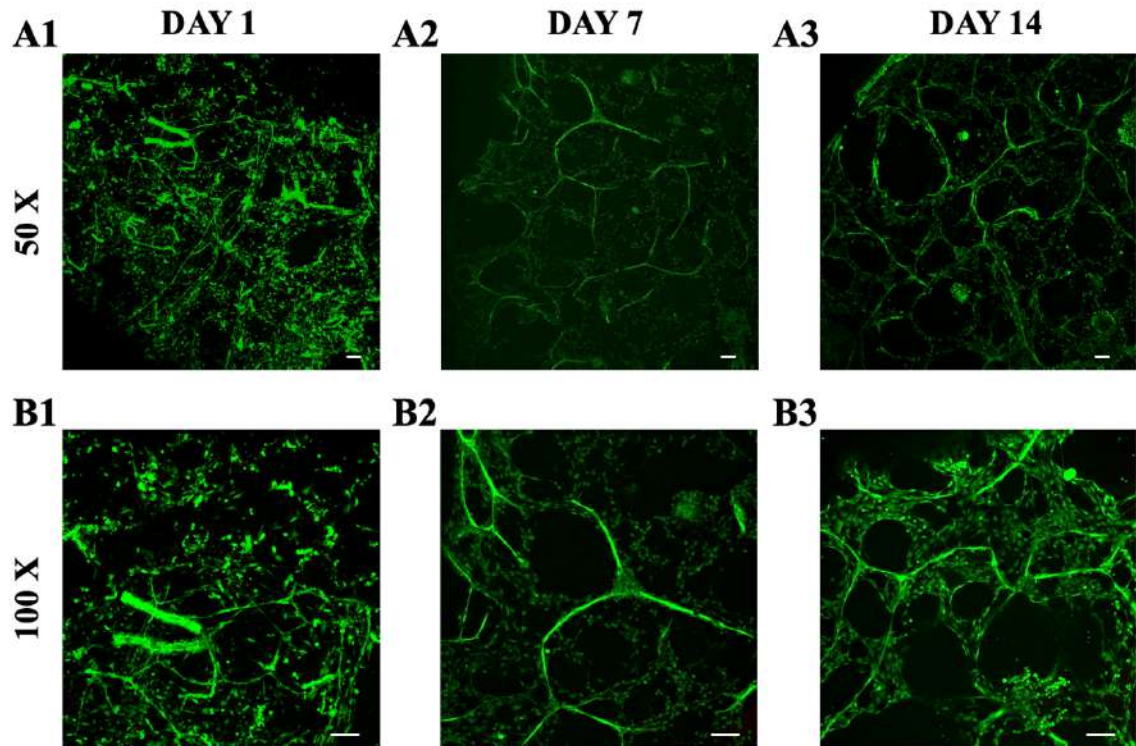


Figure 47. **Live/Dead** assay of cocultures of microvascular fragments and rat dermal fibroblasts seeded on Geltrex-coated poly(L-lactic acid) scaffolds with 300 μm pores. Images are shown at 50 $\times$ (**A** series) and 100 $\times$ (**B** series) magnifications after 1, 7, and 14 days (panels **1–3** for each time point). $N = 2$ replicates.

Immunofluorescence staining for type I collagen revealed its deposition surrounding the MVFs and along newly formed vascular structures at all observed time points. Notably, the collagen shell initially present around vessels at day 1 was preserved in newly synthesized vessels, forming a distinct peripheral layer, as evidenced by weaker staining in the central region and stronger signal along the vessel edges. This pattern suggests that collagen preferentially accumulates around the exterior of the vascular structures, contributing to their structural integrity. By day 14, the vascular architecture appeared more compact and stable compared to day 7, indicating progressive maturation (Figure 48).

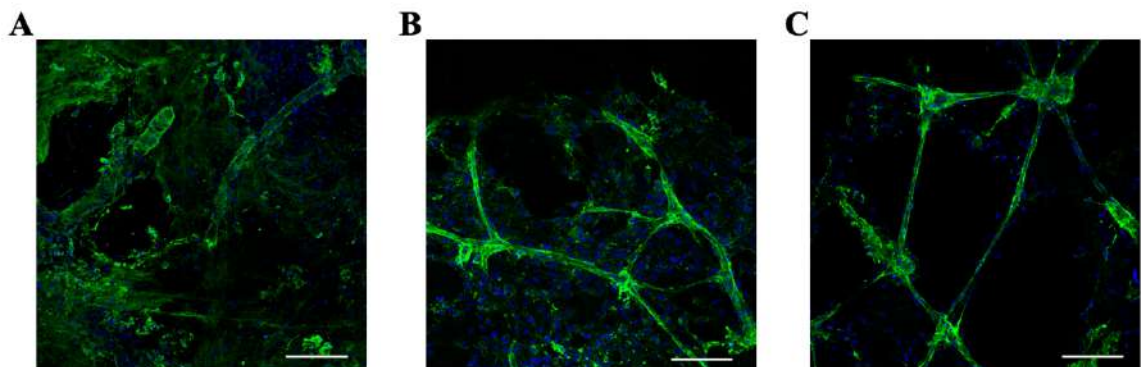


Figure 48. Immunofluorescence staining for **Collagen type I** and **DAPI** of cocultured scaffolds for 1 (**A**), 7 (**B**), and 14 (**C**) days. Scale bar 100 μm .

Although the signal in the scaffold's inner regions was lower, possibly due to limited antibody penetration, cross-sectional analyses of co-cultured scaffolds at 14 days revealed some vessels penetrating the interior (Figure 49). These observations suggest that the vascular network develops not only on the surface but also throughout the scaffold, demonstrating its capacity to support three-dimensional vessel formation.

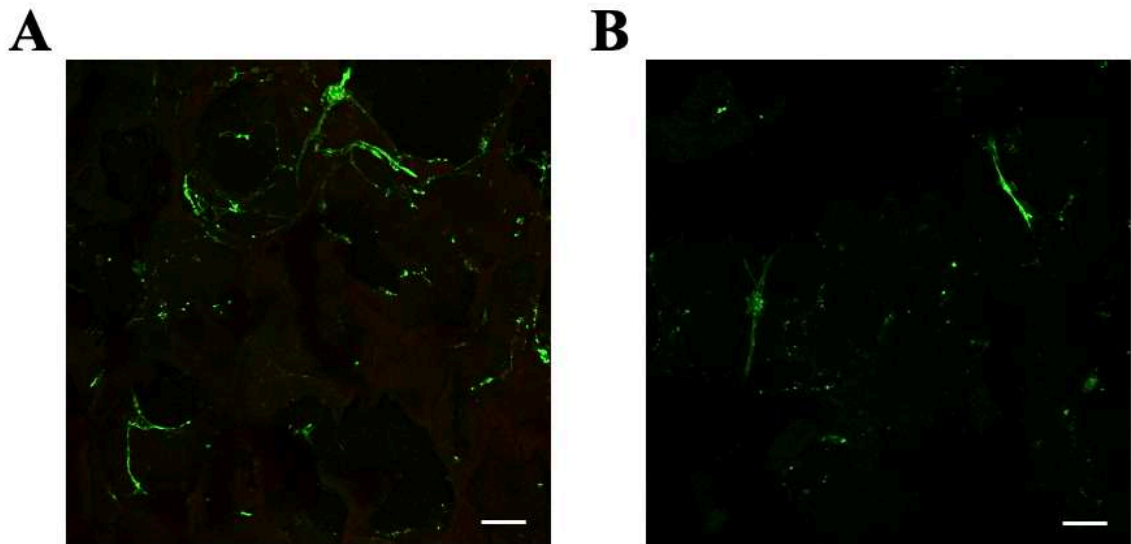


Figure 49. Immunostaining against **Collagen type I** in co-cultured scaffolds after 14 days. Figures **A** and **B** show two representative images from different regions of the scaffold cross-section. Scale bar: 100 μm .

Throughout the 14-day culture period, fibroblasts continued to proliferate robustly, colonizing the scaffold without apparent competition with MVFs. The coexistence of fibroblasts and MVFs, along with the observed vascular network formation and ECM deposition, may reflect interactions between the two cell populations. Newly formed vessels were embedded within a type I collagen matrix, providing structural support and likely contributing to vessel stabilization. In co-culture with fibroblasts, vascular networks appeared more compact and organized, particularly by day 14, displaying well-defined boundaries. This increased structural coherence may be attributed, at least in part, to ECM deposition by fibroblasts, including the formation of an external collagen layer surrounding the vessels.

Although a direct quantitative comparison with MVFs cultured alone was not performed, qualitative observations suggest that MVFs in monoculture formed more irregular, fragmented, or less cohesive structures, with partially disorganized vessel contours, further supporting this hypothesis.

Overall, these results demonstrate that Geltrex-coated PLLA scaffolds provide a permissive microenvironment that supports cell adhesion, survival, proliferation, extracellular matrix production, and the development of stable, well-organized vascular networks. The therapeutic unit is entirely populated by fibroblasts and is traversed by a well-organized, branched vascular network. Cross-sectional analysis with type I collagen immunostaining confirmed that the construct supports intraporous vascularization, as vessels were clearly visible within the sections (Figure 47). Such extensive vascularization could facilitate early anastomosis with the host circulation, promote rapid infiltration of host cells, facilitate scaffold remodeling through normal enzymatic hydrolysis of PLA during healing, and ultimately lead to effective wound closure.

Collectively, these features underscore the scaffold's potential to enhance tissue regeneration and its suitability for engineering pre-vascularized constructs for therapeutic applications.

Human Dermal Fibroblasts

Previous studies in a murine model showed that fibroblasts and microvascular fragments can coexist in three-dimensional co-culture, colonize PLLA scaffolds, and organize into an interconnected microvascular-like network. Building on these findings, the present study focuses on potential human applications.

To this end, we employed primary human dermal fibroblasts to assess their capacity to grow, survive, and remodel the same scaffold system. Since translating a model from murine to human cells requires optimizing culture conditions, we systematically evaluated parameters that influence early colonization and extracellular matrix deposition, focusing on coating conditions and cell seeding density. This approach enabled us to identify the conditions most conducive to human fibroblast integration within the 3D scaffold environment. In addition, particular attention was dedicated to the synthesis of extracellular matrix components and to the characterization of the three-dimensional matrix formed above the scaffold surface. This included a detailed evaluation of ECM molecule production, their spatial distribution, and the progressive assembly of extracellular networks, to understand how fibroblasts reshape and consolidate the scaffold microenvironment over time.

Human dermal fibroblasts (hDFs) seeded onto scaffolds: Vitality and Viability assay

Human dermal fibroblasts (hDFs) were successfully isolated from dermal explants obtained from three donors of different ages and sexes, including a six-year-old male, a twenty-four-year-old male, and a forty-year-old female from the Anatomy department of the Paracelsus Medical University (PMU), Nuremberg, Germany. The typical elongated spindle-shaped morphology of primary cells across all donors confirmed its suitability for heterogeneous human samples (Figure 50).

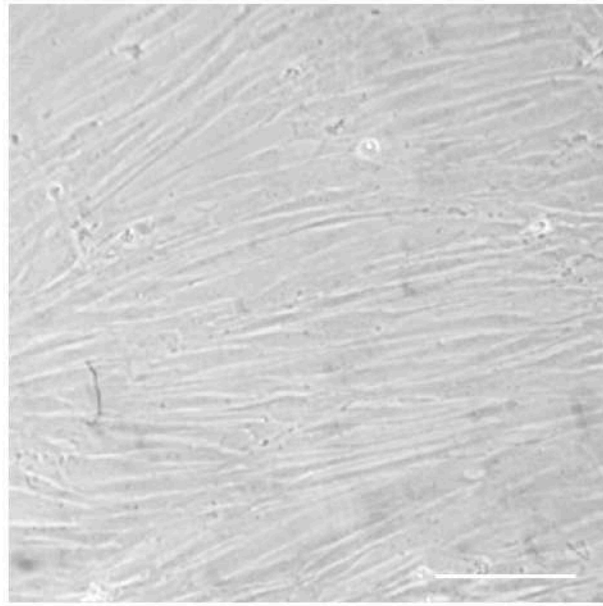


Figure 50. Primary human dermal fibroblasts in monolayer culture (Donor 2). Scale bar: 100 μm .

For subsequent experiments, we used PLLA scaffolds produced by thermally induced phase separation, maintaining the same structural parameters previously validated in the rat model: an approximate pore size of 300 μm , a thickness of 2 mm, and a diameter of 6 mm.

