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DIFFERENT TYPES OF 3D BIOPRINTING TECHNIQUES

Bioprinting skin

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ABSTRACT

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After proving its worth in multiple fields, three-dimensional printing is now bringing new possibilities into the field of regenerative medicine. This thesis presents the three most common three-dimensional bioprinting methods available and their role in the latest research regarding skin regeneration. The methods presented are extrusion-based bioprinting, inkjet-based bioprinting, and laser-assisted bioprinting. At first, a brief overview of history, skin physiology, and bioinks are presented. The printing methods are then described in detail with their working principles and the latest research on skin printing.

Human skin is made up of three layers, each with its own function. This layered structure can be considered as the blueprint for the printing of the skin. Different materials and methods can be used to mimic the functionalities of each layer. For example, keratinocytes are very often used to mimic the epidermis and fibroblasts are used to mimic the epidermis. Often, these cells are combined with a hydrogel to form a printable material.

In this thesis, it is presented that extrusion-based method has an edge over the other printing methods due to its versatility, usability, and affordable price. The final product of extrusion-based printing consists of continuous filaments, while both the inkjet-based and laser-assisted methods, consists of bioink droplets instead. The most essential difference between the inkjet-based and laser-assisted methods is the much easier usability and lower cost of the inkjet-based method. Laser-assisted printing, on the other hand, does not have a nozzle, which means that the cells inside a bioink do not experience the forces exerted by inner walls of the nozzle.

Research on extrusion-based printing has currently achieved the most promising results in skin printing, and for all methods, progress has been rapid over the past years. However, none of the methods has yet reached the clinical stage.

Keywords: 3D bioprinting, regenerative medicine, skin

TIIVISTELMÄ

Tuulia Laakso: Erilaiset biomateriaalien 3D-tulostusmenetelmät
Kandidaatintyö
Tampereen yliopisto
Lääketieteen biotekniikka
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Kuten monilla muillakin teollisuuden aloilla, myös lääketieteessä kolmiulotteinen tulostaminen tuo uusia mahdollisuuksia. Tämä opinnäytetyö esittelee kolme yleisintä kolmiulotteista biotulostusmenetelmää ja viimeisimmän tutkimustyön ihon tulostamiseksi näillä metodeilla. Esiteltävät metodit ovat pursotukseen perustuva tulostaminen, inkjet-pohjainen tulostaminen ja laser-avusteinen tulostaminen. Työn alussa esitellään aiheen kannalta oleelliset tiedot historiasta, ihon rakenteesta ja toiminnasta ja biomusteista. Tulostusmetodeista esitetään niiden toimintaperiaate ja viimeisin tutkimus ihon tulostamiseksi.

Ihmisen iho muodostuu kolmesta kerroksesta, joilla jokaisella on oma tehtävänsä. Tämä kerrosrakenne voidaan ottaa huomioon ihon tulostamisessa käyttämällä erilaisia materiaaleja ja metodeja eri kerrosten toiminnallisuuksien jäljittelemiseen. Esimerkiksi epidermiksen jäljittelyssä käytetään usein keratinosyyttejä ja epidermiksen jäljittelyssä fibroblasteja. Usein nämä solut on yhdistetty hydrogeelin kanssa biomusteeksi tulostettavan koostumuksen aikaansaamiseksi.

Tämä opinnäytetyö osoittaa, että pursotukseen perustuvan menetelmän etuna on sen monipuolisuus, helppokäyttöisyys ja edullinen hinta. Pursottamalla tuotetut tulosteet muodostuvat jatkuvasta biomustenauhasta. Inkjet-pohjaisessa ja laser-avusteisessa menetelmässä lopullinen tuote muodostuu jatkuvan nauhan sijaan biomustepisaroista. Inkjet-pohjaisen ja laser-avusteisen metodin oleellinen ero on inkjet-pohjaisen menetelmän huomattavasti helpompi käytettävyys ja alhaisempi hinta. Laser-avusteisessa tulostamisessa ei puolestaan ole kahden muun metodin tapaan suutinta, jonka paine vaikuttaisi negatiivisesti biomusteen soluihin.

Pursotukseen perustuvan tulostamisen tutkimus on tällä hetkellä saavuttanut lupaavimmat tulokset ihon tulostamisessa, ja kaikkien metodien osalta kehitys on ollut nopeaa viime vuosina. Yksikään menetelmistä ei kuitenkaan vielä ole edennyt kliiniseen vaiheeseen.

Avainsanat: 3D biotulostus, iho

CONTENTS

LIST OF ABBREVIATIONS

3D	Three-dimensional
CAD	Computer-aided design
dECM	Decellularized extracellular matrix
DNA	Deoxyribonucleic acid
DOD	Droplet on demand
EBB	Extrusion-based bioprinting
ECM	Extracellular matrix
HP	Hewlett-Packard
IJB	Inkjet-based bioprinting
LAB	Laser-assisted bioprinting
Laser	Light Amplification by Stimulated Emission of Radiation
LIFT	Laser-induced forward transfer
RP	Rapid prototyping
TE	Tissue engineering
TU	Tampere University
URL	Uniform Resource Locator
USA	United States of the America
USD	United States dollar
UV	Ultraviolet

TERMINOLOGY

allograph	A transplant from a donor site on an individual to a recipient site on a different individual.
autograft	A transplant from a donor site on an individual to a different recipient site on the same individual.
bioplotting	A fabrication method similar but not identical to bioprinting, where a plotting medium is always used.
crosslinking	A process in which covalent or ionic bonds are formed between polymers. This leads to changes in the material's mechanical and/or chemical properties.
in situ	A Latin term for "on site", which in skin bioprinting means mainly printing directly onto the wound.
resolution	An image quality factor, that is measured by the number of pixels in a certain area and affects the amount of detail visible on the picture.
scaffold	A strategically shaped growing base for cells that can be used to grow different three-dimensional tissue types.
shear-thinning	A property of non-Newtonian liquid where its viscosity decreases under shear stress. For example, whipped cream is easy to extrude, but stays in shape when not under pressure.
viscosity	A measure of a liquid's resistance to flow. The higher the viscosity, thicker the substance and higher its resistance to flow.

