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Approaches to the development of 3D bioprinted skin models: the case of natura cosmetics

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Abstract: We are close to achieving the production of a biomimetic functional skin and this advance is mainly due to the demand that is not limited to the field of regenerative medicine, the need for transplantation of this organ due to the aging of the population, but for ethical reasons related to the tests of safety and efficacy of new formulas in animal models by the cosmetic and pharmaceutical industries. The limitations involved in traditional 2D cell culture approaches and manual techniques for biomimetic generation have driven the use of innovative technologies such as 3D bioprinting. One of the main advantages of the bioprinted skin is the authenticity, scalability and reproducibility of tissues compared to conventional constructs, via precise positioning of multiple cell types and the inclusion of appendages. The models of bioprinted skins will serve as a platform for the development of new formulations, molecule testing, disease simulation, as well as an alternative to chronic wound biocuratives and clinical transplants. This paper reviews the state–of–the–art approaches available for skin model bioprinting, discusses the context of the drug–cosmetic industry in the adoption of these models and presents the characteristics of the project under development at Natura Cosmetics.

Keywords: 3D bioprinting; biofabrication; alternative methods; Natura Cosmetics; in vitro skin.

Introduction

Curiosity catches the eye when it comes to guessing what will be the first bioprinted functional organ to hit the market [1,2].The skin is among the first in this list due to a demand that is not only limited to regenerative medicine and the need for transplantation of this organ, but due to ethical issues related to animal testing[3]. Animal use has already been banned in many countries and the demand for equivalent skin models in the cosmetics and pharmaceutical industries is a worldwide trend[4–7]. Players like Natura, L'Oréal and Procter & Gamble are investing in bioprinting technology for the development of organotypic skin models.

As of 2019, the Normative Resolution of the National Council for Animal Experimentation Control (RN 18/2014, CONCEA) entered into force. This requirement obliges cosmetics manufacturers and pharmaceutical laboratories to adopt alternative methods avoiding tze use of animals for product testing. Along this line, the European Union has banned imports of animal–tested products since 2013 (Amendment 2003/15 / EC of Directive 76/768 EEC). Besides the high cost and import–related issues of these equivalent models, they are limited as they are sold as kits that can be used only in specific assays, as an accompaniment to morphological and molecular changes [8,9].

An example in the Brazilian cosmetic industry, Natura Cosmetics has not conducted animal safety and efficacy tests since 2006, nor does it purchase resources or ingredients that have been tested on animals. In 2018 the company was certified by Cruelty Free International, the first in Latin America to have this certification.

Regarding the field of regenerative medicine, Brazil today has 20.6 million elderly people, a number that represents 10.8% of the population. By 2060, the country is expected to have 58.4 million elderly (Brazilian Institute of Geography and Statistics–IBGE)[10]. With rising rates of obesity, diabetes, and aging populations, the repair of damaged or lost tissue is a worldwide concern and the demand for in vitro recreated artificial skin has grown.

Chronic lesions are followed by severe, often fatal, disorders with difficult extracellular matrix remodeling and that usually require transplantation and urgent intervention to restore tissue integrity. One possibility is the transplantation of allogeneic grafts with a high cure rate, but these are a complex process whose demand exceeds availability.

Burns, in turn, are also a major public health problem. In Brazil there are around 1,000,000 burn accidents per year, of these, 100,000 patients seek hospital care and about 2,500 die[11,12], it is the second leading cause of death in children not only in the United States and, in Brazil as well[13]. According to US statistics, about 10% of patients awaiting life–saving transplants die before they can receive donor organs[14].

Given the regulatory challenges and obstacles of agencies such as ANVISA in Brazil and the FDA in the United States, regulating products that will contact a patient's body is substantially more costly and complex than approving an in vitro skin model, which will only be used on the lab bench for simulations and testing of drugs and cosmetics. Nevertheless, the fact that there are already commercially available skin replacements for the treatment of chronic wounds such as Apligraf® and INTEGRA®, the accumulated prior knowledge and the paved roads for regulatory approval of this type of product are already established in some countries.

From a market perspective, the global size of 3D cell culture was estimated at USD 558.0 million in 2016, displaying a CAGR of 14.8% over the forecast period. The BCC Research report [16] predicts that the bioprinting market will reach $ 1.8 billion by 2021. This growth is estimated at a compound annual growth rate of 43.9% from 2016 to 2021. Another report from the consulting firm Grand View Research (2018) [17]estimated the globalbioprinting market at $ 682 million in 2016 with a forecast

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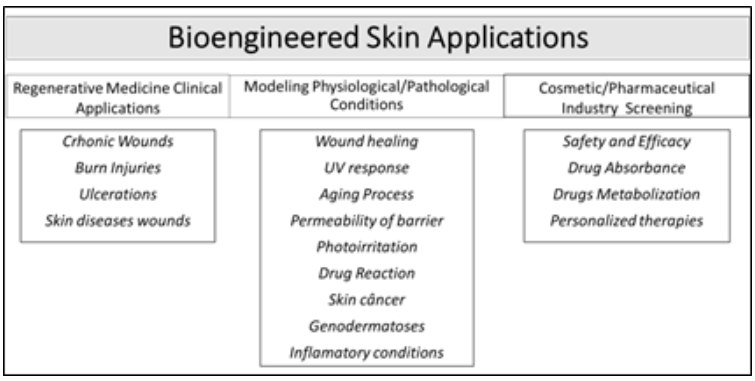


Figure 1 – Diagram of the different applications of bioengineered skin: reconstructive surgery, modeling of physiological and pathological skin conditions, pharmaceutical screening (Adapted Source15 Sarkiri *et al.* 2019).

that the market could reach $ 2.6 billion by 2024.

