Development and characterization of a human dermal equivalent with physiological mechanical properties

Gwenaël Rolin[1,2], Vincent Placet[2,3], Emmanuelle Jacquet[2,3], Hélène Tauzin[1,2], Sophie Robin[4], Lionel Pazart[5,6], Celine Viennet[1,2], Philippe Saas[1,6], Patrice Muret[1,6], Delphine Binda[5,6] and Philippe Humbert[1,2,6]

[1]Inserm UMR 645 Engineering and Cutaneous Biology Team, Univ Franche-Comté, Besançon, France,
[2]Univ Franche-Comté, Besançon, France,
[3]Department of Applied Mechanics, FEMTO-ST Institute, UMR CNRS 6174, Besançon, France,
[4]Bioexigence, Besançon, France,
[5]Clinical Investigation Center (CIC-BT 506 and CIC-IT 808), CHU, Besançon, France and [6]Univ Hospit of Besançon, F-25000, Besançon, FranceTransplantation FoundationOSEORegional Council of the Franche-Comté Region

Different models of reconstructed skin are available, either to provide skin wound healing when this process is deficient, or to be used as an *in vitro* model. Nevertheless, few studies have focused on the mechanical properties of skin equivalent. Indeed, human skin is naturally under tension. Taking into account these features, the purpose of this work was to obtain a cellular dermal equivalent (CDE), composed of collagen and dermal fibroblasts. To counteract the natural retraction of CDE and to maintain it under tension, different biomaterials were tested. Selection criteria were biocompatibility, bioadhesion properties, ability to induce differentiation of fibroblasts into myofibroblasts and mechanical characterization, considering that of skin *in vivo*. These assays led to the selection of honeycomb of polyester. CDE constructed on this biomaterial was further characterized mechanically using tensile tests. The results showed that mechanical features of the obtained dermal equivalent, including myofibroblasts, were similar to skin *in vivo*. The original model of dermal equivalent presented herein may be a useful tool for clinical use and as an *in vitro* model for toxicological/pharmacological research.

Key words: biomaterial – dermal equivalent – myofibroblasts – rigidity

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HUMAN SKIN is the largest organ of the human body and is naturally under tension (1). This complex tissue is an inhomogeneous, nonlinear viscoelastic, anisotropic material which is often subjected to large deformations (2–4). Numerous mechanical properties of skin described in the literature are collected using *in vivo* techniques, such as tensile (2), compression (5), suction (6), torsion tests (7), indentation (8, 9) or wave propagation methods (10). The review of the literature shows a significant discrepancy in reported mechanical properties. Many factors related to the human origin of this biological material (age, location, etc.) and also to experimental difficulties, influence skin properties and may explain the significant standard deviation of mechanical properties continuously observed in the literature.

Moreover, skin is described as a laminate composite material composed of three distinct layers which confer these cutaneous features (11). The deeper one, which is the hypodermis composed of adipocytes, acts as an energy reserve and also has a role in skin plasticity. The dermis covers this fat tissue, which constitutes a conjunctive tissue containing fibroblasts responsible for the synthesis and deposition of extracellular matrix (ECM) proteins. ECM is composed of a fundamental substance (glycosaminoglycan and proteoglycan), fibrous components (collagens and elastic fibers) and structural glycoproteins (fibronectin and tenascin). Collagens represent about 70% of dermal proteins and are mainly responsible for skin elasticity (rigidity and strength). Under normal conditions, skin mechanical behavior is directly related to dermal behavior (12). Nevertheless, the epidermis, which is the upper layer of the human skin, also plays an important role in skin biomechanics. It contains keratinocytes organized in a multiple

stratum, and is the final interface between the external environment and the human body. Indeed, the different mechanical forces which can be applied to the epidermis are transmitted to the dermis through the basement membrane, by cell–cell interaction and also by cell–ECM interactions.

Since the 1970s, skin has been modeled *in vitro*, first by culturing cells in monolayer, and by the development of a three-dimensional model (13, 14). Bell et al. (15) introduced a model of a three-dimensional collagen gel in which fibroblasts are embedded. These cellularized dermal **3** equivalents (CDE) were used to study fibroblast functions in an *in vivo*-like structure which is more representative than cells in monolayer. Based on this model, two major systems have been developed: free-retracted cellularized dermal equivalents (FR-CDE) and anchored cellularized dermal equivalents (A-CDE) (16). In the first one, FR-CDE, the diameter and the surface of the collagen matrix decreased during time in culture, due to the contraction of the gel by fibroblasts. Indeed, these cells migrate and remodel the ECM. In the second one, A-CDE, the structure is maintained under tension by the presence of a biomaterial which acts in opposition of cellular action described above. Consequently, the construct is maintained under tension and this mechanical state leads to the transmission of mechanical stimuli to fibroblasts embedded in CDE.