1. INTRODUCTION

Three-dimensional (3D) printing has changed the manufacturing industry. Today it is possible to ship plastic or metal spare parts to the other side of the world not only by air or by land, but by internet. A file could travel at the speed of light and be 3D-printed right where and when it is needed. [1] Advances in 3D printing present many new opportunities for tissue engineering (TE) in the form of 3D bioprinting applications. The principle described above could be applied for organ transplants in the future if 3D bioprinting lives up to the wild expectations.

A problem that could be solved by bioprinting in the future is that there is a worldwide severe lack of donor organs, including skin, in the healthcare sector. In the United States of America alone, 20 people who could have been saved by an organ transplant die each day and over 100,000 people who could benefit from a transplant are in waiting lists for a new organ. [2] 3D bioprinting could be the solution for the lack of replacing tissue, as bioprinted skin substitutes seem promising and would be beneficial especially for challenging cases such as large burns. [3]

The main benefits of additive manufacturing like 3D bioprinting instead of other fabrication methods are the ability to create unique and complex shapes significantly more affordable than before, as no moulds are needed. Bioprinting can be done using the recipient's own cells, so that the benefits of an autograft are enjoyed. On the other hand, the use of biomaterials makes it impossible to utilize high temperatures and toxic chemicals that are typically used in 3D printing.

The second chapter of this thesis will describe the history and evolution of 3D bioprinting from the first trials of additive manufacturing to this day. After this in chapter two, the differences between 3D printing and 3D bioprinting in tissue engineering are discussed. The benefits of the bioprinting approach are also presented. In Chapter three the physiology of skin is presented. Chapter four will summarize the most essential properties of bioinks and commonly used materials on printing. In chapter five, the three highlighted 3D bioprinting methods of this thesis will be discussed. These methods are extrusion-based bioprinting (EBB), inkjet bioprinting (IJB) and laser-assisted bioprinting (LAB). The chapters about operating principles are followed by the latest advances in skin tissue regeneration using the respective printing technique. Special focus is placed on how

each method could be utilised on printing skin to repair lost tissue. The conclusion section in chapter six highlights the most important aspects of each bioprinting technique for skin tissue regeneration.

2. BACKGROUND

This chapter will present a short look into the history of 3D bioprinting from the first experiments with additive manufacturing to this day. After that there follows a comparison of 3D bioprinting and 3D printing of non-living materials for TE applications.

2.1 History of 3D bioprinting

The first methods for 3D printing of non-biological materials were based on stereolithography and developed simultaneously by several groups. One of the first systems was created in 1981 by Hideo Kodama, whose goal was to develop methods for rapid prototyping (RP). This method used light to polymerize a resin to a desired shape. [4]

The first 3D printed organ implant was made in 1999, when a 3D printed polymer bladder, was coated with the recipient's cells after printing. The bladder functioned for the 11 months period of the follow up. [5] The wish to include biological particles into the printing process presented new challenges for 3D printing and created the field of 3D bioprinting. Some of the first bioprinting experiments were done with a modified inkjet printer, and in 2003 it was capable of dispersing viable cells. Inkjet-based bioprinting was then patented by Thomas Boland. [6]

The first commercially available bioprinter was NovoGen MMX, which reached the market in 2009. This device was marketed to be able to print mainly simple structures like veins and nerves. It could apply one type of cell and one supporting substance. [7]

In 2009, skin with both epidermal and dermal layers was bioprinted for the first time by Wonhye Lee et. al. This experiment was encouraging as it suggested that bioprinted skin could be used for tailored wound treating and drug testing. [8]

In 2012 Nieves Cubo et. al. tested a technology to print skin cells directly to the wound of mice. This in situ method was too labor intensive for any large-scale applications but proves that bioprinting in situ can significantly improve wound healing. [9]

The final goal for 3D bioprinting is often considered to be the printing of new organs, from the patient's own cells. This would solve the problem of lack of organ donations and the risk of rejection after a transplant. The latest advancement is not there yet, but the work continues.

2.2 Differences between 3D bioprinting and 3D printing

Both 3D printing and 3D bioprinting are used in tissue engineering applications. The main difference between these two methods is that in bioprinting, the “ink” includes biological particles like living cells or DNA and so it must be biocompatible [10]. These biological particles are often combined with a hydrogel, that provides a beneficial environment for the active components in the bioink and better mechanical properties for printing [11]. In 3D printing the bioactive ingredients are added to the product after the printing process.

Where other tissue engineering applications can create tissue with scaffolding techniques, with bioprinting it’s possible to create similar structures without the need for a scaffold. 3D printing can be used to print such scaffolds, but 3D bioprinting enables the fabrication of structures that are both biocompatible and have a decreased manufacturing time. Smaller manufacturing time can be crucial when the technology is used to treat acute wounds.

With traditional scaffolding methods, it’s challenging to control the inner structure of the material. 3D bioprinting gives more control and allows for exact placement of different cell types at the required sites.

3. PHYSIOLOGY OF SKIN

Skin is the largest human organ when measured with weight. It is composed up of three main layers, that each have their own purpose and hence a unique physiology. The outermost layer is epidermis, followed by dermis and hypodermis, respectively. [12] The layers can be seen in the Figure 1 below.