Growth is expected to be driven by new printing technologies as well as expansion of new applications in the medical field, such as blood vessels and other applications.

With regard to bioprinted skin models, there is a notable progress that has been made, especially in the last decade. Although we are close to achieving a biomimetic functional skin, dialogue and joint efforts are needed for products to leave the benches and reach patients and industry, since it is an essentially interdisciplinary area [18,19].

Brazil is the fourth largest global market for beauty products, surpassed by the United States, China and Japan. About 2.5 thousand companies in the segment had revenues of R$ 42.6 billion in 2015, according to the Brazilian Association of the Personal Hygiene, Perfumery and Cosmetics Industry (ABIHPEC) [16] (Bergin, 2016). Cosmetic products must be safe for the user and effective for the declared activity. Often, the cosmetic industry launches new products with diverse purpose appeals. Thus, testings that prove the marketing appeal and safety of these products are expected to be conducted. In a report by FAPESP Magazine: "As of 2019, any new beauty product must undergo dermatological tests on reconstructed human skin, in Brazil or abroad" [20,21].

This paper provides a review of the state–of–the–art 3D bioprinting technology for human skin reconstruction, presenting aspects and chall enges of the project (preprocessing), printing (processing), and tissue maturation (post–processing) phases for applications in the cosmetic industry. Finally, the case of the company Natura Cosmetics is presented.

Skin structure and functions

The skin is the largest organ in the human body, representing up to 16% of body weight [22]. It is the boundary between an organism and the environment, acting as a defense organ protecting against the penetration of pathogens and external toxins, controlling the damage caused by the UV rays and preventing desiccation [23]. The skin consists of three main compartments: the epidermis, the dermis and the hypodermis [24].

The epidermis, the superficial and thinner layer, consists mainly of keratinocytes and melanocytes, and because it has high cell density, acts as a vital barrier, preventing the entry of exogenous aggressors, chemical, physical or biological, and acting on water balance which avoids excessive transepidermal loss of water and protein to the environment 25. Melanin, which is a substance produced and accumulated in the epidermis, protects against ultraviolet rays, which are in turn important in the fixation of vitamin D3. The dermis located just below the epidermis is known as the core of the skin, composed mainly of collagen, elastin, glycosaminoglycans (GAGs) and fibroblasts, besides being important in the biomechanical protection of the skin. It performs sensory and immunological functions through the lymphocytes that protect against antigens and allergens that come in contact with the epidermis. The sweat glands, also present in the dermis, help in the excretion of some substances. The hypodermis is located just below the dermis and is a very vascularized layer, consisting mainly of adipose tissue, which contributes to thermal regulation and also to mechanical protection [22,26] (Figure2).

Some authors do not consider hypodermis as part of the skin; however, the hypodermic layer plays an important role in paracrine signaling of the skin, with functions related to skin protection and maintenance of homeostasis. It includes activities that help to protect bacterial infections, control of hair growth cycles, thermogenesis and plays an important role in wound healing, therefore increasing the relevance of the skin model[27]. Thus, studies show that when adipocytes are co–cultured in a monolayer with keratinocytes they stimulate their proliferation and differentiation[28], whereas adipocytes in co–culture with fibroblasts and keratinocytes demonstrate the same proliferative effect in addition to the recruitment of fibroblasts, which play an important role in wound healing[29].

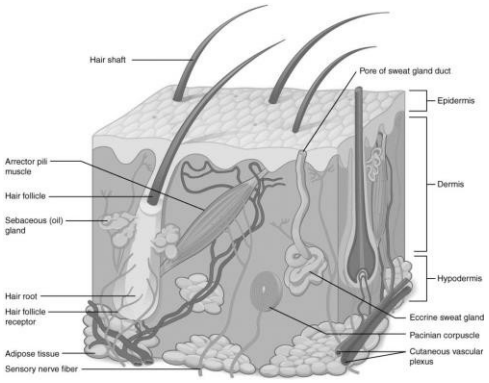


Figure 2– Skin structure model. Source: https://courses.lumenlearning.com/wmopen–biology2/chapter/structure–and–function–of–skin/ (09/09/2019).

Commercial Reconstructed Skin Models

Some reconstructed skin models are commercially available. However, the high importation cost and very long delivery times make the process unfeasible [8,30,31]. The development of new models of reconstructed skin translates to autonomy for many countries and companies.