Human fibroblasts from all three donors were seeded onto Geltrex-coated and uncoated scaffolds at two densities (150,000 and 300,000 cells) to assess how initial cell number and scaffold biofunctionalization influenced metabolic activity over time. Viability assay (Alamar Blue) analysis (Figure 51) showed that at the lower density, uncoated scaffolds exhibited nearly unchanged signals over time, indicating that early adhesion was insufficient to enable a robust proliferative response.

In contrast, coated scaffolds seeded with the same number of cells exhibited a clear and continuous increase in fluorescence from Day 1 to Day 14, reflecting sustained proliferation and metabolic activity. Across donors, statistical analysis confirmed that, despite an equal number of adhered cells at Day 1, coated scaffolds performed significantly better at all subsequent time points, whereas uncoated scaffolds showed no significant progression. These results indicate that, at low seeding densities, an adhesive and biologically active ECM is essential for stabilizing early cell–scaffold interactions.

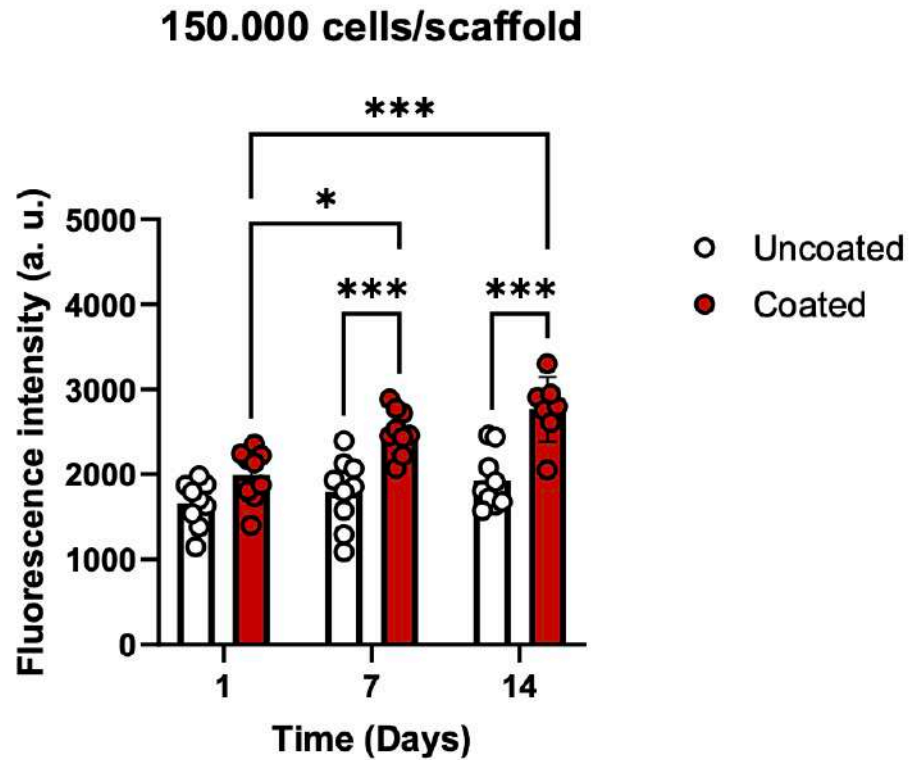


Figure 51. Alamar Blue assay of 150,000 primary human dermal fibroblasts seeded onto Geltrex-coated (red bars) and uncoated (white bars) poly L lactic acid scaffolds. Data are presented as mean $\pm$ SD. Statistical significance was determined by two-way ANOVA (* $p = 0.0332$, ** $p = 0.0021$, *** $p = 0.0002$). $N = 3$ replicates for $n = 3$ donors.

When 300,000 cells were seeded, both coated and uncoated scaffolds experienced early stress, likely due to high local cell density, limited nutrient diffusion within the porous scaffold, or temporary growth arrest as cells reorganized within the three-dimensional environment. In uncoated scaffolds, this stress led to a statistically significant reduction in cell number between Days 1 and 7 (Figure 52). In coated scaffolds, although cells did not proliferate substantially during this period, their population remained relatively stable, indicating that the coating helped preserve adhesion and survival under these initial culture conditions. However, at Day 14, fluorescence increased again in all conditions, often surpassing Day 1 values, especially in coated scaffolds. The metabolic behavior followed a parabolic trajectory that was consistent across donors, and although differences among donors were not significant, coated scaffolds again supported higher activity levels.

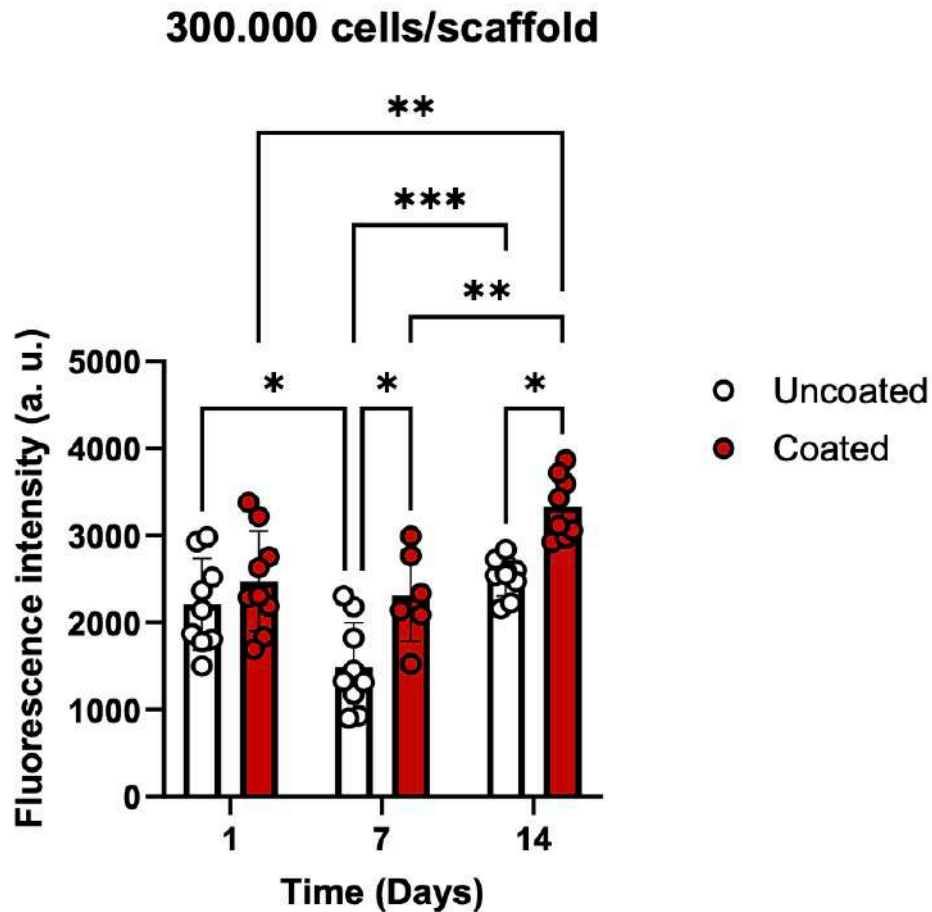


Figure 52. Alamar Blue assay of 300,000 primary human dermal fibroblasts seeded onto coated and uncoated PLLA scaffolds. Data are presented as mean $\pm$ SD. Statistical significance was determined by two-way ANOVA (* $p = 0.0332$, ** $p = 0.0021$, *** $p = 0.0002$). $N = 3$ replicates for $n = 3$ donors.

Live/Dead staining analysis (Figure 53) provided qualitative insights into the viability, morphology, and spatial distribution of fibroblasts throughout the scaffolds and confirmed the trends observed in metabolic measurements. At the lower seeding density (150,000 cells), fibroblasts on uncoated scaffolds initially formed spherical aggregates rather than adhering directly to the scaffold surface, indicating weak early interactions with the substrate and a reliance on cell–cell contacts for survival. By Days 7 and 14, these aggregates partially dispersed as cells spread and elongated, although overall cell density remained low.

In contrast, fibroblasts seeded on Geltrex-coated scaffolds adhered stably from Day 1, with some cells spreading along the scaffold surface while others remained in small aggregates. By Days 7 and 14, aggregates were no longer evident, and cells were more elongated, firmly

attached, and more evenly distributed across the scaffold. Notably, the total cell number visibly increased over this period, covering the surface.

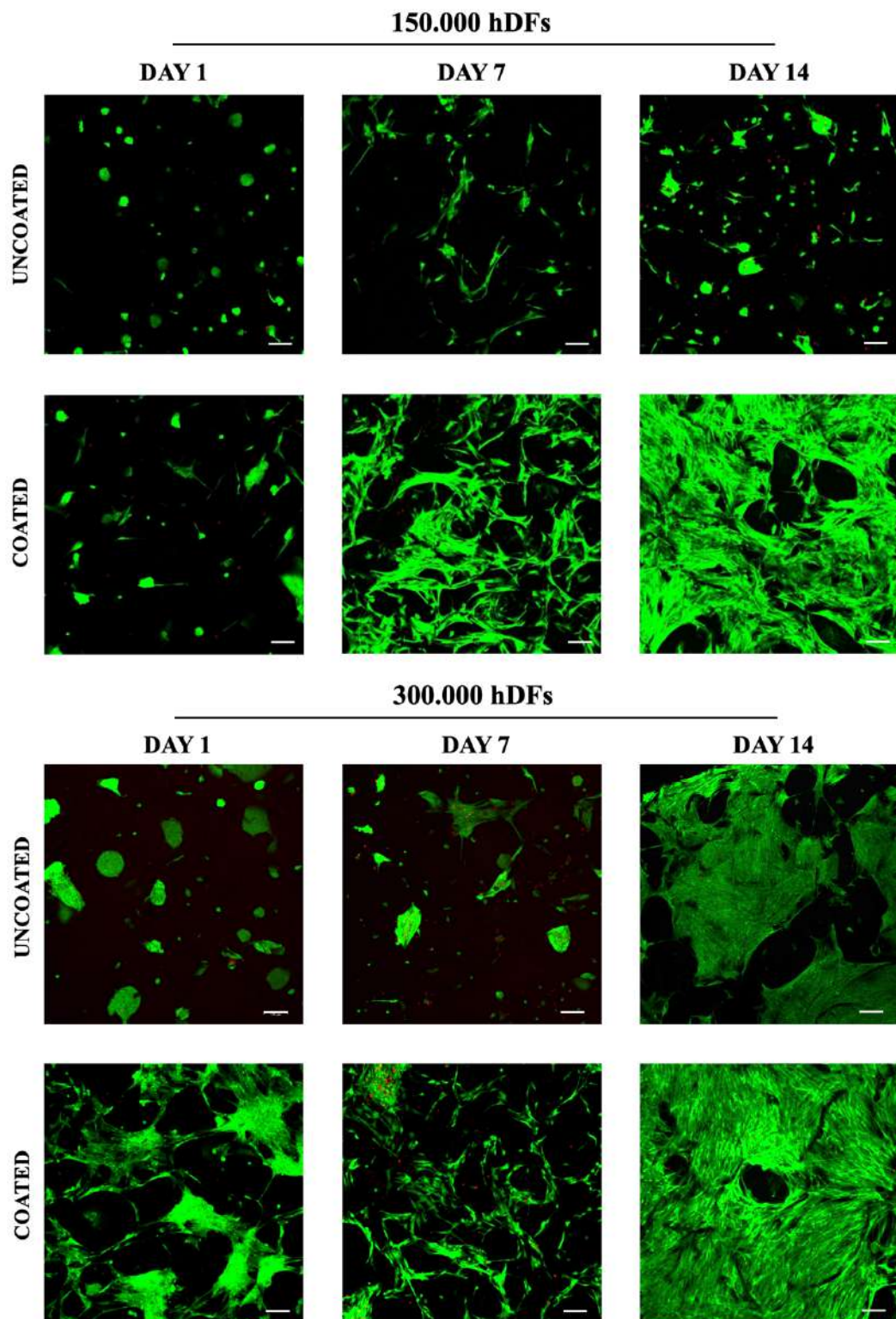


Figure 53. Live/Dead assay of 150.000 and 300.000 primary human dermal fibroblasts seeded onto coated and uncoated poly L lactic acid scaffolds. Scale bars: 100 μ m.