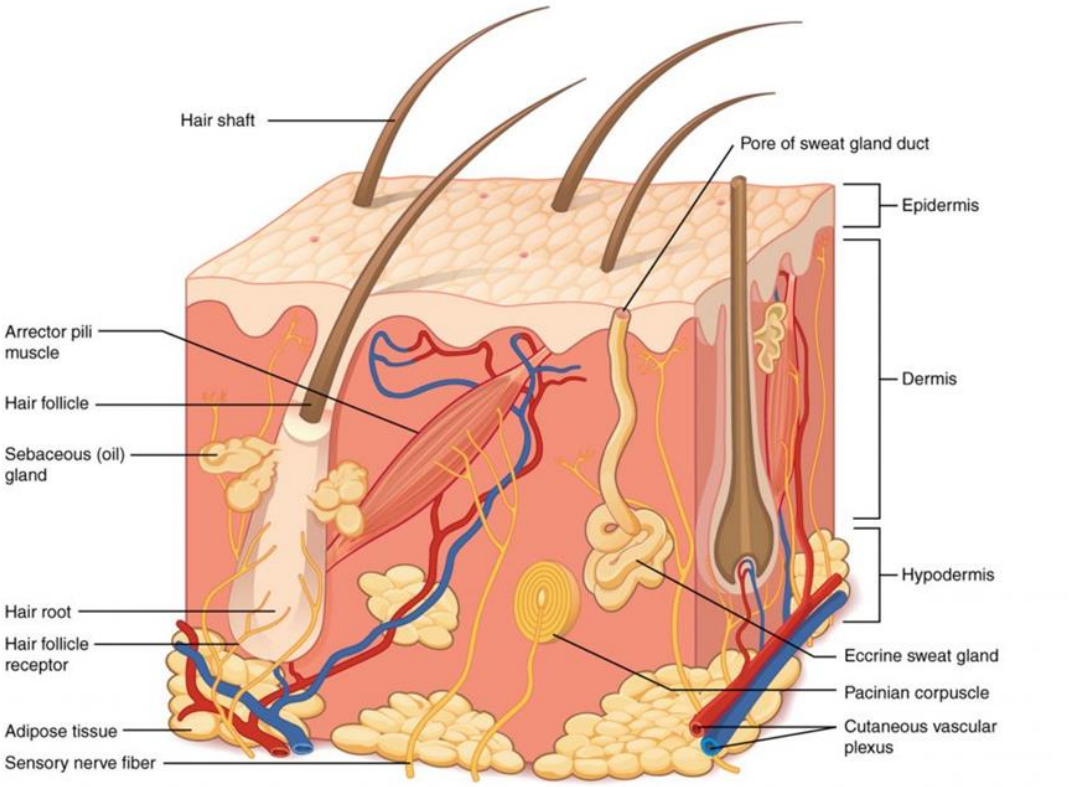

Figure 1. *The anatomy of the human skin.* [13]

Each skin layer is described in detail in the following sub sections 3.1- 3.3.

Some of the most common diseases and injuries that threaten skin are cancers and burns. The most dangerous version of skin cancer, melanoma, can be caused by UV-radiation over long exposure time. Burns are caused by heat, radiation, and corrosive chemicals. [14]

Although the human body can repair damaged skin, the remaining scar tissue can cause problems and might need further treatment [15]. Also, properties like elasticity and color of the skin depend on the person's age and family history [13]. This can affect the overall treatment of the wound further justifying the importance of patient specific methods.

3.1 Epidermis

As the surface layer, epidermis has an important role in protecting the deeper structures of the body from external hazards like UV-radiation, pathogens, and chemicals [12], [13], [14]. Melanocytes in epidermis produce the melanin pigment, which determines the color of the skin and protects from UV-radiation [12]. The protection against pathogens and chemicals results from the tight junctions between the surface layer cells [12].

Epidermis consists of at least 80% keratinocytes [12], [13]. As they are a central part of this skin layer, many bioprinting applications utilise keratinocytes to replicate the epidermis [16]. Epidermis has four or five different cell layers; the amount is determined by the specific body parts. Depending on where the keratinocyte cell is present on the layers, it will have a different function. The outermost layer lacks vascularization and consists of dead cells that serve as a protective layer for the living cells underneath. [14]

As the skin wears off, the keratinocytes move towards the surface layer and differentiate into corneocytes. During the differentiation process, they store keratin. [12] Keratin is a protein found inside the cells in the skin, nails, and hair. It hardens the tissue and makes it water resistant. [14] After the differentiation is complete, the cells go through self-induced cell death called apoptosis. This way the epidermis is constantly regenerating. [12]

3.2 Dermis

Dermis is the thickest layer in skin. It is responsible for the mechanical properties including elasticity and strength of the skin along with many other functions [12]. The dermis has a high amount of connective tissue, extracellular matrix (ECM), and structures like blood vessels, nerve endings, hair follicles and sweat glances [13], [14]. These structures are rarely considered in the bioprinting process due to challenges [17].

One of the main cell types found in dermis are fibroblasts. They produce connective tissue like ECM, and collagen. Different forms of collagen make for 70% of the total weight of the skin, excluding water. Collagen has a high resistance to tearing. [12] The other important component in dermis is elastin, which is a fibrous structure and as its name suggests, it provides elasticity. [14] Both fibroblasts and collagen are regularly used in bioprinting [16].

In addition to each cell type having a specialized function, the cooperation of different cell types is also crucial for the fabrication of a healthy skin tissue. For example, keratinocytes encourage fibroblasts to produce several important growth factors, including keratinocyte growth factors and fibroblast growth factors. [17]

3.3 Hypodermis

Hypodermis, also referred as subcutaneous tissue, consists mainly of adipose tissue. It can be seen more as a connective layer between the skin and the fascia and muscles below, than a layer of the skin itself. It is vital to the function of the upper layers and provides vascularization for them. Adipose tissue can also be bioprinted [18].

The main purpose of this layer is to work as an energy storage. This layer also helps in temperature control as an insulator and provides protective padding for the body. Hypodermis consists mostly of fibroblasts like the dermis, and macrophages and adipose cells. [13]

The thickness of hypodermis varies in the body and between individuals and genders. In general, it is thinnest on the limbs and thickest around the hips and abdomen. [19] The thickness of the hypodermis has a significant impact on the individual’s appearance [14].

