New bioprinted skin, cosmetic *in vitro* model

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Synopsis

We developed a new evolution of three-dimensional skin equivalent due to the optimization of four-dimensional laser-assisted bioprinting and skin equivalent culture protocols. This allowed us to produce fully bioprinted skin equivalents that are closed to current skin equivalents and suitable to test cosmetic ingredients. Particularly, we performed preliminary evaluation of maturogens to improve the dermis maturation before the epidermal seeding and we designed a specific "micropattern" to reproduce the nonlinear aspect of the dermal–epidermal junction. Finally an active ingredient was applied during the production of the bioprinted skin equivalent.

INTRODUCTION

Although three-dimensional (3D) printing itself is a relatively new technology, invented three decades ago, it contributes to one of the most promising medical technological advance of the century in bioscience and its market potential is only just beginning to be realized in the case of bioprinting. The first description of bioprinting occurred in 1988 when R. J. Klebe described Cytoscribing, the first two and 3D synthetic tissues construction on fibronectin substrate using ink-jet printer and computer-assisted high-precision positioning of cells. From the beginning of the technology development, the promises are to establish in a short timeframe precise spatial arrangements within large populations of cells to resemble natural tissues and organs for regenerative medicine or testing applications.

Different printing technologies have been successfully developed using either sophisticated and complementary ink-jet, bioextrusion, or laser printers. For example, the first 3D skin printing production was described in 2012 with 3D arrangement of vital cells by laser-assisted bioprinting (LaBP) as multicellular fibroblasts and keratinocytes embedded in collagen for *in vitro* testing application. In 2013, PrintAlive Bioprinter using complex microfluidic device has allowed human microtissue arrays to be routinely defined with unprecedented speed and resolution for grafting application.

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Despite recent advances and use of various technologies, the 3D printed skin lacks in dermal maturation and epidermal differentiation. Optimization of cell culture media providing maturogens and time management described as the fourth dimension need to be improved. Owning one of the most versatile skin equivalent model in terms of different skin cell types integrated yet, experience in skin functionality and LAB, we describe here the latest advances and experiments performed in our laboratories.

FOUR-DIMENSIONAL LaBP: TECHNOLOGY AND BENEFITS

TISSUE ENGINEERING EVOLUTIONS

Tissue engineering evolved from the field of biomaterials and medical devices. Conventional tissue engineering methods rely on the use of scaffolds to support and guide the subsequent cellular and tissue organization (1–3). These top-down assembly approaches greatly rely on the self-organization of cells in response to environmental cues. They do not allow a fine control over the created final structure and cell organization.

On the contrary, bottom-up approaches, like additive fabrication technologies such as bioprinting, proceed by the assembly of small units which structure and organization can be finely tuned. Bioprinting offers the ability to create highly complex 3D architectures with living cells. Bioprinting methods have been developed to effectively and rapidly pattern living cells, biological macromolecules, and biomaterials. As a consequence, this cutting-edge technique has significantly gained popularity and applicability in several fields as it facilitates physiologically relevant cell–cell and cell–matrix interactions allowing studies within an expected shorten time.

BIOPRINTING

Akin to ordinary ink printers, bioprinters have three major components to them. These are the hardware used, the type of bioink, and the material it is printed on (biomaterials). In bioprinting, there are three major types of printers that have been used. These are inkjet, laser-assisted, and extrusion printers.
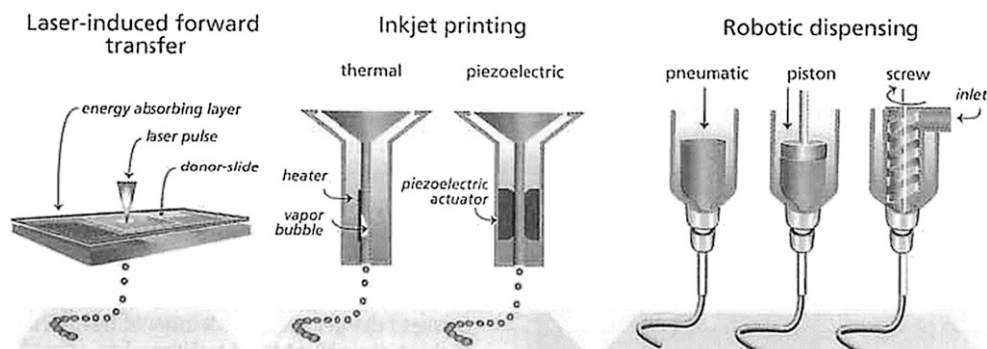

Figure 1. Selected biofabrication approaches involving the use of hydrogels in form of so-called "bioink" (4).