At the higher density of 300,000 cells (Figure 53), fibroblasts on uncoated scaffolds formed dense aggregates on Day 1 and Day 7, accompanied by an increased number of dead cells. These findings confirm that while higher density facilitates earlier scaffold occupation, it also introduces stress associated with suboptimal adhesion sites and overcrowding. By Day 14, however, the cells had largely recovered, adopting the typical spindle-shaped fibroblast morphology and spreading more evenly across the scaffold, resembling the distribution and morphology observed on coated scaffolds at earlier time points.

On coated scaffolds, despite some initial clustering and a transient constancy in cell number between Days 1 and 7, fibroblasts displayed an elongated morphology much earlier. By Day 14, they had efficiently colonized the scaffold, completely covering the seeded surface and extending to the unseeded side. The differences between coated and uncoated scaffolds highlight the role of early adhesion cues in dictating survival, spreading, and spatial arrangement in 3D culture.

Overall, these results demonstrate that both scaffold biofunctionalization and initial cell density critically influence fibroblast behavior in 3D culture. Coated scaffolds consistently promoted early and stable adhesion, enhanced metabolic activity, and supported uniform cell distribution. In uncoated scaffolds, higher seeding densities, despite initial losses in cell number, facilitated scaffold colonization and, by Day 14, fibroblasts displayed morphology and distribution similar to those observed on coated scaffolds. This recovery suggests that the cells on uncoated scaffolds may have produced their own extracellular matrix over time, indicating that higher initial cell density can stimulate more efficient matrix deposition. Moreover, these observations support the idea that the presence of an initial biofunctionalized matrix, as in coated scaffolds, can further enhance fibroblast matrix production, reinforcing scaffold colonization and tissue organization.

Importantly, these findings confirm that providing a biologically active matrix and sufficient initial cell numbers is essential for establishing a viable and functional fibroblast population, laying the foundation for effective tissue engineering and regenerative applications.

Extracellular Matrix synthesis and distribution

To further investigate this hypothesis, extracellular matrix deposition and scaffold colonization were assessed under the different culture conditions by immunofluorescence using antibodies against vimentin, fibronectin, collagen type I, and α -SMA. This panel allowed us to evaluate both fibroblast cytoskeletal organization and the production of key

ECM components, providing insight into how scaffold coating and seeding density influence early adhesion, matrix remodeling, and three-dimensional infiltration. Both the scaffold surface and cross-sections were examined at Days 1 and 14 to obtain a comprehensive view of cell distribution and ECM deposition.

In the uncoated scaffolds seeded with 150,000 cells (Figure 54), Day 1 was characterized by compact cellular aggregates, which were clearly visible on the surface and throughout the cross-section. Within these clusters, vimentin was consistently expressed, confirming fibroblast identity, whereas fibronectin expression was low and only slightly detectable, showing no evidence of extracellular network formation. The cross-section exhibited the same pattern, with strong vimentin staining but minimal or absent fibronectin.

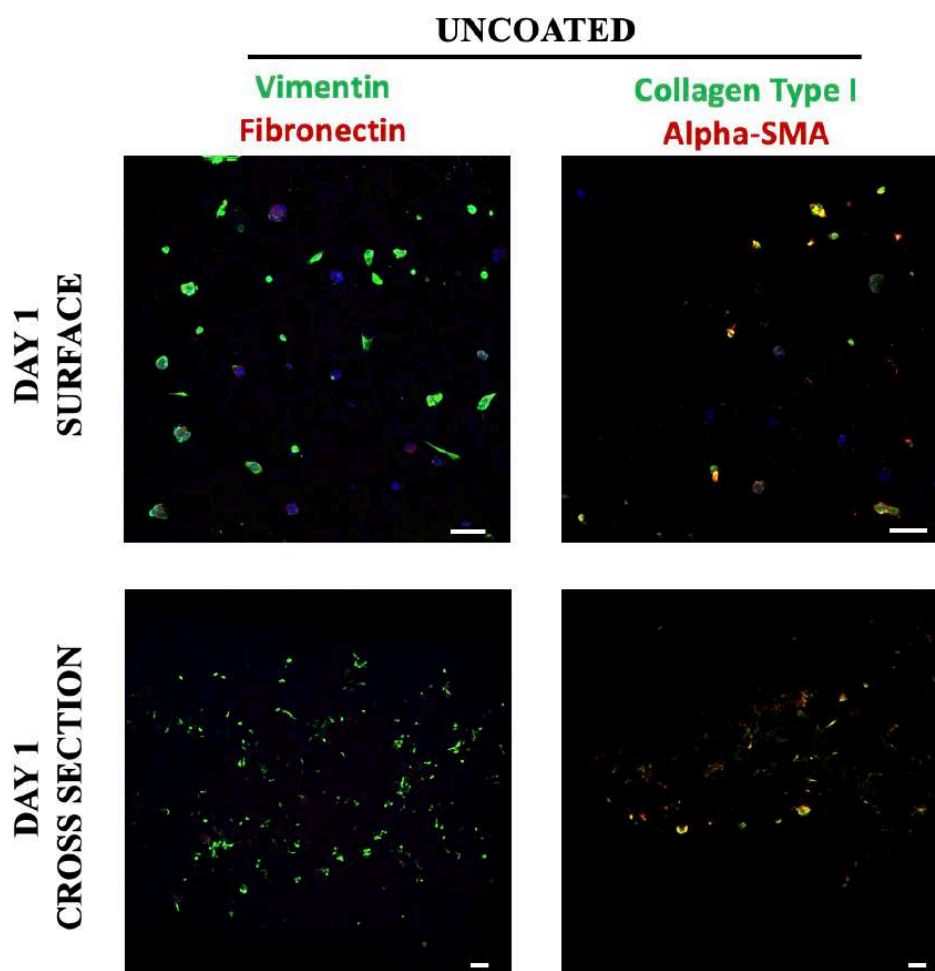


Figure 54. Immunostaining of 150,000 primary human dermal fibroblasts seeded for 1 day onto uncoated PLLA scaffolds. Scale bars: 100 μ m.

Collagen type I and α -SMA staining (Figure 54) also revealed rounded aggregates, and the overlapping red and green channels frequently produced yellow signals, indicating co-

expression of the two proteins within cellular clusters. However, the co-expression remained confined to the aggregates themselves, without organized fibrillar deposition; cross-section imaging fully reproduced this distribution.

At Day 14, the uncoated 150,000-cell scaffolds (Figure 55) exhibited a partial transition from rounded aggregates to elongated, fibroblast-like morphologies, although the cells did not fully cover the scaffold surface with clearly identifiable spots or regions of higher colonization. Within these regions, fibronectin expression was present and markedly higher than at Day 1, although it did not yet form extensive networks. A similar pattern was observed for collagen type I: although its signal increased relative to Day 1, collagen deposition remained confined to specific areas rather than being uniformly distributed. α -SMA was expressed throughout the scaffold surface, and the presence of multiple yellow and green spots indicated concomitant collagen type I production. Cross-section analysis revealed that fibronectin was barely detectable in the inner regions, whereas collagen type I was visible within deeper zones, particularly where cell aggregates had formed. Nonetheless, α -SMA was primarily localized in the outer regions of the scaffold, whereas collagen was predominantly detected in the inner areas.

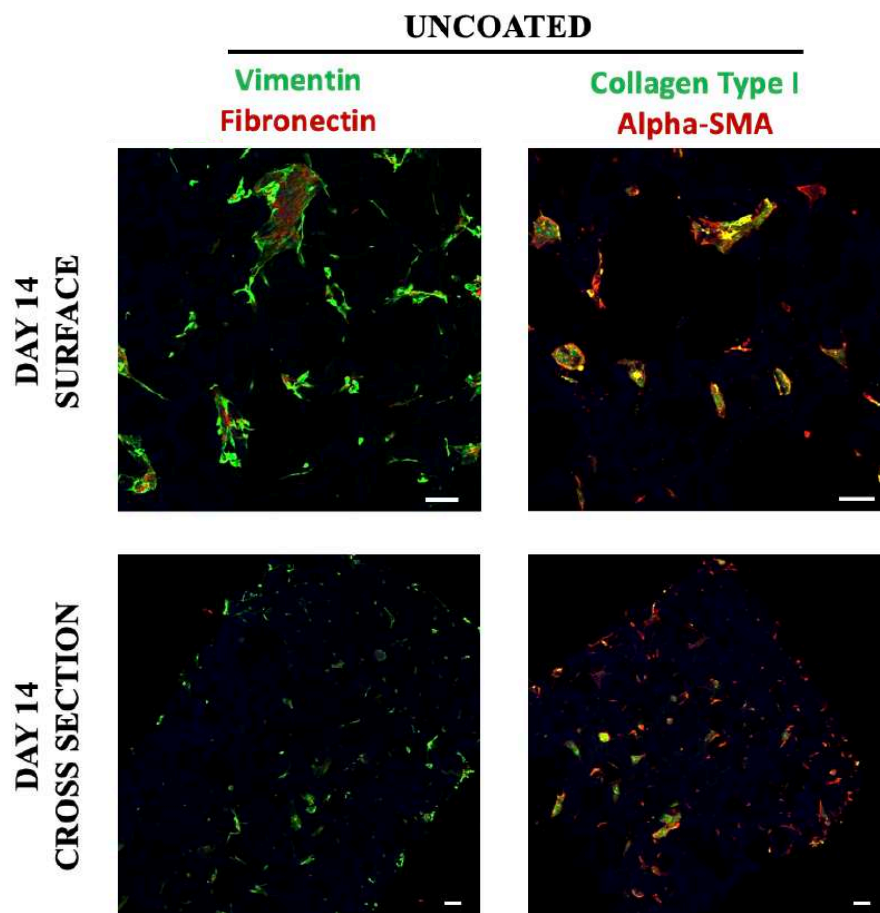


Figure 55. Immunostaining of 150,000 primary human dermal fibroblasts seeded for 14 days onto uncoated PLLA scaffolds. Scale bars: 100 μ m.

In coated scaffolds seeded with 150,000 cells (Figure 56), most cells displayed an elongated, fibroblast-like morphology after 1 day, with only small, sparse aggregates occasionally present and no large, compact aggregates observed. Vimentin expression was widespread, whereas fibronectin, although limited at this early time point, was more readily detectable than in uncoated samples. Likewise, collagen type I staining was more pronounced in coated scaffolds than in uncoated scaffolds. Consistent with these observations, cross-sectional analyses revealed a combination of elongated individual cells and small aggregates that expressed both collagen type I and α -SMA.

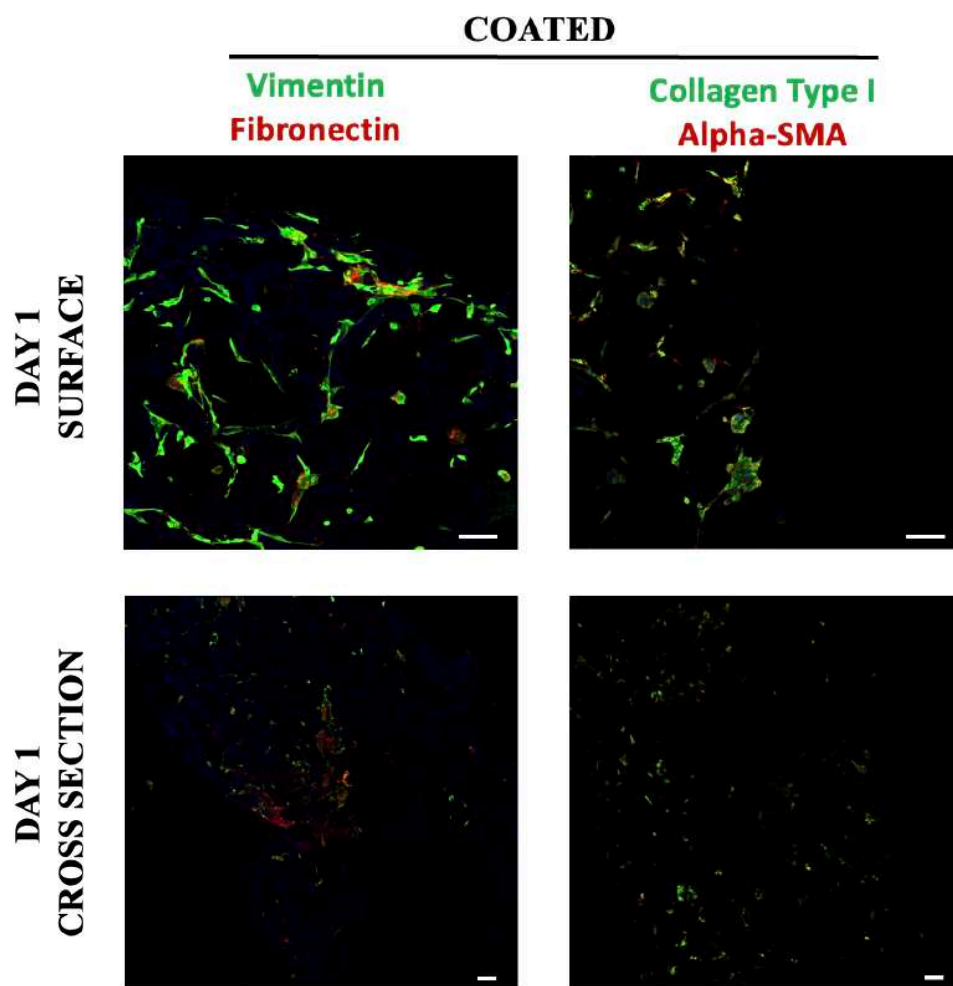


Figure 56. Immunostaining of 150,000 primary human dermal fibroblasts seeded for 1 day onto coated PLLA scaffolds. Scale bars: 100 μ m.