For fundamental research, CDE can usually be maintained under tension by attaching the matrix to the bottom of a culture dish or with the presence of a biomaterial such as stainless steel wire, glass microfiber filter, or Nylon® ring (17). However, these models of tensed CDE are very difficult to handle and present a high breakability, which makes it difficult to use them for fundamental research as well as for clinical purposes.

The aim of this study was to investigate the ability to reconstruct a cellularized dermal equivalent (CDE) using an original biomaterial allowing mechanical tension to be incorporated. Moreover, the intended tensed CDE would be easy to handle.

Several biomaterials were tested to replace Nylon®, whose thickness is about 0.25 mm. The original biomaterial, called 3D and on which this article focuses, was selected because of its apparent extensibility, its potential adaptability to a wound bed and its deformability. The biological validation of the 3D biomaterial as an alternative to Nylon® for the reconstruction of a tensed dermal equivalent was first assessed by testing the bioadhesion of CDE reconstructed on such biomaterials. This was done by checking the viability of fibroblasts embedded in CDE reconstructed on 3D and by verifying the presence of α-smooth muscle actin (α-SM actin). The resulting construct was then further mechanically characterized, by determining its rigidity using a dynamic mechanical analyzer (DMA) device.

Material and Methods

Materials

The 3D biomaterial was generously provided by Statice Santé, France. Phosphate buffer solution (PBS), Dulbecco's modified eagle's medium (DMEM), fetal calf serum (FCS), trypsin-EDTA, penicillin, streptomycin, collagenase and NaH-CO_3 were purchased from Dutscher, France; Type I collagen from Jacques Boy, France; 3-(4.5- **4** dimethylthiazol-2-yl)-2.5-diphenyltetrazoliumbromide (MTT), sodium dodecyl sulfate (SDS), dimethylformamide (DMF), NaOH, paraformaldehyde (PFA), Triton X100, bovine serum albumin (BSA), anti human α-SM actin antibody (A2547), IgG FITC-labeled antibody (F8521), anti human α-SM actin antibody FITC-labeled (F3777) and Hoechst stain solution from Sigma, France; Dako fluorescent mounting medium **5** from Dako, USA; Formol was purchased from **6** Carlo Erba, France, and Nylon® was purchased **7** from Sefar, Switzerland. **8**
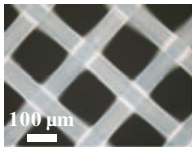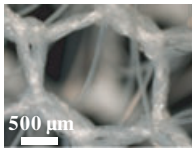
Biomaterials

The nature, morphology, thickness, and mesh size of Nylon® and 3D are described in Table 1. Before use, biomaterials were sterilized by exposure to ethylene oxide (1 h, 55°C) in a Sterivac 5XL apparatus followed by 96 h of desorption. They were then cut in sterile conditions under laminar flow and prepared specifically for the purpose of our investigation.

Fibroblast isolation and culture

Biopsies of healthy human skin were obtained, with consent from patients, from abdominal skin, which was collected during plastic surgery where neither the indication, nor the method of

TABLE 1. *Description of biomaterials used for the preparation of dermal equivalents under tension*

Designation/Composition	Description	Thickness/Mesh size (mm)
Nylon®/Nylon®	Tight square meshing 100 µm	0.25/0.15
3D/Polyester	Honeycomb 500 µm	2.8/1

intervention depends on the use or not of the operating waste. Human dermal fibroblasts (HDF) were obtained by cell outgrowth from these explants. HDFs were cultured in DMEM supplemented with 10% FCS, Penicillin (100 U/mL) and Streptomycin (0.1 mg/mL), at 37°C with 5% CO_2. When cells reached confluence, they were detached from the culture flask with trypsin (0.05%)-EDTA (0.02%) solution and subcultured at a ratio of 8.10^5 cells per flasks. For further experiments, cells were used between the fourth and eighth passage.

Extract preparation for pH measurement

Biomaterial extracts were prepared as described in the standard EN-ISO 10993-5 (18) by incubating each material for 24 h in fibroblast culture medium in cell culture conditions (37°C; 5% CO_2) at a ratio of 8 cm² of biomaterials per milliliter of medium. After incubation, extracted media were immediately used for pH measurement. Culture medium alone was used as a control. The pH of extracts was measured using a pH meter Cyberscan 500 and compared to the control, to determine whether pH was altered by the biomaterial.

Dermal equivalent mixture preparation

Cellularized dermal equivalents were reconstructed using the technique developed by Bell et al. (15) This technique consists in preparing a hydrated collagen gel containing dermal fibro-blasts (concentrations are given as final ones): DMEM medium (0.8 X), FCS (9% v/v), NaOH (0.005 N), acid-extracted type I collagen (0.6 mg/mL), HDF suspension (8.10^4 cell/mL), $NaHCO_3$ (0.3%), Penicillin (200 U/mL) and Streptomycin (0.2 mg/mL). This mixture was used for the reconstruction of CDE for the bioadhesion assay, cytotoxicity assay and α-SM actin quantification.