4. BIOINKS

The term bioink refers to the printable substance in bioprinting, which contains cells or other biological parts. Better bioinks need to be developed hand in hand with the bioprinting methods, as traditional 3D-printing inks and many materials developed for TE are not suitable for the bioprinting applications [10]. In the section 4.1, the necessary properties for a bioink are presented and in the section 4.2 the most common constituents for different types of 3D bioprinting are presented.

4.1 Bioink properties

When considering a component or compound for bioprinting, its properties should be thoroughly assessed. Meaningful properties include the following:

- biomimicry,
- resolution,
- affordability,
- scalability,
- practicality,
- mechanical and structural integrity,
- maturation time,
- degradability,
- commercial availability,
- and immunogenicity. [10]

Although, the importance of each property depends on the bioprinting method, some common demands for the bioink like rapid gelation, low to medium surface tension, and appropriate interactions are required by all bioprinting techniques. [10]

In EBB, shear thinning is the most crucial property of the bioink. It is necessary to reduce the shear stress exerted by the walls of the nozzle on the bioink during extrusion. Hence, shear-thinning hydrogels lead to increased cell viability by providing cushion to the cells during extrusion. Moreover, the bioink should also have low surface tension and adhesion. [10]

In IJB, the bioink needs to have low viscosity which should be able to support the formation of repeatable droplets. Due to this the bioink needs to be non-fibrous in nature.

Similarly, bioinks used in LAB also need to support repeatable droplet formation and be non-fibrous. In LAB, It is also important that the bioink can form an even film below an absorbing layer, that is used in the printing process as described in section 5.3.1. [10]

4.2 Common constituents

Hydrogels are a group of natural and synthetic crosslinking polymeric materials. They bind large amounts of water, and therefore water is the base of many bioinks. In general, synthetic hydrogels have a stronger mechanical structure, while natural hydrogels are biocompatible.

Cell-laden hydrogels are classified as scaffold-based bioinks. This means that the hydrogel operates as a scaffold, or as a favourable environment, for the cells and other biological particles, that bring the bioink most of its active properties. Cell-laden hydrogels are widely used in all three bioprinting methods. [10] Popular hydrogels include fibrin, gelatin, and alginate [10], [17]. Applications that include the pre-state of fibrin called fibrinogen are in average slightly more advanced than the ones without it. [20]

With the other constituents, one possible ingredient in a bioink is collagen, as it is found in all skin layers. It has been repeatedly used for skin based TE applications for a long time. This means that its biocompatibility is well tested, and it is easily available. [17]

A literature review conducted in 2017 showed that, keratinocytes and fibroblasts combined with collagen were most used active ingredients in bioinks for skin printing. [16] Figure 2 below presents how these cells have been organized in a 3D-printed skin model in an other research experiment [21].

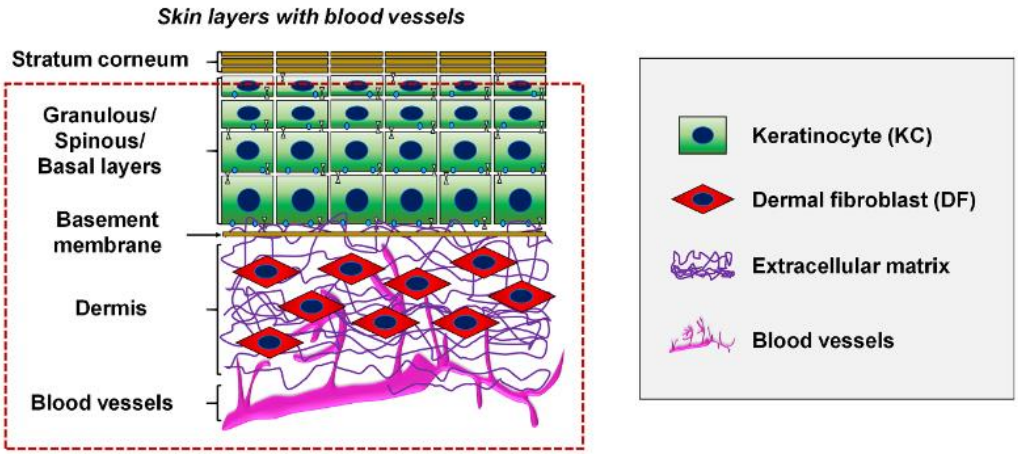

Figure 2. *The cellural structure of a 3D-bioprinted skin model.* [21]

A major problem in many TE fabrication methods is the lack of vascularization in the crafted tissue. A promising way to mimic the complexity of skin is to use decellularized

extracellular matrix (dECM) as part of the bioink [22]. The removal of cells deletes the problem with immune rejection when the dECM is an allograft [20]. Human pericytes in the bioink have shown to improve the vascularization [23]. Also, the use of human stem cells is on the rise and could provide solutions for better vascularization [16].

Besides the constituents in the bioink, the postprocessing can alter the properties of the final product. In a promising study made by Forough Hafezi et al. the crosslinking method of a bioink was altered to get higher cell viability. This bioink consisted of a polysaccharide chitosan, and genipin. By optimizing the bioink, it was possible to decrease the printing pressure, which lead to a promising cell viability of 93%. In this study, the chitosan-genipin bioink was combined with fibroblasts and dermal keratinocytes. [24]

5. COMMON 3D BIOPRINTING TECHNIQUES

The most used 3D bioprinting methods can be classified by their working principle into extrusion-based bioprinting, inkjet-based bioprinting and laser-assisted bioprinting [10]. Additionally, these three methods can be divided into subtypes based on their more detailed working principles. In EBB, the bioink can be extruded out of the nozzle onto the stage by air pressure or mechanically. In IJB, the droplet is separated from the main ink reservoir by forming an air bubble either thermally or piezoelectrically. Laser-assisted bioprinting is always based on the laser beam.