In this context, the OECD encourages the production of new reconstructed skin models by providing detailed guidance in its OECD Guide No. 439 on the quality and performance control parameters that the model should present. Such parameters include standardized criteria for cell viability, barrier function, morphology and reproducibility 4. Different approaches have been developed to achieve this goal, such as the development of reconstructed skin models, for example, reconstructed human epidermis models and full thickness skin models[32].

The first skin substitute from epidermal cells was described in 1974 by Rheinwald and Howard Green of Harvard Medical University, who cultiva–ted a small fragment of healthy skin over a wound. The success of the graft depended on the presence of dermal elements remaining or transported

to the wound, which motivated further research and triggered the development of the first commercialized product, Epicel, from the American company Genzyme (Figure 3). An epidermal substitute obtained from isolating autologous keratinocytes and co- culturing of these cells over a layer of rat mesenchymal cells, which are expanded numerous times over weeks. It is extremely fragile and is rarely used. This product has been classified by the FDA as a xenograft (derived from other non-human animal species) as it uses a rat mesenchymal cell layer as a supplement for in vitro cultivation [33,34].

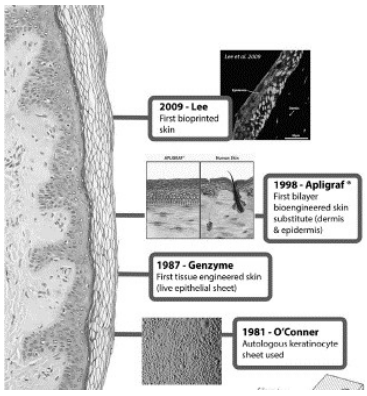


Figure 3 – Timeline of skin substitutes used in medicine [35] (Adapted from Tarassoli et al., 2017).

Another product developed by Americans from 1979–80, Integra, was not approved by the FDA for commercialization until 2002. Integra is a synthetic "acellular artificial skin" that acts as a two-layer dermal analogue, an internal matrix of Type I bovine collagen crosslinked via a controlled freeze-drying process with chondroitin-6-sulfate, glycosaminoglycan (GAG) (1-ethyl-3- (3-dimethylaminopropyl) and carbodiimide EDC, and an outer silicone layer that simulates the epidermis. Each layer performs a function, the inner layer is bioresorbable and simulates a dermal matrix, allowing the invasion of fibroblasts and capillaries (angiogenesis) from the receptor bed, enabling the repair of an equivalent dermal structure and promoting cell growth and the synthesis of a new collagen matrix. Gradually, the collagen is degraded, and over a period of 3 to 6 weeks a new matrix forms. After healing, the external silicone layer with an anti-infection and mechanical barrier function, that controls fluid loss (homeostasis) can be withdrawn [36–38].

Another group from the Massachusetts Institute of Technology (MIT) (Jim Bell, 1981) developed Apligraft® (Organogenesis Inc., USA), also marketed and approved by the FDA, since 2001. A bilaminar structure consisting of a dermal layer of human neonatal fibroblasts on a Type I bovine (calf) collagen gel, and an epidermal layer of keratinocytes from allogeneic culture [39]. The resistance and insolubility of collagen are obtained by shrinkage of the gel by the fibroblasts, resulting in the dermal equivalent [40].

Orcel® is a cellular skin substitute consisting of a bilayer cell matrix. With human donor fibroblasts grown inside a Type I bovine collagen matrix, and keratinocytes from the same donor grown outside the collagen matrix. Orcel® serves as a bioresorbable matrix, which provides a favorable environment for host cell migration due to cytokines and growth factors secreted by allogeneic fibroblasts. According to the manufacturer, after 2–3 weeks of application, no traces of allogeneic DNA are found in the wound [41].

Biobrane® is a synthetic acellular skin substitute consisting of a bilaminated membrane formed by a nylon mesh filled with porcine collagen type I (dermal analogue) and covered with a thin layer of silicone (epidermal analogue). It has small pores that allow the drainage of the transudate, being considered a semipermeable substitute. It enables fibroblasts and capillaries to invade the wound and repair the dermal defect. Reepithelization occurs from the presence of keratinocytes at the wound edge [42].

Dermagraft® (Organogenesis) is the product that most closely resembles the product that this thesis proposes to develop. It is a dermal substitute with a layer of allogeneic fibroblasts grown on a layer of Vicryl polyglycolic acid polymer. The product is cryopreserved but becomes viable and metabolically active when placed on the wound bed. For the major skin models marketed today, see Figure 4.

The Laboratoire Órganogénese Experimental (LOEX) in Quebec City, Canada, has developed a skin reconstructed from the self-assembly technique, a new approach to tissue engineering. This technique is based on the intrinsic property of cells self-organizing to form three-dimensional tissue under appropriate conditions. For example, skin fibroblasts secrete their own extracellular matrix in the presence of ascorbic acid, allowing the production of supportive dermal leaflets on which keratinocytes can be seeded [44].

