



Machine learning-assisted optimization of injectable silk fibroin hydrogels functionalized with decellularized extracellular matrix and mesenchymal stromal cell secretome

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ABSTRACT

Silk fibroin (SF) hydrogels are promising injectable scaffolds for wound repair, but optimizing their multi-parametric gelation remains challenging due to complex formulation-processing interactions. We developed a bioinspired gelation strategy combining potassium-driven β -sheet nucleation with sonication-mediated conformational activation, guided by factorial design and machine learning. Artificial neural network (NN) and Random Forest (RF) algorithms mapped non-linear relationships between SF concentration, potassium (K^+) concentration, sonication time, and gelation kinetics. Machine-learning-assisted analysis delineated an experimentally informed operational region associated with complete or near-complete gelation and generally favorable gelation kinetics, which was subsequently used to select formulations for technological characterization. All formulations showed physiological pH, acceptable injectability (extrusion forces <5 N), and sonication-tunable microstructure ranging from compact lamellar to porous sponge-like architectures. To enhance biological functionality, gels were combined with adipose-derived mesenchymal stromal cell-lyosecretome and human skin-derived decellularized extracellular matrix (dECM), with component-level testing supporting their cytocompatibility and identifying blood-interaction profiles that warrant further evaluation in the complete composite formulation. Functional analyses revealed distinct *in vitro* biological profiles: dECM produced the strongest pro-angiogenic response in the tube formation assay, accompanied by VEGFA upregulation and downregulation of NFkB1, HMGB1, and BAX in fibroblasts. Lyosecretome showed complementary effects, including selective SOD2 upregulation, FGF7 induction, and enhanced fibroblast migration when tested as a soluble component. SF-containing formulations attenuated fibroblast scratch closure relative to controls, indicating a matrix-dependent modulation of cell migration. These *in vitro* findings define distinct component-associated biological response profiles and provide a basis for subsequent mechanistic and preclinical evaluation.

1. Introduction

Deep wounds, including full-thickness skin defects, surgical cavities, pressure ulcers, and trauma-related tissue loss, represent a significant

clinical challenge. Unlike superficial injuries, they involve the disruption of multiple tissue layers and require the coordinated progression of complex biological processes, including inflammation resolution, granulation tissue formation, neovascularization, and extracellular matrix

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(ECM) remodeling [1,2]. Current treatment strategies, such as gauze dressings, vacuum-assisted closure, and skin grafts, remain suboptimal, particularly in irregular or cavitated wounds, where poor conformability, limited structural support, and inadequate antibiotic penetration can impair healing and increase the risk of infection [3–5].

These challenges highlight the need for advanced biomaterials capable of conforming to complex wound geometries while actively supporting tissue repair [6]. In this context, hydrogels have emerged as promising biomaterials for wound management due to their hydrated three-dimensional networks, which recapitulate the mechanical properties of soft tissues and facilitate nutrient diffusion, cell migration, and proliferation [7]. Injectable or in situ-forming hydrogels are particularly attractive, as they can be delivered in a minimally invasive manner, uniformly fill irregular wound cavities, and undergo local solidification, ensuring intimate contact with the wound bed without the need for surgical implantation [5,8]. However, the development of injectable hydrogel systems is not limited by the availability of bioactive materials, but by the difficulty of simultaneously controlling gelation kinetics, injectability, structural stability, and biological functionality. Small variations in formulation composition or processing conditions can markedly alter the resulting material properties, making rational navigation of the formulation space a major challenge in the development of clinically relevant injectable systems [7,9].

Among natural biomaterials, silk fibroin (SF) derived from *Bombyx mori* has attracted considerable attention owing not only to its excellent biocompatibility and tunable mechanical properties, but also to the remarkable sensitivity of its sol-gel transition to environmental and processing conditions. The assembly of SF into hydrogel networks depends on a complex interplay between protein concentration, ionic environment, and external physical stimuli, making SF an attractive yet challenging platform for the development of injectable gel formulations [10–14]. SF can be processed and functionalized with bioactive molecules, making it a versatile platform for regenerative applications [12,13]. Nevertheless, the intrinsic lack of integrin-binding motifs, such as RGD (Arg-Gly-Asp) [14], may limit cell adhesion and spreading, while the control of gelation behavior remains critical to ensure reproducible performance in injectable formulations [10,11,14]. Thus, SF is both an attractive and a technically demanding platform, because its assembly into hydrogel networks depends on a complex interplay between protein concentration, ionic environment, and external physical stimuli.

Beyond optimization of the hydrogel matrix itself, increasing attention has been directed toward the incorporation of biologically active components capable of providing regenerative signals that are not intrinsically present in SF. In particular, the mesenchymal stromal cell (MSC)-secretome and decellularized extracellular matrix (dECM) represent biologically active strategies to promote tissue regeneration through distinct and potentially complementary mechanisms. The MSC-secretome, comprising growth factors, cytokines, and extracellular vesicles (EVs), mediates paracrine tissue repair by modulating inflammation, supporting matrix remodeling, and activating antioxidant and pro-migratory responses in resident cells, while offering advantages as a cell-free therapeutic in terms of safety and scalability [15–19]. dECM provides a biomimetic microenvironment rich in structural proteins, glycosaminoglycans, and integrin-binding domains that directly regulate cell adhesion, angiogenesis, and matrix deposition [20–22]. Their incorporation within SF hydrogels may provide complementary biological inputs across different aspects of the regenerative response, including angiogenesis, cell migration, matrix remodeling, and inflammation control, with the relative weight of each component depending on the specific cellular context and delivery conditions.

Despite these advances, the successful development of injectable bioactive hydrogels requires not only the selection of suitable materials but also the rational design of formulation and processing parameters to achieve reproducible and application-relevant properties. In particular, the interplay between SF concentration, ionic conditions, and mechanical processing must be carefully controlled to modulate gelation

kinetics and final hydrogel performance. Indeed, the transition of SF from the stable Silk I (random coil) to the insoluble Silk II (β -sheet) conformation is a finely tuned physiological process. In nature, this transition is triggered within the silk gland by a precise ion gradient, where an increase in potassium (K^+) concentration, combined with mechanical shear and environmental changes, promotes protein assembly [23]. In this context, data-driven approaches, including machine learning, may serve as useful tools to explore multidimensional formulation spaces and identify candidate regions associated with desirable material properties. Rather than replacing experimental optimization, these approaches can support the rational reduction of empirical testing by highlighting promising formulation domains within complex design spaces.

Based on these premises, this study aimed to establish a formulation framework for injectable SF gels capable of integrating biologically active wound-healing components while maintaining application-relevant physicochemical properties. To achieve this, we first explored the formulation space governing K^+ - and sonication-mediated SF gelation, then identified operational conditions compatible with injectable use, and finally evaluated the individual and combined biological contributions of dECM and MSC-secretome within the resulting gel platform. Specifically, we: (i) investigated the influence of formulation and processing parameters on gelation kinetics; (ii) applied machine learning models to define an optimal design space; (iii) optimized the physicochemical and rheological properties for injectable applications; (iv) assessed cytocompatibility, qualitative cell-gel interactions, and in vitro regenerative performance; and (v) compared the functional profiles of dECM and lyosecretome as incorporated bioactive components, with the aim of defining their respective contributions to angiogenic, migratory, and transcriptional responses relevant to regeneration and redox regulation in the tested cell models.

2. Materials and methods

2.1. Materials

Cocoons from *Bombyx mori* silkworms were kindly donated by Nembri Industrie Tessili (Capriolo, BS, Italy). 3-[(3-colamidopropyl) dimetilammonio]-1-propanesulfonate (CHAPS), ethylenediaminetetraacetic acid (EDTA), HEPES, hydrochloric acid (HCl), lithium bromide (LiBr), mannitol, pepsin, potassium chloride (KCl), sodium carbonate (Na_2CO_3), sodium chloride (NaCl), sodium hydroxide (NaOH), and tris (hydroxymethyl)aminomethane (TRIS) were purchased from Merck (Milan, Italy). Dialysis membranes were obtained from Spectrum Laboratories (Milan, Italy), while easySTRAINER filters were purchased from VWR International (Milan, Italy). The dermal matrix was provided by Niguarda Hospital (Milan, Italy). Dimethyl sulfoxide (DMSO) and sodium hydroxide (NaOH) were obtained from Carlo Erba Reagenti (Milan, Italy). All reagents and plasticware for cell culture were purchased from Euroclone (Milan, Italy), while 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) was purchased from VWR International (Milan, Italy).

2.2. Preparation and technological characterization of SF gels

2.2.1. SF extraction and solubilization

SF was extracted from *Bombyx mori* cocoons through a degumming process. Briefly, 5 g of cocoons were cut into small pieces (1×1 cm) and washed under running water to remove impurities. The cocoon pieces were then immersed in a 0.02 M aqueous Na_2CO_3 solution and boiled at 100 °C for 30 min to remove sericin and obtain raw SF fibers. Following degumming, SF fibers were washed repeatedly with distilled water to eliminate residual sericin and salt and then dried at room temperature for 48 h. Subsequently, the dried SF fibers were dissolved in a 100% w/v aqueous lithium bromide (LiBr) solution at 65 °C for 4 h in a thermostatic water bath, allowing complete dissolution of the protein material

and the conformational transition from water-insoluble Silk II to soluble Silk I. After cooling to room temperature, the solution was transferred into cellulose dialysis membranes with a molecular weight cut-off of 3.5 kDa and dialyzed against deionized water at room temperature for 72 h with regular water changes to completely remove LiBr. Following dialysis, the SF solution was filtered using an easySTRAINER filter (70 μm) to remove any remaining impurities. The concentration of the SF solution was determined by gravimetric analysis after complete evaporation of water, and typically ranged between 7 and 9% w/v for each prepared batch. The SF solution was stored at 4 °C until use (maximum 2 weeks).

2.2.2. SF gel preparation

Gels were prepared by mimicking natural SF formation in the silk gland, gradually increasing K^+ concentration, and applying sonication to induce the transition from Silk I to the β -sheet-rich Silk II conformation [23]. A total of 30 experimental batches were produced by varying SF concentration, sonication time, and K^+ concentration according to a two-level, three-factor, central composite design, with each condition prepared in duplicate. In detail, the initial experimental design followed a cubic model with two levels for each of the three variables: sonication time (30 and 60 s), SF concentration (1–2.5% w/v), and K^+ concentration (0–50 mM), with the addition of a partial central point centered on SF and K^+ concentrations while using both extreme values for sonication time. The complete experimental design is summarized in Table 1.

For each batch preparation, SF was diluted with deionized water to the desired concentration. KCl was then added to reach the required final K^+ concentrations. Samples were then sonicated for 30 or 60 s using an ultrasonic probe equipped with a 0.25' steel tip at 20% amplitude (Sonics & Materials Inc., Newtown, CT, USA). During sonication, samples were immersed in ice to prevent temperature-induced SF degradation.

Gel formation was monitored by turbidimetry, measuring absorbance at 600 nm using a microplate reader (Victor Nivo, PerkinElmer, Waltham, MA, USA). For this purpose, 100 μL of the sample were transferred into a 96-well plate, and absorbance measurements were taken every minute for the first 10 min, followed by a final measurement after 24 h. Gelation was calculated using the following formula:

$$\text{Gelation} = \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{min}}}{\text{Abs}_{\text{max}} - \text{Abs}_{\text{min}}}$$

where $\text{Abs}_{\text{sample}}$ is the absorbance of the sample at time t , Abs_{min} is the absorbance of the non-gelified SF solution (same concentration as that used in the gel but without K^+ addition and sonication), and Abs_{max} is the maximum absorbance recorded for that SF concentration (gelified) after 24 h.

Gelation data were first analyzed to exclude SF and K^+ ion concentration conditions and sonication times under which SF did not exhibit gelation capacity. Subsequently, a four-parameter logistic growth model was applied to each batch prepared according to the conditions reported in Table 1 to determine the corresponding curves: the minimum value (A_{min}), the maximum value (A_{max}), the IC_{50} , and the Hill coefficient (h , indicative of gelation rate and directly proportional to the slope at time t

$= \text{IC}_{50}$). The equation of the four-parameter logistic growth model is as follows:

$$f(t, A_{\text{min}}, A_{\text{max}}, \text{IC}_{50}, h) = A_{\text{min}} + \frac{A_{\text{max}} - A_{\text{min}}}{1 + \left(\frac{t}{\text{IC}_{50}}\right)^h}$$

These parameters were used to select experimental conditions ensuring optimal gelation according to the following criteria: (i) $\sim 30\%$ gelation at 5 min, sufficient to allow homogeneous distribution of the regenerative components within the gel; (ii) $\geq 60\%$ gelation at 10 min, necessary to obtain a gel sufficiently consistent; (iii) gelation approaching 100% at 24 h. The 5- and 10-min time points represent indicative technical processing times, for which a certain tolerance was permitted.