Bioadhesion assays

For bioadhesion assays, the dermal equivalent was reconstructed in six-well cell culture plates with a volume of mixture equal to 2.5 mL for each well. Plates were then placed in the incubator (1 h, 37°C, 5% CO_2) for gel polymerization, after which 2 mL of fibroblast culture medium was added. Dermal equivalents were reconstructed and tested under different conditions ($n = 6$) to investigate the possibility of maintaining under tension a dermal equivalent with the 3D biomaterial. Dermal equivalents were prepared in the presence of a Nylon® ring (bioadhesion positive control), a ring of 3D biomaterial (tested conditions) and without biomaterial (free-retracted dermal equivalent) as a negative control. The inner and outer diameters of the ring of biomaterial were 24 and 34 mm, respectively. Dermal equivalents were scanned daily during culture time (5 days) and the diameter of each dermal equivalent was measured and expressed as a percentage of the initial diameter.

Cytotoxicity assay

The MTT assay was used as an indicator of cell viability as determined by its mitochondrial-dependent reduction to formazan in living cells. The cytotoxicity assay was performed on dermal equivalents reconstructed into 12-well plates with 1.5 mL of mixture per well. Plates were then placed in the incubator (1 h, 37°C, 5% CO_2) to allow for gel polymerization; after which 1 mL of fibroblast culture medium was added. Tested conditions ($n = 6$) were free retracted lattices, lattices tensed on Nylon® (reference condition), tensed on Nylon® in the presence of phenol [64 mg/mL, positive control of cytotoxicity (19)], and tensed on 3D biomaterial (tested condition). After 5 days of culture, the medium was discarded and the dermal

equivalent was rinsed with PBS until medium traces were completely eliminated. One milliliter of MTT (1 mg/mL in PBS) was added to each well and incubated for 4 h at 37°C. After incubation, the MTT solution was discarded and replaced by 1 mL of extraction medium (10% SDS; 0.04% DMF in sterile water) and incubated overnight at 37°C to solubilize formazan. After incubation, the solution of formazan blue was distributed in five replicates in a 96-well plate (200 µL per well). The optical density (OD) was then read at 570 nm with a **10** Multiskan RC spectrophotometer (Labsystems). Cell viability was expressed as a percentage of the reference condition.

α-SM actin expression
Flow cytometry
Dermal equivalents were reconstructed in six-well plates, with a volume of mixture equal to 2.5 mL for each well, in the presence of a ring of 3D biomaterial or in a free retracted condition. Plates were then placed in the incubator (1 h, 37°C, 5% CO_2) for gel polymerization, after which 2 mL of fibroblast culture medium was added. After 5 days of culture, collagen gels were digested in collagenase (0.33% in DMEM, 2 h, 37°C), centrifuged at **11** 1100 rpm for 5 min, washed in PBS and fibroblasts were resuspended and fixed in 10% formol (30 min, RT). Fixed cells were washed in PBS, incubated in 0.45% Triton X-100 (15 min, RT) and stained with FITC-labeled antibody directed against human α-SM actin (overnight at 4°C). Cells were washed in EDTA solution (2 mM in PBS) and analyzed by flow cytometry **12** (FC500 Beckman Coulter). Results are expressed as mean ± SEM of fluorescent intensity.

Immunostaining
Dermal equivalents were reconstructed in six-well plates, with a volume of mixture equal to 2.5 mL for each well, in the presence of a ring of 3D biomaterial or in free retracted conditions. Plates were then placed in the incubator (1 h, 37°C, 5% CO_2) for gel polymerization, after which 2 mL of fibroblast culture medium was added. After 5 days of culture, collagen gels were rinsed in PBS and fixed in PFA (3% in PBS, 30 min, RT). Fixed samples were then incubated for 15 min in permeabilization solution (triton 0.1% in PBS) and in glycine solution for 10 min (1% in PBS).

Nonspecific adhesion sites were blocked by incubation in BSA solution (0.1% in PBS, 1 h). The primary antibody directed against human α-SM actin was then added (1 : 100 in PBS) and incubated overnight at 4°C in a moist chamber. Samples were then incubated with the secondary antibody solution (anti IgG, 1 : 600 in PBS) for 1 h in a moist chamber. Cellular nuclei were countermarked with Dapi solution (10 µg/mL). Samples were then mounted in Dako fluorescent medium and observed under confocal microscope (Olympus FV1000). **13**

Mechanical tests
A DMA Bose Electroforce 3200 with an environmental chamber was used to carry out mechanical experiments (Figs 1a and b). With the versatility from static to dynamic response, this apparatus is specifically configured for material and biomaterial testing. It employs a moving magnet linear motor to apply the solicitation to the sample. The system is implemented by a high resolution system which assumes a controlled force and displacement until the values reached about 1 mN and 0.1 µm.