- Laser-assisted bioprinters are less common and use lasers focused on an absorbing substrate to generate pressures that propel cell-containing materials onto a collector substrate. Printers that utilize lasers provide high-resolution printing and because it is a nozzle-free device, clogging of the nozzle is avoided (Figure 1, left).
- Thermal ink-jet printers electrically heat the printhead to produce air-pressure pulses that force droplets from the nozzle, whereas acoustic printers use pulses formed by piezoelectric or ultrasound pressure (Figure 1, middle). Ink-jet printers are mainly used in bioprinting for fast and large-scale products.
- Microextrusion printers use pneumatic or mechanical (piston or screw) dispensing systems to extrude continuous beads of material and/or cells (Figure 1, right).

LaBP TECHNOLOGY

3D laser-assisted bioprinter has a near infrared pulse laser source and a focus system to adjust the ejecta size. A laser is beamed through a transparent slide coated with an absorbent layer, enabling light energy to be converted into kinetic energy. A thin matrix layer, containing the component to be printed and a recipient substrate, is positioned a few microns away from the first slide. Laser pulses are programmed to be sent approximately every nanosecond. This generates inkjets (cell containing mini-droplets), which are deposited layer by layer.

In this system, physical ejection conditions—energy and viscosity—as well as droplet volume to around picoliter accuracy are controlled. The biological ink cartridge scans quickly, generating over 10,000 droplets a second with a resolution of 20 µm. Compared to manual skin equivalent production, the time to make a biological structure 1 cm^2 and 200–300 µm thick useful for *in vitro* testing is reduced by two-thirds.

Our preliminary studies have shown that printable extracellular matrix and cells can be combined in a laser-assisted printing sequence to fabricate a stable and organized soft free form tissue, which can host a high cell density *de novo*. The LaBP can print versatile biological patterns such as cell clusters, cell confluent surface, and cell alignments according to computer-aided design. Also, a cell-level resolution of cell printing at a high speed (5 kHz) is achievable by this laser-assisted bioprinter. Such precision and speed were a prerequisite to apply the LaBP to cellularized tissue fabrication (5).

As a matter of facts, several advantages have been associated to the use of 3D laser-assisted bioprinter (6) (Figure 2):

- Very high resolution compared to bioextrusion (single cell printing capability);
- Very high precision (µm);
- Very high cell viability compared to ink jet (nearly 100%); and
- Very high material viscosities possible use.

However, the process is called four-dimensional (4D) bioprinting since it utilizes a fourth dimension: time. Once tissue is printed, the cells need time to communicate and self-assemble and this maturation is an important part of the biofabrication process. Indeed, the bioprinting of a 3D structure is not enough to create a functional tissue structure. Like more traditional scaffold-based methods, 4D bioprinting relies on self-organization capacities of cells and on morphogenetic processes. But unlike scaffold-based methods, bioprinting makes it possible to reproducibly control the initial 3D tissue structure. As
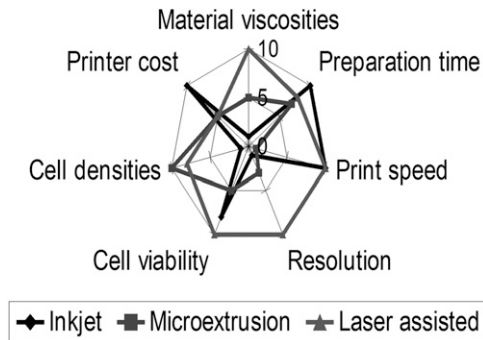
Figure 2. Laser-assisted bioprinter benefits compared to other bioprinters (6).

the final state of a dynamic system is the result of both the boundary conditions (including the initial conditions) and its dynamic characteristics, we could expect to have a more reproducible matured tissue thanks to 3D bioprinting.

SKIN EQUIVALENT STUDY DESIGN

To reproduce complex, heterogeneous functional tissues and organs found in the human body such as skin, understanding of composition and organization of their components is an essential requirement.

PREBIOPRINTING

This is the first step to generate a 3D tissue file containing the 3D structure and composition of the tissue to be bioprinted. This is the product of an ideation phase based on the observation of native tissues or imaging data and literature analysis regarding dermal and epidermal histometry. ImageMatrix is used to generate complex visual designs for tissue. The goal is to determine and design specific virtual micropatterns to engineer at first a dermis then an epidermis onto the dermis.

Dermis design. As the native dermis in the skin is mainly composed of collagen I and III, we used both collagen type I and a mixture of collagen type I and III (95–5%) to associate with normal human adult fibroblasts in our preliminary testing.

- The 3D structure was created by alternating a layer of collagen with a layer of cells.
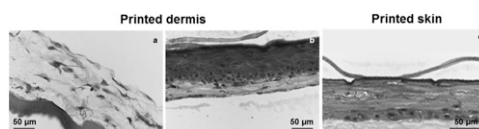- The cell pattern was chosen to ensure a uniform distribution of fibroblasts.