Subsequently, neural network (NN) and Random Forest (RF) models were used as surrogate models to explore the relationships between SF concentration, K^+ concentration, sonication time, and the gelation descriptors A_{max} and IC_{50} . Descriptive coefficients of determination (R^2) were calculated on the observations used for model fitting and were interpreted only as in-sample goodness-of-fit indicators, not as estimates of predictive performance or model generalizability. The exploratory modeling dataset comprised 12 gelling batch-level observations corresponding to 6 unique formulation conditions. Predictive utility of the exploratory NN was subsequently assessed prospectively by preparing additional model-selected formulations and comparing experimentally measured A_{max} and IC_{50} values with the corresponding predictions. The refined RF dataset comprised 13 batch-level observations corresponding to 10 unique formulation conditions. All analyses were performed using R statistical software [24].

2.2.3. Technological characterization of the gels: visual inspection, pH, resistance to penetration, viscosity, syringeability, hydrolytic stability, and morphology

For the technological characterization, formulations A-D were selected to represent distinct limiting conditions within the RF-defined operational region, rather than only its central portion (Table 2).

The organoleptic properties of the gels, including appearance, color, and overall homogeneity, were evaluated by visual inspection; pH was determined using a digital pH meter by immersing the probe directly into the preparation. Measurements were carried out both in the sol phase and in the gel phase.

Bulk gel resistance to penetration was evaluated using a constant-load penetration test [25]. Briefly, 20 mL of each formulation were allowed to gel in a cylindrical container. A calibrated weight of 70 g was placed on the gel surface and allowed to penetrate for a fixed time (10 s). Penetration depth (cm) was measured using a displacement scale, with lower penetration values indicating greater resistance to penetration under the tested conditions.

Viscosity (Centipoise, cP) was determined 24 h after preparation using a rotational viscometer (Brookfield DV-II+ Viscometer), equipped with a small cup adapter. Measurements were performed at 37.0 ± 0.5 °C with spindle 25. An aliquot of each gel was placed in a thermostatic jacket to maintain a constant temperature. Shear stress and shear rate data were simultaneously recorded to construct flow curves. The spindle rotation followed a two-phase program: an ascending ramp from

Table 1
Experimental design of the batches prepared.

Sonication time (s)	K^+ concentration (mM)	SF concentration (% w/v)
30	0	1.0
30	0	2.5
30	25	1.75
30	50	1.0
30	50	2.5
60	0	1.0
60	0	2.5
60	25	1.75
60	50	1.0
60	50	2.5

Table 2
Composition and processing parameters of gel formulations selected for technological characterization.

Formulation	K^+ concentration (mM)	SF concentration (% w/v)	Sonication time (s)
A	50	2.5	60
B	50	2.5	30
C	32.5	2.5	44
D	25	1.75	60

0.05 to 2 rpm, followed by a descending ramp from 2 to 0.05 rpm.

The syringeability test was performed following a previous study [26]. A vertical support was used to hold a 2 mL syringe filled with the gel. A support was placed on the plunger to facilitate the application of a 500 g weight (corresponding to 4.91 N). For each formulation, the time required for complete extrusion of the gel from the syringe was recorded. In the presentation of the results, syringeability was expressed as the time required to expel a given quantity of formulation, using water as a reference.

Gel stability under non-enzymatic aqueous conditions was evaluated by incubating pre-formed gels in phosphate buffered saline (PBS) at 37 °C for up to 30 days. Samples were weighed before incubation and at each predefined time point after removal of the surrounding PBS and gentle blotting/removal of excess surface liquid. Changes in gel mass were monitored over time as an indicator of bulk erosion under the tested conditions. Mass remaining (%) was calculated as $(\frac{W_t}{W_0}) \times 100$, where W_0 is the initial gel mass before incubation and W_t is the mass measured at each predefined time point after removal of excess surface liquid. The complete 30-day profile is reported in the Supplementary Information.

The morphology of the gels was characterized by scanning electron microscopy (SEM). For the morphological analysis, additional samples of formulations A-D were prepared using sonication amplitudes of 30% and 40%, while maintaining the formulation-specific SF and K^+ concentrations and sonication times reported in Table 2. Corresponding non-sonicated samples, prepared at the same SF and K^+ concentrations, were included as morphological controls. Following preparation, 100 μ L of each gel was transferred to a 96-well microplate and lyophilized at -50 °C and 8×10^{-1} mbar for 72 h using a freeze dryer (Epsilon 2-6D LSCplus, Martin Christ GmbH, Christ, Osterode am Harz, Germany). SEM analyses were performed on the lyophilized samples using a high-resolution MIRA3 instrument (Tescan, Brno, Czech Republic). Samples were observed both on their external surface and internally after being sectioned vertically. Prior to analysis, each sample was sputter-coated with a thin gold layer in an argon atmosphere. Analyses were conducted at 8 kV using an In-Beam SE detector.

2.3. Preparation and technological characterization of MSC-lyosecretome

The secretome was obtained from MSCs isolated from adipose tissue. Briefly, harvested adipose tissue was washed repeatedly with phosphate-buffered saline solution (PBS, pH 7.14) without Ca^{2+} and Mg^{2+} , and dissected into small fragments using surgical scissors. Tissue samples were enzymatically digested at 37 °C using type II collagenase (0.075% w/v dissolved in PBS supplemented with Ca^{2+} and Mg^{2+} ions, 1% w/v amphotericin B, and 1% w/v penicillin/streptomycin). After 30 min of incubation, the digested tissue was filtered through a 70 μ m cell strainer to remove undigested debris and centrifuged three times at 300 $\times$ g for 5 min. The pellet was resuspended in complete culture medium (DMEM/F12 supplemented with 10% v/v fetal bovine serum [FBS], 1% w/v amphotericin B, and 1% w/v penicillin/streptomycin). The recovered cell fraction was cultured in a monolayer (100,000 cells/cm²) until reaching sub-confluence. Subsequently, MSCs were detached using 0.05% v/v trypsin-EDTA, seeded in culture flasks (10,000 cells/cm²), and maintained at 37 °C in a humidified atmosphere with 5% CO₂ in complete culture medium.

To enhance the regenerative potential of the MSC-secretome, two different production protocols were compared: standard serum starvation and pro-inflammatory priming. Specifically, for the primed secretome, MSCs were pre-treated with interleukin-1 β (IL-1 β) at 10 ng/mL for 48 h upon reaching sub-confluence. Following the priming phase, the medium was removed, and cells were washed to eliminate residual cytokine. Both primed and unprimed MSCs were then subjected to serum starvation for 48 h to promote secretome release, with culture medium collected and replaced every 9 and 12 h. The collected

conditioned medium was centrifuged at 2800 $\times$ g for 10 min to remove cellular debris and apoptotic bodies, and subsequently ultrafiltered using the KrosFlo Research 2i system (Spectrum Laboratories, Milan, Italy) equipped with a filtration cartridge with a 5 kDa molecular weight cut-off. This procedure enabled secretome concentration followed by diafiltration with deionized water. After ultrafiltration, each batch was diluted with deionized water to achieve a target protein concentration of 115 μ g/mL, consistent with previously described protocols [27]. Finally, the purified secretome was supplemented with mannitol as a cryoprotectant at a final concentration of 0.5% w/v and lyophilized at 8×10^{-1} mbar and -50 °C for 72 h (Christ Epsilon 2-16D LSCplus, Christ, Osterode am Harz, Germany). The obtained lyosecretome was stored at -20 °C until use (within 12 months). The lyosecretome was fully characterized for total protein and lipid content using a BCA Protein Assay Kit (Thermo Fisher Scientific, Milan, Italy) and a Nile red assay, as well as particle size distribution analysis by nanoparticle tracking analysis, as previously reported [27,28].

2.4. Preparation and characterization of decellularized dermal matrix (dECM)

2.4.1. Dermal matrix decellularization

Dermal tissue was decellularized at Niguarda Hospital following a previously established protocol [29,30]. Briefly, human skin samples were harvested from the lumbar region and proximal thigh areas of 8 different donors (age range: 18–70 years) after obtaining informed consent, using a dermatome (Zimmer) set at a thickness between 0.8 and 1 mm. Following collection, samples were stored in PBS solution at 4 °C for a maximum of 48 h, and then cut into 2×2 cm² fragments and subsequently decellularized using 0.033 M CHAPS in 3 M KCl and 1 M HEPES. Each tissue fragment was incubated with 10 mL of detergent solution (pH 8) at 37 °C for 16 h under constant agitation provided by an orbital shaker set at 300 rpm. Subsequently, samples underwent a series of washing steps: 20 min in hypotonic solution (pH 8) containing 10 mM TRIS, 0.1% NaCl, and 1 mM EDTA; 40 min in PBS; 40 min in sterile water for injection; 20 min in PBS; and a final 20-min rinse in sterile water for injection. Decellularized dermal samples were finally lyophilized using a Martin Christ LSC Plus freeze-dryer (Christ GmbH, Christ, Osterode am Harz, Germany) at -50 °C and 8×10^{-1} mbar for 96 h.

2.4.2. Digestion and lyophilization of dECM

The dermal matrix, in solid form, was mechanically fragmented into small pieces ($< 0.1 \times 0.1$ cm) to reduce digestion time. The obtained fragments were placed in a glass vial, and a 2% w/v solution of 0.1 M HCl was added along with pepsin at a quantity equal to 20% w/w of the tissue weight. The vial was subjected to continuous magnetic stirring at a constant temperature of 37 °C for 48 h. Upon complete tissue digestion, the pH was neutralized with 1 M NaOH to inactivate the pepsin. Subsequently, the process was optimized to achieve enzyme inactivation not only through pH adjustment but also by thermal treatment at 60 °C or 70 °C for 1 h, in order to render the dECM powder cytocompatible. Finally, the solution was lyophilized after adding mannitol (0.5% w/v) as a cryoprotectant. dECM was characterized for total protein and lipid content using a BCA Protein Assay Kit (Thermo Fisher Scientific, Milan, Italy) and a Nile red assay, as previously reported [27,28].

2.5. In vitro biological activity

2.5.1. Cytocompatibility of SF gel and dECM

The cytocompatibility of SF gels was evaluated on human fibroblasts using an indirect contact assay. Samples were prepared by varying the SF and K^+ ion concentrations and sonication time within the optimized operational region defined by the RF model (Table 2), while untreated cells were used as controls. Fibroblasts were seeded in 24-well plates at a density of 5000 cells/cm² and cultured for 72 h in complete medium (DMEM high glucose supplemented with 10% v/v FBS, 1% v/v

penicillin/streptomycin, and amphotericin B). After 72 h, Transwell® inserts (polycarbonate membrane, 3 µm pore size) containing 100 µL of pre-formed gel were placed in each well to allow indirect exposure via diffusion. Following 24 h of incubation, inserts were removed, and cell metabolic activity was assessed using an MTT assay. Briefly, cells were incubated with MTT solution (0.5 mg/mL) for 3 h at 37 °C; the resulting formazan crystals were dissolved in DMSO, and absorbance was measured at 570 nm using a microplate reader (Victor Nivo, PerkinElmer, Waltham, MA, USA). Cell metabolic activity was expressed as a percentage relative to untreated controls according to the following equation:

$$\text{Metabolic activity (\%)} = 100 \times \left(\frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \right)$$

where Abs_{sample} is the absorbance of treated samples and Abs_{control} is the absorbance of the control (untreated cells, corresponding to 100% viability).

The cytocompatibility of lyophilized dECM was evaluated on fibroblasts, MSCs, and human umbilical vein endothelial cells (HUVECs). Cells were seeded in 96-well plates at a density of 5000 cells/cm² and cultured for 72 h in complete medium. Culture conditions were as follows: DMEM/F12 supplemented with 10% v/v FBS and 1% v/v penicillin/streptomycin for MSCs; DMEM high glucose supplemented with 10% v/v FBS and 1% v/v penicillin/streptomycin for fibroblasts; and endothelial growth medium supplemented with growth factors and 1% v/v penicillin/streptomycin for HUVECs. After 72 h, the medium was replaced with fresh medium containing lyophilized dECM dissolved in culture medium at concentrations ranging from 0.16 to 20 mg/mL, corresponding to approximately 0.11–13.8 mg/mL of matrix component based on its 69% w/w matrix content. Following 24 h of incubation, cell metabolic activity was assessed by MTT assay as described above. All experiments were performed in triplicate, and results are reported as mean ± standard deviation.

2.5.2. Blood compatibility

Blood compatibility was assessed by hemolytic and hemagglutination tests. Fresh human blood samples were collected from at least three consent-informed healthy individual donors (female blood). In detail, the hemolytic assay was performed as reported by Maji et al. with some modifications [31]. Red blood cells (RBCs) were separated from plasma by centrifugation at 1500 × g for 5 min. The pellet was washed twice with PBS without Ca²⁺ and Mg²⁺ (pH = 7.4). 90 µL of cell suspension were co-incubated with 10 µL of samples, resuspended in PBS, and incubated at room temperature for 60 min. RBCs treated with PBS were considered the negative control, while RBCs treated with distilled water were considered the positive control (100% hemolysis). After incubation, samples were centrifuged at 3000 × g for 10 min, and the absorbance of the recovered supernatants was measured at 540 nm (NanoPhotometer NP80 IMPLM), which corresponds to the maximum absorption of hemoglobin. Hemolysis (%) was evaluated with the following equation:

$$\text{Hemolysis (\%)} = \left(\frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}}{\text{Abs}_{\text{positive CTR}}} \right) \times 100$$

where Abs_{sample} is the absorbance of samples, Abs_{blank} is the absorbance of the negative control, and Abs_{positive CTR} is the absorbance of RBCs treated with distilled water.

Hemagglutination activity was evaluated following the procedure reported in [32,33] with slight modifications. Briefly, 100 µL of the RBC pellet was mixed with 10 mL of PBS (pH 7.4). SF gel, dECM, and lyosecretome were tested separately at the indicated concentrations or dilutions. Because the three tested materials differed substantially in their physical form, sample preparation was material-specific: the pre-formed SF gel was tested at volumetric dilution ratios (1:1 to 1:10), whereas lyosecretome and dECM, being lyophilized powders resuspended in PBS,

were tested at defined mass concentrations (mg/mL). In all cases, the assay evaluated direct contact between the tested material and the RBC suspension in the same aqueous vehicle, with 50 µL of each prepared sample combined with an equal volume (50 µL) of RBC suspension in a 96-well U-bottom plate; PBS served as the negative control. Samples were incubated at 37 °C for 2 h and visually assessed for RBC sedimentation patterns, whereby formation of a compact central “button” was taken to indicate the absence of hemagglutination. In contrast, a diffuse sedimentation pattern was taken to indicate hemagglutination. A dedicated excipient-only control (mannitol and/or pepsin) was not included.

2.5.3. In vitro scratch test and angiogenesis assays

The in vitro migratory response of human fibroblasts and the angiogenic response of HUVECs were evaluated using scratch and tube formation assays, respectively. Specifically, the IL-1β-primed lyosecretome was used for the scratch assay, while the serum-starved lyosecretome was employed for the tube formation assay [30]. Three experimental conditions were investigated: (i) unloaded SF gels, (ii) individual components (lyosecretome and dECM), and (iii) loaded gels incorporating lyosecretome and dECM. For gel-based conditions, samples were incubated in complete culture medium without FBS for 24 h to obtain conditioned media, which were subsequently used for cell treatment. In contrast, lyosecretome and dECM alone were directly dissolved in culture medium without FBS at the desired concentrations prior to cell exposure.

For the in vitro scratch test, GFP-expressing fibroblasts were prepared according to the procedures previously described [34], seeded at a density of 5000 cells/cm² in a 96-well plate, and then scraped with a sterile pipette tip to create a linear “scratch”. 100 µL of conditioned medium obtained after 24 h of incubation with gel samples, or medium containing lyosecretome or dECM alone at the desired concentrations, were then added to each well and incubated at 37 °C and 5% CO₂ for 48 h. Images were acquired using a Leica THUNDER Imager 3D Live Cell with a peak fluorescence emission of 509 nm, taken every 2 h. To quantify scratch closure, scratch assay images were processed using a custom-developed Matlab® script as described previously [30]. Initially, a binary mask of the scratch region was manually selected on the time-zero (t₀) image to establish a baseline for the subsequent time-course analysis. The script then sequentially analyzed the remaining files in a batch-processing manner, applying the initial mask parameters across the entire time series. For each time point, the green channel was isolated, and a median filter was used to suppress salt-and-pepper artifacts. To enhance the contrast of cell structures against the background, a Fast Fourier Transform bandpass smoothing filter was implemented, retaining spatial features within a 3–40 pixel range. Segmentation was achieved by adjusting the image histogram around its primary intensity peak, followed by Otsu's thresholding. Then, morphological dilation (disk element, radius 10) was applied, and isolated background voids smaller than 3000 pixels were removed. The total scratch area was quantified by summing the three largest detected regions. Finally, scratch areas (in pixels) were determined across the time series and normalized against the t₀ baseline to compute the percentage of wound closure.

For the tube formation assay, 50 µL of undiluted Corning Matrigel® Basement Membrane Matrix was added to a 96-well plate and incubated for 1 h at 37 °C to allow for gel formation. HUVECs were seeded at a density of 30,000 cells/cm² in low-serum endothelial cell basal medium without growth factors and supplemented with 1% w/v amphotericin B and 1% w/v penicillin/streptomycin. 100 µL of conditioned medium obtained after 24 h of incubation with gel samples or medium containing lyosecretome or dECM alone at the desired concentrations were then added; complete medium (with growth factors) was used as a positive control. Images were acquired every hour for 10 h using the Leica THUNDER Imager 3D Live Cell and subsequently processed in ImageJ, using the angiogenesis analyzer plugin, to quantify tube formation

parameters. Phase contrast images of HUVECs were analyzed using the HUVEC phase contrast software to assess network structures automatically. Each analysis was performed in triplicate.

2.5.4. Gene expression

Changes in gene expression were evaluated in human fibroblasts. Three experimental conditions were investigated: (i) unloaded SF gels, (ii) the individual components administered separately (serum-starved lyosecretome or dECM), and (iii) loaded SF gels incorporating both serum-starved lyosecretome and dECM.

Cells were seeded in 96-well plates at a density of 5000 cells/cm² and cultured for 72 h in complete medium (DMEM high glucose supplemented with 10% v/v FBS and 1% v/v penicillin/streptomycin). After 72 h, the medium was replaced with fresh medium. For gel-based conditions, 100 µL of each gel formulation (unloaded or composite) was loaded into Transwell® inserts (polycarbonate membrane, 3 µm pore size) and placed in the wells to allow indirect exposure through diffusion. In contrast, lyosecretome and dECM alone were directly dissolved in culture medium. Following 7 days of incubation, cells were harvested, and total RNA was extracted using the QIAcube automated system (QIAGEN, Hilden, Germany) and the RNeasy Mini Kit, including DNase I treatment, according to the manufacturer's instructions.

Reverse transcription was performed using the iScript™ gDNA Clear cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer's protocol to synthesize complementary DNA (cDNA) and remove residual genomic DNA contamination. Quantitative real-time PCR (qRT-PCR) was then performed using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories) following the manufacturer's instructions. Gene expression data were analyzed using the CFX Maestro software (Bio-Rad Laboratories Inc., Hercules, CA, USA), and relative expression levels were calculated after normalization to the reference gene. In detail, the 13-gene panel covered five functional domains relevant to wound healing: inflammation (IL6, NFKB1, HMGB1), redox-associated response (NQO1, SOD2), ECM remodeling (COL3A1, FN1, MMP2, TIMP1, TM7SF2), apoptosis (BAX), and angiogenic signaling (FGF7, VEGFA). HPRT1 was used as the endogenous reference gene for normalization based on its established stability in human fibroblasts under the tested conditions.

2.5.5. Cell-gel interaction and qualitative Z-stack fluorescence imaging

Qualitative cell-gel interactions were evaluated using GFP-expressing fibroblasts. Briefly, GFP-expressing fibroblasts were seeded onto gels with or without the incorporation of dECM and lyosecretome. For each sample, 10000 cells were deposited as a droplet directly onto the gel surface and incubated at 37 °C with 5% CO₂ for 30 min before the addition of complete culture medium. Fluorescence distribution within the gel constructs was qualitatively observed by Z-stack fluorescence imaging at 4 and 14 days after seeding. Images were acquired using a Leica THUNDER Imager 3D Live Cell microscope, with a maximum fluorescence emission at 509 nm. Z-stacks were acquired along the Z-axis to visualize fluorescent cells at different focal planes within the gel constructs. The imaging approach was intended for qualitative visualization only and was not used to quantify cell morphology, proliferation, cell density, or infiltration depth.

2.6. Statistical analysis

Raw data of gel strength and syringeability, metabolic activity, scratch migration, angiogenic potential, and gene expression were processed using Statgraphics XVII (Statpoint Technologies, Inc., Warrenton, VA, USA) or GraphPad Prism (version 8.02, GraphPad Software, Boston, MA, USA). A linear generalized analysis of variance (ANOVA) model was applied to analyze the data with a normal distribution, and the differences between the groups were evaluated using Tukey's or least significant difference (LSD) procedure, as detailed in the caption of the figures. The statistical significance was set at $p < 0.05$.

3. Results

3.1. Gelation kinetics and definition of the accessible SF gelation domain

SF gelation was induced by combining sonication with K⁺ to recapitulate the physiological process occurring in the silk gland of *Bombyx mori* during spinning (Fig. 1A). The initial objective was not to identify an optimal formulation, but to delineate the experimental domain within which controllable gelation could be achieved under application-relevant conditions. To this end, a cubic experimental design was implemented to systematically explore the effect of three formulation variables across a broad gelation domain: sonication time (30 and 60 s), SF concentration (1–2.5% w/v), and K⁺ concentration (0–50 mM). Partial central points were included by varying SF and K⁺ concentrations at both extreme sonication times. All conditions were prepared in duplicate, yielding an initial dataset of 20 formulations (batches 1–20); formulation parameters and derived kinetic descriptors are reported in Table 3. This dataset was subsequently used both to map the boundaries of the gelation domain and, as described in Section 3.2, to train a first-pass surrogate model of the formulation space.

Preliminary qualitative assessment of gelation revealed that SF solutions lacking K⁺ (batches 1, 2, 5, 6, 11, 12, 15, and 16) did not undergo gelation even after 24 h and were therefore excluded from further analysis (Fig. S1), confirming that ionic strength is a prerequisite for sol-gel transition under the tested conditions and defining the first boundary of the experimentally accessible gelation space.

Gelation kinetics of the 12 gelling batches, corresponding to 6 formulation conditions, were modeled using a four-parameter logistic growth function, enabling the extraction of the minimum signal ($A_{\min}$), maximum gelation extent ($A_{\max}$), half-gelation time (IC_{50}), and Hill coefficient (h), the latter describing the steepness of the sol-gel transition (Table 3). A broad spectrum of gelation behavior was observed across this dataset. Formulations characterized by low SF concentration and short sonication times failed to gel, whereas a combination of high SF concentrations ($\geq 2.0\%$ w/v), elevated K⁺ levels (≥ 25 mM), and prolonged sonication times (≥ 50 s) consistently produced rapid gelation, with IC_{50} values below 10 min (e.g., batches 8, 18, and corresponding replicates). Replicate batches prepared under identical conditions demonstrated good reproducibility; for instance, batches 8, 18, and 23 yielded IC_{50} values of 4.52, 5.51, and 7.18 min, respectively. After 24 h, the majority of gelling batches reached complete network formation ($A_{\max} \approx 1$), indicating relatively limited variability in final gelation extent compared with gelation kinetics. The magnitude of the Hill coefficient further differentiated transition profiles, with larger absolute values corresponding to steeper fitted transitions. Because the sign of h depends on the adopted logistic-function parameterization, transition steepness was interpreted from $|h|$ rather than from its sign. This pronounced variability suggested the existence of a highly sensitive formulation space, motivating a modeling-assisted exploration of the parameter space (Section 3.2) prior to any experimental refinement.

3.2. Surrogate modeling of formulation space

The dataset of 12 gelling batches (batches 3–4, 7–10, 13–14, and 17–20), together with their logistic-model-derived $A_{\max}$ and IC_{50} values, was used to develop a first-pass surrogate model relating the three formulation parameters (SF concentration, K⁺ concentration, and sonication time) to gelation outcomes. An artificial NN was trained on this exploratory dataset to generate continuous response hypersurfaces for $A_{\max}$ and IC_{50} across the explored parameter space (Fig. S2). At 2.5% w/v fibroin, NN-predicted $A_{\max}$ values remained uniformly high (0.9–1.1), forming a smooth response surface indicating consistently high predicted $A_{\max}$ values across this modeled section of the formulation space. At 1.75% w/v SF, greater variability was observed, with reduced gelation efficiency mainly at elevated K⁺ concentrations and improved gelation achieved at intermediate K⁺ levels combined with prolonged

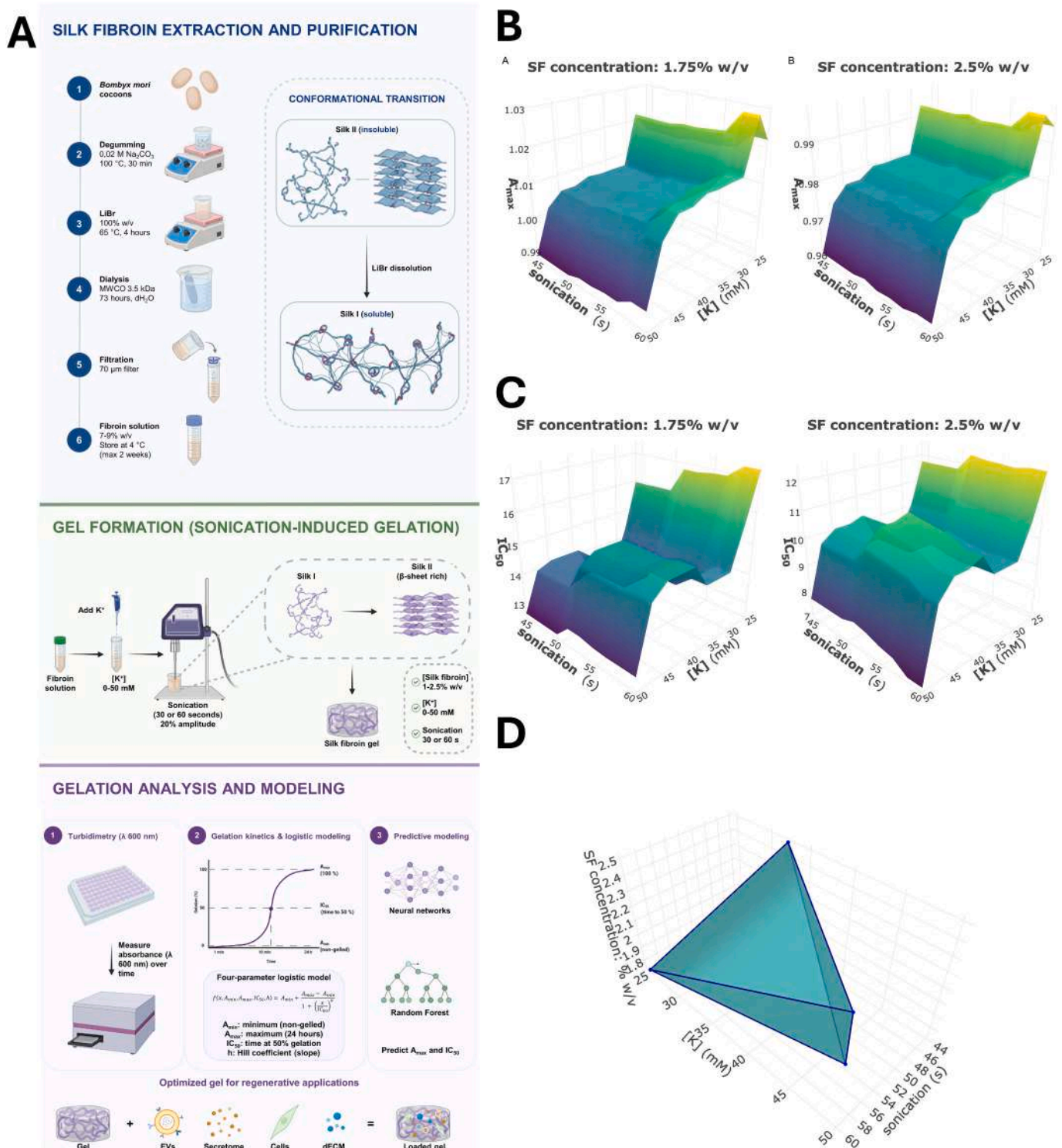


Fig. 1. Multi-parametric formulation development and surrogate modeling of SF gelation. (A) Scheme of the gel preparation. (B) Cross-sections of the RF-predicted A_{max} hypersurface as a function of K^+ concentration and sonication time at fixed SF concentrations (2.5% and 1.75% w/v). (C) Corresponding IC_{50} hypersurfaces predicted by the RF model. (D) Optimized operational region for gel formulation identified by RF modeling in the three-dimensional parameter space (SF and K^+ concentrations, and sonication time).

sonication. NN-predicted IC_{50} surfaces further highlighted the non-linear interaction between sonication and ionic strength. At 1.75% w/v SF, IC_{50} exhibited a pronounced non-monotonic dependence on both variables, with localized maxima at intermediate processing conditions and substantially lower values at prolonged sonication. At 2.5% w/v SF, IC_{50} values remained globally lower, with the lowest predicted values

occurring at higher K^+ concentrations and longer sonication times, indicating that increased SF concentration broadens the processing window while reducing sensitivity to individual parameter variations. Descriptive in-sample goodness of fit differed markedly between the two gelation descriptors. For A_{max} , R^2 values were 0.950 and 0.940 for the NN and RF models, respectively, whereas corresponding values for IC_{50}

Table 3

Experimental design and gelation parameters of SF formulations. Experimental conditions for all batches generated through a factorial design varying sonication time, SF, and K^+ concentrations. Reported parameters were obtained by fitting gelation kinetics with a four-parameter logistic model and include minimum signal ($A_{\min}$), maximum gelation ($A_{\max}$), half-gelation time (IC_{50}), and Hill coefficient (h). / indicates absence of measurable gelation. The last column identifies the role of that formulation in the surrogate modeling workflow.

Batch n.	Sonication time (s)	K^+ concentration (mM)	SF concentration (% w/v)	$A_{\min}$	$A_{\max}$	IC_{50}	h	Role in surrogate modeling workflow
1	30	0	1	/	/	/	/	Excluded (no gelation)
2	30	0	2.5	/	/	/	/	Excluded (no gelation)
3	30	50	1.00	-0.02	0.67	119.71	-1.82	NN training set
4	30	50	2.5	0.19	1.02	8.39	-2.06	NN training set
5	60	0	1.00	/	/	/	/	Excluded (no gelation)
6	60	0	2.5	/	/	/	/	Excluded (no gelation)
7	60	50	1	0.27	1.00	24.57	-1.99	NN training set
8	60	50	2.5	0.25	0.84	4.52	-1.81	NN training set; also RF training set
9	30	25	1.75	0.01	0.95	309.89	-1.06	NN training set
10	60	25	1.75	0.3	1.00	11.26	-2.23	NN training set; also RF training set
11	30	0	1	/	/	/	/	Excluded (no gelation)
12	30	0	2.5	/	/	/	/	Excluded (no gelation)
13	30	50	1	-0.15	0.81	125.36	-1.86	NN training set
14	30	50	2.5	0.04	1.01	62.36	-1.45	NN training set
15	60	0	1	/	/	/	/	Excluded (no gelation)
16	60	0	2.5	/	/	/	/	Excluded (no gelation)
17	60	50	1	-0.12	1.05	136.11	-1.32	NN training set
18	60	50	2.5	0.27	1.01	5.51	-1.82	NN training set; also RF training set
19	30	25	1.75	0.03	1.01	84.4	-1.52	NN training set
20	60	25	1.75	0.15	0.99	24.58	-1.72	NN training set; also RF training set
21	60	27.5	2.05	0.18	1.11	15.47	-2.62	Experimental validation; RF training set
22	56	32.5	2.05	0.15	1.00	8.19	-2.2	Experimental validation; RF training set
23	60	50	2.5	0.14	1.00	7.18	-2.12	Experimental validation; RF training set
24	44	32.5	2.5	0.13	1.00	9.98	-2.04	Experimental validation; RF training set
25	50	32.5	2.13	0.13	0.98	7.77	-2.22	Experimental validation; RF training set
26	50	27.5	2.13	0.08	1.00	10.03	-2.36	Experimental validation; RF training set
27	60	50	2.2	0.1	1.00	10.13	-2.15	Experimental validation; RF training set
28	56	45	2.28	0.08	1.00	16.12	-2.11	Experimental validation; RF training set
29	41	27.5	2.43	0.03	1.13	169.56	-0.95	Experimental validation; excluded from RF training set
30	51	40	2.35	0.1	1.00	16.81	-1.92	Experimental validation; RF training set

were -0.495 and 0.049. Because these R^2 values were calculated on the same batch-level observations used for model fitting, they are reported only as descriptive goodness-of-fit measures and should not be interpreted as independent validation of predictive performance or generalizability. The markedly poorer fit observed for IC_{50} further supports a cautious interpretation of quantitative gelation-kinetics predictions.

Based on these NN-predicted $A_{\max}$ and IC_{50} hypersurfaces, a candidate operational region associated with near-complete gelation and relatively rapid kinetics was identified within the explored parameter space. A set of candidate formulations located within this region was extracted from the model predictions, from which ten formulations (batches 21–30) were selected for experimental preparation and characterization, with the aim of testing the predictive accuracy of the NN model against real gelation data and using the resulting experimental dataset to build an experimentally refined model of the operational region (Section 3.3).

3.3. Experimental validation and RF-based definition of the optimal region

The ten candidate formulations (batches 21–30) selected from the NN-predicted optimal region were prepared experimentally, and their gelation kinetics were characterized using the same logistic-model fitting procedure described in Section 3.1. Predicted and experimentally measured $A_{\max}$ and IC_{50} values for these batches are compared in Table 4.

$A_{\max}$ predictions were generally consistent with complete or near-complete final gelation, with most experimentally tested batches reaching $A_{\max}$ values close to 1. IC_{50} predictions showed substantially greater variability. The absolute predicted-to-experimental IC_{50} differences were 2.16 min for batch 23, 1.64 min for batch 26, and 6.13 min

Table 4

Validation of NN predictions. Comparison between NN-predicted and experimentally measured IC_{50} and $A_{\max}$ values for validation batches (21–30).

Batch	Predicted values		Experimental values	
	IC_{50}	$A_{\max}$	IC_{50}	$A_{\max}$
21	6.97	1.06	15.47	1.11
22	9.83	0.96	8.19	1.00
23	5.02	0.93	7.18	1.00
24	6.99	0.83	9.98	1.00
25	5.39	0.84	7.77	0.98
26	8.39	0.81	10.03	1.00
27	8.43	0.95	10.13	1.00
28	9.99	0.86	16.12	1.00
29	6.09	0.84	169.56	1.13
30	8.44	0.82	16.81	1.00

for batch 28; among batches 21–28 and 30, deviations ranged from 1.64 to 8.50 min. Batch 29 represented a marked prediction failure, with an experimental IC_{50} of 169.56 min compared with a predicted value of 6.09 min, despite reaching complete final gelation ($A_{\max} = 1.13$). Because its experimentally measured gelation kinetics fell far outside the rapid-gelation region targeted for RF refinement, batch 29 was not included in the subsequent RF training dataset. Importantly, it was retained in Table 4 and in the prospective assessment of NN predictive performance.

These nine experimentally tested batches were combined with four batch-level observations from the original design (batches 8, 10, 18, and 20), yielding a refined dataset of 13 batch-level observations corresponding to 10 unique formulation conditions. Unlike the initial NN, which was trained solely on logistic-model fits derived from the original design-of-experiments dataset, this refined dataset uniquely combined

model-predicted candidates with their corresponding experimentally measured outcomes. This dataset of 13 batch-level observations was used to train an RF model dedicated to the final definition of the optimal formulation window. Within this refined dataset, in-sample descriptive

R^2 values for IC_{50} were 0.664 for NN and 0.811 for RF, whereas corresponding values for A_{max} were 0.365 and 0.555, respectively. These values are reported as descriptive goodness-of-fit measures only and not as independent estimates of predictive accuracy or generalizability. RF

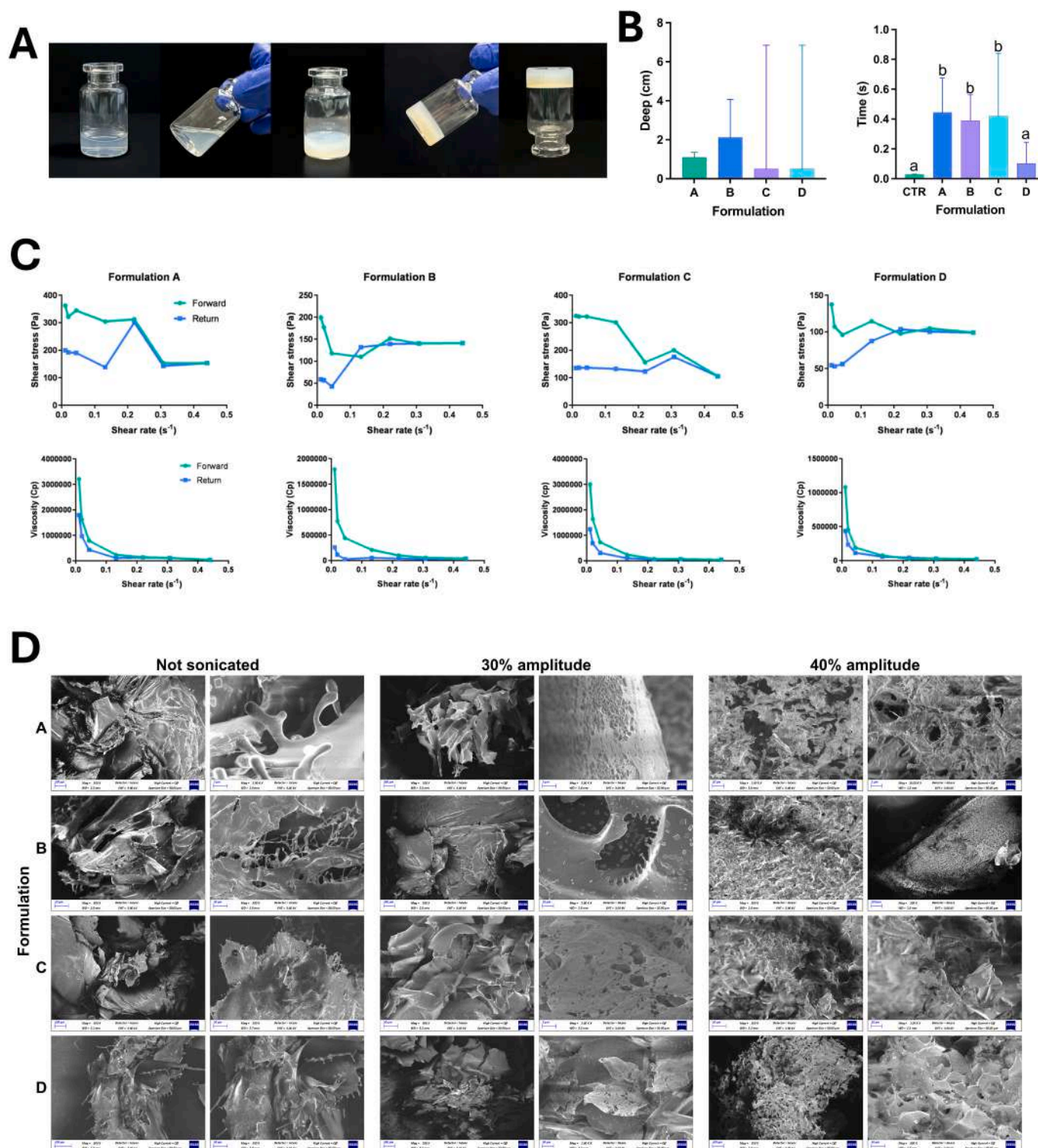


Fig. 2. Technological and morphological characterization of optimized SF gels. (A) Time-dependent evolution of gel appearance, showing the transition from a transparent, low-viscosity solution immediately after preparation to a turbid, self-supporting gel within 10 min, confirmed by vial inversion. (B) Resistance to penetration and syringeability of formulations A, B, C, and D. Data are reported as mean values $\pm$ LSD; Multifactor ANOVA, $n = 3$. Different letters (a, b) indicate a significant difference between the means ($p < 0.05$). (C) Viscosity measurements of gels under defined conditions. (D) SEM micrographs showing surface and cross-sectional morphology of gels prepared under different conditions. Non-sonicated samples are compared with gels obtained using sonication at 30% and 40% amplitude and different processing parameters. Scale bars and magnification as indicated.

modeling generated the definitive four-dimensional hypersurface for $A_{\max}$; two-dimensional cross-sections at fixed SF concentrations (2.5% and 1.75% w/v) are shown in Fig. 1B and supplementary files F1 and F2. At 2.5% w/v fibroin, $A_{\max}$ values were uniformly high, indicating relatively stable final gelation extent across the refined formulation space. At 1.75% w/v SF, the response surface showed reduced but still acceptable variability compared to the exploratory NN model, consistent with the restricted range of $A_{\max}$ values within the experimentally refined formulation space. RF-predicted IC_{50} surfaces are shown in Fig. 1C and supplementary files F3 and F4, again as cross-sections at 2.5% and 1.75% w/v SF. Predicted IC_{50} values were 14–17 min at 1.75% w/v SF and 8–12 min at 2.5% w/v SF. For both SF concentrations, IC_{50} decreased with increasing K^+ up to approximately 31.25 mM, followed by a slight increase and subsequent decline at higher ionic strengths, confirming the non-linear interplay between ionic and mechanical inputs already suggested by the exploratory NN analysis, but now examined within a narrower, experimentally refined formulation region. Inspection of experimentally repeated conditions corresponding to the limits of the RF-defined region further showed that final gelation extent was comparatively stable, whereas gelation kinetics were substantially more variable. At 2.5% w/v SF, 50 mM K^+ , and 60 s sonication (formulation A), repeated preparations reached near-complete gelation with IC_{50} values of 4.52, 5.51, and 7.18 min. At the same SF and K^+ concentrations but with shorter sonication (30 s; formulation B), near-complete final gelation was retained, whereas IC_{50} values varied from 8.39 to 62.36 min. Similarly, at 1.75% w/v SF, 25 mM K^+ , and 60 s sonication (formulation D), $A_{\max}$ remained close to 1 while IC_{50} ranged from 11.26 to 24.58 min. Thus, the tested limiting conditions generally retained the ability to reach complete or near-complete gelation, while kinetic robustness decreased at specific boundaries of the operational region.

The resulting RF model defined an operational region associated with complete or near-complete gelation and generally favorable gelation kinetics (Fig. 1D, supplementary File F5), geometrically approximated by a tetrahedron in the three-dimensional parameter space (SF and K^+ concentrations, and sonication time). Consistent with the exploratory NN analysis, this region expanded toward higher K^+ concentrations and longer sonication times, and narrowed at lower ionic strengths; however, being trained on experimentally validated data rather than purely on model predictions of the initial design, it provided a conservative estimate of the formulation space associated with favorable gelation behavior, while acknowledging increased kinetic variability at specific boundary conditions.

3.4. Physicochemical and structural characterization

The temporal evolution of gel formation is illustrated in Fig. 2A. Immediately after preparation, samples appeared transparent with a slightly increased viscosity, consistent with the early stages of network assembly. After 24 h, gels exhibited characteristic turbidity and a whitish appearance, consistent with extensive network formation and self-supporting behavior, as confirmed by vial inversion. Visual inspection in multiwell plates (Fig. S3) further highlighted the impact of formulation optimization: early formulations displayed marked heterogeneity, whereas gels within the optimized region exhibited uniform appearance and reproducible gel formation.

The pH of all formulations ranged between 7.3 and 7.4, remaining within the physiological range and therefore compatible with subsequent biological investigations.

Bulk gel resistance to penetration was assessed by penetrometry. Formulation B (K^+ 50 mM, sonication 30 s) displayed the greatest penetration depth (~ 2.2 cm), whereas formulations C (K^+ 32.5 mM, sonication 44 s) and D (K^+ 25 mM, sonication 60 s) exhibited the lowest values (~ 0.5 cm each). Formulation A (K^+ 50 mM, sonication 60 s) showed an intermediate penetration depth (~ 1 cm). No statistically significant differences were observed among the four formulations,

indicating no detectable differences in resistance to penetration under the investigated conditions.

Syringeability was assessed by measuring injection time through a standardized syringe setup (Fig. 2B). The water control (CTR) exhibited near-zero injection time. Formulations A, B, and C showed significantly higher injection times (~ 0.40 – 0.45 s; $p < 0.05$ vs CTR), with no significant differences among them. Formulation D exhibited a significantly lower injection time (~ 0.1 s), comparable to CTR ($p > 0.05$), suggesting a more favorable extrusion profile despite its gel-like consistency.

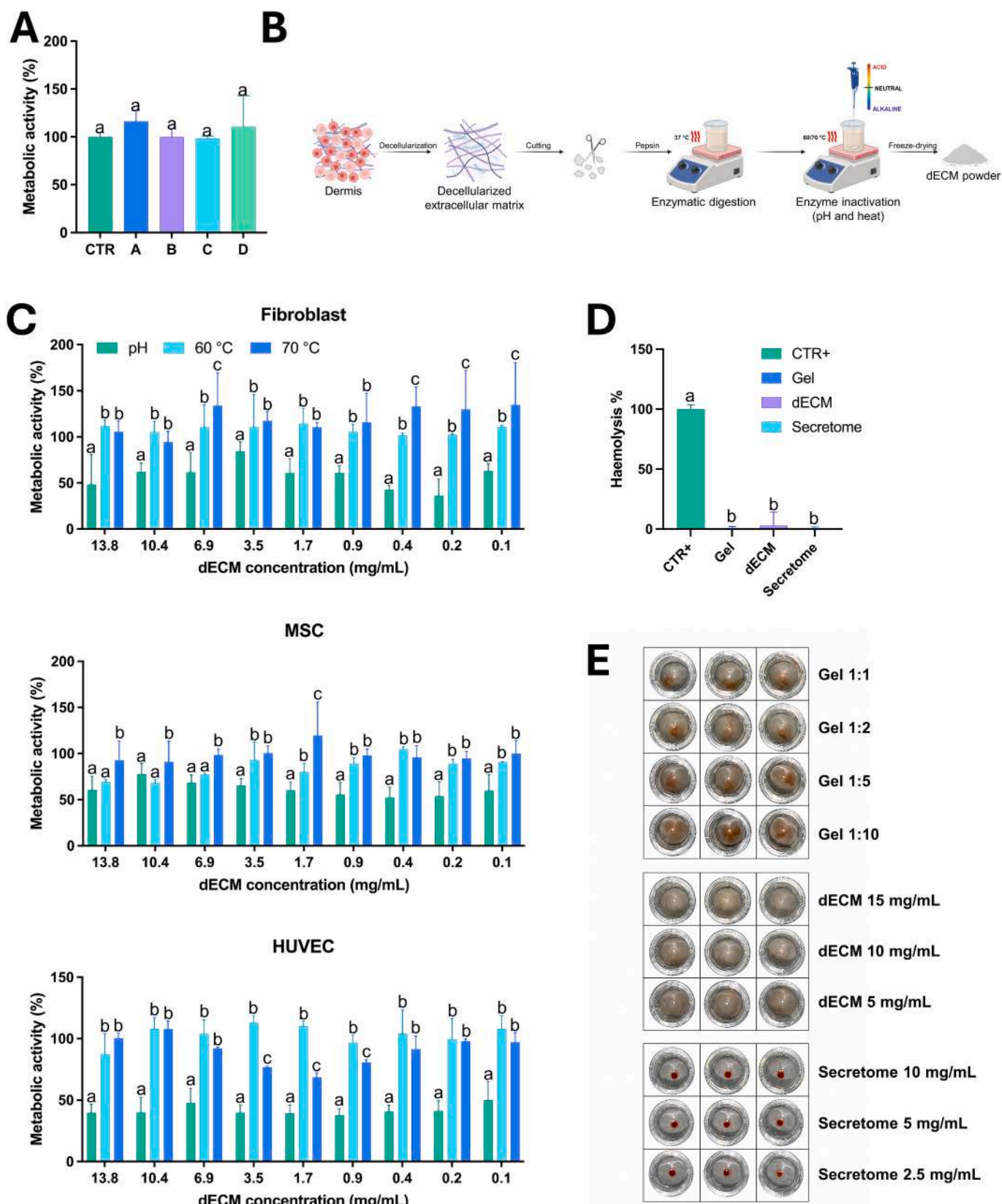
Rheological characterization revealed a pronounced shear-thinning behavior in all formulations, a feature generally considered advantageous for injectable systems (Fig. 2C). Viscosity decreased markedly with increasing shear rate, confirming the pseudoplastic nature of the gels. Formulations A and C exhibited the highest apparent viscosities at low shear rates, reaching values above 3×10^6 cP, whereas formulations B and D showed lower initial viscosities (approximately 1.8×10^6 and 1.1×10^6 cP, respectively). As the shear rate increased, the viscosity values progressively decreased and tended to converge among formulations. Comparison of forward and return sweeps revealed hysteresis loops of different magnitudes among formulations, indicating shear-history-dependent rheological behavior.

Hydrolytic stability was further assessed by incubating the gels in PBS at 37 °C for 30 days. No measurable bulk erosion was detected over the investigated period under these non-enzymatic conditions (Fig. S4), indicating prolonged stability of the SF network in PBS.

Morphological characterization by SEM revealed marked differences in microstructure as a function of formulation composition and processing conditions. (Fig. 2D). Non-sonicated controls, obtained by lyophilization of ungelled solutions at identical SF and K^+ concentrations, displayed relatively compact lamellar structures with limited visible porosity. In contrast, sonicated gels exhibited progressively more open architectures. At 30% sonication amplitude, structures became more porous and discontinuous, characterized by irregular voids and fragmented lamellar organization. At 40% amplitude, porosity appeared to increase further, yielding a more open sponge-like morphology. Analysis of representative sample groups (rows A–D) confirmed a combined effect of formulation and processing parameters. Row A (2.5% w/v SF; 50 mM K^+ ; 60 s) displayed the most pronounced morphological changes at increasing amplitude. Row B (same SF and K^+ ; 30 s) produced comparatively more cohesive structures, suggesting a contribution of sonication duration to microstructural organization independent of amplitude. Row C (2.5% w/v SF; 32.5 mM K^+ ; 44 s) showed intermediate morphology, with reduced matrix compactness relative to row B but less disruption than row A, likely attributable to the lower ionic strength. Row D (1.75% w/v SF; 25 mM K^+ ; 60 s) exhibited the most pronounced structural heterogeneity, with large cavities and a markedly irregular sponge-like architecture, further intensified at 40% amplitude. Collectively, these findings demonstrate that SF concentration, K^+ concentration, sonication time, and sonication amplitude jointly influence gel microstructure, modulating the transition from relatively compact lamellar architectures to progressively more porous sponge-like structures. The observed microstructural variability suggests that formulation and processing parameters can be used to tailor gel architecture over a relatively broad range while maintaining acceptable injectability and gel formation characteristics.

3.5. Biological performance of SF gels and integration of bioactive components

Gels prepared within the optimized region, including formulations containing the highest investigated K^+ concentration (50 mM), did not induce significant reductions in fibroblast metabolic activity compared to untreated controls (Fig. 3A), supporting the cytocompatibility of the complete K^+ -containing formulations under the tested in vitro conditions.



(caption on next page)

Fig. 3. Gel components, in vitro cytocompatibility, and component-level blood compatibility. (A) Metabolic activity of fibroblasts after 24 h of exposure to SF gels (formulations A-D). Untreated cells (CTR) were considered to have 100% metabolic activity. Data are reported as mean values $\pm$ LSD; one-way ANOVA with Tukey's post hoc test, $n = 3$. Different letters indicate statistically significant differences between the means ($p < 0.05$). (B) Schematic representation of dECM preparation from human skin, including decellularization, enzymatic digestion, pepsin inactivation (pH and thermal treatment), and lyophilization with mannitol. (C) Metabolic activity of fibroblasts, MSCs, and HUVECs after 24 h of exposure to dECM processed under different inactivation conditions and concentrations (0.11–13.8 mg/mL). Untreated cells (CTR) were considered as 100% metabolic activity. Data are reported as mean values $\pm$ LSD; two-way ANOVA with Tukey's multiple comparisons test, $n = 3$. Different letters indicate statistically significant differences between the means ($p < 0.05$). (D) Hemolysis assay of SF gel, dECM, and lyosecretome; distilled water (CTR+) was used as the positive control, while PBS served as the negative control. Data are reported as mean $\pm$ LSD; two-way ANOVA with Tukey's multiple comparisons test, $n = 3$. (E) Representative hemagglutination assay of the individual components of the composite system. The SF gel was tested at volumetric dilution ratios, whereas dECM and lyosecretome were tested at defined mass concentrations, reflecting their different physical forms. These different concentration metrics were not intended to represent dose-equivalent exposures across groups. For all conditions, equal volumes of prepared sample and RBC suspension were brought into direct contact, with PBS used as the negative vehicle control. Formation of a compact central erythrocyte button was interpreted as absence of hemagglutination.

To investigate whether biologically active components could complement the SF matrix, gels were functionalized with two components: lyosecretome derived from adipose-derived MSCs and dECM from human skin (Fig. 3B). Lyosecretome was produced by ultrafiltration and diafiltration of conditioned medium, followed by lyophilization in the presence of mannitol as a cryoprotectant. Biochemical characterization of the serum-starved secretome yielded a total protein content of $23 \pm 0.28 \mu\text{g}/\text{mg}$ and a lipid content of $21.8 \pm 1.06 \mu\text{g}/\text{mg}$ of lyophilized material; the IL-1 β -preconditioned secretome contained $27.3 \pm 1.11 \mu\text{g}/\text{mg}$ protein and $38.3 \pm 1.36 \mu\text{g}/\text{mg}$ lipid. Nanoparticle tracking analysis confirmed a size distribution consistent with EVs (data not shown). The dECM was obtained through a sequential detergent-based decellularisation, enzymatic digestion, and lyophilization protocol, yielding a standardized composition of 69% matrix, 13.8% pepsin, and 17.2% mannitol (w/w). The lyophilized dECM contained $32.3 \pm 0.41 \mu\text{g}/\text{mg}$ protein and $266.4 \pm 44.83 \mu\text{g}/\text{mg}$ lipid. Particular attention was devoted to pepsin inactivation, which was achieved either by pH neutralization alone or by combining pH adjustment with thermal treatment at 60 or 70 °C. Cytocompatibility of dECM preparations was evaluated in fibroblasts, MSCs, and HUVECs across a concentration range of 0.11–13.8 mg/mL (Fig. 3C). pH neutralization alone resulted in marked cytotoxicity in all cell types, with significant viability reductions observed even at 0.11 mg/mL. Combined pH neutralization and thermal treatment at 60 °C restored full cytocompatibility; under these conditions, metabolic activity remained at or above control levels across the tested range in all three cell types, with minor decreases only at the highest concentrations (6.9–13.8 mg/mL) in MSCs. An increase in metabolic activity at intermediate concentrations, particularly in fibroblasts and MSCs, suggests a bioactive rather than purely structural role of the dECM components. Based on the cytocompatibility screening, two cytocompatible dECM concentrations (1 and 10 mg/mL) were selected to investigate concentration-dependent biological responses in subsequent functional assays.

Component-level blood compatibility screening showed that none of the individually tested constituents induced hemolysis above the threshold of biological relevance at any tested concentration (Fig. 3D). In the hemagglutination assay, lyosecretome produced a compact central RBC button at all tested concentrations, consistent with the absence of hemagglutination. SF gel and dECM instead showed a diffuse sedimentation pattern (Fig. 3E); however, given the intrinsic turbidity of these two matrix-based preparations, this reading should be interpreted with caution and cannot be unambiguously attributed to true erythrocyte agglutination versus sample opacity interfering with visual assessment. PBS was used as the vehicle and negative control. No dedicated mannitol-only or pepsin-only control was included, so a possible excipient-related contribution cannot be formally excluded.

3.6. Functional bioactivity of composite gels

The in vitro biological activity of the optimized formulations was assessed using multiple cell-based assays. Formulation A, loaded with lyosecretome and dECM, was used as the reference loaded formulation

throughout. Its technological properties, including pH, resistance to penetration, and rheological profile, remained comparable to those of the unloaded gel described in Section 3.4, with no appreciable differences observed under the tested conditions (data not shown).

Scratch assay analysis over 24 h demonstrated that SF gel significantly attenuated fibroblast scratch closure relative to the untreated control (CTR; Fig. 4). A concentration-dependent effect was observed for dECM alone: at 1 mg/mL, fibroblast migration was significantly accelerated compared to CTR ($p < 0.05$), whereas at 10 mg/mL, migration was markedly impaired. Lyosecretome alone similarly enhanced scratch closure relative to CTR ($p < 0.05$). Incorporation of either component into the gel did not recover migratory activity; scratch closure remained significantly slower than CTR ($p < 0.05$) in all loaded formulations, indicating that the gel matrix exerted a dominant modulatory effect on fibroblast motility that was not overcome by the incorporated bioactive components. Loaded gels containing dECM at 1 mg/mL showed partial recovery relative to the unloaded gel, whereas loaded gels containing dECM at 10 mg/mL displayed a closure profile comparable to dECM 10 mg/mL alone ($p > 0.05$).

Tube formation assay on HUVECs (Matrigel®, 10 h) was assessed by confocal microscopy and quantification of mesh number, junction number, total tube length, and master segment length (Fig. 5). It is noted that the positive control (CTR+) consisted of complete endothelial growth medium supplemented with pro-angiogenic growth factors, representing an established pro-angiogenic stimulus, whereas all other conditions were tested in growth factor-free medium. No condition induced a significant reduction in any angiogenic parameter relative to CTR+ ($p > 0.05$), indicating that neither the SF matrix nor the incorporated bioactive components impaired endothelial network formation under the tested conditions. dECM at 10 mg/mL induced the greatest number of meshes and junctions, significantly exceeding all other groups, including CTR+ ($p < 0.05$), and was the only condition to significantly increase master segment length ($p < 0.05$). Loaded gel with dECM at 10 mg/mL also exhibited significantly elevated mesh and junction counts compared to CTR+ ($p < 0.05$), and a significant increase in total tube length comparable to dECM 10 mg/mL alone ($p > 0.05$). Remaining conditions, including unloaded gel, dECM at 1 mg/mL, secretome, and loaded gel with dECM at 1 mg/mL, maintained angiogenic parameters at levels comparable to CTR+ ($p > 0.05$), consistent with preservation of pro-angiogenic activity even in the absence of exogenous growth factor stimulation. Notably, lyosecretome alone, despite the absence of growth factor supplementation, was sufficient to sustain angiogenic parameters comparable to CTR+, indicating an intrinsic pro-angiogenic capacity of the secretome that matched the effect of exogenous growth factor stimulation, although it did not reach the magnitude of dECM-driven enhancement.

RT-qPCR analysis of a 13-gene panel in fibroblasts revealed treatment-specific transcriptional profiles (Fig. 6A-E). Regarding inflammatory signaling, the secretome was the only condition to significantly upregulate IL6 ($p < 0.05$), whereas dECM, gel, and loaded gel significantly downregulated both NFKB1 and HMGB1 ($p < 0.05$), indicating reduced transcript levels of these inflammation-associated genes.

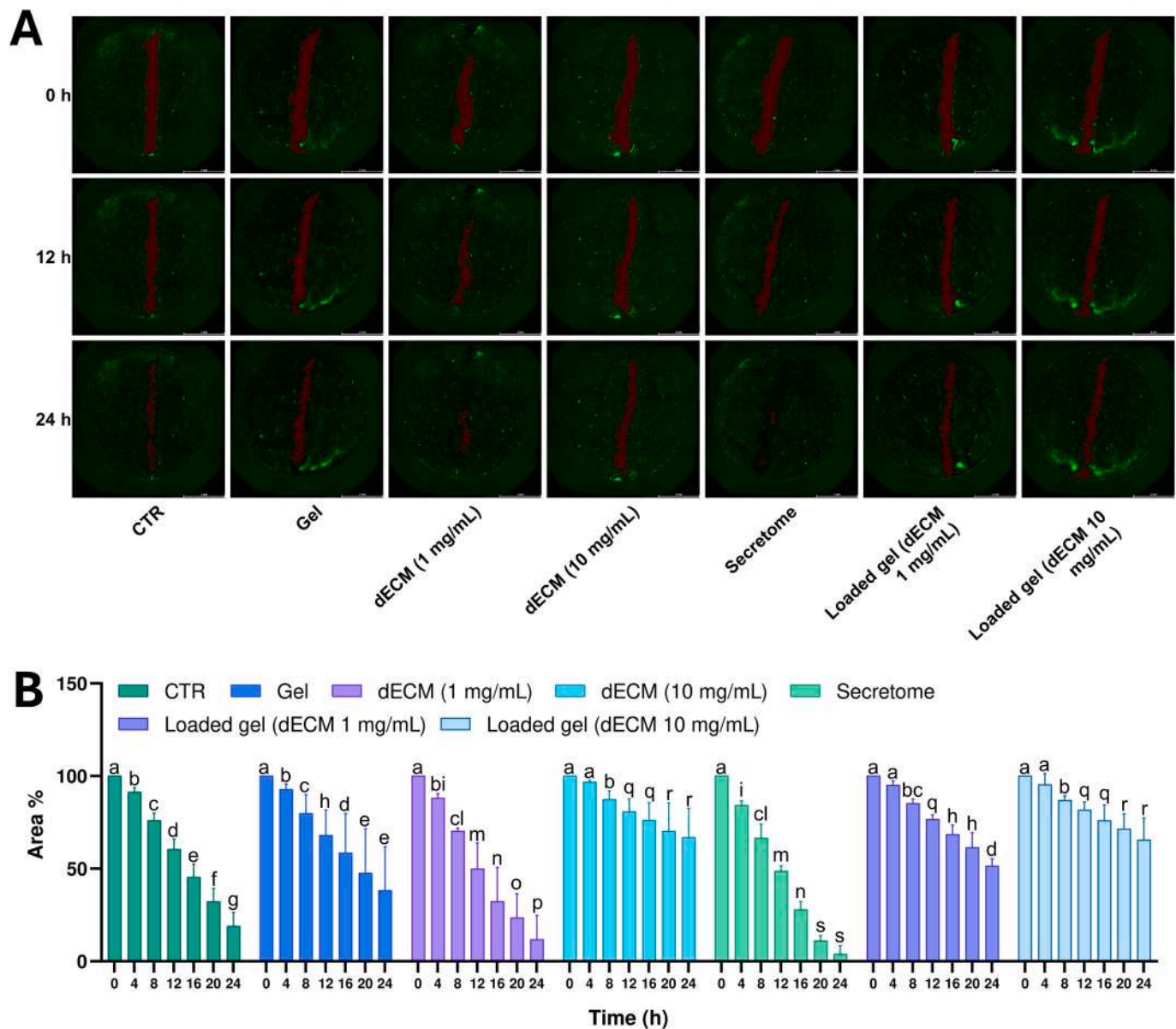


Fig. 4. In vitro scratch migration assay of human dermal fibroblasts treated with SF gel, dECM, secretome, and gels loaded with dECM and lysosecretome. (A) Representative fluorescence microscopy images of the scratch assay at 0, 12, and 24 h for each experimental condition: CTR, Gel, dECM (1 mg/mL), dECM (10 mg/mL), Secretome, Loaded gel (dECM 1 mg/mL), and Loaded gel (dECM 10 mg/mL). GFP-expressing fibroblasts were used. Red masks overlay the remaining open scratch area at each time point, enabling visual comparison of scratch closure kinetics across conditions. Scale bar: 2 mm. (B) Quantitative analysis of scratch closure expressed as the percentage of the remaining open area relative to the initial scratch area at t_0 , measured at 0, 4, 8, 12, 16, 20, and 24 h. Data are reported as mean $\pm$ LSD; Multifactor ANOVA, $n = 3$. Different letters above bars indicate statistically significant differences between groups at each time point ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Regarding redox-associated genes, NQO1 and SOD2 were significantly reduced by gel, dECM, and loaded gel ($p < 0.05$). Lysosecretome maintained NQO1 expression at control levels while markedly increasing SOD2 expression ($p < 0.05$), indicating a selective transcriptional response involving this mitochondrial antioxidant-defense-associated gene. ECM remodeling genes displayed divergent regulation: COL3A1 was upregulated by gel but downregulated by secretome and loaded gel ($p < 0.05$); FN1 was significantly induced by dECM and loaded gel ($p < 0.05$); MMP2 remained largely unchanged across conditions, with the exception of loaded gel, which significantly reduced its expression ($p < 0.05$); TIMP1 was strongly induced by dECM and moderately by secretome ($p < 0.05$), whereas loaded gel significantly decreased its expression below control levels ($p < 0.05$); TM7SF2 was selectively upregulated by secretome ($p < 0.05$). The pro-apoptotic marker BAX

was significantly downregulated by gel, dECM, and loaded gel ($p < 0.05$), consistent with modulation of apoptosis-associated transcriptional responses. Angiogenesis-related genes showed divergent regulation: FGF7 was increased by secretome but reduced by gel and loaded gel ($p < 0.05$); VEGFA was significantly downregulated by gel, secretome, and loaded gel ($p < 0.05$), whereas dECM was the only condition to significantly enhance VEGFA transcription ($p < 0.05$), providing an angiogenesis-related transcriptional response complementary to the tube-formation findings.

Z-stack imaging was used to qualitatively assess the presence of GFP-expressing fibroblasts at different focal planes within the gel constructs (Fig. 6F). Fluorescence signal was detectable across multiple optical sections in all tested conditions, as illustrated by the maximum intensity projection and representative focal planes A-C (Fig. 6F), demonstrating

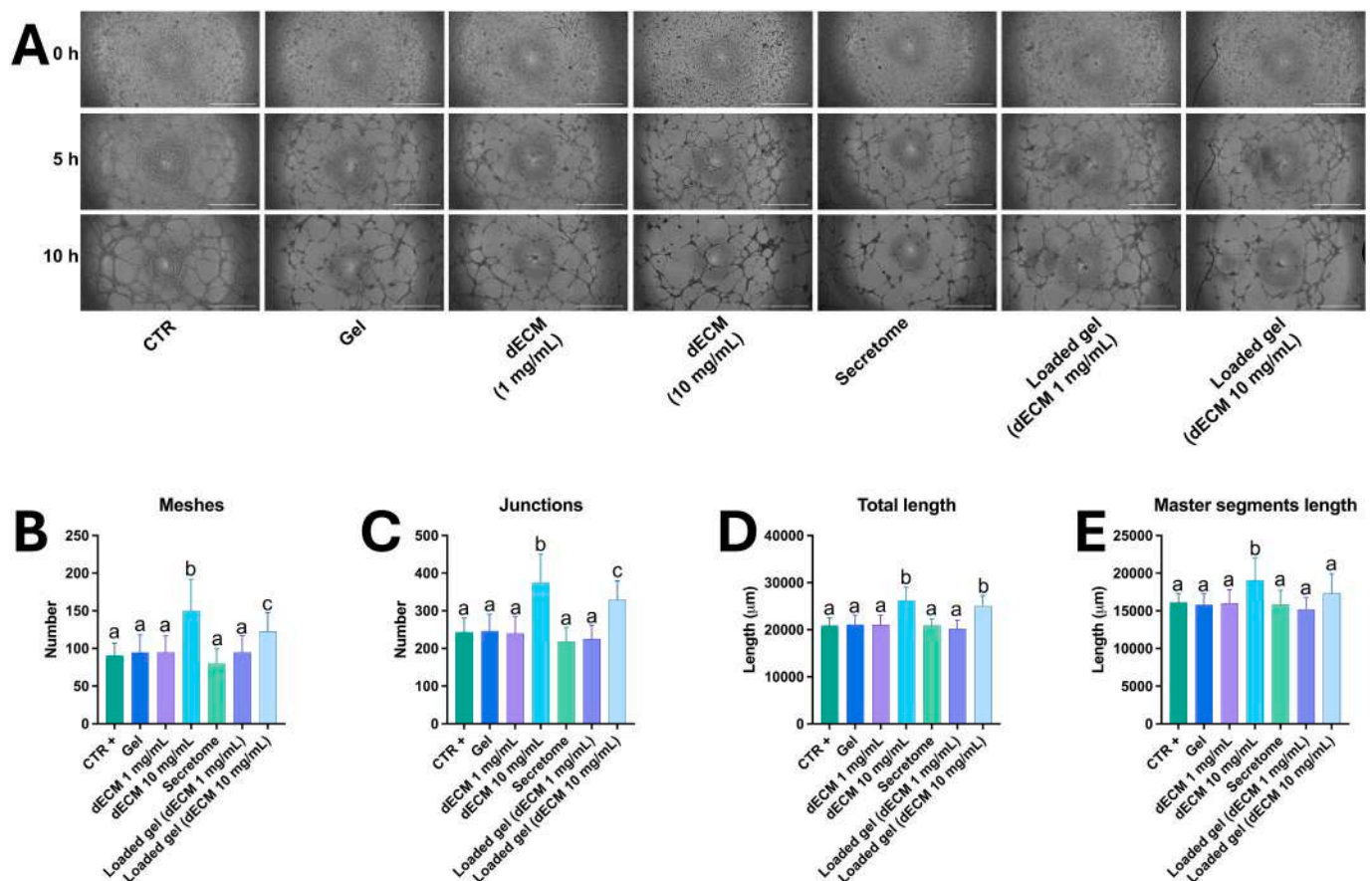


Fig. 5. In vitro angiogenic potential assessed by tube formation assay. (A) Representative microscopy images of human endothelial cells at 0, 5, and 10 h after treatment with CTR+, Gel, dECM (1 mg/mL), dECM (10 mg/mL), Secretome, Loaded gel (dECM 1 mg/mL), and Loaded gel (dECM 10 mg/mL). Scale bar: 1 mm. Quantitative analysis of tube formation parameters, including (B) number of meshes, (C) number of junctions, (D) total tube length (μm), and (E) master segments length (μm). Data are reported as mean ± LSD, Multifactor ANOVA, $n = 3$. Different letters indicate statistically significant differences among groups ($p < 0.05$).

that fluorescent cells could be visualized at different focal planes within the gel constructs. No quantitative analysis of cell morphology, proliferation, density, spatial distribution, or infiltration depth was performed; therefore, the images should be interpreted as qualitative observations only.

4. Discussion

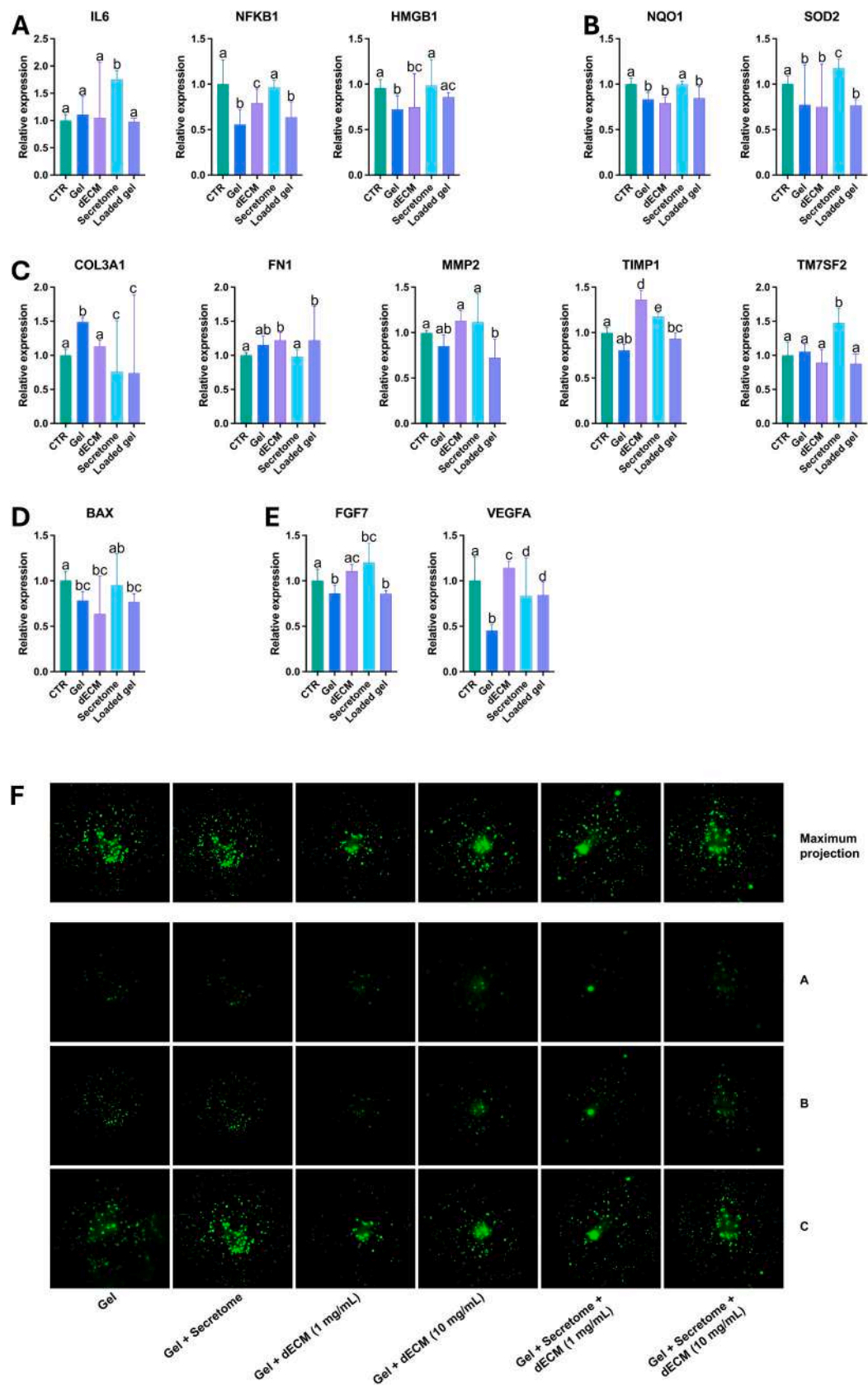
The development of injectable SF-based gels for regenerative wound healing requires balanced control over formulation variables governing sol-gel transition, flow and handling properties, and biological performance. In the present study, we addressed this challenge by integrating a biomimetic K^+ -triggered gelation mechanism with sonication-induced conformational transitions and data-driven formulation optimization. The resulting platform was further functionalized with adipose-derived MSC-secretome and human dermis-dECM to evaluate whether these components provide complementary biological inputs within an injectable SF matrix.

Gelation of SF is primarily driven by the transition from Silk I (random coil/helix-rich) to Silk II (β -sheet-rich) structures, a process accelerated by mechanical energy input. In our system, sonication acted as the main physical trigger, promoting chain rearrangement and hydrophobic domain association [35–37]. K^+ played a complementary role by modulating electrostatic interactions and facilitating macromolecular assembly [38–40]. The complete absence of gelation in K^+ -free formulations across all SF concentrations and sonication conditions indicates that ionic strength is a necessary condition for sol-gel transition under the present experimental framework, consistent with the role of

ionic gradients in natural silk spinning [11]. The combination of formulation variables generated a highly non-linear gelation landscape, where small variations in ionic strength or mechanical input produce disproportionate changes in gelation behavior. From a formulation perspective, this sensitivity is particularly relevant for injectable systems: it defines a narrow but exploitable processing window in which injectability and in situ stabilization can be simultaneously achieved, provided that formulation parameters are rationally controlled – a challenge that motivates the machine learning-assisted approach adopted here.

Machine learning models were thus employed as surrogate tools to explore this multidimensional design space. The RF model consistently identified an operational region characterized by intermediate K^+ concentrations and sufficient mechanical input, supporting the existence of a tunable formulation window rather than a single optimal composition. It should be noted that the dataset size limits the generalizability of the surrogate models beyond the explored design domain; their value in this study lies in formulation space navigation rather than universal predictive capability. The marked endpoint-dependent variation in model performance, together with the limited stability obtainable from resampling of such a small dataset, further supports this conservative interpretation. Experimental assessment of limiting conditions further indicated that final gelation extent was comparatively robust, whereas gelation kinetics showed greater variability toward specific boundaries of the RF-defined region. The tetrahedral region should therefore be interpreted as an operational formulation window rather than as a sharp deterministic boundary of uniformly reproducible gelation kinetics.