The dermal equivalents reconstructed in the presence of the selected biomaterial were characterized using quasi-static tensile tests. In this way, specific samples of biomaterials were designed. The first ones were made of a rectangular piece of biomaterial (3D) of about 29×36 mm in dimension (Fig. 1c), and the second ones, which were hollowed (H3D), included a rectangular window of 22×15 mm
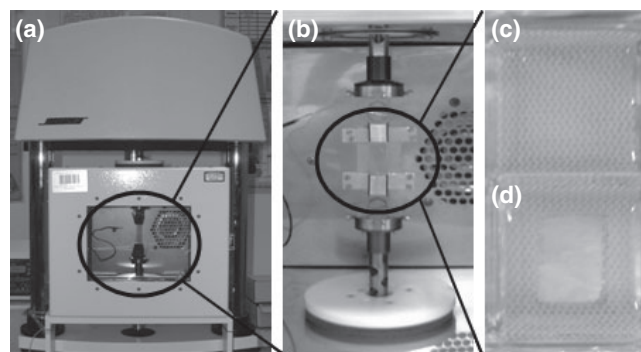
Fig. 1. *Dynamic mechanical analyser (DMA) with the environmental chamber (a) where a tensed dermal equivalent is placed for mechanical measurements (b): tested samples tensed on 3D biomaterial hollowed (d) or not (c).*
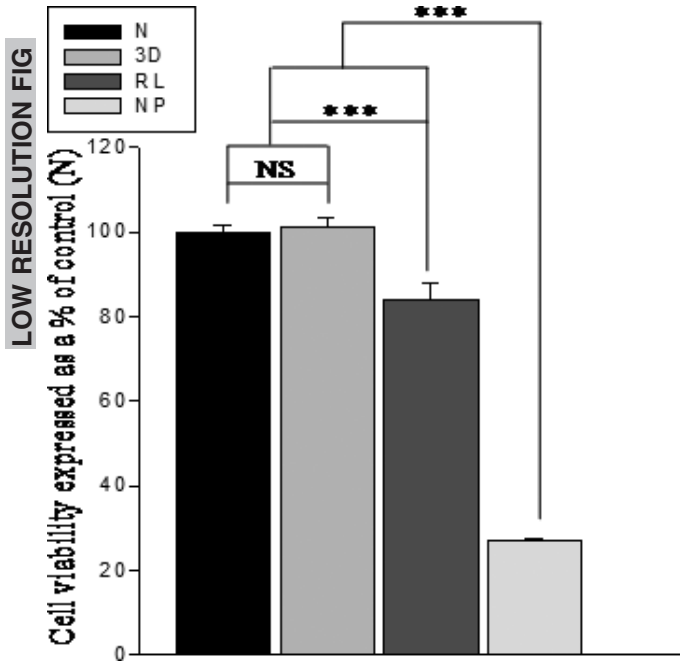
Fig. 2. Fibroblasts viability in three dimension culture condition: N, CDE tensed on nylon ring (control); 3D, CDE tensed on 3D biomaterial; RL, CDE free retracted; NP, CDE tensed on nylon with phenol (cytotoxicity control). Results are expressed as a percentage of control. Significant difference is marked as ***P < 0.001.

in the middle of the sample (Fig. 1d). Dermal equivalents were reconstructed on these different samples and tested in tension (Fig. 2b) with a controlled displacement, at a rate of 0.05 mm/s. The clamping length was 22 mm.

Statistical analysis

For biological investigations, data were expressed as means with standard errors (mean ± SEM) of at least three different measurements. SigmaStat software was used to assess differences between groups using a one way analysis of variance. Differences were considered as significant for P values <0.05.

For mechanical assays, reproducibility was assessed in triplicate.

Results

pH measurement

The pH levels obtained after incubation of the culture medium with the different biomaterial were 7.49 for control, 7.50 for Nylon®, and 7.47 for 3D, respectively. No critical pH variation was observed and no pH adjustment was necessary for further investigations.
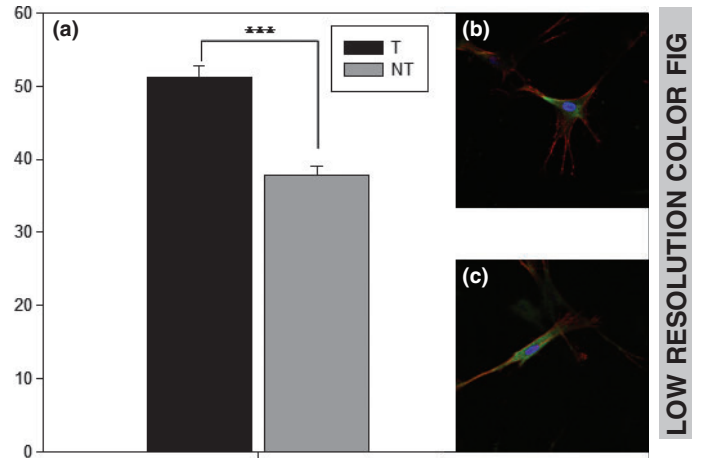
Fig. 3. (a) Quantification by flow cytometry of the expression of α-SM actin by fibroblasts cultured in CDE maintained under tension on 3D (T) or free retracted (NT). Significant difference is marked as ***P < 0.001. (b) and (c) Confocal microscopy pictures of fluorescence staining of fibroblasts cultured in free-retracted CDE (a) or CDE maintained under tension on 3D biomaterial (b): α-SM actin (green), F-actin fibers (red) and nucleus (blue), magnification: ×60.