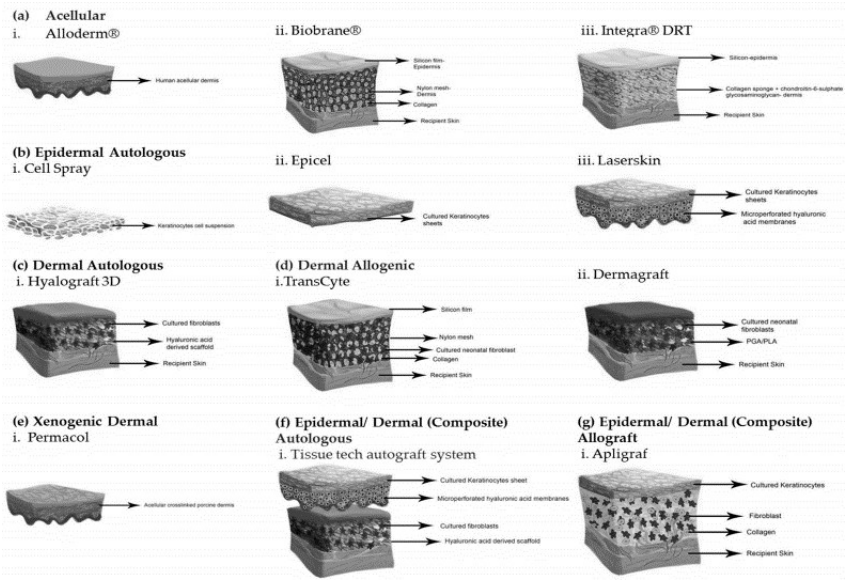


Figure 4 – Tissue engineered skin substitutes. (a) Acellular: i. Karoderm ii. Biobrane iii. Integra (b) Epidermal Autologous: i. Cell Spray ii. Epicel iii. Laserskin (c) Dermal Autologous: i. Hyalograft 3D (d) Dermal Allogenic: i. TransCyte ii. Dermagraft (e) Dermal Xenogenic: i. Permacol (f) Epidermal/ Dermal (Composite) Autologous i. Tissue tech Autograft system (g) Epidermal/Dermal (Composite) Allograft i. Apligraf. 43 (Source with pemission for use: Vig et al., 2017).

3D Bioprinting: The Additive Manufacturing

Before we start the discussion on the state of the art of bioprinted skin models, it is important to situate and define some key terms for this area. Automated skin reconstruction is part of a large area called biofabrication. The term biofabrication is defined as "the automated generation of functional biological products with structural organization of living cells, bioactive molecules, biomaterials, cell aggregates (such as micro–tissues, or hybrid cell–material structures) through bioprinting or bioassembly, followed by a tissue maturation process" [45,46]. In either case, additive manufacturing may be used in some of the fabrication stages of these structures.

Additive manufacturing, or more commonly known as 3D printing, is a process of controlled deposition of materials layer–by–layer to generate a three–dimensional structure (ISO – INTERNATIONAL ORGANIZATION FOR STANDARDIZATION, 2017). These technologies bring with them unique capabilities of rapid prototyping, repeatability and high accuracy 47,48. Bioprinting is a subarea of additive manufacturing and an emerging and revolutionizing field of technology that is part of the wider field of tissue engineering and regenerative medicine [49].

Bioprinting is used to fabricate three–dimensional structures of biological materials, generally cells and biomolecules, through layer–by–layer precise positioning [50,51]. The printing process is controlled by a computer instruction, usually a computer–aided design (CAD) file of the respective tissues structures [51]. The advantage of this technology is the ability to manufacture biomimetic tissues to meet specific needs related to in vitro models or patients, the so–called personalized medicine.

The set of bioprinting techniques that allow living cell deposition includes: inkjet printing, which is subdivided into two types, continuous inkjet (CIJ) 52,53 and drop–on–demand (DoD) 54,55, direct ink writing (DIW),which can be controlled by mechanic pistons or pneumatic control 56–58 and laser printing or stereolithography (Stereolithography–SLA) or laser–induced forward transfer (LIFT) [59–62] (Figure 5).

Today, microextrusion and inkjet techniques are the most used [48,58]. Microextrusion bio printers are one of the conventional 3D printheads that use high temperature fused polymeric filament reels (FDM Fused Deposition Modeling mechanism). The micro–extrusion mechanism is the most widespread in literature and the most used worldwide as it offers greater flexibility in the rheological conditions of bioinks. It also allows working with high densities and cell types in the same construction and allows the deposit of pre–differentiated cells in three– dimensional spherical (spheroids) structures. These advantages are of paramount importance for the production of complex structures since it is necessary to manipulate several types and large cell densities to simulate the heterogeneous environment of complex tissues [50].

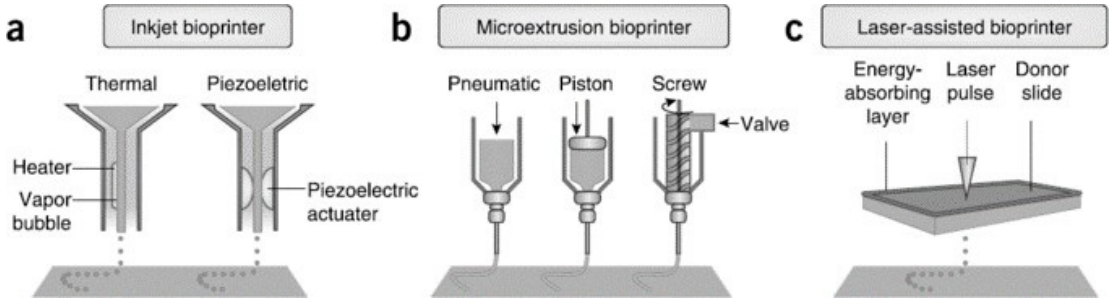


Figure 5 – Bioprinting mechanisms of inkjet, microextrusion, and laser–assisted. A) Inkjet printers, the print head is electrically heated to produce air–pressure pulses that force droplets from the nozzle, while acoustic printers use pulses formed by piezoelectric or ultrasound pressure. B) Microextrusion printers use pneumatic or mechanical dispensing pistons systems to extrude continuous beads of material and/or cells. C) Laser–assisted printers use lasers focused on an absorbing substrate to generate pressures that propel cell–containing materials onto a collector substrate 50. Reprinted by permission from: Murphy and Atala, 2014.

The Challenges Associated with Skin Bioprinting

Challenges for tissue, and more specifically, skin bioprinting, are primarily associated with the selection of bioinks, which are the primary input for bioprinters.

Bioinks, defined as biomaterials that carry cells during the bioprinting process [63–65].Therefore, cells are a mandatory component in the formulation of bioinks, which may or may not also carry biomolecules; otherwise, these inks are called hydrogels or biomaterial ink. Biomaterial inks produce cell––free scaffolds that can be seeded with the cells of interest or combined in bioink hybrid systems to produce more complex tissues. Another example of biomaterial inks is the case of sacrificial materials which when interspersed with bioinks are subsequently washed away leaving clear spaces such as channels (ex.: the synthetic Pluronic F127, gelatin), as illustrated in Figure 6.

Therefore, the bioinks to be printed must meet certain requirements taking into account rheological (physicochemical) and biocompatibi–lity properties, such as: printability, biocompatibility, degradation and byproduct kinetics, structural and mechanical properties, and material biomimicry[50]. Moreover, the rheological properties of gelation and pre–servation of the three–dimensional post–impression structures must be in synergy with the biocompatibility properties, also allowing the growth, differentiation and cellular preservation of the dermis–epidermis layers in air–liquid interface (Figure 7).

Some important characteristics for a bioink are summarized in Table 1. Note that the requirements for obtaining a printable material are asso–ciated with the characteristics that it must have before and after printing.

Smart hydrogels and bioinks are emerging materials that act in res–ponse to external stimulus, such as temperature, pressure or pH, and have been developed to accompany the so–called 4D Bioprinting, where the Time variable is also considered. While bio–printing of functional organs is still a Herculean task to be achieved, the good news is that this is happening faster than we ever imagined. We already have scientific reports of orga

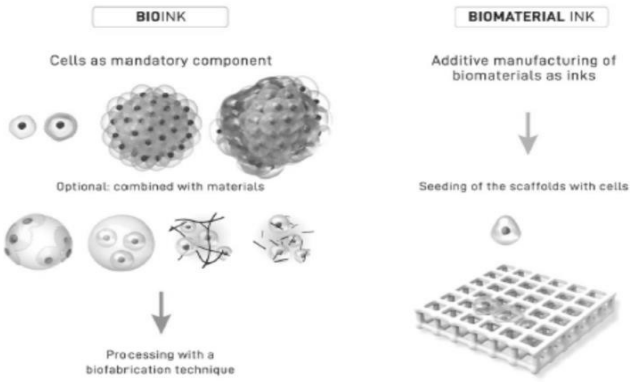


Figure 6 – Distinction between a bioink (left side), where cells are a mandatory component of the bioink formulation, loaded with individual cells, cell aggregates (spheroids), one or more cell types, and biomaterial ink (right side), where a bioma–terial is used for printing and the cells may or may not be seeded after manufacture [65] (Source: Groll et al., 2018).

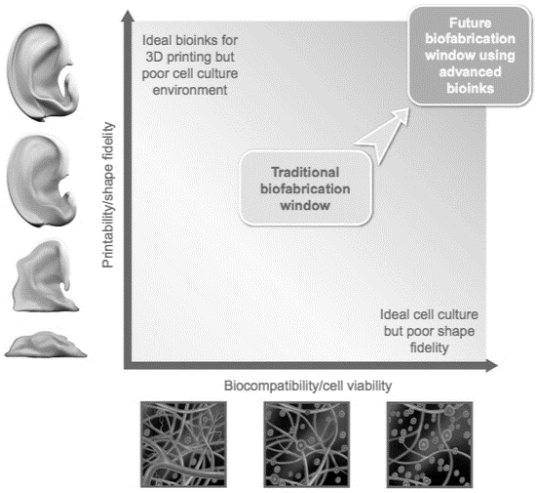


Figure 7 – The challenge for the development of new bioinks aimed at tissue reconstruction. Bioinks must have dual functionality: *rheological properties* that maintain the printed structure and *biocompatibility* that promote cell viability 66 (Source: Kyle et al., 2018).