At 14 days, cells had markedly colonized a large portion of the scaffold surface (Figure 57). Vimentin was uniformly expressed across the entire surface, confirming complete fibroblast coverage. Fibronectin production was robust and clearly detectable as a dense, intricate extracellular network surrounding the cells. This was especially evident on the scaffold surface, where fibronectin appeared to envelop the cells, indicating substantial matrix

secretion and maturation. Cross-sectional images corroborated these findings, showing widespread fibronectin deposition across most regions, with lower intensity in some internal areas, consistent with the expected diffusion and matrix assembly gradient. The extensive surface coverage observed in the vimentin–fibronectin staining was fully supported by the collagen type I– α -SMA staining. The entire scaffold surface was occupied by α -SMA-positive cells, and numerous yellow and green spots indicated active secretion of collagen type I. Collagen deposition at Day 14 was markedly higher than both collagen expression at Day 1 and in uncoated scaffolds. In the internal regions, collagen type I was present but localized to specific cell-dense zones, whereas α -SMA expression remained widespread across the cross-section.

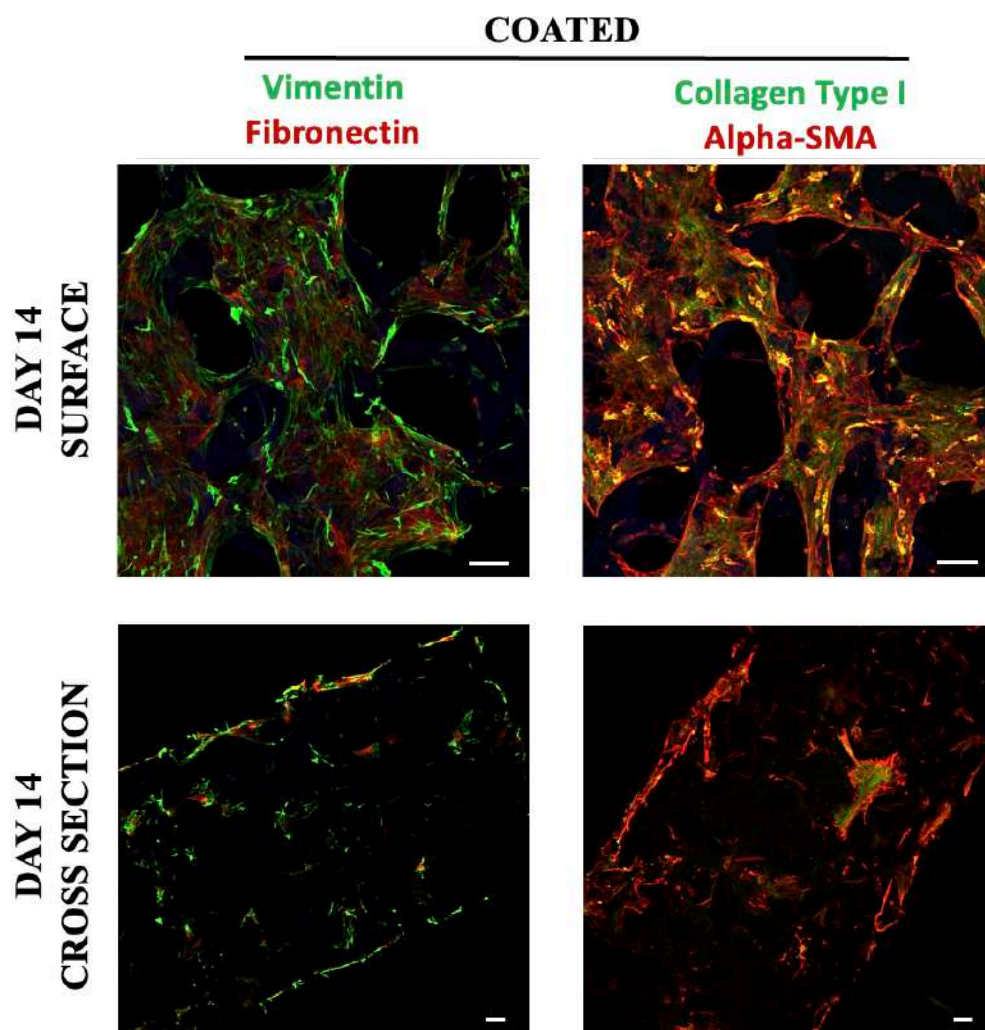


Figure 57. Immunostaining of 150,000 primary human dermal fibroblasts seeded for 14 days onto coated poly L lactic acid scaffolds. Scale bars: 100 μ m.

Overall, the coated 150,000-cell scaffolds exhibited superior colonization, greater ECM production, and more advanced matrix organization than the uncoated scaffolds.

In the uncoated scaffolds seeded with 300,000 cells, the Day 1 pattern showed a combination of compact cellular aggregates, similar to those observed in the 150,000-cell uncoated condition, alongside a population of elongated fibroblast-like cells (Figure 58). Almost all cells expressed both α -SMA and vimentin, indicating that the high seeding density promoted a widespread myofibroblast-like phenotype. Despite this, the production of extracellular matrix components remained limited at this early stage, with only sparse collagen type I and fibronectin detectable. The cross-sectional analysis provided a more nuanced view: while the scaffold surface was dominated by intense α -SMA staining, with only occasional yellow spots indicating minimal collagen type I production, the inner regions contained distinct cellular aggregates that actively produced abundant collagen type I. This created a clear contrast between the seeded surface, rich in α -SMA but poor in ECM, and the interior, where collagen deposition was more pronounced. A similar distribution pattern was observed for fibronectin, although the overall intensity was lower; fibronectin expression was

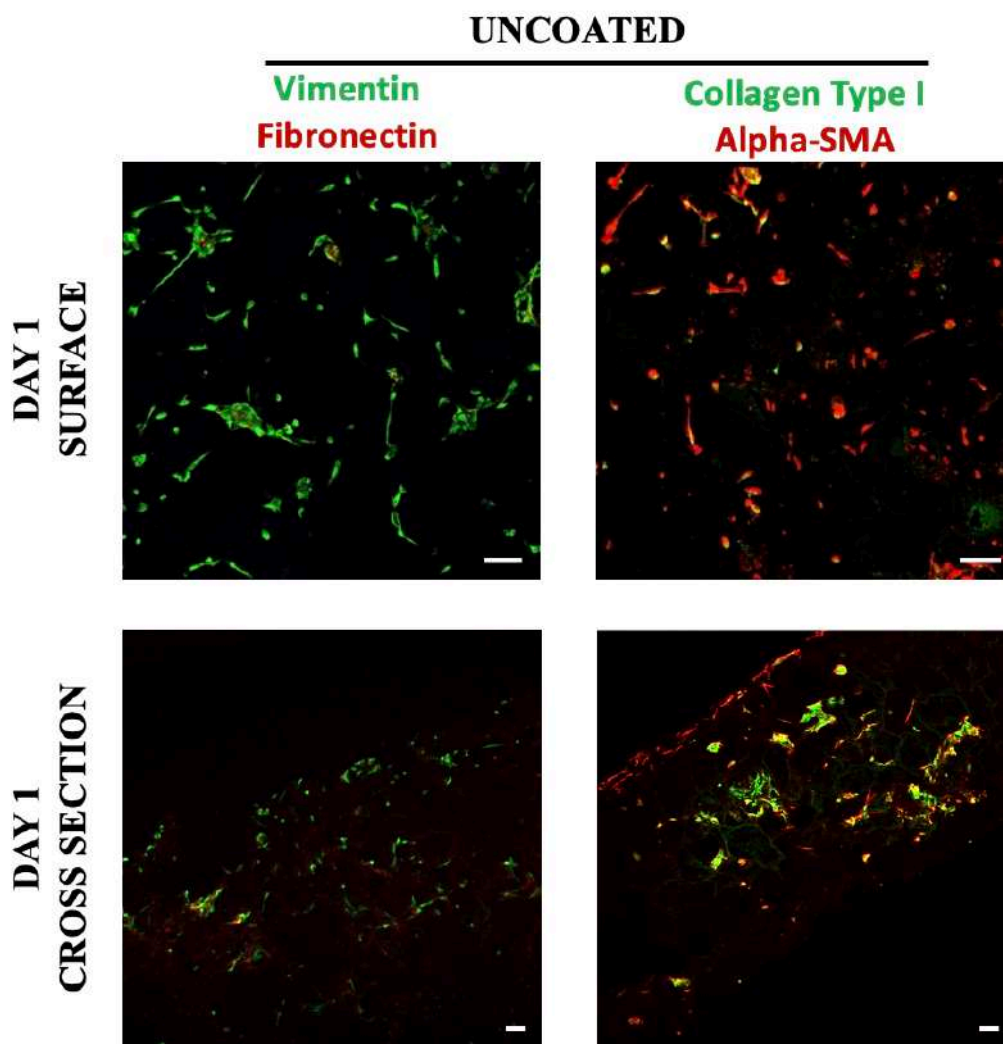


Figure 58. Immunostaining of 300,000 primary human dermal fibroblasts seeded for 1 day onto uncoated poly L lactic acid scaffolds. Scale bars: 100 μ m.

nevertheless more evident in the inner regions than on the surface, suggesting earlier or more sustained matrix synthesis by cells that had migrated or settled deeper within the structure.

At Day 14 (Figure 59), cells had spread extensively across the scaffold surface, covering it almost entirely except for the pores. In cell-rich regions, collagen type I was abundantly expressed, largely intracellularly, whereas fibronectin was clearly visible extracellularly and organized in a more coherent pattern. This was supported by cross-sectional analyses, which revealed a mixture of elongated fibroblasts and residual aggregates that actively produce ECM components, with collagen type I particularly prominent in these internal clusters. Although the overall pattern was less homogeneous than in coated scaffolds, the cells had clearly transitioned from an aggregate-dominated structure to a more organized fibroblast-like distribution supported by ECM deposition.