In the last 5 years, research on extrusion-based bioprinting has been growing rapidly while laser-assisted bioprinting has been slowly decreasing and inkjet has not experienced any significant changes in its popularity. This can be seen in the Figure 3 below. The graph also shows that the research around 3D bioprinting in general started after the year 2000.

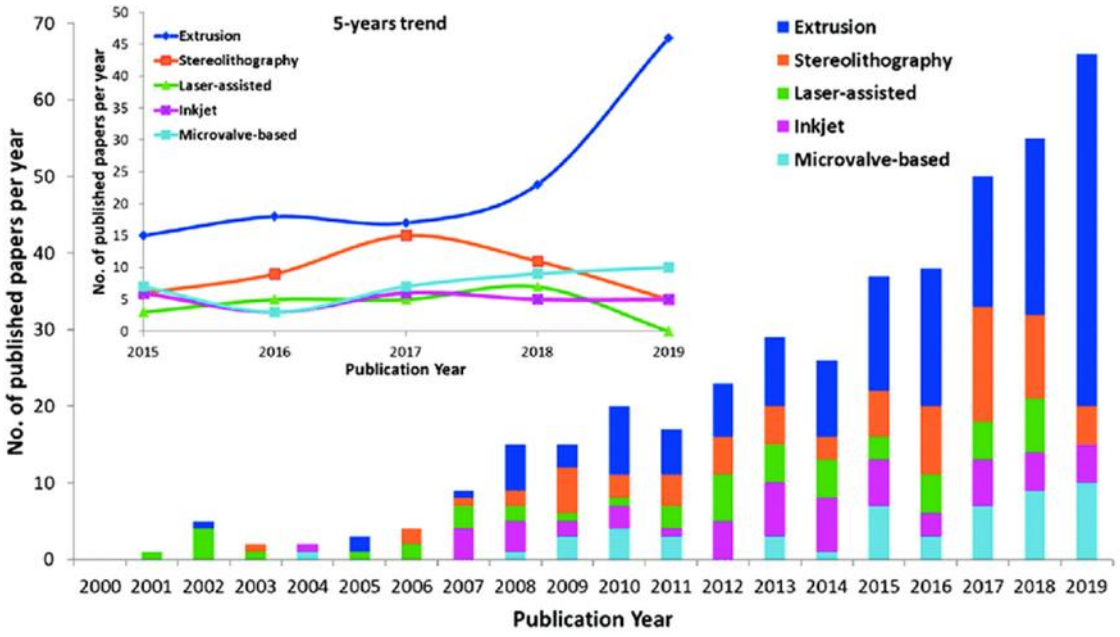

Figure 3. *Publication trend of different 3D bioprinting techniques shows the growth and popularity of EBB compared to other modalities* [25].

All 3D bioprinting techniques share some similarities such as each can be divided into three phases; preprocessing, processing, which is the printing part itself, and postprocessing [10]. All these methods work on a layer-by-layer principle, where two-dimensional planes are stacked to form a three-dimensional shape.

When considering the whole fabrication path of a 3D bioprinted object, at first, the model of the final product is prepared using computer-aided design (CAD). At this part, medical

imaging techniques can be used to map out the shape of the wound site. The operating mechanism in the processing phase is different for each type of printing method which are discussed in detail later in this chapter. Finally, the printed product undergoes post printing treatment including incubation or cell seeding, which depends on the end application. In this phase, the printed cells can form bonds and incubate to form a solid construct. [10]

5.1 Extrusion-based bioprinting

The commercial supply for extrusion-based bioprinters is larger than for laser- or inkjet-bioprinters. As stated in the previous chapter, EBB is also the most utilised method in research [25]. This could be because extrusion-based bioprinters are more versatile and user-friendly, and significantly more affordable than laser-assisted printers [10]. Inkjet-printers on the other hand can be self-made with moderate effort [26].

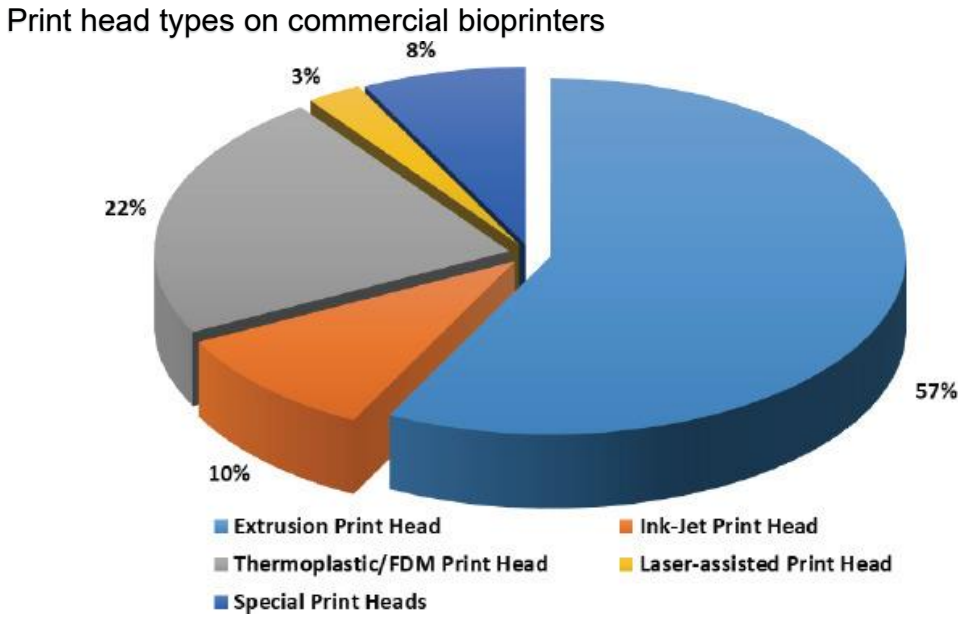

Figure 4. *Most commercial bioprinters in 2018 were based on EBB. Modified from* [27].