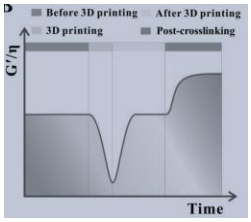
Bioink requirements	**Goal**	**Desired Value or Condition**	**References**
Biocompatibility	Must support cell viability	Cellular viability superior to 70%	(50,67,68)
Viscoelasticity (Fiber shape fidelity)	reproduce shear stress during the printing process, as increasing the shear rate reduces the viscosity by up to an order of magnitude	30 when over 6.10^7 mPa.s; 764 mPa.s for Nivea Cream, considered adequate for purpose	. (50,64,67,68)
Shear–thinning behavior (printability)	reproduce shear stress during the printing process, as increasing the shear rate reduces the viscosity by up to an order of magnitude	K = 26,1 e n = 0,552 for Nivea Cream	(50,64,67,68)
Storage Modulus (Temporary structure fidelity)	Increases the stability of the structure. It can be physical, chemical or a combination of both.		(50,64,68)
Gelation/crosslinking (Permanent structure fidelity)	Must be of the same order of magnitude as native skin.	Physical Crosslink (ionic) due to mild conditions	(50,64).
Crosslinking process	Increases the stability of the structure. It can be physical, chemical or a combination of both.	Thermal or chemical crosslinking, thrombin and calcium chloride	(69)
Biocompatibility	Must be Biocompatible	Cellular viability superior to 70%	(50)

Table 1 – Requirements of useful bioinks formulations for application in tissue engineering in bioprinting processes.

noids such as liver, heart, bladder, skin and bioprinted cartilage tissues[70].

Bioprinted Skin Models

The role of biomimetics is crucial for tissue engineering. Unraveling the secrets of nature and trying to mimic them, starting from the observation and understanding of their forms and biogenesis is the main challenge in this field[71]. Despite the traditional approach of simplifying highly complex structures, in nature, structures are complex and nano-and micro-metrically designed. Both industry and academia are driving the development of new approaches to human skin engineering and in vitro skin models for research, not just compartmentalizing it in the dermis and epidermis, using only two cell types, keratinocytes and fibroblasts, and 3D bioprinting technology is supporting this evolution.

Didactically, skin bioprinting can be divided into three major stages, preprocessing, processing and post- processing. These three steps are subdivided into five activity centers: 1) project image acquisition and 3D generation and modeling of a digital (.gcode) file, 2) bioink selection, 3) cell selection, 4) selection of the bioprinting technique to be used and, 5) maturation of this tissue in the time variable (Figure 8).

1) 3D modeling: Image preprocessing for bioprinting purposes is the phase in which the capture of the bioimaging and the representation of this image in 3D occurs, using CAD-compatible software (eg InVesalius, BioCAD software)[72]. If the intended application of the bioprinted skin is for wound healing and transplantation in humans or animals, it is necessary to know the type, size and depth of the wound. Capturing characteristics such as skin color and texture are often important. For cosmetic testing, this approach to complex designs is not required as a basic organotypic pattern for testing is more interesting. However, a database of various skin types (normal, dry, oily, mixed or sensitive), texture, age and color is relevant for skin models focused on product efficacy testing[73,74].

2) Bioink: Next is the stage of materials and bioinks selection, natural polymers, synthetic polymers or decellularized cell matrices. Natural polymers such as collagen gelatin, fibrin and chitosan resemble the native extracellular matrix and are more cell compatible, while synthetic polymers have better mechanical properties and help promote structural integrity.

3) Cell: Concomitant with the materials, we have the stage of cell selection, primary, from an immortalized lineage, autologous, heterologous or xenographic, this will depend on the application. To make a multilayer skin mimetic, it is necessary to use more than one cell type (fibroblasts, keratinocytes, melanocytes, mesenchymal cells)[73].

4) Printing: After the preprocessing steps are completed, we reach the processing step, when one of the three possibilities (ex.: micro-extrusion, inkjet or laser-based) of bioprinting is selected to achieve the best resolution, precision and required scalability.

5) Post-processing: Post-processing of the bioprinted construct involves maturation of the tissue in the time variable (currently known as the 4D dimension), in a greenhouse or bioreactor. Finally, the biomimetic skin is transplanted to a patient or used for drug or and cosmetic testing.