Figure 3. (A) Printed dermis after 5 days, (B) epidermized printed dermis (keratinocytes manually deposited) after 15 days, and (C) printed skin after 14 days.
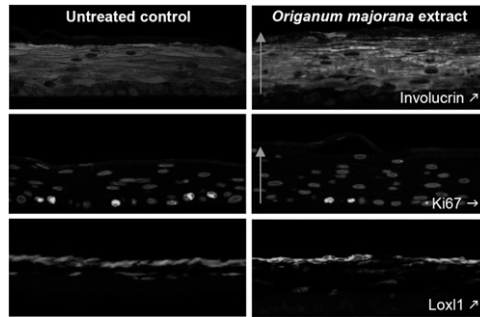
Figure 4. Immunostaining of untreated and treated models after 14 days.

- In each plane, the cell pattern was a square network with a characteristic distance of 300 μm between spot of cells.
- Number of layers is designed to print a minimal initial dermis thickness of 200 μm.

Epidermis design.

- The epidermis basal layer was designed with pattern was chosen to ensure a uniform distribution of normal human adult keratinocytes.
- In each plane, the cell pattern was a square network with a distance of 100–300 μm between spot of cells.
- Number of layers is designed to reach a minimal initial epidermal basal layer thickness.

BIOFABRICATION

Two different technologies were combined to 3D print the dermis equivalent, a microvalve technology was used to print the collagen layers, whereas LaBP was used for the cell layers.

Culture media for fibroblasts is a DMEM/F12 base containing antibiotics and 20% SVF for seeding then 10% during growth period. Green medium (7) supplemented by 50 μg/ml ascorbic acid and antibiotics is used for keratinocyte seeding and DMEM/F12 supplemented by (0.8% BSA, 0.12 UI/ml insulin, 0.4 μg/ml hydrocortisone, 50 μg/ml ascorbic acid and antibiotics) for keratinocytes differentiation.

Kinetic of dermis maturation was performed to define the best timing for dermis maturation before epidermal printing. One active ingredient (*Origanum majorana* leaf extract 0.04%—BASF) was added in the culture media to evaluate the benefit on dermis maturation extracellular matrix synthesis, dermal–epidermal junction quality) and quality of epidermal anchorage and differentiation.

ANALYSIS

Dermis and epidermis quality was studied by histology and immunostaining. Results were compared to human skin biopsies and in house skin equivalent performed manually (Mimeskin®).

Bioprinted skin models are suitable for dermocosmetic evaluations and allows to observe some changes induced by the treatment with an *O. majorana* extract used at 0.04% (in the dermis: LOXL1 elastin cross-linking enzyme—in the epidermis: thickness and involucrin).

CONCLUSION

We succeeded to develop a new evolution of 3D skin equivalent thanks to the optimization of 4D LaBP. This has now allowed us to produce a fully bioprinted skin that is close to current skin equivalents. It presents various advantages such as reproducibility and time for production. This model was used for the first time to evaluate the efficacy of a cosmetic ingredient (*O. majorana* leaf extract).

Due to this recent advancement in the area of 3D bioprinted skin using laser-assisted bioprinter, we now anticipate the implementation of this model in the future with different cell types such as epidermal and/or AD stem cells in a specific pattern and define micro-environment that will enable to be closer to human skin. Thus, we expect that these models will allow more predictive evaluation of active ingredient performance before clinical trials.

REFERENCES

(1) E. Bell, H. P. Ehrlich, D. J. Buttle, and T. Nakatsuji, *Science*, **211(4486),** 1052–1054 (1981).
(2) V. Yannas IV and J.F. Burke, *J. Biomed. Mater. Res.*, **14(1),** 65–81(1980).
(3) S.T. Boyce, Christianson DJ, Hansbrough JF, *J. Biomed. Mater. Res.,* **22(10),** 939–957 (1988).
(4) J. Malda, J. Visser, F. P. Melchels, T. Jüngst, W. E. Hennink, W, J. A. Dhert, J. Groll, and D. W., Hutmacher, *Adv. Mater.*, **25(36),** 5011–5028, (2013).
(5) B. Guillotin, A. Souquet, S. Catros, M. Duocastella, B. Pippenger, S. Bellance, R. Bareille, M. Rémy, L. Bordenave, J. Amédée, and F. Guillemot, *Biomaterials,* **31(28),** 7250–7256 (2010).
(6) S. V. Murphy and A. Atala, *Nat Biotechnol.*, **32(8),** 773–785 (2014).
(7) H. Green, O. Kehinde, and J. Thomas, *Cell Biol.*, **11,** 5665–5668 (1979).