Technological characterization of the selected formulations focused



(caption on next page)

Fig. 6. Gene expression profile of fibroblasts and Z-stack fluorescence imaging analysis. (A–E) RT-qPCR analysis of a 13-gene panel in fibroblasts treated with different formulations (Gel, dECM, Secretome, and Loaded gel) relative to the control (CTR). Investigated functional pathways include: (A) inflammation (IL6, NFKB1, HMGB1); (B) redox-associated response (NQO1, SOD2); (C) ECM remodeling (COL3A1, FN1, MMP2, TIMP1, TM7SF2); (D) apoptosis (BAX); and (E) angiogenic growth factors (FGF7, VEGFA). Data are presented as mean $\pm$ LSD; Multifactor ANOVA, $n = 3$. Different letters (a, b, c, d) indicate statistically significant differences among groups ($p < 0.05$). (F) Representative Z-stack fluorescence images, showing GFP-expressing fibroblasts at different focal planes within the gel constructs (maximum projection and representative planes A–C) for Gel, Gel + Secretome, Gel + dECM (1 mg/mL and 10 mg/mL), and Gel + Secretome + dECM (1 mg/mL and 10 mg/mL) combinations. Images are presented for qualitative visualization only; no quantitative analysis of cell morphology, proliferation, density, or infiltration depth was performed. Scale bar: 2 mm.

on properties directly relevant to preparation and administration of an injectable, in situ-forming system rather than on a comprehensive mechanical qualification. All formulations showed suitable extrusion behavior under the tested conditions [41] and pronounced shear-thinning profiles, while penetration testing did not reveal significant differences in bulk resistance among formulations. In contrast, formulation and processing parameters markedly affected microstructural organization, indicating that substantial architectural differences can occur without detectable differences in resistance to penetration under the conditions of this assay. In detail, increasing mechanical input produced a progressive transition from compact lamellar morphologies to highly porous sponge-like structures, a feature relevant to wound repair as it may facilitate oxygen diffusion, nutrient transport, and cell infiltration [42–44]. The SF gels also showed prolonged stability in PBS, with no measurable bulk erosion over 30 days under the tested non-enzymatic conditions. This finding should be interpreted as evidence of hydrolytic stability rather than as a complete assessment of biodegradation, since SF degradation in biological environments is expected to depend substantially on proteolytic and cell-mediated processes.

Biocompatibility studies confirmed the intrinsic cytocompatibility of SF gels, consistent with the well-established safety profile of SF [10,11]. The performance of dECM was strongly dependent on pepsin inactivation: pH neutralization alone was insufficient to eliminate cytotoxic effects, whereas combined pH adjustment and thermal treatment at 60 °C restored full cytocompatibility across all tested cell types. Notably, dECM at intermediate concentrations stimulated cell metabolic activity beyond control levels, suggesting a bioactive rather than purely structural contribution of the matrix components, consistent with the known instructive role of dECM in directing cell behavior [45,46]. Hemolysis remained below the 2% international safety threshold across all conditions. Blood compatibility was investigated as component-level screening of SF gel, dECM, and lyosecretome rather than as a qualification assay of the complete composite. Lyosecretome displayed a compact RBC button, consistent with absence of hemagglutination, while SF gel and dECM showed a diffuse sedimentation pattern whose interpretation is complicated by the intrinsic turbidity of these matrix-based preparations. To our knowledge, hemagglutination has not been previously reported for either SF or dECM, so this observation cannot presently be attributed to a defined mechanism. Because both dECM and lyosecretome contain mannitol, and residual pepsin is associated with the dECM preparation, differential behavior between preparations offers only contextual information; moreover, neither mannitol nor residual pepsin has, to our knowledge, been previously associated with erythrocyte agglutination, which further argues against a major excipient-driven contribution, although dedicated mannitol-only and pepsin-only controls remain the only way to exclude it formally. As such, these findings are preliminary and warrant confirmation on the complete gel, with dedicated excipient controls and a readout less sensitive to sample opacity.

Functional evaluation revealed distinct and complementary roles for each component. In the scratch assay, dECM exhibited a concentration-dependent response, enhancing fibroblast migration at 1 mg/mL while inhibiting it at 10 mg/mL. Such divergent responses may reflect concentration-dependent differences in cell-matrix signaling, potentially involving integrin-binding matrix motifs and bioactive matrix-derived fragments [47–50]; however, the molecular pathways responsible were not directly investigated in the present study. The secretome

accelerated scratch closure, consistent with its known repertoire of pro-migratory paracrine mediators, including HGF, TGF- β , and multiple chemokines secreted by adipose-derived MSCs [51–53]. SF-containing formulations significantly attenuated fibroblast migration relative to untreated controls. Since gels were physically separated from the cell monolayer through the indirect exposure system, the observed reduction in scratch closure is consistent with an effect mediated by soluble species released from the formulation. K^+ represents one biologically plausible candidate contributor, given its presence in the gel and previous evidence that elevated extracellular K^+ can modulate fibroblast migration and phenotype [54]. However, as K^+ release was not quantified and no concentration-matched free- K^+ control was included, these data do not establish K^+ as the specific causal mediator of the reduced fibroblast migration. Importantly, the clinical context for which this platform is intended – deep, cavitated, and chronic wounds – is characterized by dysregulated fibroblast activity and persistent inflammatory signaling, both of which can contribute to impaired healing and fibrotic remodeling. In this context, the observed reduction in fibroblast migration, together with the downregulation of NFKB1, HMGB1, and BAX in fibroblasts exposed to SF-containing formulations, suggests a distinct cellular response profile that may be relevant to wound-associated remodeling. However, the present study did not assess myofibroblast differentiation or fibrosis-related markers, and therefore no conclusions regarding antifibrotic or anti-scar activity can be drawn. Dedicated validation through antifibrotic marker analysis will be required to further characterize this aspect in future studies.

The tube formation assay identified dECM at 10 mg/mL as the condition producing the strongest endothelial network response under the tested in vitro conditions, significantly exceeding both the untreated controls and the growth factor-supplemented positive control for selected network parameters. The associated VEGFA upregulation further supports an effect of dECM on angiogenesis-related pathways under the tested in vitro conditions. Matrix-associated growth factors, adhesive motifs, and bioactive peptide fragments represent plausible contributors to this response [55], but their individual contributions were not determined in the present study. The divergence between dECM pro-angiogenic activity and its simultaneous inhibition of fibroblast migration at 10 mg/mL suggests a context-dependent, cell-type-specific mode of action, reflecting the distinct integrin repertoires of fibroblasts and endothelial cells. Notably, lyosecretome alone maintained angiogenic parameters at levels comparable to CTR+ under the present assay conditions, despite the absence of exogenous growth factor supplementation, indicating an intrinsic pro-angiogenic capacity sufficient to functionally replace growth factor stimulation, even though it did not reach the magnitude of dECM-driven enhancement. This finding reinforces the asymmetric but complementary functional profile of the two components: dECM acts as the dominant angiogenic driver, exceeding even growth factor-supplemented conditions, while the secretome provides an independently sufficient angiogenic stimulus of its own.

Gene expression profiling provided molecular resolution to the functional observations. Downregulation of NFKB1, HMGB1, and BAX by SF gel, dECM, and loaded gel indicates transcriptional modulation of inflammation- and apoptosis-associated genes, a pattern of potential relevance to chronic wound biology [56–58]. Selective IL6 upregulation by the secretome without concomitant NFKB1 activation suggests NF- κ B-independent transcription via JAK-STAT or AP-1 pathways; a

controlled IL-6 elevation may benefit wound healing by promoting the inflammatory-to-proliferative phase transition [59]. SOD2 upregulation by lyosecretome indicates a transcriptional response involving a mitochondrial antioxidant-defense pathway at the transcriptional level [60–63]. However, as SOD1 and other complementary redox markers were not assessed, and no direct measurement of intracellular oxidative status was performed, this finding should be regarded as a transcriptional indication rather than evidence of a global antioxidant effect or comprehensive modulation of cellular redox status. The loaded gel uniquely suppressed TIMP1 below control levels while simultaneously reducing MMP2; regardless of the underlying mechanism, this pattern is consistent with a shift toward a globally attenuated proteolytic state that may limit excessive ECM degradation within the gel construct [64,65]. The robust induction of FN1 by dECM and loaded gel suggests active matrix remodeling rather than mere scaffold tolerance [50,66,67], while secretome-driven FGF7 upregulation points to a complementary pro-regenerative axis centered on keratinocyte activation and re-epithelialization [68–70].

Comparison of the individual components with the loaded formulations further indicates that incorporation within the SF matrix does not simply preserve or add their independent biological effects. In the scratch assay, the pro-migratory response of dECM at 1 mg/mL was attenuated after incorporation, although partial recovery relative to the unloaded gel was retained, whereas the inhibitory profile of dECM at 10 mg/mL was largely preserved in the corresponding loaded formulation. In contrast, the strong endothelial network response associated with dECM at 10 mg/mL remained evident after incorporation, indicating that matrix loading did not uniformly attenuate component activity. A similarly non-additive response pattern emerged at the transcriptional level: increases in VEGFA and TIMP1 associated with dECM and in SOD2 and FGF7 associated with lyosecretome were not reproduced in the loaded gel, whereas FN1 induction was retained and MMP2 reduction emerged specifically in the composite condition. Collectively, these findings indicate that the biological response of the loaded system is component-, dose-, and readout-dependent rather than a simple superposition of the effects observed for the free components. Differential sequestration within the SF network, altered diffusional availability, or convergence of component-derived signals represent possible explanations; however, these mechanisms cannot be distinguished without component-specific release and mechanistic studies.

Finally, z-stack imaging provided qualitative evidence that GFP-expressing fibroblasts could be visualized at different focal planes within the gel constructs. Owing to the resolution and acquisition characteristics of the imaging platform, these observations do not allow quantitative conclusions regarding cell morphology, proliferation, density, or infiltration depth. Dedicated three-dimensional imaging and quantitative analysis will therefore be required to characterize these parameters in future studies.

Taken together, the data reveal distinct biological profiles for dECM and lyosecretome, together with matrix-dependent modulation of these responses following incorporation into the composite gel. dECM showed pro-angiogenic activity, inflammation- and apoptosis-associated transcriptional changes, and biologically relevant matrix-derived cues. The lyosecretome contributed a distinct complementary layer, characterized by selective SOD2 upregulation, pro-migratory paracrine signaling, and FGF7-mediated keratinocyte-directed activity – a combination relevant to the re-epithelialization phase of wound repair. Machine learning-assisted optimization served as a resource-efficient strategy for narrowing the complex formulation space and as an exploration tool within the tested domain. A major limitation of the present study is that biological performance was assessed exclusively using *in vitro* models, which provide information on cellular responses but cannot establish regenerative efficacy or predict functional wound repair *in vivo*. In particular, the observed pro-angiogenic and pro-migratory responses require validation in appropriate preclinical wound-healing models. Future studies should therefore address enzymatic and cell-mediated gel

degradation, release kinetics of K^+ , the availability and release of selected dECM- and secretome-derived bioactive factors, long-term cellular responses, antifibrotic marker profiling, and *in vivo* performance, including tissue integration, vascularization, inflammatory response, matrix remodeling, and wound closure. The present findings nonetheless establish an experimentally informed formulation and biological screening framework for multifunctional SF-based gels and provide a basis for subsequent *in vivo* evaluation.

5. Conclusions

This study demonstrates that machine learning-assisted exploration of a bioinspired, K^+ - and sonication-driven gelation strategy can support navigation of the non-linear formulation space of injectable SF gels, reducing empirical screening within the investigated design domain. The resulting materials combined controllable gelation kinetics, physiological pH, tunable microstructure, and acceptable injectability. Functionalization with dECM and MSC-derived lyosecretome revealed distinct biological profiles *in vitro*: dECM produced the strongest angiogenesis-related response and was associated with VEGFA upregulation and reduced NFKB1, HMGB1, and BAX expression, whereas lyosecretome showed selective SOD2 and FGF7 upregulation and enhanced fibroblast migration when tested as a soluble component. These findings identify distinct component-associated response patterns *in vitro*; further mechanistic and *in vivo* studies will be required to determine their biological basis and translational relevance.

CRediT authorship contribution statement

Edoardo Bertania: Writing – review & editing, Writing – original draft, Visualization, Investigation. **Angelo Modena:** Investigation. **Maurizio Rinaldi:** Writing – review & editing, Visualization, Methodology, Formal analysis. **Marta Cecilia Tosca:** Investigation. **Ivana Miletto:** Investigation. **Dmitry Lim:** Investigation. **Ilenia Rita Renzulli:** Software. **Alice Maria Balderacchi:** Investigation. **Ilaria Ferrarotti:** Investigation. **Angelo Guido Corsico:** Investigation. **Elia Bari:** Writing – review & editing, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Marzio Sorlini:** Investigation. **Giovanni Sesana:** Investigation. **Maria Luisa Torre:** Writing – review & editing, Formal analysis, Conceptualization.

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Declaration of competing interest

Maria Luisa Torre and Marzio Sorlini are co-founders and members of the company's advisory board Pharmaexceed S.r.l. This company had no role in the design of the study, in the collection, analysis, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioadv.2026.215193>.

Data availability

Data will be made available on request.

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