Bioadhesion test

Bioadhesion assays lead to the conclusion that the 3D biomaterial is able to maintain dermal equivalents under tension in a similar way to Nylon®. Indeed, after 5 days of culture, lattice diameters were equal to their initial diameter when reconstructed in the presence of these two biomaterials. In contrast, the diameter of dermal equivalents freely retracted significantly decreased during time in culture, and mainly between day 0 and day 1 (about 65.7%).

Cytotoxicity assay

Results of cytotoxicity in the different conditions are presented in Fig. 2. The presence of phenol in the culture medium clearly led to a critical reduction of human dermal fibroblast viability (about 75%). No difference in cell viability was observed in lattices maintained on 3D biomaterial compared to Nylon®. In contrast, cell viability was significantly lower (about 20%) in free retracted lattices compared to tensed lattices.

Myofibroblastic differentiation

As shown above, the 3D biomaterial allowed dermal equivalents to be maintained under tension as efficiently as Nylon®. The expression of α-SM actin in tensed conditions vs. free retracted conditions and the quantification of
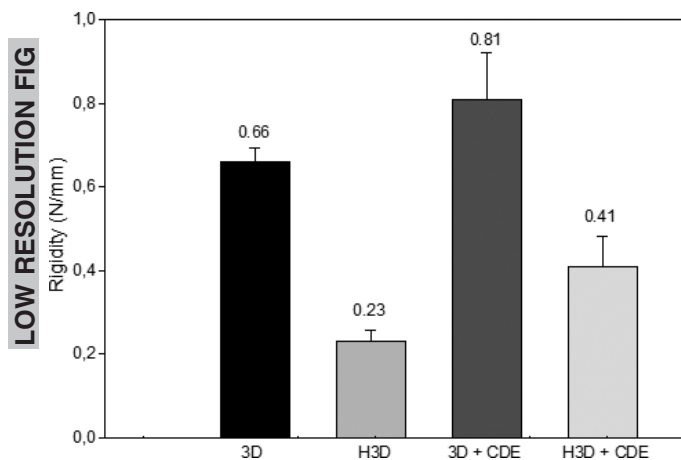
Fig. 4. Measurements of rigidity (N/mm) of the 3D material with [19] or without CDE and hollowed (H3D) or not (3D).

this expression by flow cytometry are presented in Fig. 3. As expected from the observation with confocal microscopy, the quantification showed that the expression of α-SM actin by myofibroblasts in CDE tensed on the 3D biomaterial was significantly higher (about 15%) than in free retracted conditions (Fig. 3a). Moreover, the shape of fibroblasts was quite different in tensed conditions compared to free retracted conditions. Indeed, in tensed conditions (Fig. 3c) fibroblasts were elongated whereas in FR-CDE, fibroblasts displayed a stellar shape with numerous pseudopodia (Fig. 3b).

Mechanical behavior of dermal equivalent

To characterize tensed CDE, measurements of rigidity were determined using tensile tests on 3D material in different configurations, sometimes hollowed and with or without CDE (Fig. 4). When the 3D material was hollowed, its rigidity decreased by 65.15%. When a CDE was reconstructed on the H3D, its rigidity was superior to H3D alone (+78.26%) representing 62.22% of the rigidity of 3D alone. In addition, the rigidity of 3D + CDE increased by 22%. Taken together, these results show that the presence of cellularized collagen gel reinforces the rigidity of the biomaterial (3D and H3D). The increase in rigidity, which is related to the presence of CDE, is about 0.2 N/mm.

Discussion

We report the reconstruction of CDE in the presence of a material which counters the natural retraction of the cellular dermal equivalents. In fundamental research, CDE can be maintained under tension with the presence of a Nylon® ring. However, the dermal equivalent obtained remains quite breakable (low thickness) and difficult to handle. The 3D (honeycomb of polyester) biomaterial was thus tested as an alternative to Nylon®. The results showed that the 3D biomaterial was highly biocompatible and could therefore potentially be used in medical devices intended for implantation in contact with cutaneous tissues. This biomaterial, in the same way as Nylon®, was efficient in maintaining the dermal equivalent under tension. This mechanical status led to the differentiation of fibroblasts into myofibroblasts expressing α-SM actin at significantly higher levels in tensed lattices than in free retracted conditions. Our dermal equivalent therefore appears to allow cells to function, which is essential in cutaneous wound healing in to contract granulation tissue and bring wound edges together (20). These results are in accordance with data in the literature which show that mechanical forces, in particular tension forces, act on dermal fibroblasts by inducing their differentiation into myofibroblasts, and also by improving their migration, proliferation, and collagen synthesis (21–23). The functionalization of fibroblasts could be useful in the case of chronic wounds, such as venous leg ulcers where the senescence of fibroblasts is responsible for a delay in wound healing. Nowadays, of the numerous available models, few bioengineering studies (24–26) focus on their intrinsic mechanical properties compared to those of skin *in vivo*.