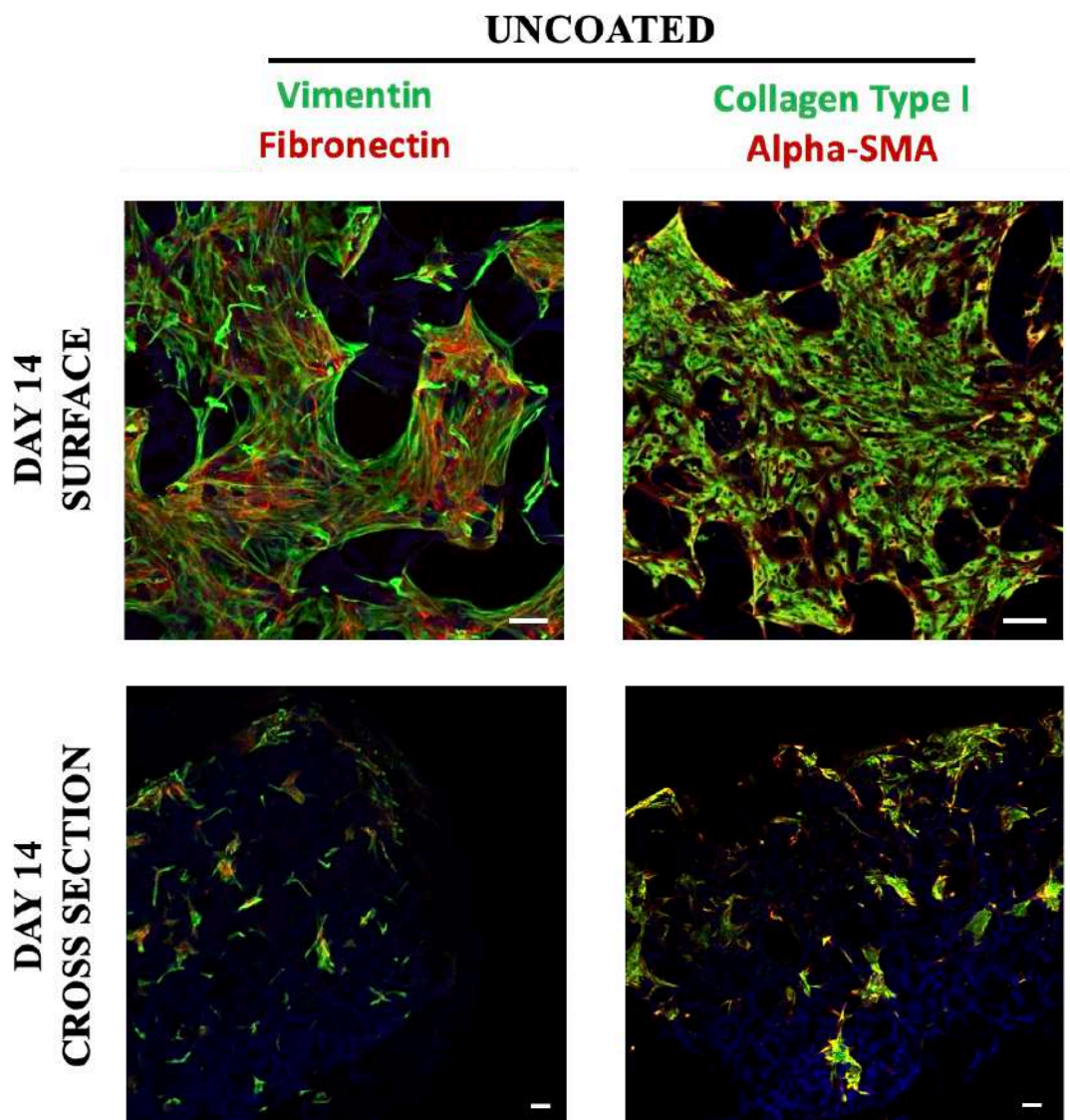


Figure 59. Immunostaining of 300,000 primary human dermal fibroblasts seeded for 14 days onto uncoated PLLA scaffolds. Scale bars: 100 μ m.

The coated scaffolds seeded with the same density exhibited a different scenario at Day 1 (Figure 60). Cells uniformly covered the entire scaffold surface, leaving only the pore openings unoccupied, and no aggregates were present. All cells displayed an elongated spindle-shaped morphology characteristic of fibroblasts. Both α -SMA and vimentin were strongly expressed throughout the scaffold. Fibronectin was already detectable not only intracellularly but also as an extracellular network extending around the cells, indicating enhanced and accelerated matrix deposition compared to uncoated samples. Collagen type I was also expressed by nearly all cells, although predominantly intracellularly at this stage. Cross-sectional imaging confirmed that elongated fibroblast-like cells were present not only near the surface but also deep within the scaffold, including the unseeded side, demonstrating effective early infiltration and uniform colonization. No major differences in expression patterns were detected between the surface and the interior; both collagen type I and fibronectin were consistently expressed across the scaffold thickness.

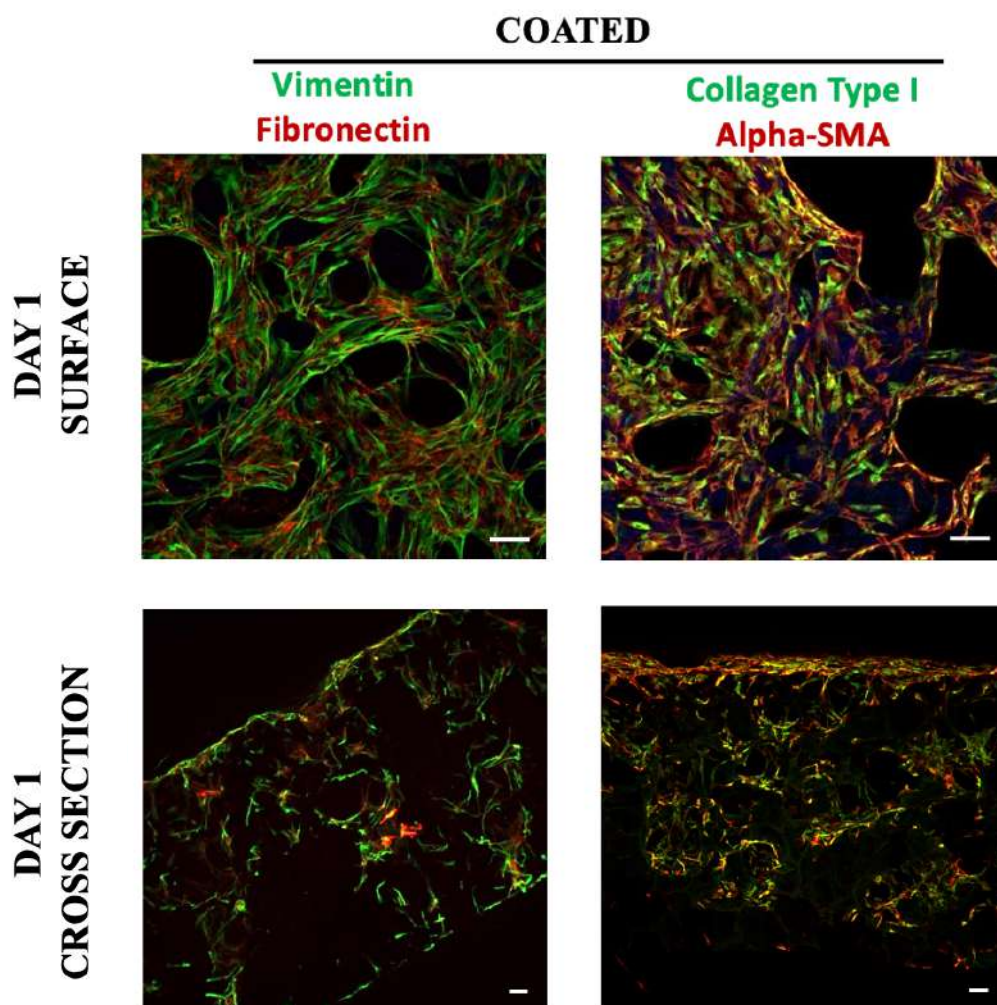


Figure 60. Immunostaining of 300,000 primary human dermal fibroblasts seeded for 1 day onto coated PLLA scaffolds. Scale bars: 100 μ m.

The coated scaffolds at Day 14 showed the most advanced stage of colonization and matrix production (Figure 61). On the seeded surface, cells formed a continuous, dense layer that partially occluded the pores, indicating extensive proliferation and matrix-driven stabilization. On the unseeded side, cells were also present, though they did not form the thick layer observed on the seeded surface, likely because contact with the well bottom limited expansion. Fibronectin formed a fully developed extracellular network covering the entire surface, while collagen type I, though still predominantly intracellular, was also visible extracellularly in several regions, indicating ongoing matrix maturation. Cross-sectional imaging revealed a thick, continuous cell layer occupying nearly the entire scaffold thickness, with cells expressing vimentin, α -SMA, fibronectin, and collagen type I. The overall impression was that of a fully colonized scaffold in which fibroblasts had produced and organized substantial extracellular matrix, confirming that coating in combination with high seeding density leads to optimal infiltration and matrix deposition.

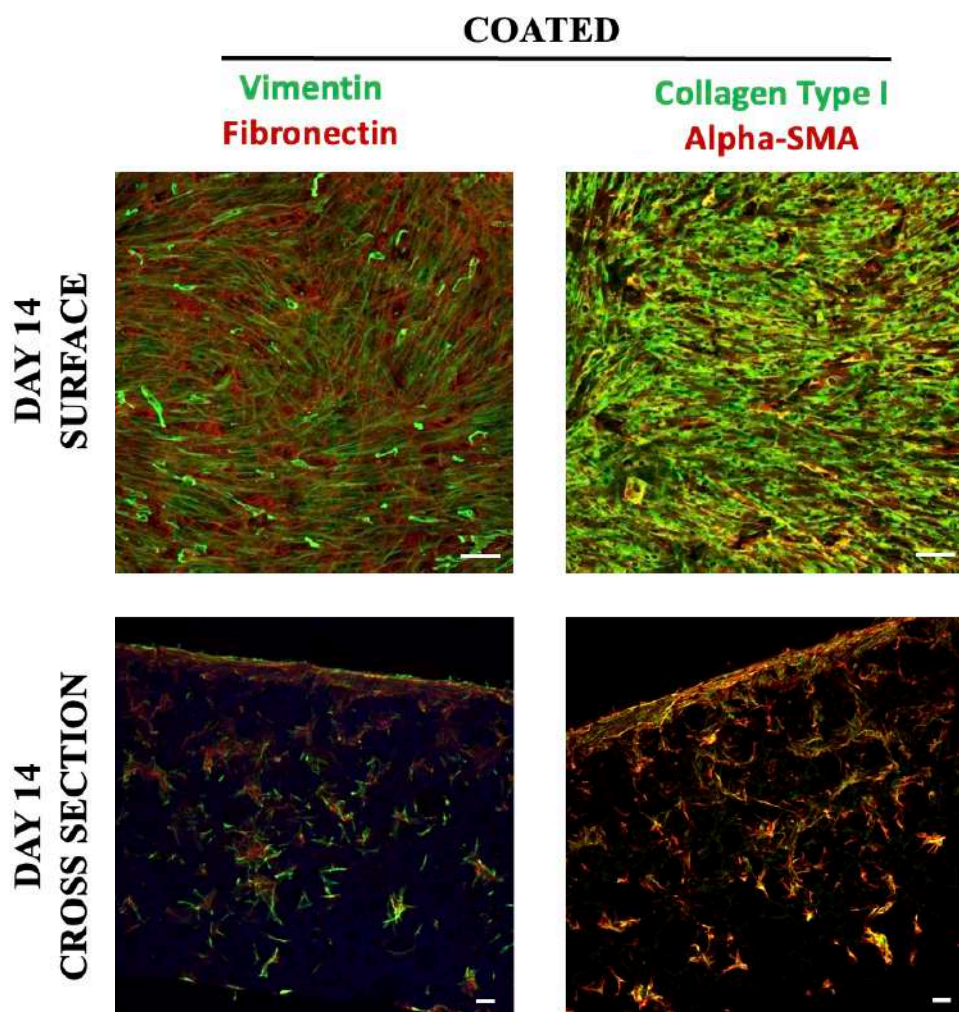


Figure 61. Immunostaining of 300,000 primary human dermal fibroblasts seeded for 14 days onto coated PLLA scaffolds. Scale bars: 100 μ m.

To further characterize ECM organization, we acquired high-magnification images focused on coated scaffolds seeded with 300.000 RDFs, which had shown the most advanced colonization and ECM deposition. These images enabled visualization of the fine structure of extracellular networks at much higher resolution. Fibronectin, labelled in red, appeared as a highly intricate and continuous meshwork surrounding the cells, with minimal overlap with intracellular regions (Figure 62). This distribution strongly suggests that fibronectin was efficiently secreted and assembled extracellularly, rather than remaining confined within the cells. Notably, fibronectin was detectable on the surface of coated scaffolds as early as Day 1 (Figure 61 A1-2), indicating early activation of ECM deposition under these conditions. Fibronectin expression persisted over time, and by Day 14 (Figure 61 B1-2) the network had matured substantially. At this later time point, fibronectin production appeared largely stabilized, with the network exhibiting a dense and well-structured organization.