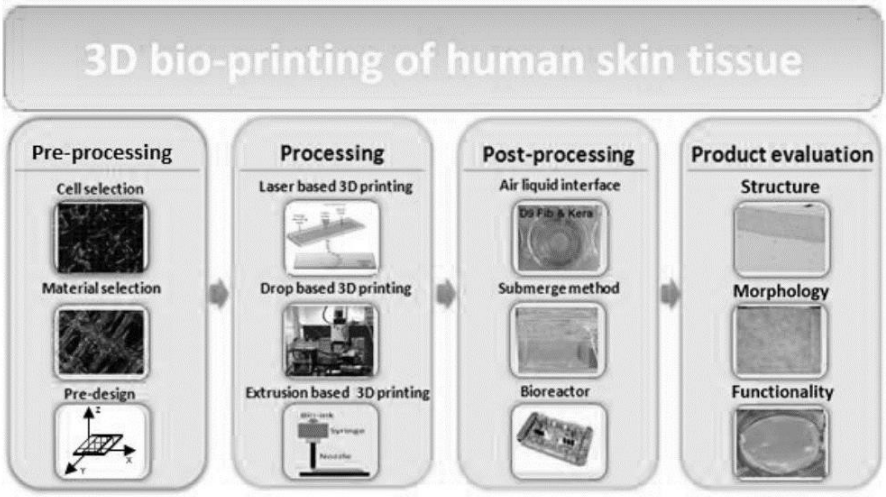


Figure 8 – Schematic of the overall 3D bioprinting process for human skin tissue. Preprocessing, processing and post-processing, which is divided into five main activities: 1) project image acquisition and 3D generation and modeling of a digital (.gcode) file, 2) bioink selection, 3) cell selection, 4) selection of the bioprinting technique to be used and, 5) maturation of this tissue in the time variable[19]. Source adapted from Wei-Cheng, 2018.

Bioprinted Skin Mimetics: State of the Art

About the state of the art of bioprinted biomimetic skin , mouse fibroblast (NIH3T3) and human immortalized keratinocyte (HaCat), well-established cell lines have been widely combined in studies to print 3D skin constructs [75,76]. In these researches were confirmed high viability of printed cells in hydrogel, secretion of collagen by the fibroblasts, and cytokeratin (CK14) expression of keratinocytes[77]. Collagen type I (from rat tail), the main extracellular matrix (ECM) protein in skin, was used as a bioink, embedding cells to print skin structures and approximating native skin as far as possible[78,79]. . Koch and collaborators generate dermis-epidermis structure with 20 layers of keratinocytes embedded in collagen printed by a Laser-assisted bioprinter on a sheet of Matriderm® (decellularized dermal matrix)[62], to generate dermis-epidermis structure. The researchers labeled the fibroblasts and keratinocytes using fluorescent cell membrane markers. In another study, dermal/epidermal-like distinctive layers were successfully printed by an extrusion printer with primary adult human dermal fibroblasts and primary adult human epidermal keratinocytes in a 3D hydrogel scaffold. Ten layers of type I collagen precursor (rat tail origin) were printed. These constructs were able to generate dermis and epidermis structures; however, the construct did not show tissue generation or the establishment of intercellular junction[78].

Abaci and collaborators built a 3D bioprinted vascular perfusion network from the generation of channels embedded in an alginate layer simulating the dermis[80]. Endothelial primary cells and induced pluripotent stem cells (IPS) were employed to obtain a permeable endothelial barrier. This model enables the study and systemic administration of medications, as well as being a developing platform for drug screening. Michael *et al.* (2013)[75], printed a laser bilayer skin and implanted the substitute in the skin fold of rats. In addition to dermis and epidermis formation, a small amount of neovascularization was observed at the site of the wound 11 days after implantation. Similarly, Cube and collaborators (2016)[81] used microextrusion bioprinting and implanted it in mice. They demonstrated that equivalent skin is similar to native skin in structural and functional terms.

However, despite these progresses in skin bioprinting, a scaffold-free model bioprinted with human primary skin cells is still not available.

Natura Skin Model Case

The company Natura Cosmetics in 2018 signed a research collaboration agreement with the Faculty of Pharmaceutical Sciences of the State University of São Paulo and since then it has been developing its own skin models using 3D bioprinting technology[74]. The company has not used animal models for efficacy and safety tests since 2006 and had been importing reconstructed models and mostly using the skin model developed by University of São Paulo at the laboratory of Prof. Silvya Stuchi Maria-Engler (USP / Sao Paulo).

To conduct this research, Natura is using the 3D bioprinting platform. The goal is to translate the technology used by USP of manually fabricated full thickness skin models to an automated model and biofabricated via 3D bioprinting [82,85]. The research uses a bioink composed of collagen Type I [6,9] alginate[86,87] gelatin[87] and fibrinogen[69,88] developed in conjunction with optimal printing conditions and 3D printer functions. Two bioprinters are being used in the research, the Inkredible model, from the Swedish company Cellink and the GenesisII™model from the Brazilian startup 3D Biotechnology Solutions – 3DBS. Both work as a microextrusion-based mechanism with two printheads, with the exception that Inkredible uses air pressure extrusion and Genesis uses mechanical pistons. They are an open source machine developed to fully comply with laboratory safety standards (Figure 9).

The bioink formulation has three purposes: (i) maintaining an appropriate gel rheology during the extrusion process, (ii) enabling the consolidation of the printed object during the post-processing step, and (iii) allowing the adequate development of the 3D cell network leading to a correct organization and function of the maturate tissue. These three functions are supported by the following biomaterials. Type I collagen, the main component of the dermal layer, is soluble in acidic pH. Gelatin, a collagen-based polymer, with a phase transition temperature at 35°C, is used as a rheological component giving the bioink its strength once printed on a cooled substrate but still being soluble and then eliminated in the subsequent steps of the process. Alginate, a carbohydrate-based polymer, with the ability to form hydrogel in the presence of calcium, is was used as a structural component giving the printed bioink mechanical stability once the gelatin solubilized[69]. Fibrinogen, a glycoprotein with the ability to form hydrogel was used both as a structural and a maturation component thanks to its cellular adhesion RGD pattern [89].

For the generation of a dermo-epidermal equivalent, the bioinks are loaded with primary cell lines, fibroblasts and keratinocytes and melanocytes, in the case of pigmented skin models. Once we have obtained the three-dimensional structure, the stratum corneum should meet the requirements of OECD Guide 439 [90] (Figure 10).

Initially, the ability to extrude the material is rheologically evaluated by evaluating its pseudoplasticity. Next, the printed structure should be stabilized immediately after printing using a material that does not collapse after deposition. This stability can also be assessed by material rheology, through viscosity and yield stress [67]. The crosslinking after printing is done due to the need to manipulate the printed structure without losing its geometry and to impart some mechanical resistance to the printed scaffold. Among the possible types of crosslinking, ionic is used because it has milder conditions during the process [46,50].

The study is focused on the demonstration of the capability of both 3D printers to produce full thickness skin equivalents in an effective way

Figura 9 – The two bioprinters used under the project to develop skin models to meet the cosmetic industry. A) Cellink, Inkredible model (Natura Cosmetics). Source: Cellink / EU. B) 3DBS, Genesis II model (Faculty of Pharmaceutical Sciences / USP). Source: 3DBS site/BR.

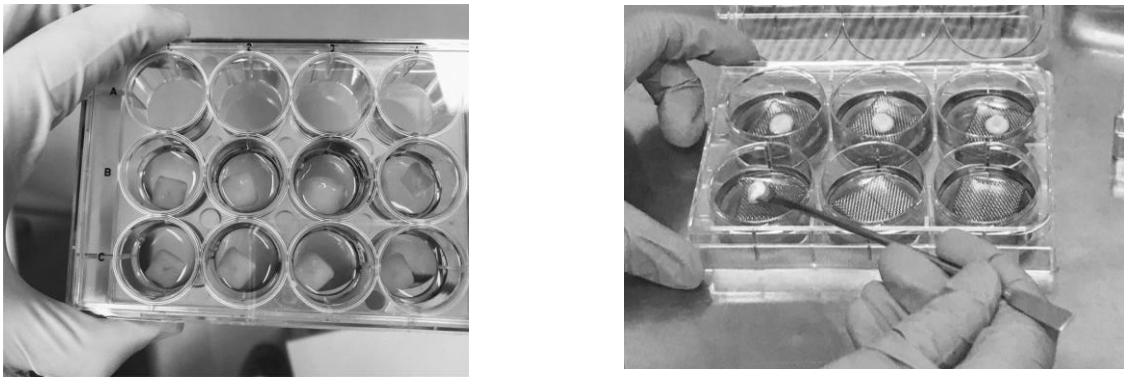


Figure 10 – a) Dermal-epidermal skin model printed prior to the passage to air-liquid surface (Source: Natura Cosmetics / 2019), b) Full thickness skin on an air-liquid surface (Lab. Prof. Silvya Maria-Engler Stucchi / USP).

and with a cellular architecture of the mature tissue highly similar to the *in vivo* skin composition and organization. The final aim of the developed technique is of course, the production of highly complex skin models.

Conclusions

Despite the advances, there are still challenges in the reconstruction of this organ, such as the inclusion of appendages, hair follicles, hypodermic layer, microvessels and immune cells. Recently, researchers at Rensselaer Polytechnic Institute/USA have developed a full thickness 3D print skin with microvessels [91], also, a group of scientists from Japan developed a 3D skin with hair follicles and sebaceous glands[92], a major leap in the Bioprinting field. Even so, more effort is needed to create functional skin with sufficient vascularity, innervation, and functions such as sensation of touch and perception. Reproducing the color and texture of native skin is another major challenge. Ex vivo skin is a valuable model for skin penetration studies but due to logistical and viability limitations the development of in vitro alternatives is required[93].

Now the challenge is to produce dry, oily skin with different texture, pigmented with different shades of European white, moderate Asian and dark African tones [93]. Scalability and accessibility are still two other obstacles to overcome. There are some ethical, social and legal challenges that need attention before the technology and product can be successful.

On a timescale, the bioprinted skin mimetics will follow logic of market regulation and maturation:

•Cosmetics Industry: Cosmetic companies would first test their products on skin models as an alternative to animal testing;

•Pharmaceutical / Chemical Industry: Pharmaceutical industries will test their medicines and chemical products using these *in vitro* skin models (microfluidic systems);

•Organ Transplantation: treatment of burns and injuries using bioprinted skins; cells taken from the patient himself (autologus) will be used in this bioprinting process;

•*In vitro* 3D tumor models: Tumor models with tumor microenvironment designed to study the *modus operandi* of cancer proliferation, metastasis and response to drugs; and

•Precision Medicine: With the 3D skin / tumor model, the effectiveness of the medicine can be studied for each patient and thus help in personalized medicine.

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