Dermal equivalents and biomaterials intended for therapeutic use must have properties in accordance with *in vivo* human skin and also a high adaptability to the wound bed. Indeed, human skin is inhomogeneous, nonlinear viscoelastic, and an anisotropic material. The rigidity of our CDE constructed both on 3D and hollowed 3D material (H3D-CDE), is about 0.81 and 0.41 N/mm, respectively. Wijn (27) measured a ratio of 3.8 and Manschot and Brakee (28, 29) a ratio of anisotropy of 2.8 at the level of calf. To refer to intrinsic material property, the tensile elasticity modulus may be considered to quantify the relationship between the stress and strain field. The tensile elasticity modulus of human skin may be approximated

to 1 MPa (4, 11). Other authors give a large range for this value: from 0.3 to 30 MPa for total skin (30, 2), depending on the location and the system used for this measure. Concerning the dermis, the elasticity modulus is somewhere between 0.05 and 2 MPa (31), which corresponds, in our measurement conditions, to a rigidity between 0.1 and 3.2 N/mm. Consequently, mechanical properties of our model are within this range of values, meaning that our CDE presents physiological mechanical properties. Results have also shown that the presence of CDE confers rigidity to the structure by itself, both for 3D-CDE and H3D-CDE. Moreover, the use of the hollowed 3D allows only for the presence of biological components in the middle part of the construct and an optimal adaptability to the wound bed. Modulations of the respective concentrations of fibroblasts and collagen could further be performed to estimate the local rigidity of the grafted area and to take into account inter individual variability for personalized therapy.

Conclusion

The feasibility of an original model of cellular dermal equivalent (CDE), with mechanical characteristics close to those of the dermis *in vivo* is reported herein. This CDE reconstructed with an original biomaterial, including functionalized cells (myofibroblasts), may be a useful tool for clinical use, namely not only in chronic wounds but also in an *in vitro* model for toxicological/pharmacological research. This original model could be further epidermized with keratinocytes to obtain a dermo-epidermal equivalent maintained under tension which could be used as an alternative to animal models.

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References

1. Jacquet E, Josse G, Khatyr F, Garcin C. A new experimental method for measuring skin's natural tension. Skin Res Technol 2008; 14: 1–7.
2. Khatyr F, Imberdis C, Vescovo P, Varchon D, Lagarde J. Model of the viscoelastic behaviour of skin in vivo and study of anisotropy. Skin Res Technol 2004; 10: 96–103.
3. Retel V, Vescovo P, Jacquet E, Trivaudey F, Varchon D, Burtheret A. Nonlinear model of skin mechanical behaviour analysis with finite element method. Skin Res Technol 2001; 7: 152–158.
4. Elsner P, Beradasca E, Wilhelm KP, Maibach HI. Bioengineering of the skin – Skin biomechanics. Boca Raton, FL: CRC Press, 2002.
5. Pailler-Mattéi C, Zahouani H. Study of adhesion forces and mechanical properties of human skin in vivo. J Adhes Sci Technol 2004; 18: 1739–1758.
6. Khatyr F, Imberdis C, Varchon D, Lagarde J, Josse G. Measurement of the mechanical properties of the skin using the suction test. Skin Res Technol 2006; 12: 24–31.
7. Salter DC, McArthur HC, Crosse JE, Dickens AD. Skin mechanics measured in vivo using torsion: a new and accurate model more sensitive to age, sex and moisturizing treatment. Int J Cosmet Sci 1993; 15: 200–218.
8. Delalleau A, Josse G, Lagarde J, Zahouani H, Bergheau J. Characterization of the mechanical properties of skin by inverse analysis combined with the indentation test. J Biomech 2006; 39: 1603–1610.
9. Pailler-Mattéi C, Bec S, Zahouani H. In vivo measurements of the elastic mechanical properties of human skin by indentation tests. Med Eng Phys 2008; 30: 599–606.
10. Liang X, Boppart SA. Biomechanical properties of in vivo human skin from dynamic optical coherence elastography. IEEE Trans Biomed Eng 2010; 57: 953–959.
11. Agache P, Varchon D. Skin mechanical function. In: Agache P and Humbert P, eds. Measuring the skin: non-invasive investigations, physiology, normal constants. Berlin: Springer, 2004: 429–445.
12. Silver FH, Siperko LM, Seehra GP. Mechanobiology of force transduction in dermal tissue. Skin Res Technol 2003; 9: 3–23.
13. MacNeil S. Progress and opportunities for tissue-engineered skin. Nature 2007; 445: 874–880.
14. Shevchenko RV, James SL, James SE. A review of tissue-engineered skin bioconstructs available for skin reconstruction. J R Soc Interface 2010; 7: 229–258.
15. Bell E, Ivarsson B, Merrill C. Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro. Proc Natl Acad Sci U S A 1979; 76: 1274–1278.
16. Dallon JC, Ehrlich HP. A review of fibroblast-populated collagen lattices. Wound Repair Regen 2008; 16: 472–479.
17. Bride J, Viennet C, Lucarz-Bietry A, Humbert P. Indication of fibroblast apoptosis during the matura-