Some commercially available EBB-devices include EnvisionTEC, which has a line of three printers, the first one being available since 2000. This USA-based company states that their popularity among researchers is due to its user-friendliness and flexibility of the printer, which is in line with the general pros of extrusion-based bioprinting. The simplest model of EnvisionTEC's printers is shown in the Figure 5 below [28]. Another popular

provider for extrusion-based bioprinters is the Swedish Cellink, which has numerous bioprinters and provides bioinks and accessories all well [29].

Figure 5. *An extrusion-based bioprinter manufactured by EnvisionTEC* [28].

In the chapter 5.1.1, the general working principles of mechanical and pneumatic extrusion-based methods will be presented. The chapter 5.1.2 will present the research around skin printing with extrusion-based bioprinters and will focus on in situ -bioprinting and the biomimicking approach.

5.1.1 Operating principles of extrusion-based bioprinters

In extrusion-based bioprinting, the bioink is extruded through a nozzle tip by pressure. The bioink extrudes as a continuous filament and is printed layer-by-layer on top of a substrate placed on the stage. Depending on the sub-type of EBB, the pressure can be created mechanically or pneumatically. Mechanical methods include screw- and piston-based extrusion. [10][30] The Figure 6 below shows three sub-types of extrusion-based bioprinting.

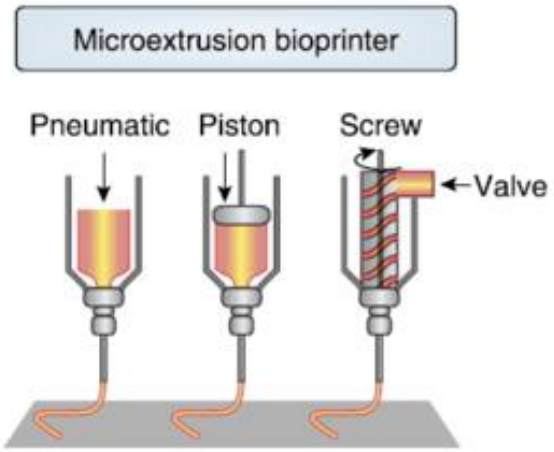

Figure 6. *The bioink can be extruded by pneumatic pressure, a piston or by turning a screw.* [11]

In EBB the flow of the bioink is controlled by the pressure, a valve (if one is used), the inner diameter of the nozzle and its shape, and the distance of the nozzle from the stage. With very low viscosity bioinks, the flow out of the nozzle can happen even without added pressure. High viscosity bioinks on the other hand can stick to the nozzle and block it without reaching the stage. [10]

Printers with mechanical working principle are simpler compared to pressure-based printers and thus more affordable and transportable. When pneumatic pressure is used, the need for an air compressor and purification adds more complexity to the system. However, mechanical methods are better for near-solid bioinks. [10]

Pneumatic printing can be done with or without a valve. Whether one should be used depends largely on the viscosity of the bioink. A valve increases the precision but adds more complexity to the system. In general, low viscosity bioink behave better with a valve as it allows better control over the flow of the bioink. [10]

Due to the continuous filament structure, EBB has greater structural strength compared to other methods, where the bioink is printed as droplets. The type of the bioink plays a big role in the EBB system, because different phenomenon can be utilised to crosslink and thus stabilise the printed layer. The crosslinking can be done using heat, light or added chemical crosslinking substance. If a chemical substance is used, it can be bought to contact with the bioink in several different ways such as crosslinking bath, aerosol spray or core-shell morphology. [10]

5.1.2 Extrusion-based bioprinting of skin

Extrusion-based bioprinting is used in many skin printing applications. Due to its ability to print substances ranging from low to high elasticity, EBB can also support higher cell densities compared to inkjet or laser-assisted bioprinting. The variety of nozzle tip designs also adds to the versatility of this method and allows for different methods of crosslinking. [10]

Due to its good usability and portability, EBB is a preferred method for in situ bioprinting. Extrusion-based bioprinters can be made into handheld machines, as presented in the Figure 7 below. This method requires the bioink to crosslink rapidly, so that the movement of the patient's breathing and contact with wound dressings do not damage the printed layers. [31]

Figure 7. *A handheld bioprinter in use on a porcupine.* [31]

In 2020, an overview of the recent research on EBB of skin was constructed. In that study it was noted that in 61% of the studies only one type of cell was used. Most often that was dermal fibroblasts. This is not enough considering the important roles and cooperation mechanisms of the different cell types in skin, as discussed in chapter 3. The remaining 39% of the cases combined a wide variety of materials including fibrinogen, decellurized extracellular matrix (dECM), collagen, alginate and gelatin. [20]

Extrusion based bioprinting is a balancing act between the material's structural strength and suitability for cell growth. Vascularization and perfusability are necessary properties for the skin tissue, and how to archive them is a major challenge in TE that still needs more research. [32] Despite the struggles some articles do report success in creating all

the necessary skin layers [23], [33], [34]. Most of these experiments seem to be made on nude mice.

One of the big downsides in EBB is its poor resolution compared to IJB and LAB [30]. The best accuracy with this method is still 100 micrometres in diameter [10].

In all methods that use a nozzle, the shear stress near the nozzle tip causes loss of living cells. This can be reduced when the bioink has a significant amount of shear-thinning, but it also makes the use of non-shear-thinning substances impossible. Even though EBB is quite fast, the printing of usable-sized skin pieces takes so long that the number of living cells decreases [10].

5.2 Inkjet-based bioprinting

Inkjet-based bioprinting was one of the first methods used in bioprinting. It falls under the umbrella of different droplet-based bioprinting methods. Figure 8 below presents the starting point and the final product in creating a 3D bioprinter from a HP inkjet printer. The pictures are from an open community project, in which free instructions were created to guide anyone through the modification process [26].