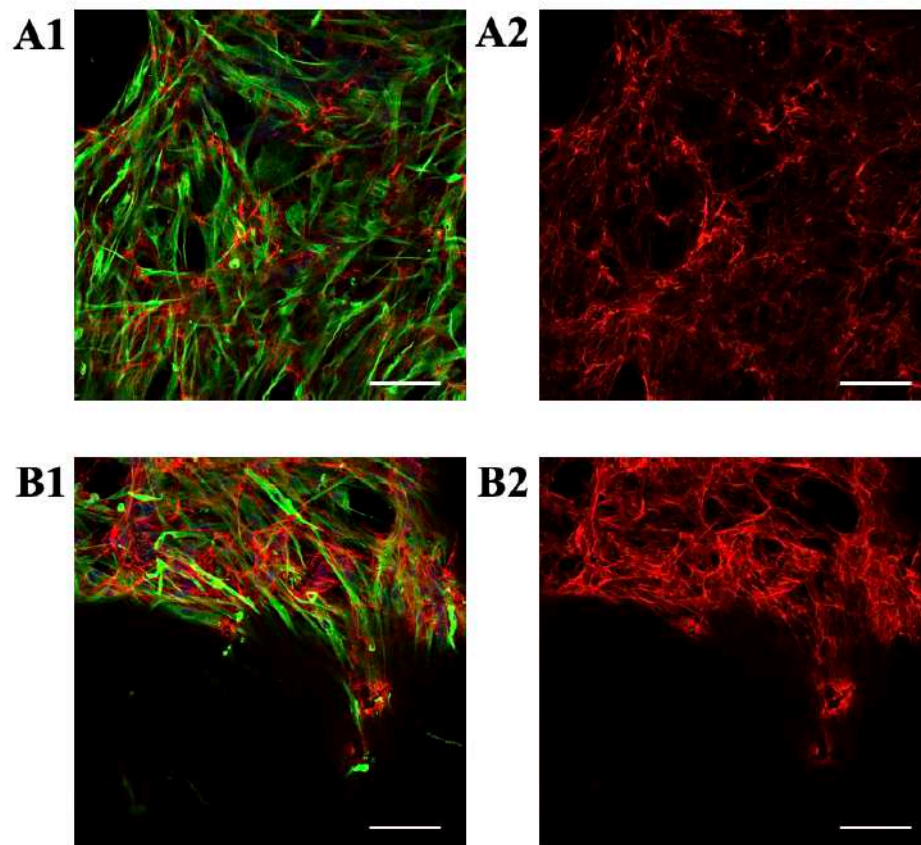


Figure 62. Immunostaining of 300.000 primary human dermal fibroblasts seeded for 1 (**A 1-2**) and 14 (**B 1-2**) onto coated PLLA scaffolds: **Fibronectin** and **Vimentin**. Scale bars: 100 μ m.

Collagen type I exhibited a distinct pattern compared to fibronectin (Figure 63). After 1 day of culture, it was predominantly intracellular, often appearing as discrete granular structures (Figure 63A 1–2). These punctate patterns are consistent with intracellular processing steps, in which collagen is synthesized as pre-procollagen, converted to procollagen within the secretory pathway, and subsequently packaged into vesicles prior to secretion. In contrast, after 14 days, collagen was observed both intracellularly and extracellularly (Figure 63B 1–2). The extracellular collagen network, although less dense and less extensive than that of fibronectin, was clearly present and appeared to become more organized over time.

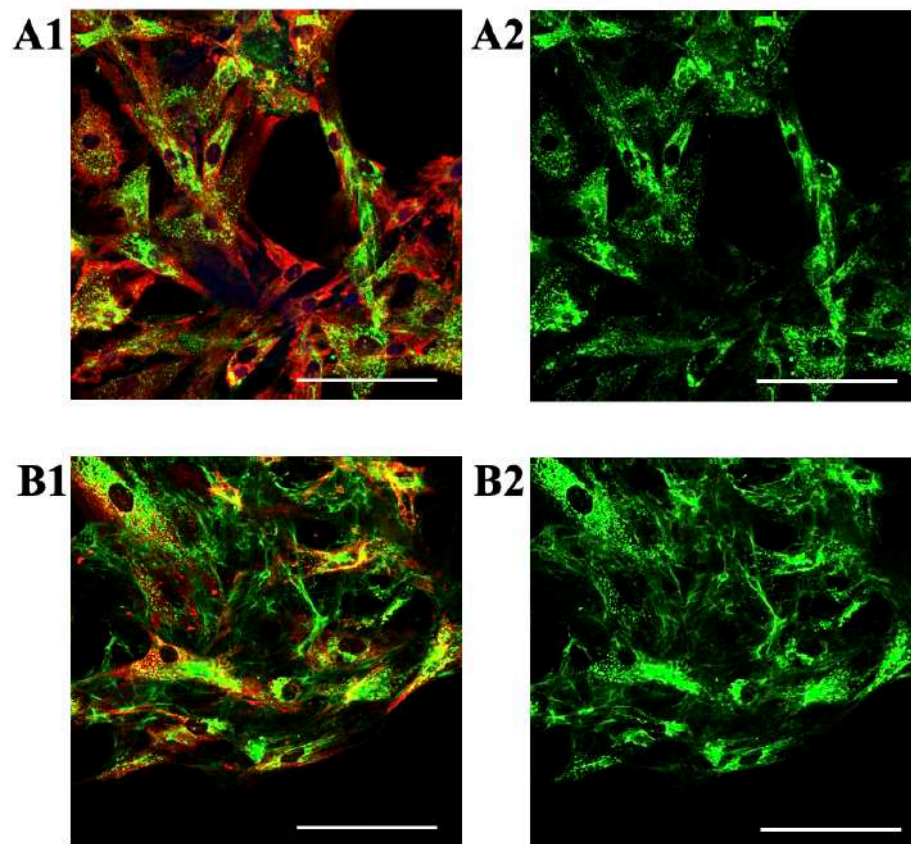


Figure 63. Immunostaining of 300,000 primary human dermal fibroblasts seeded for 1 (A 1-2) and 14 (B 1-2) days onto coated PLLA scaffolds: **Alpha-SMA** and **Collagen type I**.

Overall, these results demonstrate that both scaffold coating and seeding density are key determinants of fibroblast colonization, morphology, and ECM deposition in 3D culture. Coated scaffolds consistently promoted early and uniform adhesion, enhanced ECM secretion. They facilitated scaffold infiltration, while higher seeding densities accelerated surface coverage and promoted a more homogeneous distribution throughout the construct. Fibroblasts on coated scaffolds rapidly adopted an elongated, spindle-shaped morphology,

actively produced fibronectin and collagen type I, and established interconnected ECM networks, indicating effective matrix remodeling and functional colonization. In contrast, uncoated scaffolds exhibited delayed adhesion, patchy coverage, and slower ECM organization, highlighting the importance of biochemical cues in guiding cellular behavior. Collectively, these findings underscore that the combination of scaffold biofunctionalization and sufficient initial cell numbers is essential for achieving robust fibroblast integration, matrix maturation, and the establishment of a structurally and functionally competent 3D tissue construct.

Histological analyses

To complement the immunofluorescence analyses and provide a broader overview of cell colonization and ECM deposition within the scaffolds, additional histological stainings were performed on paraffin-embedded sections from all experimental conditions. Based on the results of previous experiments, only scaffolds seeded with 300,000 fibroblasts on coated and uncoated surfaces, which exhibited the most advanced colonization and matrix production, were analyzed in this set of experiments. Hematoxylin and Eosin were used to visualize tissue morphology and cell distribution, Sirius Red to highlight collagen fibers, and Alcian Blue to detect glycosaminoglycans. Because the orientation of the scaffolds during embedding could not be controlled, and because sections were collected at different depths with a thickness of 5–7 μm , cells were not consistently present in every section. For this reason, the images shown are qualitative, representative examples illustrating the presence of cells and ECM components where detectable, rather than enabling quantitative comparisons between samples or time points.

Hematoxylin & Eosin

Histological analysis using hematoxylin and eosin (H&E) staining (Figure 64) demonstrated that the presence of the coating significantly influences fibroblast behavior during the early stages of scaffold colonization. In particular, at Day 1, coated scaffolds promoted a more homogeneous cell distribution and an elongated fibroblastic morphology, with cells appearing closely associated with the scaffold surface. These features are commonly associated with improved cell adhesion and a more physiologically relevant interaction with the material. In contrast, uncoated scaffolds exhibited compact cellular aggregates with limited contact with the surrounding substrate, suggesting suboptimal initial cell–material interactions.

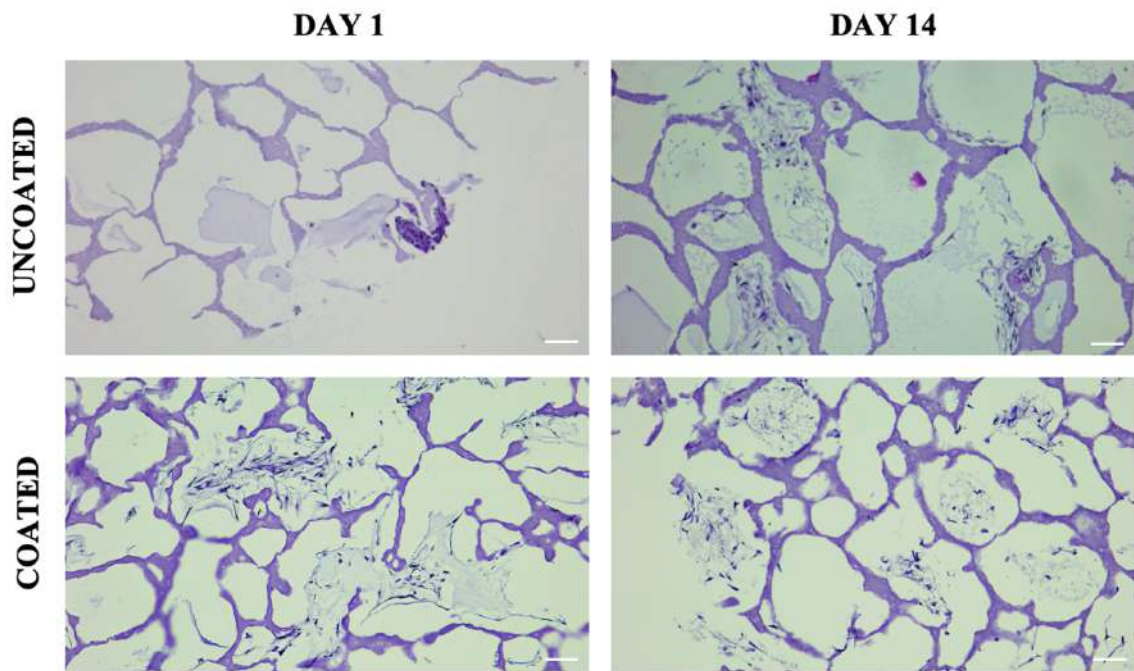


Figure 64. Hematoxylin/Eosin histological staining of coated and uncoated scaffolds at 1 and 14 days. Scale bars: 100 μm .

At later time points, however, these differences became progressively less pronounced. By Day 14, fibroblasts cultured on both coated and uncoated scaffolds displayed a similar elongated morphology and a more dispersed, non-aggregated organization. This convergence in cellular behavior indicates that fibroblasts possess a strong capacity to adapt to their microenvironment and to actively remodel the scaffold surface through de novo extracellular matrix deposition. Such matrix production likely compensates for the absence of the bioactive cues provided by the coating, ultimately enabling stable and sustained cell–substrate interactions.

Overall, these findings suggest that while the coating plays a critical role in enhancing early cell adhesion, spreading, and spatial organization, its influence diminishes over time as cell-mediated remodeling processes become dominant. From a tissue engineering perspective, this highlights the importance of surface functionalization for applications requiring rapid cellular integration and early biological performance, while also indicating that uncoated scaffolds may still support long-term cell adhesion and organization due to intrinsic cellular remodeling mechanisms.

Sirius Red

A similar temporal progression was observed in Sirius Red staining (Figure 65). On Day 1, the coated scaffolds already exhibited small extracellular collagen fibers surrounding the cells, suggesting early matrix deposition. In contrast, collagen fibers were not detectable in uncoated scaffolds at the same time point, where only aggregates were observed, consistent with the delayed establishment of extracellular matrix organization in the absence of surface functionalization.

By Day 14, collagen deposition was clear in both coated and uncoated scaffolds. In both conditions, collagen fibers appeared more abundant and organized, frequently aligned with the elongated cellular morphology. Notably, the increase in collagen deposition observed in uncoated scaffolds between Day 1 and Day 14 was particularly pronounced, ultimately reaching levels comparable to those observed in coated scaffolds.

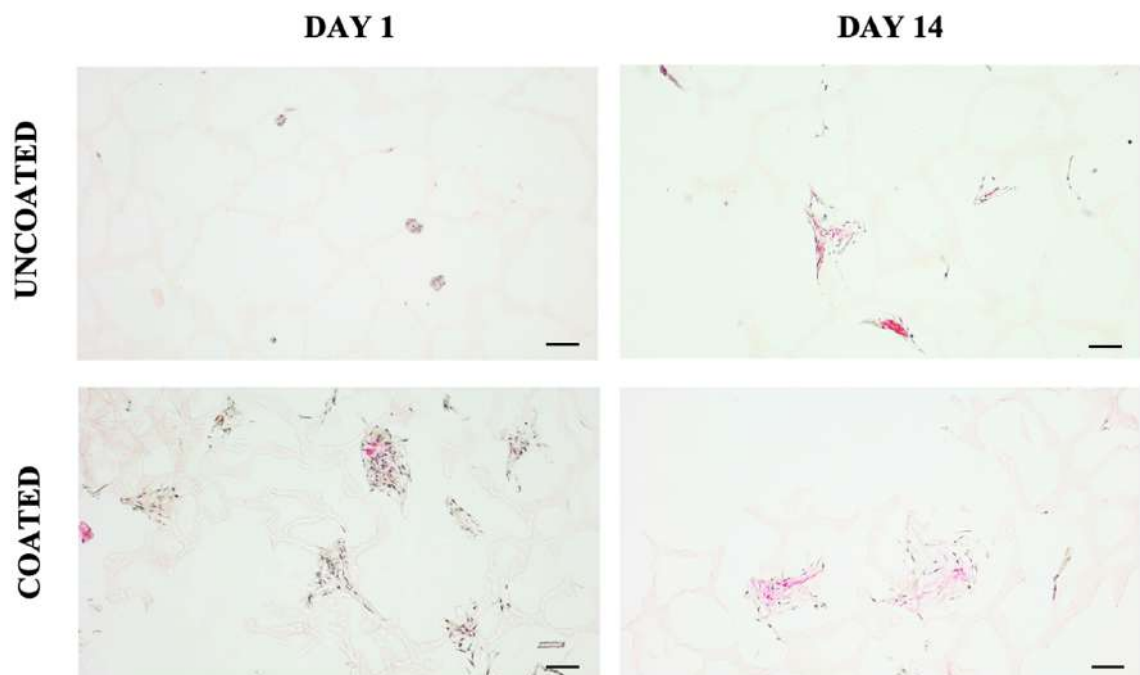


Figure 65. Sirius red histological staining of coated and uncoated scaffolds at 1 and 14 days.
Scale bars: 100 μ m.

Overall, these findings further support fibroblasts' ability to progressively remodel the scaffold microenvironment through ECM deposition, resulting in a structured collagen network over time, regardless of the initial presence of a coating.

Alcian Blue

Alcian Blue staining provided additional insights into the distribution of glycosaminoglycans (GAGs). Because the coating solution contained chondroitin sulfate, the analysis focused on regions with visible cells, excluding areas where staining might reflect the coating rather than cell-derived GAGs. Moreover, only day 1 samples were analysed due to a lack of samples. Both coated and uncoated scaffolds displayed a generally intense turquoise background, likely due to intrinsic interactions between the dye and the scaffold material (Figure 66). On Day 1, uncoated scaffolds exhibited compact cellular aggregates that stained more intensely than elongated fibroblasts in coated scaffolds. This could reflect either increased GAG production within the aggregates or a simple concentration effect due to the close packing of cells. Because both conditions were sectioned at the same thickness, differences could not be attributed to section depth.

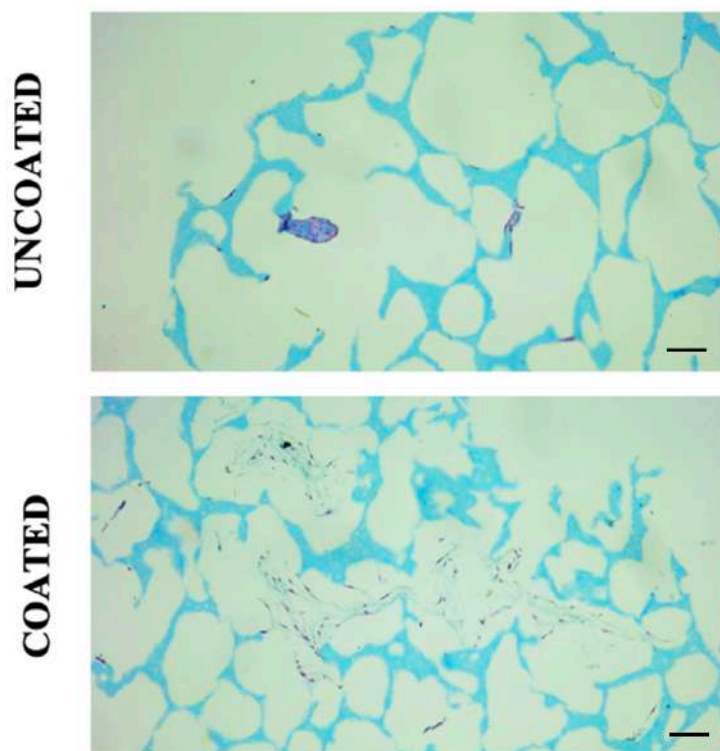


Figure 66. Alcian blue histological staining of coated and uncoated scaffolds at 1 and 14 days. Scale bars: 100 μm .

GAGs quantification

To further clarify these observations, GAG quantification was performed and normalized to total DNA content, which was used as a proxy for cell number. Before performing

quantitative analyses of glycosaminoglycan (GAG) production, total DNA was isolated from each scaffold condition to normalize ECM production per cell. Specifically, 300,000 cells were seeded on three scaffolds per condition (coated and uncoated), and DNA content was measured using the CyQuant assay at each time point. This approach allowed the quantification of GAGs relative to cell number, providing a more accurate assessment of matrix deposition independent of variations in cell proliferation or scaffold colonization (Figure 67).

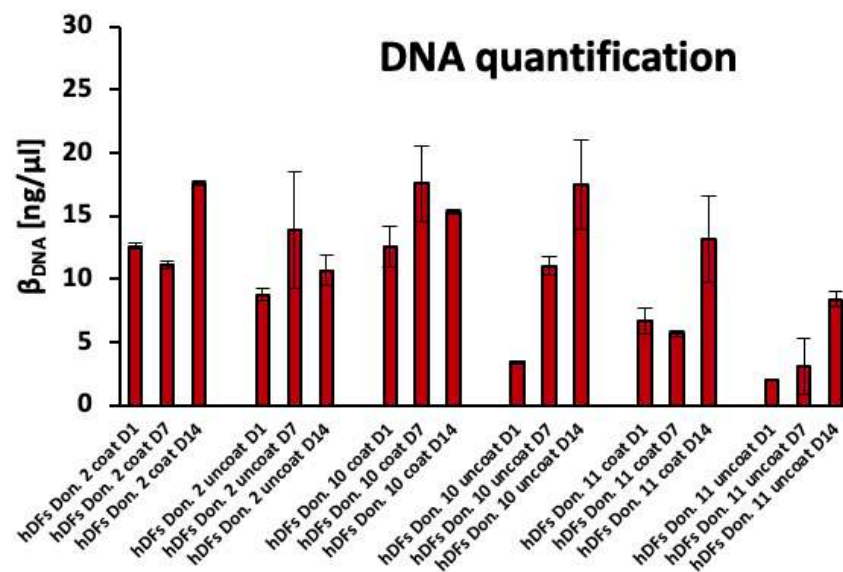


Figure 67. CyQuant DNA quantification of fibroblasts from different donors (Don.) seeded on coated (Coat.) and uncoated (Uncoat.) for 1 (D1), 7 (D7), and 14 (D14) days.

Quantitative analyses confirmed that fibroblasts produced measurable amounts of GAGs in both coated and uncoated scaffolds, following a characteristic temporal pattern (Figure 68). GAG levels were similar between conditions at Day 1 and increased markedly by Day 7. In uncoated scaffolds, levels then declined by Day 14, returning close to Day 1 values. In contrast, coated scaffolds showed a steady increase in GAG content over time, nearly doubling from Day 1 to Day 14, indicating sustained matrix production.

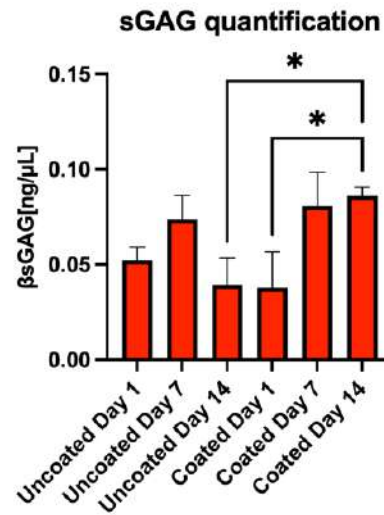


Figure 68. GAGs quantification.

Overall, these results suggest that although Alcian Blue staining initially appeared more intense in uncoated scaffolds, likely due to cell aggregation and compaction, the quantitative GAG analysis, normalized to DNA, clearly demonstrates that coated scaffolds support higher and more sustained ECM production. This finding highlights the beneficial effect of surface coating on promoting early and robust matrix synthesis at the single-cell level.

CONCLUSION

The present study comprehensively presents the development and characterization of a multifunctional, three-dimensional tissue-engineered construct designed for regenerative applications, particularly targeting chronic wound healing. By integrating porous PLLA scaffolds with primary dermal fibroblasts and microvascular fragments, the study establishes a model that supports both cellular colonization and vascular network formation in a controlled three-dimensional environment. Importantly, the use of thermally induced phase separation (TIPS) PLLA scaffolds allows for an interconnected porous architecture that supports sustained cell infiltration, promotes extracellular matrix deposition, and provides mechanical resistance suitable for resisting contraction forces such as those exerted by the panniculus carnosus in rodent models.

Chronic wounds represent a complex pathological context characterized by impaired cellular activity, reduced vascularization, persistent inflammation, and hypoxia. Addressing these multifactorial deficiencies requires a tissue-engineered strategy that simultaneously promotes structural support, extracellular matrix deposition, and rapid angiogenesis, all of which are achieved in the present construct through the complementary roles of fibroblasts and MVFs. The combination of fibroblasts and MVFs offers a synergistic advantage, as fibroblasts provide ECM deposition and pro-angiogenic signals, while MVFs serve as pre-assembled vascular units capable of sprouting, branching, and forming interconnected networks, which in other studies have been shown to inosculate rapidly *in vivo*, offering a clear translational potential for rapid tissue perfusion once implanted.

RDFs were successfully isolated using both explant and enzymatic digestion, yielding pure, spindle-shaped cells with stable mesenchymal identity, as evidenced by vimentin and collagen type I expression. These cells maintained their functional phenotype, producing ECM components and demonstrating phenotypic activation, particularly upon seeding into three-dimensional scaffolds. Scaffold architecture is a key determinant of fibroblast behavior: PLLA scaffolds fabricated via thermally induced phase separation consistently supported optimal cell adhesion, proliferation, and multilayered colonization. Smaller pores facilitated rapid early expansion but quickly reached saturation. In contrast, pore sizes of approximately 300 μm enabled sustained growth and a uniform spatial distribution, highlighting the importance of matching scaffold microarchitecture to the intended cellular and regenerative application. These features ensure effective cell-matrix interactions and facilitate uniform tissue regeneration throughout the scaffold volume.

Seeding strategy and initial cell density were critical for early retention and long-term colonization. Low cell densities resulted in progressive cell loss, whereas seeding 300,000 cells per scaffold maintained stable populations capable of sustained proliferation. Biochemical optimization further enhanced fibroblast performance: medium supplemented with EGF promoted adhesion, spreading, and survival, enabling fibroblasts to penetrate scaffold pores, form multicellular aggregates, and initiate functional remodeling. Confocal microscopy and SEM analyses confirmed that fibroblasts effectively colonized the internal porous architecture, forming multilayered structures and spherical aggregates that occupied scaffold pores, demonstrating strong cell-matrix interaction and spatial organization.

Functionally, fibroblasts exhibited enhanced expression of vimentin and α -SMA within the three-dimensional environment, reflecting cytoskeletal organization, contractile potential, and acquisition of a myofibroblast-like phenotype critical for extracellular matrix remodeling and wound stabilization, effects not achievable in standard 2D culture. These observations indicate that the 3D PLLA scaffold not only supports cell adhesion and proliferation but also induces a wound-like microenvironment, promoting fibroblast differentiation toward a myofibroblast phenotype and driving ECM deposition patterns that more closely recapitulate in vivo tissue repair. By enabling fibroblasts to adopt a physiologically relevant morphology and functional behavior, the scaffold facilitates the early stages of wound healing, including contractile force generation and matrix remodeling, thereby providing a more predictive in vitro model of regenerative processes.

MVFs isolated from adipose tissue retained structural integrity and exhibited robust angiogenic potential. Adipose tissue was confirmed as an optimal source due to its compliant architecture, ease of enzymatic dissociation, and preservation of native microvascular morphology. Visceral and perirenal depots yielded the highest number of intact MVFs, with lengths of 30-500 μ m, diameters of 10-30 μ m, branching morphologies, and occasional luminal erythrocytes, confirming vascular integrity. In 2D culture, MVFs reorganized from fragmented segments into multicellular aggregates that sprouted and interconnected into extensive endothelial networks within seven days, with collagen type IV and VE-cadherin staining confirming endothelial identity, basement membrane deposition, and formation of stable cell-cell junctions. In 3D collagen hydrogels, MVFs retained their tubular morphology, exhibited sustained viability, sprouting, branching, and anastomosis, resulting in dense, interconnected vascular networks across multiple depths. While our study was conducted in vitro, these observations demonstrate the intrinsic potential of MVFs to establish pre-vascular networks within scaffolds, a strategy which has been shown in

literature to outperform single-cell vascularization approaches, enabling faster and more stable network formation.

Integration of MVFs into PLLA scaffolds revealed that scaffold biofunctionalization and biochemical supplementation are essential for sustained angiogenesis. Uncoated scaffolds permitted initial adhesion but failed to support the formation of organized vessels over time. Geltrex coating promoted early network formation; however, this effect was transient, with most vascular structures disappearing by day 14. Supplementation with VEGF was critical for stabilizing and maintaining well-defined, lumen-containing vessels over two weeks, as evidenced by SEM and fluorescence imaging, which confirmed the presence of continuous lumenized vascular networks and perivascular-like cells, indicative of progressive maturation and stabilization. Notably, this is the first demonstration of MVFs seeded onto PLLA scaffolds, representing a novel and translationally relevant platform that combines a mechanically robust synthetic polymer with pre-vascularized microtissues, enabling controlled investigation of microvascular integration and fibroblast–vessel synergy.

The co-culture of RDFs with MVFs on Geltrex-coated, VEGF-EGF-supplemented PLLA scaffolds represents the culmination of the model and highlights the synergistic interplay between stromal and vascular components. In this setting, fibroblasts not only adhered to, proliferated within, and infiltrated the scaffold effectively but also actively deposited type I collagen around developing vascular structures, thereby stabilizing nascent vessels and supporting the formation of dense, interconnected, intraporous vascular networks by day 14. This co-culture system integrates the structural and functional contributions of fibroblasts with the intrinsic angiogenic capacity of MVFs, producing a pre-vascularized construct that closely mimics the cellular and matrix complexity of native tissue. The resultant environment provides both a mechanically and biochemically competent microarchitecture, demonstrating that the combination of scaffold biofunctionalization, optimized seeding density, biochemical cues, and coordinated cellular interactions is essential for achieving functional tissue constructs. These results highlight the added value of pre-assembled microvascular units over dispersed endothelial or progenitor cells, providing an intrinsic vascular blueprint that can support rapid network formation in patient-specific constructs.

To evaluate translational relevance, primary human dermal fibroblasts (hDFs) from donors of various ages were seeded onto PLLA scaffolds. The results confirmed that hDFs effectively adhered to, proliferated on, and remodeled the scaffolds, with scaffold coating and initial seeding density as decisive factors. Coated scaffolds consistently enhanced early

adhesion, uniform spreading, and metabolic activity, particularly at lower seeding densities. In comparison, higher cell densities promoted faster surface coverage and scaffold infiltration, despite a transient initial stress.

Immunofluorescence revealed early and robust production of fibronectin and collagen type I, widespread vimentin and α -SMA expression, and the formation of interconnected extracellular networks. By day 14, coated scaffolds exhibited dense, fully developed ECM networks, with fibronectin forming an intricate extracellular mesh and collagen type I increasingly matured intracellularly and extracellularly, demonstrating effective matrix remodeling and functional scaffold colonization. Histological analyses, including H&E, Sirius Red, and Alcian Blue staining, confirmed progressive fibroblast adhesion, infiltration into inner scaffold parts, and ECM deposition, with coated scaffolds consistently showing accelerated and more uniform matrix formation. Quantitative GAG analysis normalized to cell number further highlighted the beneficial effect of scaffold biofunctionalization on ECM synthesis at the single-cell level. These results demonstrate the potential to generate patient-specific dermal constructs that combine optimized scaffold architecture with functional cellular components, supporting translational relevance for autologous wound repair applications.

Overall, the study provides a comprehensive framework demonstrating that structural, biochemical, and cellular cues act synergistically to produce a functional, vascularized, and ECM-rich three-dimensional tissue construct, offering a promising strategy for accelerating wound healing and tissue regeneration. The novelty of this work lies in the integration of TIPS-fabricated PLLA scaffolds with intact microvascular fragments and primary dermal fibroblasts, an approach that, to our knowledge, has not been previously reported. Consequently, this study represents the first investigation of PLLA scaffolds combined with MVFs for functional vascularization, providing a novel platform to evaluate microvessel dynamics and scaffold integration within a clinically relevant biomaterial system. This approach provides a unique platform to study microvascular dynamics, scaffold integration, and stromal-vascular synergy in vitro, establishing a basis for future patient-specific and clinically translatable tissue-engineered skin constructs.

FUTURE PROSPECTIVES

Given the robust performance of primary human dermal fibroblasts on PLLA scaffolds, a key future direction is the development of a fully human-based therapeutic unit. In this context, collaboration with a clinical partner would enable access to human adipose tissue obtained from surgical procedures, allowing the isolation of human MVFs. The integration of human fibroblasts and human-derived MVFs within the optimized PLLA scaffold would permit the generation of a fully human pre-vascularized construct. This step is essential for evaluating cellular compatibility, angiogenic potential, and ECM remodeling in a human-specific context, thereby strengthening the platform's translational relevance and supporting its suitability for clinical application.

However, a significant limitation that must be addressed before any *in vivo* experimentation concerns the use of Geltrex as a scaffold coating. Geltrex is derived from murine tumor cells and, therefore, could not be suitable for *in vivo* applications or translational use. Consequently, identifying a biologically compatible and clinically relevant alternative will be necessary before proceeding to animal studies. Potential strategies include the formulation of a synthetic or semi-natural matrix reproducing the main components of Geltrex at comparable proportions, thereby preserving its pro-adhesive and pro-angiogenic properties. Alternatively, extracellular matrix derived from autologous decellularized adipose tissue represents a promising candidate, as it retains native biochemical cues, supports both stromal and vascular cells, and aligns well with the use of adipose-derived microvascular fragments.

Once a suitable Geltrex-free biofunctionalization strategy is established and validated *in vitro*, *in vivo* studies will represent the next critical phase of development. Planned experiments will employ the Wistar rat as a preclinical model to evaluate the efficacy of the therapeutic unit in chronic ulcers, with a particular focus on diabetic ones. Full-thickness wounds will be generated on the dorsal region, followed by standardized cleaning and enzymatic debridement before application of the complete construct (Figure 69). Healing outcomes will be compared with untreated controls and with groups receiving incomplete versions of the therapeutic unit, enabling systematic evaluation of the contribution of each component.

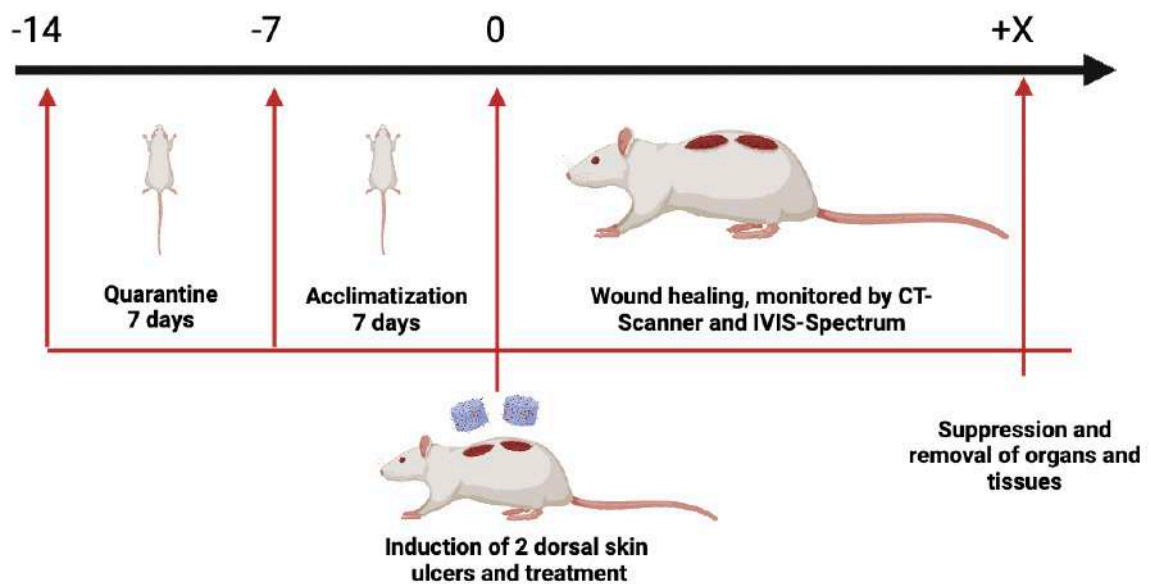


Figure 69. Treatment plan for rat-induced non-diabetic ulcers.

To model impaired healing conditions, diabetes will be induced via Streptozotocin administration, and ulcer formation will be initiated once sustained hyperglycemia is confirmed (Figure 70). This model will allow assessment of construct performance in an environment characterized by reduced angiogenesis, altered fibroblast activity, and chronic inflammation. Longitudinal monitoring of wound healing and scaffold integration will be conducted using X-ray axial tomography and IVIS Spectrum imaging, enabling non-invasive evaluation of structural regeneration and vascular dynamics. At defined time points, regenerated tissue will be harvested for histological and immunohistochemical analyses to assess extracellular matrix organization, collagen maturation, vascular integration, and overall tissue architecture.

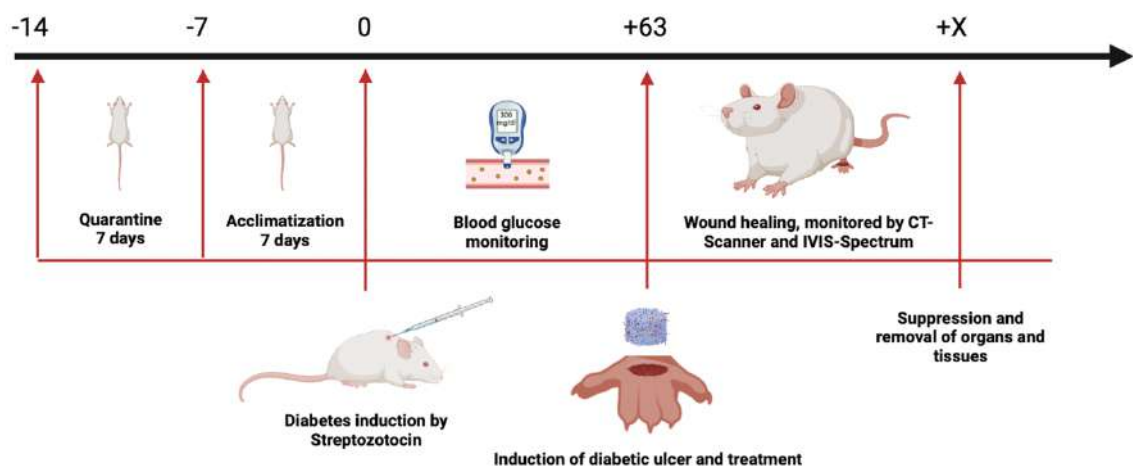


Figure 70. Treatment plan for rat-induced diabetic ulcers.

Collectively, this stepwise strategy provides a rigorous, clinically oriented framework for translational development. These studies will establish the safety, functionality, and regenerative capacity of the construct, supporting its advancement toward therapeutic application in the treatment of chronic and non-healing wounds.

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