tion of disc-shaped mechanically stressed collagen lattices. Arch Dermatol Res 2004; 295: 312–317.

18. ISO document 10993-5. Biological evaluation of medical devices. Test for cytotoxicity: in vitro methods. 1992.

19. Schappacher M, Le Hellaye M, Bareille R, Durrieu M, Guillaume SM. Comparative in vitro cytotoxicity toward human osteoprogenitor cells of polycaprolactones synthesized from various metallic initiators. Macromol Biosci 2010; 10: 60–67.

20. Clark RAF. Wound repair: overview and general considerations. In: Clark RAF, ed. The molecular and cellular biology of wound repair, 2nd edn. New York: Plenum Press; 1996: 14–17.

21. Parsons M, Kessler E, Laurent GJ, Brown RA, Bishop JE. Mechanical load enhances procollagen processing in dermal fibroblasts by regulating levels of procollagen C-proteinase. Exp Cell Res 1999; 252: 319–331.

22. Kessler D, Dethlefsen S, Haase I, Plomann M, Hirche F, Krieg T, Eckes B. Fibroblasts in mechanically stressed collagen lattices assume a "synthetic" phenotype. J Biol Chem 2001; 276: 36575–36585.

23. Junker JP, Kratz C, Tollbock A, Kratz G. Mechanical tension stimulates the transdifferentiation of fibroblasts into myofibroblasts in human burn scars. Burns 2008; 34: 942–946.

24. Clark RAF, Ghosh K, Tonnesen MG. Tissue engineering for cutaneous wounds. J Invest Dermatol 2007; 127: 1018–1029.

25. Jachowicz J, McMullen R, Prettypaul D. Indentometric analysis of in vivo skin and comparison with artificial skin models. Skin Res Technol 2007; 13: 299–309.

26. Zahouani H, Pailler-Mattei C, Sohm B, Vargiolu R, Cenizo V, Debret R. Characterization of the mechanical properties of a dermal equivalent compared with human skin in vivo by indentation and static friction tests. Skin Res Technol 2009; 15: 68–76.

27. Wijn PFF, Brakkee AJM, Kuiper JP, Vendrik JH. The alinear viscoelastic properties of human skin in vivo related to sex and age. In: Marks R and Payne PA, eds. Bioengineering and the skin. Lancaster: MTP Press; 1981: 135–145.

28. Manschot JF, Brakkee AJ. The measurement and modelling of the mechanical properties of human skin in vivo–II. The model. J Biomech 1986; 19: 517–521.

29. Manschot JF, Brakkee AJ. The measurement and modelling of the mechanical properties of human skin in vivo–I. The measurement. J Biomech 1986; 19: 511–515.

30. Daly CH, Odland GF. Age-related changes in the mechanical properties of human skin. J Invest Dermatol 1979; 73: 84–87.

31. Tran HV, Charleux F, Rachik M, Ehrlacher A, Ho Ba Tho MC. In vivo characterization of the mechanical properties of human skin derived from MRI and indentation techniques. Comput Methods Biomech Biomed Engin 2007; 10: 401–407.

Address:
Pr. P. Humbert
Department of Dermatology
CHU St Jacques
2 Place St Jacques
25030 Besançon Cedex
France
Tel: 0033(3) 81 21 83 01
Fax: 0033(3) 81 21 82 79
e-mail: philippe.humbert@univ-fcomte.fr

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15	AUTHOR: Please provide the name of the publisher, city location of publisher for reference [18].	

16	AUTHOR: Figure 1 has been saved at a low resolution of 136 dpi. Please resupply at 600 dpi. Check required artwork specifications at http://authorservices.wiley.com/submit_illust.asp?site=1
17	AUTHOR: Figure 2 has been saved at a low resolution of 103 dpi. Please resupply at 600 dpi. Check required artwork specifications at http://authorservices.wiley.com/submit_illust.asp?site=1
18	AUTHOR: Figure 3 has been saved at a low resolution of 149 dpi. Please resupply at 600 dpi. Check required artwork specifications at http://authorservices.wiley.com/submit_illust.asp?site=1
19	AUTHOR: Figure 4 has been saved at a low resolution of 139 dpi. Please resupply at 600 dpi. Check required artwork specifications at http://authorservices.wiley.com/submit_illust.asp?site=1