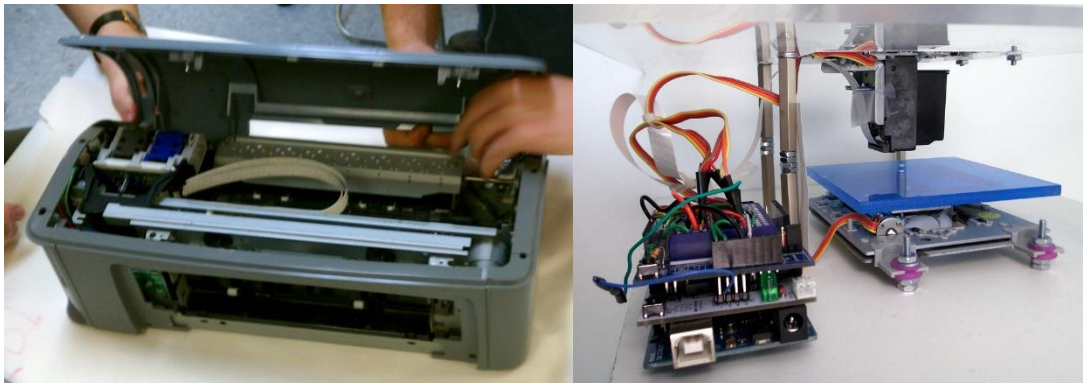

Figure 8. *On the left an unaltered inkjet-printer ready to be modified and on the right a finished 3D-bioprinter. Modified from* [26].

Besides the do-it-yourself -inkjet-printer, droplet-based bioprinters are also available commercially. They include the RASTUM by Inventia and a mixed-method printer from Regenhu. Inventia specifies on their website, that the printer supports printing skin cells like dermal fibroblasts [35]. Regenhu combines the benefits of extrusion-based and droplet-based printers in their device, which is also optimized for printing skin among other tissues [36].

The chapter 5.2.1 will describe the operating principles of inkjet-printers. In the chapter 5.2.2 it is discussed how the non-contact nature of inkjet-based printing can be beneficial to the printing of skin, especially in situ.

5.2.1 Operating principles of inkjet-based bioprinters

Inkjet-based bioprinting is a form of droplet-based bioprinting, in which the bioink is dropped on the stage in small portions. This working principle is the same as in some ink cartridge office printers, and those can be easily modified for bioprinting [32], [37].

In IJB, different methods can be used to separate the droplet from the main reservoir of bioink. These methods include the use of a heating element or piezoelectric material [10]. The thermal and piezoelectric systems are presented in the Figure 9 below.

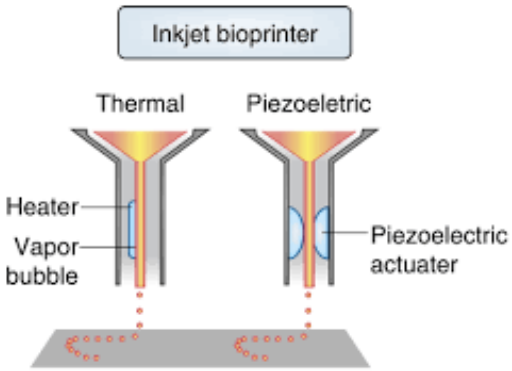

Figure 9. *The thermal and piezoelectric methods differ in how the droplet is separated from the ink reservoir.* [11]

If a heating element is used, the rapid rise in temperature will create a vapor bubble at the nozzle tip. This bubble will separate the droplet from the main reservoir and push it out. With piezoelectric printheads, there is a piece of material that will increase in size when voltage through it changes. This increase in size will cause the same result as the vapour bubble that is formed with heat. Thermal and piezoelectric methods do not differ from each other in any other ways. [10]

A unique feature of IJB is that several different materials can easily be printed at once. For example, with a modified HP printer, there are four different bioinks, as the printer has separate cartridges for black, magenta, yellow and cyan. Also, one advantage of droplet-based methods compared to EBB is that there is no contact needed between the printer and the stage. Additionally, IJB allows for very high precision printing compared to EBB. [10]

5.2.2 Inkjet-based bioprinting of skin

In inkjet and extrusion-based bioprinters, clogging of the nozzle tip can be a problem. One way this is solved in IJB is the use of a separate ink reservoirs and printing nozzle.

This way, if the nozzle fails, the whole reservoir of valuable cells is not lost. This also allows for the same nozzle tip to print from different ink reservoirs. [37]

In 2019 Mohammed Albanna et al. conducted a small experiment on nude mice where they showed that the wounds that were treated with in vivo bioprinting healed in three weeks whereas, the controls took five weeks to heal. [38] This can be considered a significant result.

5.3 Laser-assisted bioprinting

Laser-assisted bioprinting has been a popular method for creating two-dimensional cell patterns, because of its great resolution and high cell density. The development of laser-assisted 3D methods is, however, hindered by the method's slow speed, limited bioink selection, high cost and homogeneity. [10]

Laser-assisted bioprinters add up to a total of 3% of all available bioprinters in the market [27]. As stated by one manufacturer, Poietis, their model NGB-R is the first commercially available laser-assisted bioprinter. The device shown in the **Virhe. Viitteen lähdettä ei löytynyt.** combines LAB with EBB and IJB. [39]

Figure 10. *A 3D bioprinter based on laser-assisted method is bulky and non-transportable compared to extrusion or inkjet-based printers.* [40]

As shown in the figure, compared to devices made for EBB or IJB, NGB-R requires a significant amount of space.

The chapter 5.3.1 will present the working principles of one laser-based bioprinting method, laser-assisted forward transfer (LIFT). In chapter 5.3.2 the recent progress in the field of bioprinting skin with this method will be presented.

5.3.1 Operating principles of laser-assisted bioprinters

In laser-assisted bioprinting, the bioink is stored in a donor slide. On top of the donor slide and in direct contact with it is an energy absorbing layer, which can be made from a metal such as gold. When a strictly controlled laser is pulsated into the absorbing layer, the shockwave from the laser separates a droplet of bioink from the donor slide. The droplet then falls to the collector slide due to gravity. This operating principle is presented in the Figure 11 below and is referred as laser-induced forwards transfer (LIFT).

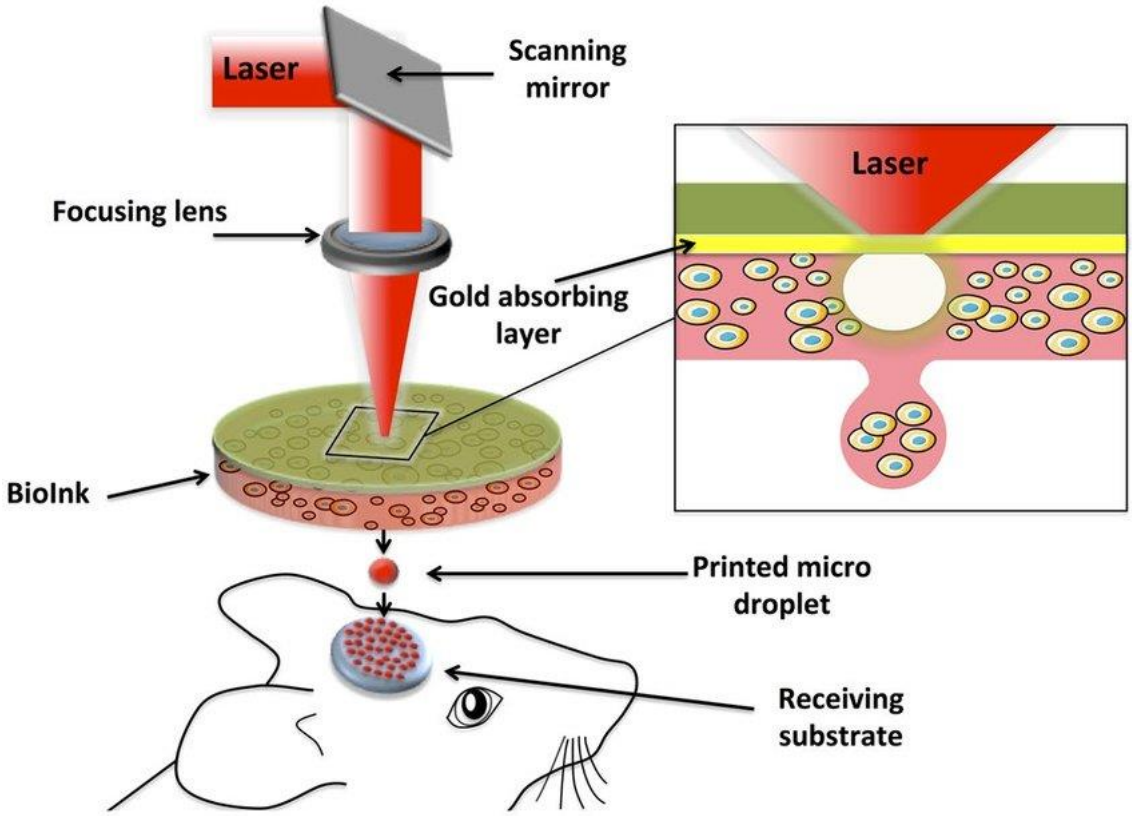

Figure 11. *The arrangement of a laser-assisted bioprinter.* [41]

LAB has many similar properties as IJB, as both share the droplet-based transferral of bioink. LAB however, differs in that there is no nozzle tip and therefore no similar shear stress. [10] This is the main benefit of LAB compared to EBB and IJB.

5.3.2 Laser-assisted bioprinting of skin

The resolution of LAB can match the size of an individual cell, which makes it a promising method for mimicking the heterogenic and specific structure of skin [10], [42] .As LAB is especially used to print cells, it is important to understand what kind of effect the laser can have on the cells. In a study conducted by Sylvain Catros et. al. it was noted, that in general, a higher energy laser will increase cell mortality for endothelial cells. Increasing the bioink viscosity however, will decrease mortality. [43]

The popular combination of keratinocytes, fibroblasts, and collagen has also been used in LAB. For example, a study by Lothar Koch et. al. stated that after LAB the keratinocytes and fibroblasts have a viability rate of 98%. These cells also showed the ability to proliferate and keep their original phenotype. This indicates that the cells were not harmed by the laser significantly [18], [44].

The NGB-R device by Poietis has been used to bioprint skin. In a study by Valérie Andre-Frei et. al. the laser-assisted method was used to print fibroblasts, and then collagen was printed with a microvalve-based method. As can be expected from a nozzle-free method, the cell viability was high. This skin model was claimed to be usable for drug testing purposes. [45]

In another study, LAB was used to print fibroblasts and keratinocytes on mice skin in vivo. The bioprinted skin presented better vascularization than similar experiments before. The study claimed that to reach full vascularization, the maturation time should be increased up from the 18 days used in this experiment. [42]

SUMMARY

The main properties of each three method are presented in the Table 1 below.

Table 1. *The summary of the three 3D bioprinting methods*

	Extrusion	**Inkjet**	**Laser**
Price	Moderate (e.g. 6 000 USD, CELLINK)	Low (e.g. 400 USD, modified HP)	High (e.g. 300,000 USD, Poietis)
Speed	Slow	Fast	Moderate
Usability	Easy	Easy	Complex
Resolu-tion	Moderate: 5 um	Low: 50 um	High: 0,5 um
Cell viability	Low: 40-80%	High: >85%	High: >95%
Refer-ences	[3], [9], [46], [45]	[3], [9], [26], [46], [45]	[3], [9], [46], [45]

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