

Development of a 3D bioprinted human skin model for predictive toxicology

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ARTICLE INFO

Keywords:

Bioprinting
Skin
Biocompatibility
Testing
Regeneration

ABSTRACT

Three-dimensional (3D) cultures represent an advanced approach for mimicking the architecture and cellular complexity of human tissues, providing a valuable alternative to conventional two-dimensional models. In particular, 3D bioprinting enables the generation of precise, standardized and biomimetic tissue constructs, with controlled spatial organization. The aim of the study was to design a novel 3D bioprinted full-thickness human skin model 3D protocol able to replicate human skin layers. The model is based on a high-concentration type I collagen bioink combined with dermal fibroblasts and keratinocytes, in which the use of dense collagen I enhances the mechanical properties and biomimetic features of the construct, providing dermal and epidermal contributions, and a physiologically relevant extracellular matrix. The skin like tissue (SLT) obtained was evaluated using established irritant and sensitizer controls, demonstrating its ability to respond to chemically induced stimuli without implying quantitative performance comparisons. Overall, this 3D skin model provides a biologically relevant and reproducible platform for early-stage testing of topically applied compounds, with potential applications in cosmetic safety assessment, drug screening, and alternative in vitro testing strategies.

1. Introduction

Three-dimensional (3D) models are currently used to mimic the architecture and complexity of human tissues in physiological or pathological conditions. The use of 3D models is considered a good compromise between two-dimensional (2D) cell culture and animal models. Both systems are limited and, in several approaches, cannot be used as faithful study models: cell culture is too simple and does not reproduce the spatial organization, cell-cell or cell-matrix connection, or the interplay between different cell types [1]. On the other hand, in vivo studies are expensive and involve ethical concerns as well as inter-species variability [2]. The development of 3D in vitro models

offers great potential to overcome the shortcomings of traditional cell cultures and animal studies.

Methods such as 3D bioprinting, spheroids, organoids, and organ-on-chip have been introduced to reproduce in vivo microarchitecture and to model chemical gradients and cell interactions, thereby replicating physiological microenvironment configurations [3].

3D bioprinting has significantly revolutionized the field of tissue and disease modeling. Using a computer-assisted motorized device, bioprinting enables the layer-by-layer deposition of biomaterials, cells, and biomolecules to create 3D construct with precise geometry, capable of structurally and functionally mimicking native tissue [4]. Over time, bioprinting has made rapid progress toward the fabrication of functional

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organ, enabling the printing of human liver, heart, bladder, skin and cartilage [5–9]. Bioprinted tissues not only mimic human architecture but also serve as platform for drug development, toxicity screening, and regenerative medicine [10]. The first bioprinted tissue was skin, the largest and most complex organ on the surface of the human body. It plays essential roles in temperature regulation, evaporation control, vitamin D synthesis, and fluid balance [11]. As a protective interface between internal organs and the external environment, the skin functions as an active immune organ, mediating key processes such as irritation and sensitization following exposure to external agents [12].

In 2009, for the first time, Lee and colleagues used bioprinting to produce a dermis equivalent by mixing human fibroblasts into a collagen hydrogel [13]. In the following years, a collagen-based bioink was used to provide a 3D architecture for keratinocytes and fibroblasts [14–16], resembling the human epidermis and dermis. Building on this preliminary evidence, several strategies have been developed to increase the complexity and biomimetic properties of reconstructed skin, enabling the use of bioprinted skin as a platform for screening cosmetic and pharmaceutical formulations [17].

Most bioprinted skin constructs are based on bioink made of collagen I, which constitutes approximately 90% of the proteins in mammalian connective tissue [18]. However, collagen concentrations commonly used for bioinks range between 5% and 10%, which are relatively low and lead to poor mechanical properties [18]. As a consequence, these systems frequently require the incorporation of supportive hydrogels to maintain structural stability [19], affecting the biomimesis of the reconstructed tissue. Increasing the storage modulus—for example by raising collagen concentration—would be desirable, yet only a limited number of mechanically robust collagen formulations are currently available on the market [19].

In parallel, several reconstructed human epidermis models, such as EpiDerm™, EpiSkin™, and SkinEthic™, have been extensively validated for regulatory toxicology applications and are primarily based on keratinocyte-only epidermal architectures [20]. While these systems provide robust and standardized platforms for specific endpoints, their simplified cellular composition limits the investigation of dermal–epidermal interactions. In parallel, full-thickness skin models generated through manual cell deposition approaches have been reported; however, these systems are often labor-intensive, poorly automatable, and difficult to integrate into controlled manufacturing environments, such as class-grade isolators, thereby limiting reproducibility and large-scale standardization.

Collectively, these limitations reveal a gap between highly standardized epidermal models used for regulatory purposes and emerging bioprinted skin constructs, both of which remain insufficient to fully replicate the structural and cellular complexity of human skin.

Existing skin models are predominantly generated through manual cell deposition approaches, resulting in limited reproducibility and poor suitability for automation. Literature reports how employing bioprinting strategies resulting in more standardizable and automatable.

In this context, the present study introduces a bioprinted full-thickness skin model incorporating dermal fibroblasts within a high-density collagen matrix, enabling the exploration of cell–cell and cell–matrix interactions that are not addressed by epidermis-only reconstructed models. Here, we described a novel protocol for generating a 3D skin culture that biomimics the main layers of human skin—epidermis and dermis—and serves as a platform for testing components used in cosmetic formulations. In line with internationally recognized standards for in vitro skin irritation and sensitization testing, the irritant and sensitizing potential of tested compounds was evaluated using Sodium Dodecyl Sulfate (SDS) and the sensitizer trinitrobenzene sulfonic acid (TNBS). The use of these standard controls demonstrates the model's ability to respond appropriately to chemically induced irritation and sensitization. Overall, this study aims to establish a robust and reproducible bioprinted full-thickness human skin model as a versatile in vitro testing platform, enabling the investigation of skin responses

such as irritation and sensitization and supporting the development of alternative, animal-free testing strategies.

2. Results

2.1. Extrusion-based bioprinting technology was able to resemble a multistratified skin like tissue

The bioprinted skin constructs were first evaluated for cell viability, tissue organization, and epidermal structural features. As shown in Fig. 1a–b, the majority of cells were viable and homogeneously distributed throughout the scaffold. The diffuse and continuous green fluorescence signal, together with the limited presence of red staining, indicated a high level of cell survival following the bioprinted process. Quantitative image analysis confirmed these qualitative observations, revealing a cell viability of $97.62 \pm 5.68\%$, with only $2.38 \pm 1.63\%$ of the total fluorescent area corresponding to dead cells (Fig. 1c).

Hematoxylin and Eosin (H&E) staining revealed the presence of two overlapping and clearly distinguishable layers. As shown in Fig. 1d, the bottom layer appeared as a light orange, dense matrix containing dermal fibroblasts with elongated and stretched morphology. This layer is primarily composed of the type I collagen used in the bioink formulation. In the upper region, a multilayered structure was observed, consisting of keratinocytes that were well adhered to one other, closely resembling the human epidermis.

H&E staining analysis revealed that the thickness of the reconstructed skin (supplementary figure 1a) was comparable to that of native human skin (supplementary figure 1b). Specifically, the epidermal thickness of healthy human skin was $99.21 \pm 21.71 \mu\text{m}$, while the reconstructed skin showed a thickness of $108.20 \pm 28.43 \mu\text{m}$. Statistical analysis indicated no significant difference between the two groups ($p = 0.49$; supplementary figure 1d)). Similarly, cell density analysis demonstrated comparable numbers of epidermal cells between native and bioprinted skin. Healthy human skin exhibited an average of 299 ± 32 cells, while the reconstructed skin showed 261 ± 23 cells per analyzed area ($p = 0.23$; supplementary figure 1c). Overall, these results indicate a high degree of similarity between reconstructed and native human skin with respect to epidermal thickness and cell density, supporting the structural equivalence of the epidermal layer.

To investigate the differentiation status of keratinocytes, specific immunofluorescence staining was performed. As shown in Fig. 1e, keratin 10 was expressed in the supra-basal layers of keratinocytes, consistent with its localization in normal skin. The positive staining for claudin 1, reported in Fig. 1g, indicates the presence of tight junctions between keratinocytes, suggesting the formation of continuous and organized layers. Finally, loricrin, a protein specific to the cornified envelope, was correctly expressed in the uppermost part of the construct, as shown in Fig. 1h. Quantitative analysis, reported in Fig. 1h), revealed widespread claudin-1 expression ($98.0\% \pm 1.0$), keratin 10 positivity in $67.0\% \pm 6.4$ of cells, and loricrin expression in $32.0\% \pm 4.9$ of the total cell population. This expression pattern is consistent with the physiological localization of these markers in the human epidermis and reflects progressive epidermal differentiation in the bioprinted skin model.

To further validate keratinocyte differentiation, Real Time-qPCR analysis of key epidermal markers was performed. A strong upregulation of keratin 10 expression (791.13 ± 75.46 -fold; p -value < 0.0001) was observed at the final time point compared to T1, indicative of active suprabasal differentiation. Consistently, increased expression of the late differentiation markers loricrin (12.25 ± 1.19 -fold; p -value < 0.0001) and filaggrin (5.53 ± 0.16 -fold; p -value < 0.0001) was detected, supporting the progressive maturation and stratification of the epidermal layer (Fig. 1i).

To investigate whether the observed epidermal organization translated into functional barrier properties, a permeability assay using fluorescent dextran was performed (supplementary figure 2). The

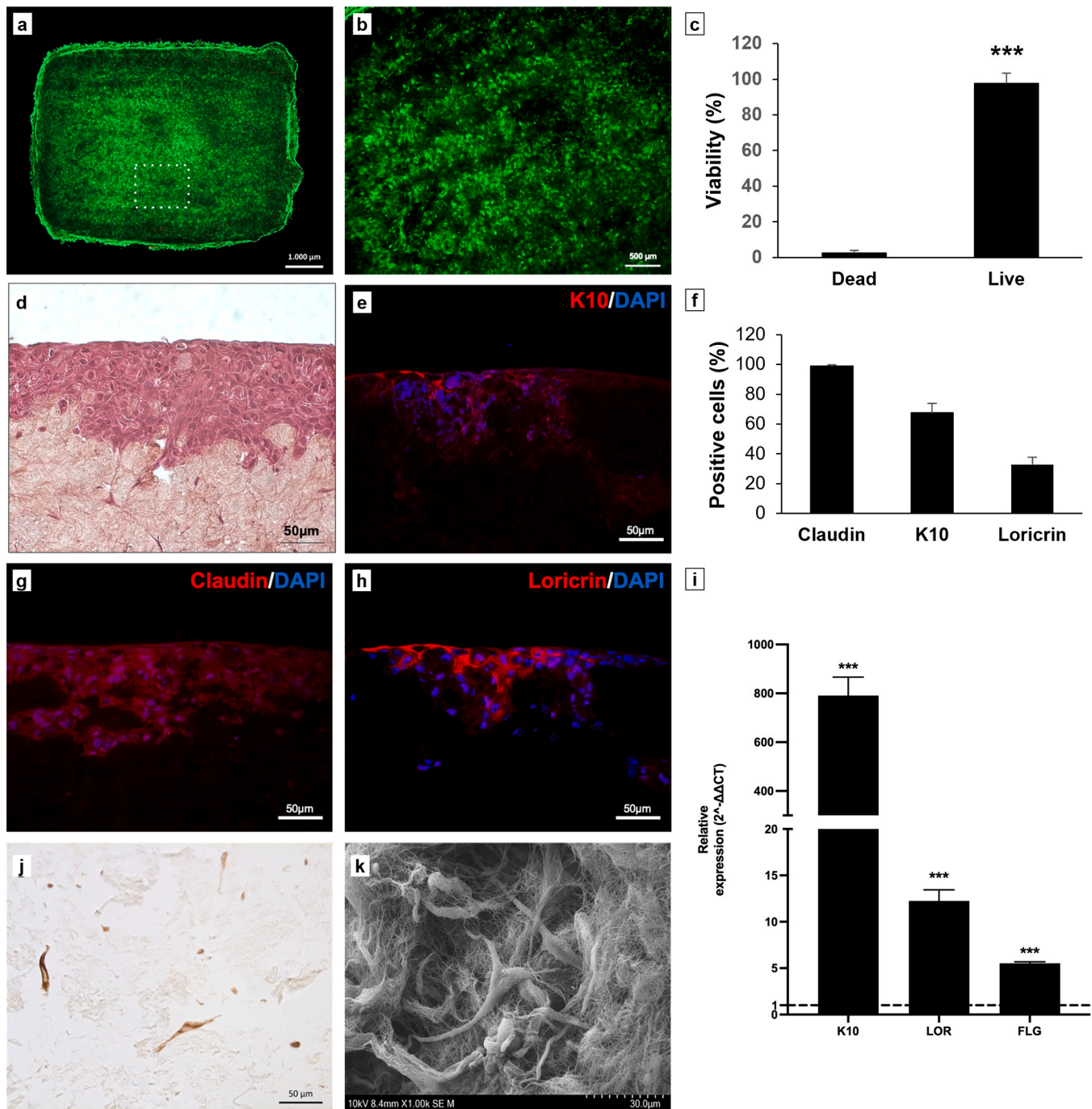


Fig. 1. Live & dead assay and characterization of SLT scaffolds. (a-b) Live & dead assay of SLT scaffolds, showing viable cells stained in green and dead cells in red. Bar: 1000 μm and 500 μm respectively (c) Live & dead assay quantification. Representative fluorescence images showing live (green) and dead (red) cells within the bioprinted constructs. For quantitative analysis, the green (live cells) and red (dead cells) fluorescence channels were independently thresholded, and the total fluorescent area (μm^2) was measured for each channel. Cell viability was calculated as the percentage of live or dead fluorescent area relative to the total fluorescent area (live + dead). Quantitative data are reported as viability percentages and presented as mean $\pm$ standard deviation. Statistical significance was evaluated using a Student's t-test. (d) Hematoxylin and Eosin (H&E) staining of SLT scaffolds, highlighting a thick, dark purple layer corresponding to HaCaT cells and a pink/orange matrix layer populated by HFF. Bar: 50 μm (e-g) Immunofluorescence staining of SLT sections showing the expression of epidermal markers: cytokeratin 10 (K10) (e), claudin (g) and loricrin (h), all labelled in red. Nuclei were counterstained with DAPI (blue). Bar 50 μm (f) Quantification of epidermal markers. K10, claudin and loricrin were quantified as the percentage of marker-positive cells relative to the total number of nuclei. Quantitative data are reported as viability percentages and presented as mean $\pm$ standard deviation. (i) Real-time PCR analysis of Keratin 10, Filaggrin, and Loricrin expression at the end of scaffold maturation. Data were normalized to the endogenous control GAPDH and to the expression levels at the day of keratinocyte seeding ($\Delta\Delta CT$). The dashed line represents RNA levels at day 1. Statistical analysis is reported as p-values. (j) Alpha-smooth muscle actin (α -SMA) staining identifying fibroblasts embedded in collagen matrix. Bar 50 μm (k) Scanning Electron Microscopy (SEM) of the bioprinted dermal scaffold showing collagen fibers forming a fibrous matrix within the bioink. 30 μm .

70 kDa fluorescent dextran rapidly diffused across dermal-only constructs (T0), showing a steep increase within the first hours followed by a plateau. In contrast, constructs with a fully developed epidermis (T14) exhibited a markedly reduced and time-dependent dextran permeation. These results indicate that the presence of a mature epidermal layer significantly limits molecular diffusion, supporting the establishment of a functional barrier.

To assess the contribution of the dermal compartment, we analyzed the fibroblast population embedded within the dermis of the skin model.

We performed immunohistochemical staining for alpha-smooth muscle actin (α -SMA). The results confirm the presence of fibroblasts within the dermal compartment, supporting their correct localization in the collagen I-based dermal layer (Fig. 1j).

To further explore the behavior of fibroblasts, we performed Scanning Electron Microscopy (SEM) analysis. The SEM images demonstrated that fibroblasts dynamically interact with collagenous matrix, adhering to and spreading along the collagen fibers present in the bio-ink. Moreover, we observed close proximity and contact between fibroblasts, suggesting potential cell–cell communication (Fig. 1k). These

observations reinforce the structural and functional integration of fibroblasts within the scaffold. Given the pivotal role of the ECM in maintaining skin homeostasis, architecture and cell signaling, we explored its temporal evolution in the 3D models.

Histological assessment of ECM deposition was performed using Masson's Trichrome and Picrosirius Red staining at day 1 and day 14 (post keratinocytes seeding) to evaluate ECM maturation within the dermal compartment of the bioprinted construct. In Masson's Trichrome staining, a marked increase in collagen content was observed over time. At day 1, the scaffold displayed minimal blue staining, indicative of low collagen presence (Fig. 2a). By day 14, a denser and more widespread blue staining pattern was evident, consistent with active collagen deposition and ECM remodeling (Fig. 2b).

Picrosirius Red staining analyzed under polarized light further confirmed the enhancement of collagen content. At day 1, the birefringence signal was limited and sparsely distributed (Fig. 2c), whereas by day 14, the signal appeared more intense and homogeneous, highlighting an increased and more organized collagen fiber network (Fig. 2d). Quantitative analysis of Picrosirius Red staining revealed a

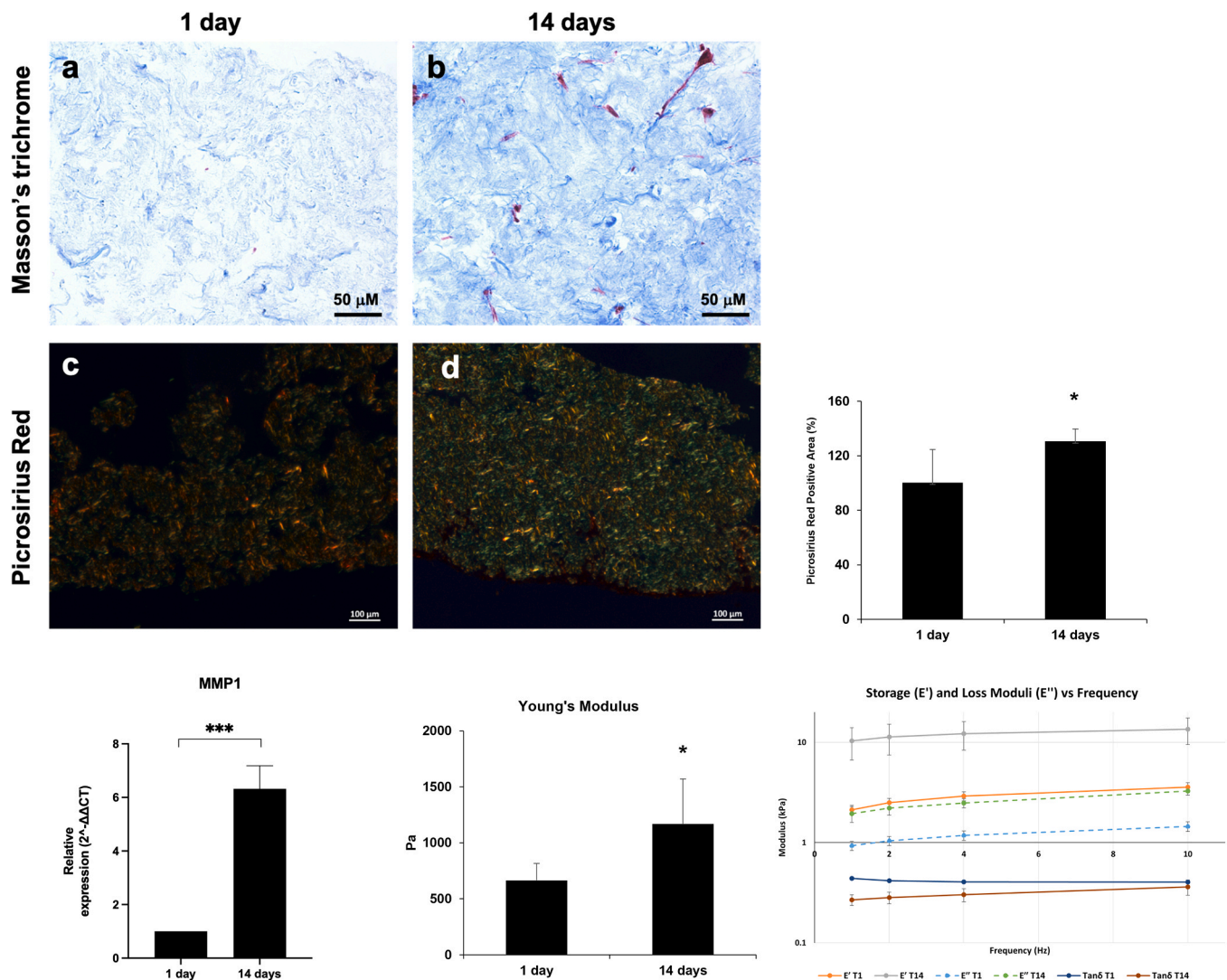


Fig. 2. Histological analysis of extracellular matrix scaffold (a-b). Representative images of Masson's Trichrome staining at day 1 (left) and day 14 (right) post keratinocytes seeding. Collagen is stained in blue. Scale bar: 50 μ m (c-d). Representative images of Picrosirius Red staining visualized under polarized light at day 1 (c) and day 14 (d), showing enhanced birefringence corresponding to increased and organized collagen fibers. Scale bar: 100 μ m (e) Quantification of Picrosirius Red staining. Data are expressed as percentage (%). Statistical significance was set at $p < 0.05$. Scale bar: 100 μ m. (f) Real-time PCR analysis of MMP1. Data were normalized to the endogenous control GAPDH and to the expression levels at the day 1 ($\Delta\Delta$ CT). Statistical analysis is reported as p-values. (g) Young's modulus at day 1 and day 14. Data were reported as mean $\pm$ SD ($n = 5$). Statistical significance was assessed by Student's t-test ($p < 0.05$) (h) Dynamic Mechanical Analysis (DMA) at day 1 and day 14. Data are presented on a logarithmic scale as mean $\pm$ SEM ($n = 5$).

significant increase in collagen content during scaffold maturation. At day 1, the normalized collagen signal was $100.0 \pm 24.7\%$, whereas at day 14 it increased to $130.3 \pm 9.3\%$, corresponding to an approximate 30% increase in collagen deposition. This result supports the progressive remodeling and maturation of the extracellular matrix (ECM) within the scaffold (Fig. 2e).

To further investigate the molecular mechanisms underlying ECM remodeling, the expression of matrix metalloproteinase-1 (MMP1) was evaluated by RT-qPCR. A significant upregulation of MMP1 expression was observed at day 14 post keratinocyte seeding, reaching a 6.32 ± 0.86 -fold increase compared to day 1 (Fig. 2f). This finding supports the presence of an active remodeling process within the construct, consistent with the dynamic turnover of collagen-based matrices.

In line with these observations, ECM remodeling was accompanied by a significant increase in mechanical stiffness of the construct. The Young's modulus increased from 666.24 ± 148.88 – 1169.86 ± 399.89 after 14 days of culture, with a statistically significant difference ($p = 0.016$), suggesting progressive structural maturation of the construct (Fig. 2g). To further characterize the mechanical behavior of the scaffold, dynamic mechanical analysis (DMA) was performed to

assess its viscoelastic properties. Both storage (G') and loss (G'') moduli increased with frequency and culture time, indicating a progressive stiffening and enhanced resistance to deformation. Additionally, a decrease in $\tan \delta$ was observed over time, reflecting a shift in the viscoelastic balance of the construct.

These results indicate a transition from a predominantly viscous behavior at early time points, consistent with a highly hydrated hydrogel with limited matrix deposition, toward a more elastic-like behavior at later stages (Fig. 2h). This shift is in agreement with the progressive ECM remodeling and collagen organization observed in the construct.

Altogether, these results confirm the ability of the cells to self-assemble into a 3D structure, generating a full thickness model that closely resembles both the epidermis and dermis of human skin. Given its capacity to mimic native skin architecture and function, the construct will hereafter be referred to as Skin Like Tissue (SLT).

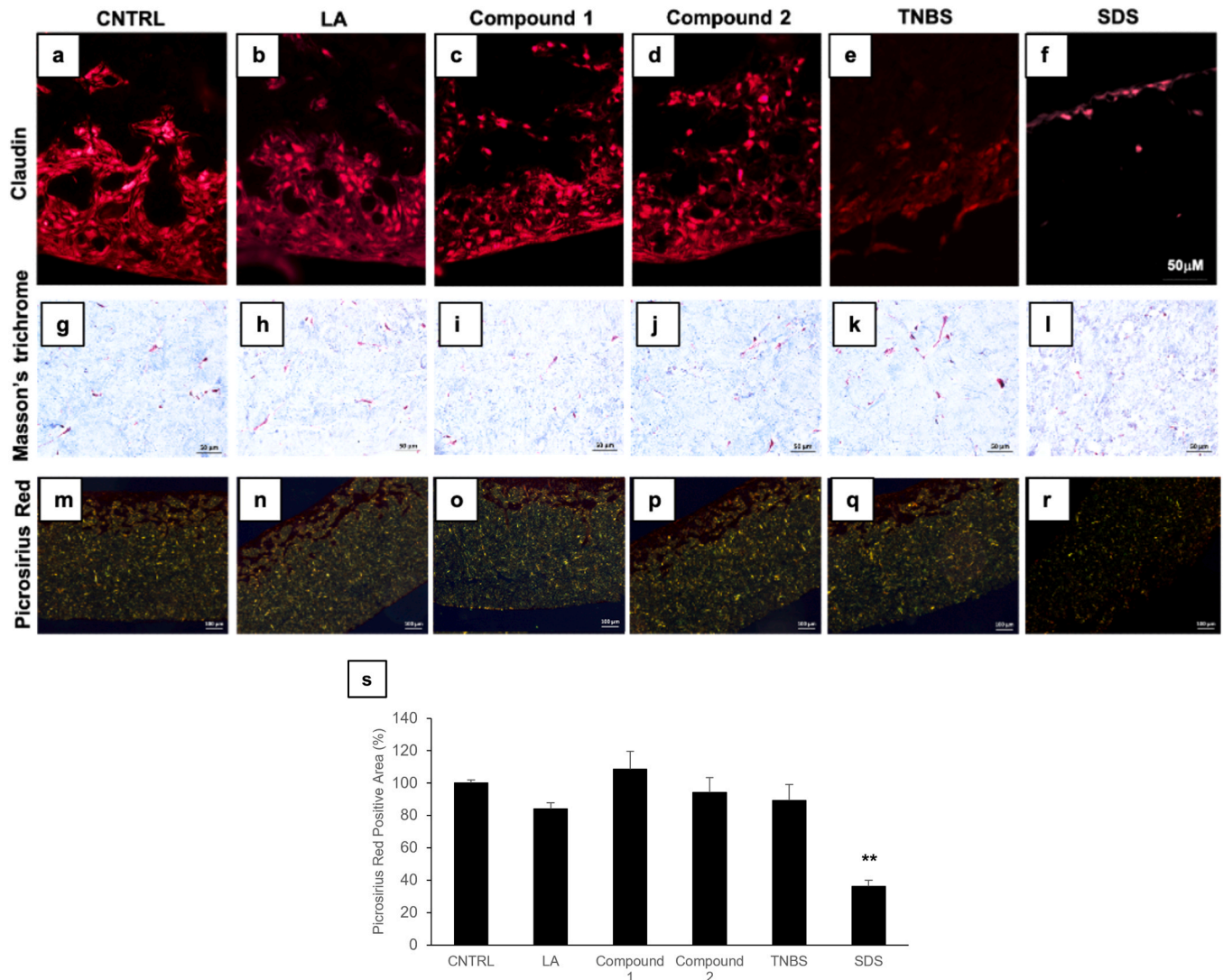


Fig. 3. (a-f) Immunofluorescence staining of claudin after treatment with Compound 1 (c) and Compound 2 (d), compared to positive (e-f) and negative controls (a, b). Scale bar: 50 μ m. (e-k) Representative images of Masson's Trichrome after treatment with Compound 1(h) and Compound 2 (i), compared to positive (j,k) and negative controls (e,g). Collagen is stained in blue. Scale bar: 50 μ m. (l-q) Representative images of Picrosirius Red staining after treatment with Compound 1 (n) and Compound 2 (o), compared to positive (p,q) and negative controls (n, m). (r) Quantification of Picrosirius Red staining. Data are expressed as percentage (%). Statistical significance: $p < 0.01$. Scale bar: 100 μ m.

2.2. SLT could be used as platform to evaluate tissue alteration, cytotoxicity and inflammation

To evaluate the response of the SLT model to commercially available cosmetic compounds, the platform was stimulated by compounds 1 and 2 for 24 h, alongside negative and positive controls.

Immunofluorescence for claudin 1 was assessed to evaluate the integrity of tight junctions (Fig. 3a–f). In the untreated control (Fig. 3a) and in the LA stimulated sample (Fig. 3b), claudin 1 expression appeared continuous and well-distributed along cell–cell borders, indicating preserved tight junction architecture in the epidermal layers. In samples treated with Compound 1 and Compound 2, claudin 1 distribution was comparable to control, suggesting a negligible impact on tight junction stability (Fig. 3c, d). Conversely, TNBS exposure led to a partial disorganization of claudin staining, indicating a moderate tight junction disruption (Fig. 3e). As expected, treatment with SDS caused a severe loss of claudin 1 signal and structural disintegration, reflecting the extensive breakdown of keratinocyte cell–cell junctions (Fig. 3f).

To assess the impact of the different treatments on tissue structure, histological analyses were performed using Masson's Trichrome staining. In negative control samples (Fig. 3g), the tissue architecture appeared well preserved, with distinct cellular layers and an intact ECM. Similarly, samples treated with Compound 1 (Fig. 3i) and Compound 2 (Fig. 3j) maintained normal histological features, with no evidence of structural disorganization or collagen degradation, indicating the absence of cytotoxic or tissue-damaging effects.

In contrast, tissues exposed to SDS displayed pronounced histological alterations, including a marked loss of structural organization and fragmentation of the ECM (Fig. 3l). LA (Fig. 3h) and TNBS (Fig. 3k) treatments showed no evident signs of collagen degradation or tissue disorganization compared to the untreated control.

To further evaluate ECM integrity, Picrosirius Red staining was performed to specifically assess collagen fiber content (Fig. 3m–r). Quantitative analysis revealed a significant reduction in collagen fibers exclusively in SDS-treated samples (Fig. 3r), while collagen levels remained comparable to controls in tissues treated with Compound 1 and Compound 2 (Fig. 3s). The concordance between the results obtained with Masson's Trichrome and Picrosirius Red staining reinforces the reliability of the model in detecting structural damage of Compound 1 and Compound 2.

In addition to morphological changes, we assessed cytotoxicity by MTT assay to further characterize the response of the SLT to treatment.

As expected, LA, due to its inert nature, did not significantly affect cell viability, in contrast, TNBS (1.5 mg/mL) induced a moderate reduction in viability, with a value of $74.12 \pm 5.91\%$ (p-value < 0.001), while SDS caused a pronounced and statistically significant decrease, reducing viability to $4.09 \pm 3.11\%$ (p-value < 0.001).

The MTT assay revealed that neither of the tested compounds

exhibited cytotoxic potential. Compound 1 showed a viability rate of $102.19 \pm 11.05\%$ (p-value > 0.05), while Compound 2 resulted in a slight but statistically significant reduction to $88.63 \pm 8.29\%$ (p-value < 0.001), as shown in Fig. 4a. These findings were further supported by Live & dead immunofluorescence analysis performed on SLT, followed by confocal imaging (Fig. 5), confirming cell viability and tissue integrity following compound exposure.

To evaluate the potential of the 3D model for quantifying the secretion of inflammatory mediators, IL-1 α - a key marker of innate immunity and inflammation - was analyzed using an ELISA assay [21] following exposure to known inert or irritant compounds.

As expected, LA did not significantly stimulate IL-1 α secretion compared to the blank control (p-value > 0.05). In contrast, cells treated with TNBS (1.5 mg/mL) and SDS, compounds known for their pro-inflammatory and irritant properties respectively, exhibited significantly elevated IL-1 α levels. Specifically, a 5.99 ± 0.18 -fold and 3.95 ± 0.10 -fold increase was observed compared to the negative control (p-value < 0.001).

No significant differences in IL-1 α secretion were detected following stimulation with Compound 1 and 2, indicating a lack of inflammatory response to these agents (Fig. 4b).

3. Discussion

Skin bioprinting is a promising technology for reconstructing biometric skin models as reliable platforms for testing products from various industrial sectors, including cosmetics, textiles, pharmaceuticals, and medical devices [5,22].

Despite advances in 3D bioprinted skin models, current systems remain limited by poor reproducibility, low automation, and insufficient mechanical robustness, often due to low collagen concentrations and the need for supportive hydrogels [18,19]. As a result, both standardized epidermal models and emerging full-thickness constructs fail to fully recapitulate the structural complexity and dermal–epidermal interactions of native human skin [23].

Dermal fibroblasts interact with keratinocytes indirectly through the secretion of growth factors and cytokines, as well as via cell–matrix interactions across the basement membrane [24].

For this reason, the development of full thickness skin models has involved the inclusion of multiple cell types to better recapitulate the structural and functional features of human skin [13,25].

This study aimed to expand the range of available skin models for product testing by employing bioprinting technology. Type I collagen I was selected as the hydrogel component, as the ECM of connective tissue is primarily composed of collagen types I and III [26]. Moreover, collagen I is an excellent biomaterial for extrusion-based 3D printing due to its favorable printability and its ability to undergo self-assembly into stable fibrillar structures at 37°C after extrusion, resulting in a robust

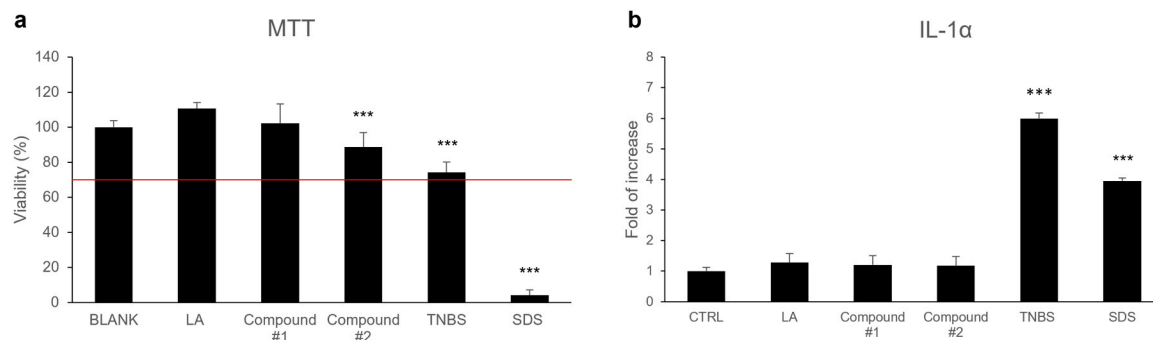


Fig. 4. Evaluation of cell viability and inflammatory response upon treatment with Compound 1 and Compound 2. (a) cell viability assessed by MTT assay after treatment with Compound 1 and Compound 2, compared to positive and negative controls. The red line indicates the 70% viability threshold, which serves as the acceptance criterion for cytotoxicity. (b) Quantification of IL-1 α secretion by ELISA following treatment with Compound 1 and Compound 2, in comparison to positive and negative controls. Statistical significance was defined as ***p < 0.001.

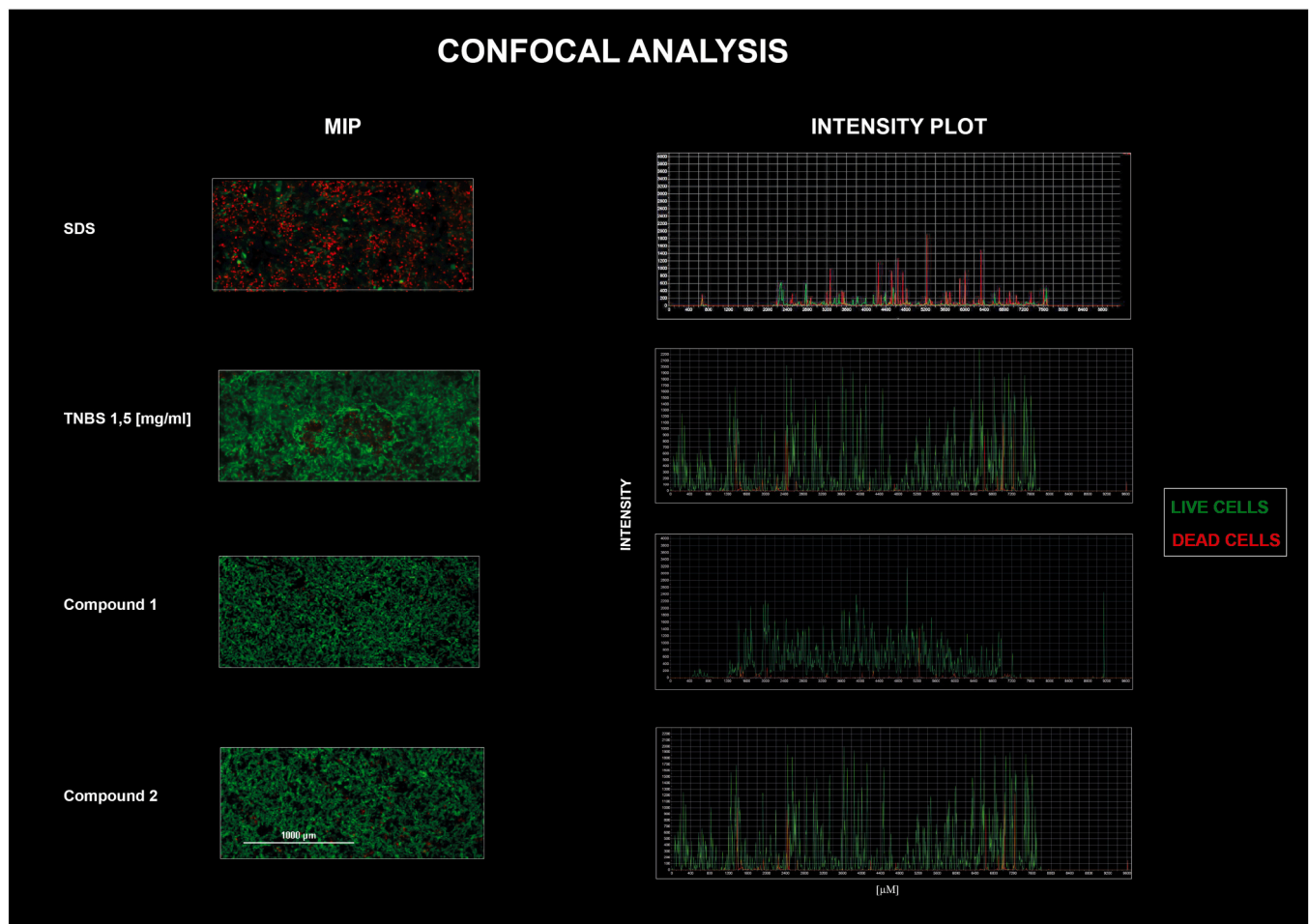


Fig. 5. Live/Dead fluorescence assay performed on SLT scaffold following exposure to different cytotoxic agents. Confocal imaging was carried out using a Nikon A1 confocal laser scanning microscope. Analysis was performed on a minimum of three biological replicates.

and biologically relevant 3D construct.

Most studies have been conducted using hydrogels with low collagen concentrations (typically no more than 5–10 mg/mL), resulting in limited mechanical strength [19,27,28].

However, stiffness is a critical parameter in tissue engineering, as it influences mechanotransduction pathways that affect gene expression. In bioprinting skin, an increase in the storage modulus -and consequently mechanical robustness- can be achieved by adjusting sodium chloride (NaCl) concentration, processing temperature, or by increasing collagen content.

In this study, a highly concentrated type I collagen hydrogel (35 mg/mL) was mixed with dermal fibroblast and used as a bioprinting bioink to rapidly generate a stable, cell-laden hydrogel. In addition, the construct printed is highly reproducible, and showed no significant batch-to-batch variability in geometry, stratification, or cell viability. This approach improved the mechanical properties of the scaffold, enabling precise spatial cell layering with consistent cellular composition, homogeneous distribution and organized ECM architecture. Furthermore, previous studies have shown that the use of high-concentration collagen confers advantages in long-term culture by significantly reducing scaffold contraction, as demonstrated by quantitative dimensional analyses over time, compared to conventional low-concentration collagen-based models [29]. Importantly, this bioprinted model recapitulates a stratified architecture resembling native skin, comprising both epidermal and dermal components. The progressive organization of the ECM observed by Masson's Trichrome and Picrosirius Red staining reflects the establishment of a dermal

microenvironment that supports fibroblast organization and contributes to dermal homeostasis and dermal-epidermal crosstalk. A structured and collagen-rich dermal matrix is known to provide essential mechanical and biochemical cues that influence keratinocyte organization and differentiation, supporting the biological relevance of the observed epidermal stratification.

ECM remodeling is an early and active response to toxic insult that directly influences fibroblast behavior. Changes in matrix composition and mechanics promote fibroblast activation through mechanotransduction pathways. Activated fibroblasts adopt a pro-remodeling and pro-inflammatory phenotype, marked by increased ECM deposition and sustained cytokine release. This cytokine signaling amplifies local inflammatory responses and reinforces tissue stress. The resulting ECM-fibroblast-cytokine feedback loop precedes irreversible tissue damage. Therefore, these interconnected events provide mechanistically grounded and sensitive endpoints for predictive toxicology [30].

In our model, additional cellular components, including immune, endothelial, and perivascular cells, are not included in order to focus on keratinocyte-fibroblast interactions. While this reductionist design improves reproducibility, it could represent a structural limitation of the dermal compartment, which is restricted to fibroblasts. However, the use of a simplified fibroblast-only dermal compartment allows the investigation of cell-specific responses and enables a more controlled evaluation of fibroblast-driven extracellular matrix remodeling and cytokine signaling, without interference from additional cellular populations. The absence of endothelial and perivascular cells may reduce the physiological accuracy of ECM remodeling and cytokine cross-talk.

Future model iterations could incorporate vascular components to better recapitulate dermal complexity.

Keratinocytes are known to play a central role in the early phases of skin sensitization and inflammatory responses by acting as immune-active cells. Upon exposure to sensitizing agents, keratinocytes can release cytokines, chemokines, and danger-associated signals that initiate and modulate downstream immune activation [31]. In line with ISO 10993–23 guidelines, IL-1 α was selected as the primary inflammatory biomarker, as it represents the most standardized and robust endpoint for in vitro skin irritation assessment. While keratinocytes are known to produce additional cytokines such as IL-6, TNF- α , and IL-8, the release of these mediators is highly context-dependent and not linearly correlated with irritation outcomes, limiting their suitability as quantitative endpoints within a regulatory testing framework [32]. Therefore, the proposed model is particularly suited to investigate early events of cutaneous sensitization rather than later immune cell-mediated responses.

The MTT assay demonstrated that the platform responded as expected to both positive and negative controls, as well as to the tested compounds, confirming the suitability of the model for cytotoxicity assessment. Although Compound 2 produced a statistically significant decrease in the MTT signal ($\sim$ 12%), overall cell viability remained above 70%, and the material was therefore classified as non-cytotoxic according to ISO 10993–5. To better interpret this result within the context of a 3D tissue-like system, it is important to consider the intrinsic limitations of metabolic assays. As the MTT assay primarily reflects cellular metabolic activity rather than direct cell death, this modest reduction is likely indicative of a transient metabolic modulation rather than cytotoxicity. In addition, in a 3D microenvironment, metabolic readouts may be influenced by factors such as compound diffusion dynamics, cell–matrix interactions, and temporary metabolic adaptation, which are known to affect assay signals without necessarily compromising cell viability.

The tissue-like skin constructs produced via bioprinting technology represent a rapid and reliable platform for safety profiling of topically applied compounds or drugs during the early phases of research.

Upon exposure to treatment, the model exhibits measurable alterations in cell–cell junctions—an essential indicator of skin barrier integrity—as well as changes in fibroblast organization and ECM remodeling, both of which are crucial for maintaining skin homeostasis. These features underscore the model's suitability for investigating early tissue responses and barrier-related endpoints in dermatological and toxicological studies.

A potential limitation of the model relates to oxygen and nutrient diffusion within the construct, particularly considering its thickness ($\sim$ 0.96 mm). Diffusion constraints are well documented in dense collagen-based 3D scaffolds and may affect central cell viability. However, the high cell viability and homogeneous cell distribution observed in our model suggest that diffusion was sufficient to sustain cellular activity under the applied culture conditions.

Nevertheless, further improving the physiological relevance of the model could be achieved by incorporating a functional vascular network. Although still a major technological challenge, vascularization would enable more accurate modeling of nutrient and oxygen diffusion, immune cell trafficking, and systemic drug absorption, thereby further enhancing the utility of the model for both research and preclinical testing.

4. Conclusions

In this study, we developed a full-thickness human skin model using a bioprinting-based approach that enables the fabrication of a tissue-like construct with a high degree of biomimicry.

The model is generated using a biomimetic type I collagen-based bioink with enhanced mechanical properties, allowing not only the spatial organization of keratinocytes and fibroblasts but also the

recreation of key ECM features characteristic of native human skin. This full-thickness architecture supports physiologically relevant dermal–epidermal interactions and provides a reliable platform for assessing the safety and efficacy of topical compound, cosmetics, and pharmaceutical drugs during the early stages of research and development.

Importantly, the bioprinting process allows automated and standardized manufacturing within controlled class-grade isolator environments, thereby reducing operator-dependent variability, personnel requirements, and associated costs.

Overall, this work aims to provide a biologically relevant, mechanically robust, and scalable in vitro skin model that supports early safety and efficacy testing while contributing to the advancement of more predictive, human-relevant, and ethically responsible alternatives to animal experimentation.

5. Materials and methods

5.1. Cell culture

The spontaneously transformed keratinocyte line HaCaT (American Type Culture Collection ATCC, Manassas, VA, USA) was kindly provided by prof. Alessandra Marconi (DermoLAB, University of Modena and Reggio Emilia). HaCaT keratinocytes, isolated from shoulder skin, were used between passages 9 and 13. Cells were seeded at the density of 10,000 cell/cm² and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS; Corning, Woodland, CA, USA), 2% L-Glutamine (200 mM; Gibco, Paisley, UK) and 1% Penicillin/Streptomycin (Gibco). Primary human foreskin fibroblasts (HFFs; ATCC, Manassas, VA, USA) were seeded at the density of 8000 cells/cm² and cultured in DMEM supplemented with 15% FBS (Corning, Woodland, CA, USA), 2% L-Glutamine (200 mM; Gibco) and 1% Penicillin/Streptomycin (Gibco). HFFs were used between passages 3 and 5. For both cell types, the medium was changed every 2–3 days. Upon reaching confluence, keratinocytes and fibroblasts were detached using Trypsin/EDTA (0.05%/0.02%, Gibco) by incubation at 37°C and 5% CO₂ for 10 and 5 min respectively. Cells were then counted using trypan blue (Sigma-Aldrich, Gillingham, UK) and re-seeded at the appropriate densities in the respective culture media, as described above.

5.2. Bioprinting

A total of 2.5×10^6 HFF were resuspended in type I collagen (35 mg/mL, Lifeink®200, Advanced BioMatrix, Carlsbad, CA, USA) and loaded into 3D BIOPLOTTER (EnvisionTEC GmbH, Gladbeck, Germany). The bioink composed of HFFs and collagen, was extruded according to a digital 3D model generated using Computer Aided Design (CAD) software (Perfactory RP 3.2.2945, EnvisionTEC) through a four-layer deposition process. Printing parameters (pressure, temperature, and needle size) were selected based on preliminary optimization tests to ensure stable and continuous extrusion, suitable ink rheological properties, and an optimal balance between printing resolution and filament

Table 1

Summary table of the parameters used during the 3D printing process of collagen and HFF-based constructs.

Parameter	Value
Slicing	240 μ m
Speed of printing	5–6 mm/s
Layer	4
BioInk Temperature	4°C
Printing table temperature	39°C
Pneumatic Pressure	0.7–0.9 bar
Crosslinker	Chemical crosslink (T° 20–37°C)
Needle size	250 μ m

stability. Parameters used during the 3D printing process are reported in Table 1.

Collagen gelation was achieved exclusively through temperature-induced physical crosslinking. The bioink was maintained at 4°C prior to extrusion to preserve printability, while printing was performed onto a temperature-controlled platform maintained at 39°C. Following extrusion, the constructs were immediately immersed in pre-warmed culture medium (37°C), promoting rapid collagen fibrillogenesis and stable hydrogel formation. No chemical crosslinking agents were used at any stage of the process, thereby avoiding potential cytotoxic effects associated with chemical crosslinkers. The thermal gelation conditions (temperature and incubation time) were selected based on preliminary optimization experiments and ensured reproducible gelation, shape fidelity, and high cell viability.

Once extruded, the scaffolds were maintained in fibroblasts culture medium supplemented with 1% Fluconazole (Fresenius Kabi Italia S.r.l., Isola della Scala, Verona, Italy) at 37°C and 5% CO₂ for 3 days. Keratinocytes were resuspended in DMEM medium and manually seeded on top of the scaffold. Each dermal components received 10 µL of the cell suspension and was incubated for 30 min at room temperature, followed by 1 h at 37°C and 5% CO₂ to allow keratinocytes adhesion.

The selected seeding densities were defined based on preliminary internal optimization experiments in which different cell concentrations were evaluated. In particular, the keratinocyte seeding density was chosen to promote proper epidermal stratification while maintaining a balanced proliferation–differentiation profile. The fibroblast seeding density was selected to support dermal layer formation and matrix deposition without inducing excessive cell crowding.

To promote cell proliferation, the scaffolds were cultured for 7 days in 12-well Transwell® inserts (Costar, Corning incorporated, Kennebunk, ME, USA) using an enriched culture medium (referred to as 3D skin medium) composed of DMEM and HAM'S F12 (Dominique Dutcher, Brumath, France) supplemented with 10% FBS (Corning), 4 mM L-Glutamine (Gibco), 100 µg/mL penicillin/ streptomycin (Gibco), 0.18 mM Adenine, 5 µg/mL insulin (from bovine pancreas); 5 µg/mL apo-Transferrin human, 0.4 µg/mL hydrocortisone, 2 nM 3,3',5-Triiodo-L-thyronine sodium salt, 10 ng/mL epidermal growth factor and 0.1 nM cholera Toxin (from *Vibrio cholerae*) (all from Sigma-Aldrich). After maturation, the scaffolds were maintained at the air-liquid interface for an additional 7 days in the 3D skin medium without 10 ng/mL epidermal growth factor (Sigma-Aldrich).

Cultures were maintained at 37 °C in a humidified incubator with 5%

CO₂ and approximately 95% relative humidity, achieved through a water pan-based humidification system. During air–liquid interface culture, evaporation was minimized by filling peripheral wells with sterile PBS, maintaining adequate medium volumes, and performing medium changes every 2–3 days.

A schematic representation of the process is shown in Fig. 6.

5.3. Mechanical characterization of the scaffold

Nanoindentation of the biological scaffold was performed using a Piuma nanoindenter (Optics11 Life, Amsterdam, Netherlands) equipped with a spherical indenter with a radius of $R = 48.5 \mu\text{m}$. Samples were fixed with cyanoacrylate glue in a Petri dish and tested fully immersed in $1 \times \text{PBS}$ at room temperature. Indentations were carried out in indentation mode to a maximum depth of 20 µm, applied at a loading rate of 5 µm/s. A holding time of 1 s was maintained at the maximum load to minimize viscoelastic creep. For each sample, three indentations were performed in areas at least 500 µm apart to avoid interactions between plastically deformed regions. Force–displacement data were analyzed using the Hertz contact model for a spherical indenter, assuming a Poisson's ratio of $\nu = 0.5$. For the DMA, frequency sweeps were performed on the same samples applying cyclic oscillations at 1, 2, 4, and 10 Hz. The analysis yielded the frequency-dependent Storage Modulus (E'), Loss Modulus (E''), and Loss Tangent ($\tan\delta$).

Analysis was performed using the manufacturer's software, which provides automatic fitting of the loading–unloading curves according to the Hertz contact model. Data are reported as mean $\pm$ standard deviation calculated over all indentations for each sample ($n = 5$). Differences between groups were evaluated using the student's t test statistical test, with significance set at $p < 0.05$.

5.4. Live/Dead fluorescence assay

The Live/Dead assay (LIVE/DEAD™ Viability/Cytotoxicity Kit for mammalian cells; Invitrogen) was performed according to manufacturer's instructions. Briefly, after removal of the TNBS-containing medium, the scaffolds were incubated for 40 min with 1 mL of staining solution consisting of 5 µL of calcein AM (Component A) and 20 µL of ethidium homodimer-1 (Component B) diluted in 10 mL of DPBS.

Following staining, samples were rinsed in PBS and immediately imaged using an AxioZoom V16 microscope (Carl Zeiss, Oberkochen, Germany) at 15 $\times$ and 50 $\times$ magnification to assess scaffold

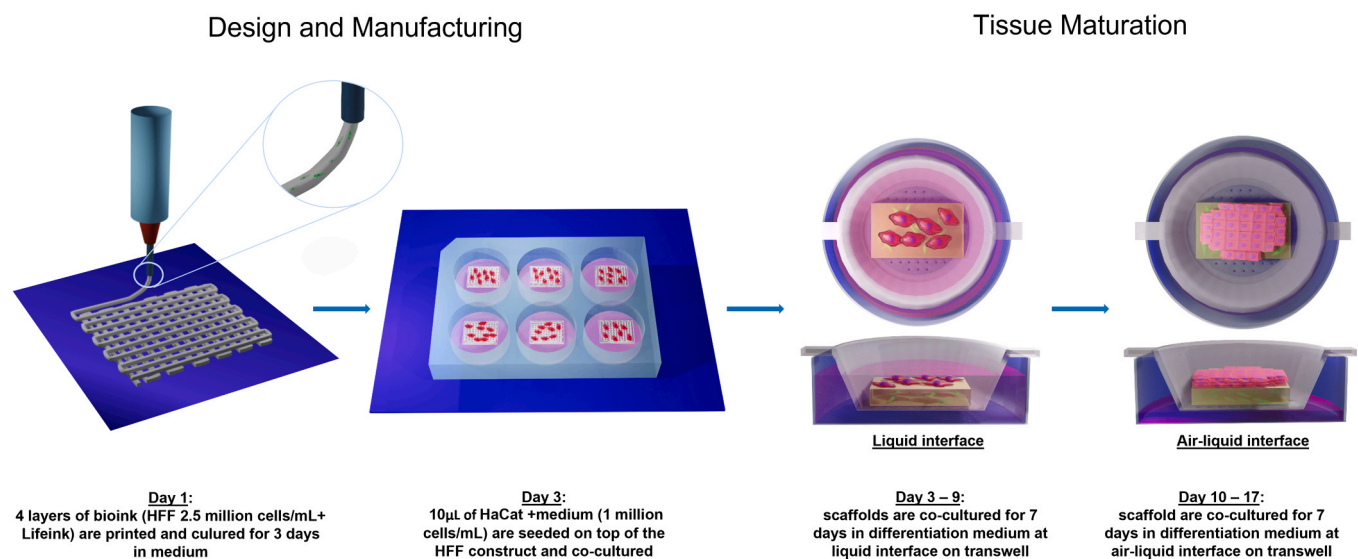


Fig. 6. Schematic of the SLT reconstruction process. The process is based on a bioprinting technique combined with classical cell culture. The hybrid technique starts with the extrusion of a 3D HFF-based scaffold with the addition of HaCat cells, which undergoes a tissue maturation phase during which the SLT model is formed.

colonization.

Large-area imaging was performed with a Nikon A1 confocal laser scanning microscope equipped with a 4X objective (0.10 NA), employing 488 and 561 nm laser lines. Image analysis, including Intensity profile plotting, was conducted using NIS-Elements Advanced Research software (Nikon).

Cell viability was quantified using the Image Analysis plugin (ZEN software, Zeiss). Height independent bioprinted skin constructs were analyzed. For each construct, fluorescence images were acquired using identical acquisition settings (laser power, gain, and exposure time). The green (live cells) and red (dead cells) fluorescence channels were independently thresholded, and the total fluorescent area (μm^2) was quantified for each channel. Cell viability was determined by quantifying the area of live cells and expressing it as a percentage of the total cell area, obtained from the sum of green (live) and red (dead) fluorescence signal areas. Quantitative results are reported as mean $\pm$ standard deviation (SD). Statistical analysis was performed using a student's *t*-test, with statistical significance set at $p < 0.05$.

5.5. Hematoxylin and eosin (H&E) staining

Scaffolds were washed in $1 \times$ phosphate-buffered saline (PBS; Gibco) and fixed in 2.5% (v/v) glutaraldehyde solution (Sigma-Aldrich) in $1 \times$ PBS for 24 h. After dehydration through a graded ethanol series and clarification in HistoClear (Histo-Line Laboratories, Milan, Italy), the samples were embedded in paraffin (Histo-Line Laboratories). For histological analyses, 4 μm sections were obtained using a microtome (Leica RM2255, Leica, Wetzlar, Germany) and mounted onto glass slides. Histological stainings were performed on five independent constructs per experimental condition, with three non-consecutive sections analyzed for each sample. Sections were deparaffinized in two changes of HistoClear (10 min each) and rehydrated through a descending ethanol series (ethanol 100, 90, 80%, followed by 70 e 50%; 5 min per step). Specimens were stained by incubating in hematoxylin solutions (Histo-Line Laboratories) for 5 min, then rinsed twice in tap water and once in double-distilled water. After brief incubation in 95% ethanol, samples were counterstained with eosin Y solution (Carlo Erba reagents, Arese, Milan, Italy) for 20 s, followed by incubation in 95% and 100% ethanol for 40 s each, and in HistoClear for 1 min. Slides were mounted using DPX Mountant (Sigma-Aldrich). Staining was visualized using an Axio Imager.M2 (Zeiss, Oberkochen, Germany) and images were acquired at $40 \times$ magnification.

5.6. Masson's trichrome staining

Paraffin-embedded tissue Section (6 μm) were subjected to Masson's Trichrome staining using the Masson Tricromica Kit (Histo-Line Laboratories, Italy), following the manufacturer's instructions. Masson's Trichrome staining was performed on five independent constructs per experimental condition, with three non-consecutive sections analyzed for each sample. Briefly, sections were deparaffinized in HistoClear, rehydrated through graded ethanol solutions, and incubated in Bouin's solution for 60 min at 56°C to enhance staining intensity. After cooling and rinsing, nuclei were stained with Weigert's iron hematoxylin for 15–20 min, followed by staining with Biebrich scarlet-acid fuchsin for 20 min. Sections were then treated with phosphotungstic/phosphomolybdic acid solution for 10 min and counterstained with aniline blue for 5–7 min. A final differentiation step was performed using 1% acetic acid for a few seconds. Sections were dehydrated in ethanol, cleared in xylene, and mounted with a synthetic resin-based medium.

The staining allowed clear differentiation of collagen fibers (blue), muscle and cytoplasm (red), and nuclei (blue-black). Staining was visualized using an Axio Imager.M2 (Zeiss) and images were acquired at $40 \times$ magnification.

5.7. Picrosirius red

Scaffolds were fixed in 2.5% glutaraldehyde, dehydrated through a graded ethanol series, and embedded in paraffin. Masson's Trichrome staining was performed on five independent constructs per experimental condition, with three non-consecutive sections analyzed for each sample. Sections of 4 μm thickness were stained for 1 h with a 0.1% solution of Direct Red (Sigma Aldrich) prepared in saturated aqueous picric acid (Sigma Aldrich). After staining, sections were washed in 0.5% acetic acid (Carlo Erba), dehydrated through ascending concentrations of ethanol, cleared in xylene, and mounted using a synthetic resin.

Collagen fibers were visualized under both brightfield and circular polarized light microscopy to visualize red and yellow-green birefringent fibers. Imaging was performed on five randomly selected fields per sample using the AxioZoom V16 microscope (Zeiss). Quantification of Picrosirius red-positive areas was carried out using the Image Analysis plugin of ZEN Pro software (Zeiss). Data were expressed as percentage area of positive signal, normalized to scaffold at initial time point (set as 100%).

5.8. Immunohistochemistry

Sections of 4 μm thickness were retrieved in citrate buffer (pH 6) for 15 min at 96°C and then 20 min at room temperature. Primary rabbit polyclonal anti α -SMA antibody (1:200; ab5694, Abcam Cambridge, UK,) was incubated overnight at 4°C . Slides were then incubated with biotinylated goat anti-rabbit IgG (H+L) (1:200; BA1000; Vector Laboratories, Burlingame, CA) for 1 h at room temperature. α -SMA positive cells were visualized by diaminobenzidine (DAB, Vector Laboratories). All slides were counterstained with hematoxylin (Bio Optica, Milan, Italy). Sections were examined by Axio Observer Z1 (Zeiss). Images were acquired at $40 \times$ magnification.

5.9. Immunofluorescence

To assess the maturation and differentiation status of the full-thickness construct, immunofluorescence staining was performed. Paraffin-embedded 4 μm -sections, obtained using a microtome (Leica RM2255, Leica, Wetzlar, Germany) and mounted on glass slides, were de-paraffinized in HistoClear (Histo-Line Laboratories) and re-hydrated through a descending ethanol series. Antigen retrieval was carried out by incubating the section in citrate buffer (pH 6.0) at 96°C for 15 min, followed by cooling at room temperature for 20 min. The samples were then incubated for 30 min with a blocking solution composed of maleic acid (Sigma-Aldrich), blocking reagent (Roche, Basilea, CH) and newborn calf serum (Euroclone) in a 3:1:1 volumetric ratio. Subsequently, the sections were incubated overnight at 4°C with primary antibodies: anti-human claudin (rabbit, Abcam, cat. no. ab140349 1:150); anti-loricrin (rabbit, Abcam, cat. no. ab240385 1:250) and anti-cytokeratin 10 (rabbit, Abcam, cat. no. ab76138 1:200). Antibody specificity was validated based on manufacturer-provided documentation and previously published studies [33–35].

After 3 washes in PBS, samples were incubated for 1 h at room temperature with a red fluorescent anti-rabbit secondary antibody (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA; cat. no. A21207, 1:500). Nuclei were counterstained with DAPI (Thermo Fisher Scientific) for 5 min at room temperature. Stained sections were visualized, and images acquired using an Axio Imager.M2 optical microscope (Zeiss, Oberkochen, Germany) at $40 \times$ magnification.

Quantitative analysis of keratin 10, loricrin, and claudin immunofluorescence was performed on five independent samples using the ZEN Image Analysis plugin (Carl Zeiss). The number of marker-positive cells was normalized to the total number of DAPI-positive nuclei. Results are reported as mean percentage $\pm$ standard deviation.

5.10. RNA extraction and quantitative real-time PCR (qPCR)

At each time point ($n = 10$), scaffolds were processed for cell isolation. Samples were minced using Noyes surgical scissors and digested in 1 mL of DMEM medium (Gibco) containing 1.52 U/mL Collagenase P (Roche), 1% sodium pyruvate, 1% L-glutamine, and 1% penicillin-streptomycin (all from Gibco). Digestion was performed using an IncuShaker Mini (Benchmark Scientific) at 37°C and 95 rpm for 10 min, followed by mechanical dissociation through gentle pipetting. Enzymatic activity was neutralized by adding an equal volume of FBS-supplemented medium, and cells were pelleted by centrifugation at 1200 rpm for 5 min. Cell pellets were resuspended in 500 μ L of TRIzol (Ambion, Thermo Fisher Scientific) and stored at –80°C until further processing. Total RNA was extracted using the Direct-Zol RNA Microprep Kit and Direct-Zol RNA Miniprep Kit (Zymo Research) according to the manufacturer's instructions, and RNA concentration and purity were assessed using a NanoDrop One spectrophotometer (Thermo Fisher Scientific). Complementary DNA (cDNA) was synthesized from 1 μ g of total RNA using the RevertAid RT Reverse Transcription Kit (Invitrogen, Thermo Fisher Scientific), following the manufacturer's protocol. Quantitative PCR (qPCR) was performed using the SYBR Green method on a QuantStudio 1 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). Each reaction (10 μ L final volume) contained 10 ng of cDNA, 0.3 μ L of forward primer, 0.3 μ L of reverse primer, and 5 μ L of 2 $\times$ SYBR Green master mix, and samples were analyzed in technical triplicates including no-template controls. Thermal cycling conditions consisted of an initial denaturation step at 95°C for 20 s, followed by 40 cycles of 95°C for 1 s and 60°C for 20 s, with a final melt curve analysis. Relative gene expression levels of Keratin 10 (KRT10), Loricrin (LOR), Filaggrin (FLG), and Matrix Metalloproteinase 1 (MMP1) were calculated using the $\Delta\Delta C_t$ method, with GAPDH as the housekeeping gene. Primer sequences were as follows: KRT10 forward 5'-AAACCATCGATGACCTTAAAAATC-3' and reverse 5'-GCGCAGAGCTACCTCATTCT-3'; LOR forward 5'-AGTGGACTGCGTGAAGACCT-3' and reverse 5'-AGC-CAGAACCGTGCTAC-3'; FLG forward 5'-CACAAGATTCTGCGTATCACTCAGG-3' and reverse 5'-GCCTTTCAGTGCCCTCAGATTG-3'; MMP1 forward 5'-GGTCTCTGAGGGTCAAGCAG-3' and reverse 5'-AGTTCATGAGCTGCAACACG-3'; and GAPDH forward 5'-ACATCGCTCAGACCCATG-3' and reverse 5'-TGTAGTTGAGGTCAATGAAGGG-3'.

5.11. Scanning electron microscopy (SEM)

Scaffolds were rinsed in PBS, fixed overnight in a solution of 4% paraformaldehyde and 2% glutaraldehyde, then dehydrated through a graded ethanol series (50%, 70%, 90%, and two changes of 100%, 15 min each). Following dehydration, samples were immersed twice in hexamethyldisilazane (1 mL for 10 min each) and left to air dry overnight (approximately 10 h).

Dried samples were mounted on aluminum SEM stubs using conductive carbon tape and sputter-coated with a gold/palladium alloy to improve surface conductivity. SEM imaging was performed using a Hitachi TM4000 II Plus microscope (Mot 55E-0312). Surface morphology was analyzed in secondary electron mode (SE), using a 5 kV accelerating voltage, under medium vacuum conditions.

5.12. Assessment of biological effect of compounds administrate to tissue like 3D model

Two commercially available skin care compounds were selected to evaluate their biological effects. The 3D scaffolds were treated for 24 h at 37°C and 5% CO₂ with these compounds, referred to as Compound 1 and Compound 2. The cosmetic functional compounds were kindly provided by Ophera s.r.l. and were administered after dissolution in the culture medium. The following treatments were used as negative controls: blank is DMEM supplemented with 10% FBS (Corning) and 1% Penicillin/Streptomycin (Gibco), used a solvent medium for the test

compounds and LA (Sigma-Aldrich) at the concentration of 0.2 mg/mL, in culture medium. Positive controls are TNBS (.5 mg/mL, Sigma-Aldrich) and SDS (0.2% v/v in culture medium Sigma-Aldrich). SDS, an anionic surfactant able to disrupt cell membranes by solubilizing membrane lipids and denaturing proteins, is the recommended positive control for in vitro cytotoxicity test, according to ISO 10993-5:2009 guidelines [26]. TNBS is an hapten capable of inducing immune activation and inflammation and it is used as positive control for in vitro sensitization assays, as recommended by OECD 442/E guidelines [27].

The biological effect was evaluated using the thiazolyl blue tetrazolium bromide (MTT, Sigma-Aldrich) assay and by quantifying IL1 α secretion. For the MTT assay, scaffolds were incubated with 1 mg/mL thiazolyl blue tetrazolium bromide (Sigma-Aldrich) diluted in DMEM for 2 h at 37°C. After incubation, scaffolds were transferred into 1.5 mL conical tubes (Eppendorf, Hamburg, Germany) prefilled with 1 mL of isopropanol (Sigma-Aldrich). To ensure complete dye extraction, scaffolds were chopped using surgical scissors and vortexed for 30–60 s until the dye was fully released. The resulting solution was transferred into a 96 wells plate (100 μ L/well, in sextuplicate) and optical density (OD) was measured using a multiplate reader (Enspire, PerkinElmer, Hopkinton, MA, USA) at 570 nm. The cell viability rate was calculated using the following formula:

$$\text{Cell viability(\%)} = \frac{OD570e}{OD570b} \times 100$$

where $OD570e$ is the mean value of the measured optical density of the 100% extract, and $OD570b$ is the mean value of the measured optical density of the blank. A viability lower than 70% of the blank was evaluated as cytotoxic. For ELISA assay, supernatant was collected and stored to –20°C until analysis was performed. IL-1 α was detected by Quantikine® ELISA Human IL-1 α /IL-1F1 (R&D Systems, Minneapolis, MN, USA) according to manufacturer instructions.

5.13. Statistical analysis

Statistical analysis was performed using the T-test (Microsoft® Excel, Office 2016). Results were considered statistically significant at ($p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***)). All experiments were conducted in triplicate.

CRedit authorship contribution statement

Luisa Pecorari: Resources. **Valentina Bergamini:** Validation, Formal analysis, Data curation. **Anna Tesesi:** Validation, Conceptualization. **Alberto Portone:** Formal analysis, Data curation. **Elena Veronesi:** Writing – original draft, Supervision, Conceptualization. **De Biagi Leone Alberto:** Methodology. **Massimo Dominici:** Writing – original draft, Data curation, Conceptualization. **Elisa Resca:** Visualization. **Noemi Marino:** Data curation. **Francesco Ganzerli:** Methodology, Formal analysis, Data curation. **Martina Bedeschi:** Validation. **Tiziana Petrachi:** Writing – original draft, Supervision, Methodology, Conceptualization. **Giada Lodi:** Software. **Alberto Ferrari:** Resources. **Luca Accorsi:** Methodology. **Rebecca Voltan:** Methodology. **Simona Sbardelatti:** Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The work was financially supported by the grants POR FESR 2014–2020 [ID: CUBIBOX] and by 'Progetto Dipartimenti Eccellenti

MIUR 2017 and 2022'. The authors acknowledge OPHERA srl for the supply of the cosmetic compounds used for the study.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.biopha.2026.119305](https://doi.org/10.1016/j.biopha.2026.119305).

Data availability

Data will be made available on request.

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