WILEY-BLACKWELL

USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

Required software to e-Annotate PDFs: <u>Adobe Acrobat Professional</u> or <u>Adobe Reader</u> (version 8.0 or above). (Note that this document uses screenshots from <u>Adobe Reader X</u>)
The latest version of Acrobat Reader can be downloaded for free at: <u>http://get.adobe.com/reader/</u>

Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:
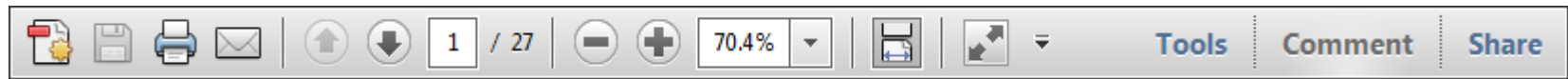
This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We've picked out some of these tools below:

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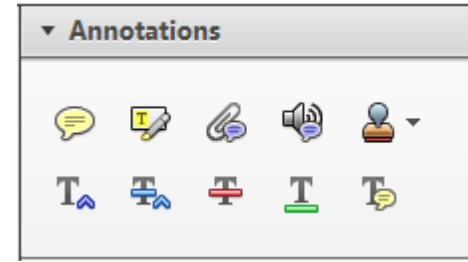
1. Replace (Ins) Tool – for replacing text.

 Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
- Click on the Replace (Ins) icon in the Annotations section.
- Type the replacement text into the blue box that appears.

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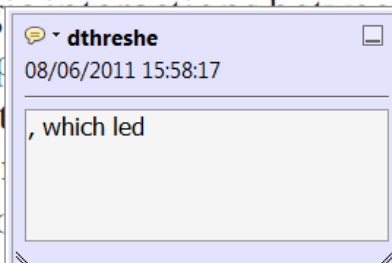
2. Strikethrough (Del) Tool – for deleting text.

 Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

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3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

 Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

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4. Add sticky note Tool – for making notes at specific points in the text.

 Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.

# USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

How to use it

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.
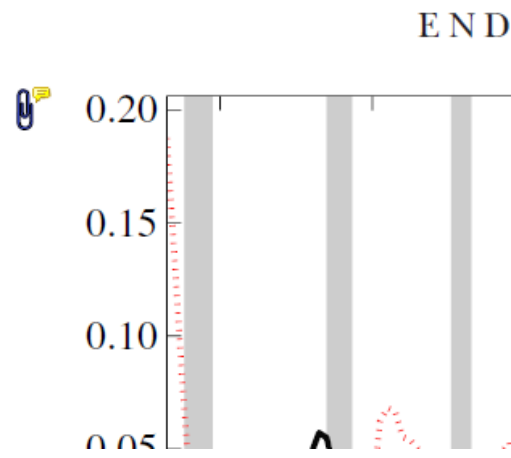
## 6. Add stamp Tool – for approving a proof if no corrections are required.

Inserts a selected stamp onto an appropriate place in the proof.

How to use it

- Click on the Add stamp icon in the Annotations section.
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
- Click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).
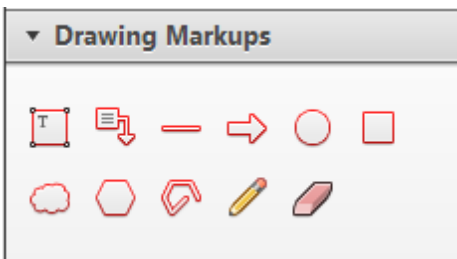
7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..

How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.
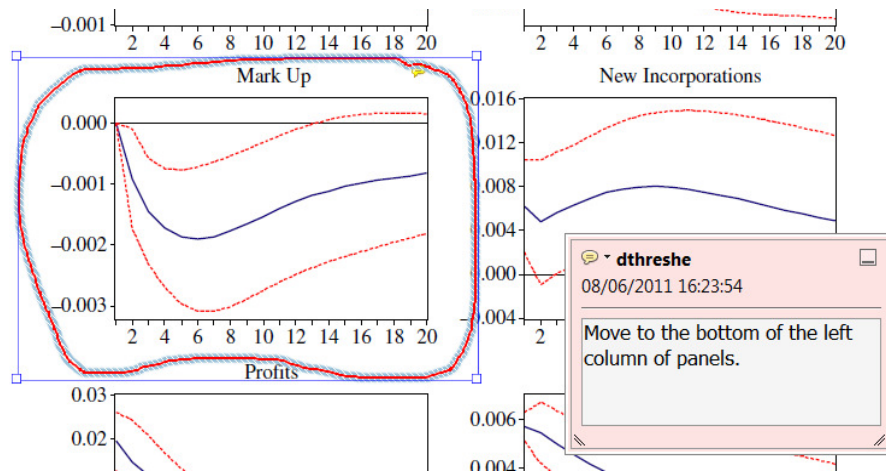
For further information on how to annotate proofs, click on the Help menu to reveal a list of further options: