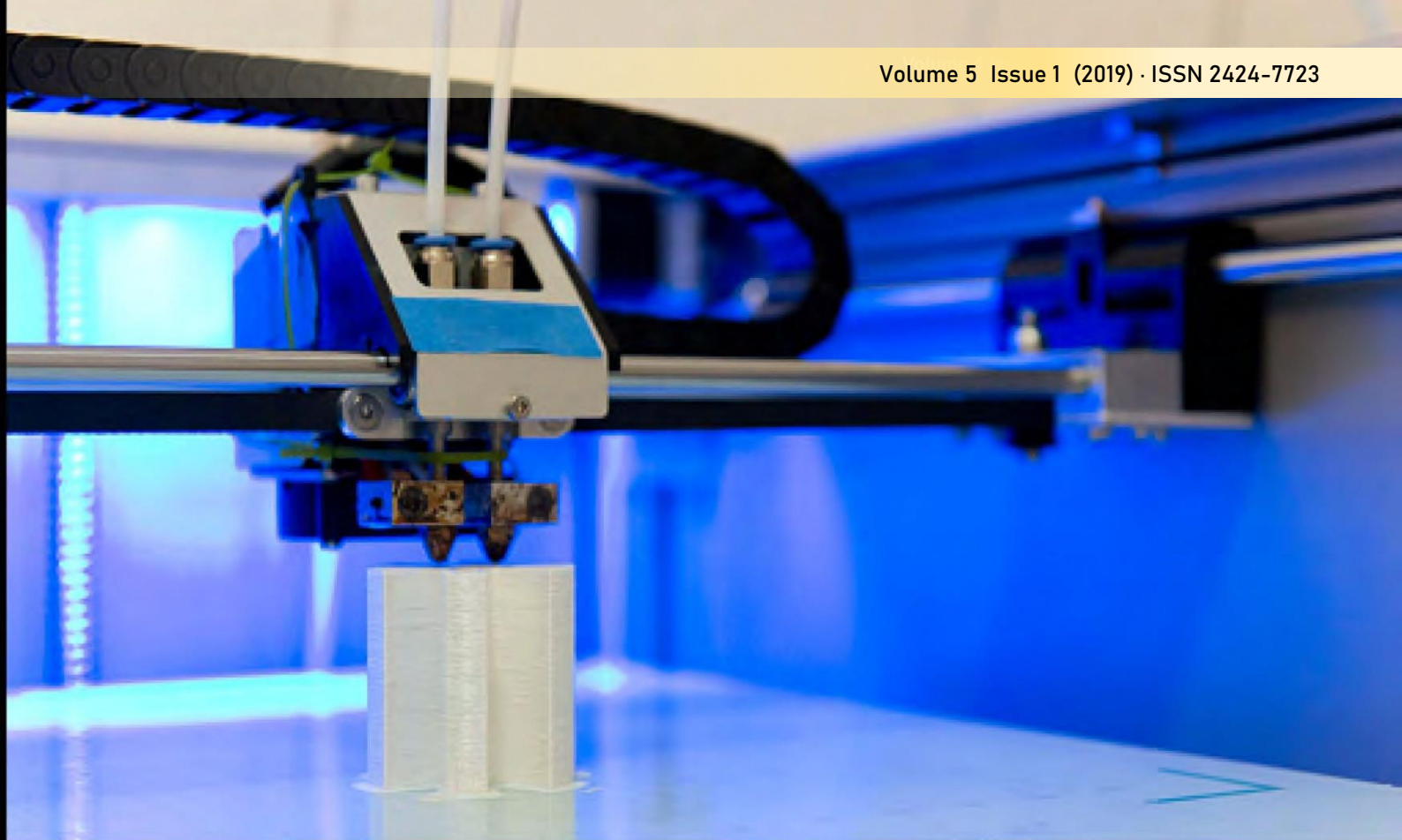




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Nanyang Technological University, Singapore



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EDITORIAL

Bioprinting in space and International Journal of Bioprinting stays ahead of the game

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<http://dx.doi.org/10.18063/ijb.v5i1.E>.

Now, bioprinting in space is no longer a dream but a reality!

Early December 2018, a magnetic three-dimensional (3D) bioprinter was successfully sent to International Space Station by Russian spacecraft Soyuz MS-11. Since then, various tissue/organ printing experiments have been conducted, and the results are expected to be released in February 2019. The scientific question whether living tissues printed under zero gravity matures faster than that printed on earth will soon be answered. These extraordinary experiments conducted in space could shed light on new strategies of quality tissue construction while achieving rapid tissue maturation.

The other exciting news is the continued development of International Journal of Bioprinting (IJB). The table below shows the achievement of IJB in 2018. According to Clarivate Analytics, IJB is on track to be evaluated for inclusion into SCI around October 2019. When accepted, IJB will receive official impact factor in June 2020.

Web of Science (ESCI)	2017 Mock Impact Factor	5.143
	2018 Mock Impact Factor	4.429 (26 Nov 2018)
Scopus 2017 CiteScore	2017 CiteScore	N.A.
	2018 Citescore tracker	4.5 (8 Dec 2018)

In addition, five special issues have been initiated in 2019 to highlight the latest development of bioprinting technology globally. This will definitely increase the visibility of IJB in various countries. I would like to thank Makoto, Ming, Jerry, Boris, Shoufeng, and Jing for their strong support. I also welcome more special issue proposals.

- Special issue: Bioprinting in Japan (Guest Editor: Makoto Nakamura).

- Special issue: Bioprinting in the USA (Guest Editor: Ming Leu).
- Special issue: Bioprinting and biofabrication for tissue engineering in Asia (Guest Editor: Jerry Fuh).
- Special issue: Bioprinting in Germany (Guest Editor: Boris N. Chichkov).
- Special issue: Bioprinting in Europe (except Germany) (Guest Editors: Shoufeng Yang and Jing Yang).

Being one of the leading journals in bioprinting, the January 2019 issue of IJB is proud to present a total of 12 original research articles and reviews. Four of them report on a new and emerging bioprinting process, namely the electrohydrodynamic printing technology. Kolan *et al.* printed 3D biomimetic structures using a polymer/bioactive glass composite^[1], while Sun *et al.* developed a microscopic image identification technique to monitor the printing process^[2]. Gao and Zhou reviewed the mechanisms of electrohydrodynamic printing^[3] as well its state-of-the-art designs and applications^[4]. Cheptsov *et al.* reviewed new microorganism isolation techniques with emphasis on laser printing^[5]. Khan *et al.* presented the optimization outcome of printing novel ultrashort peptide bioinks^[6]. Moreover, Liu *et al.* reported a hybrid fabrication approach of printing polymer/hydrogel scaffolds but with in-process plasma treatment^[7]. Similarly, Moldovan *et al.* reviewed the potential of hybrid biofabrication of 3D tissue analogs in future^[8]. Both works suggest that hybrid bioprinting could be a new direction in research. In addition, Yang *et al.* reviewed additive manufacturing of bone scaffolds^[9], and Mir *et al.* offered a perspective on how to overcome the obstacles and challenges in tissue engineering through bioprinting^[10]. Finally, Rodriguez-Salvador and her colleagues, report two bibliometric studies on recent trends in bioprinting, one in the dentistry applications^[11] and the other in the optical tissue phantoms^[12].

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Biofabrication offers future hope for tackling various obstacles and challenges in tissue engineering and regenerative medicine: A Perspective

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Abstract: Biofabrication is an emerging multidisciplinary field that makes a revolutionary impact on the researches on life science, biomedical engineering, and both basic and clinical medicine, has progressed tremendously over the past few years. Recently, there has been a big boom in three-dimensional (3D) printing or additive manufacturing (AM) research worldwide, and there is a significant increase not only in the number of researchers turning their attention to AM but also publications demonstrating the potential applications of 3D printing techniques in multiple fields. Biofabrication and bioprinting hold great promise for the innovation of engineering-based organ replacing medicine. In this mini review, various challenges in the field of tissue engineering are focused from the point of view of the biofabrication - strategies to bridge the gap between organ shortage and mission of medical innovation research seek to achieve organ-specific treatments or regenerative therapies. Four major challenges are discussed including (i) challenge of producing organs by AM, (ii) digitalization of tissue engineering and regenerative medicine, (iii) rapid production of organs beyond the biological natural course, and (iv) extracorporeal organ engineering.

Keywords: Biofabrication; bioprinting; tissue engineering; regenerative medicine

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1. Introduction and Backgrounds of Biofabrication (Bioprinting)

Organ failure is a critical issue in the health-care sector and there are currently millions of people waiting for organ transplants throughout the world^[1-7]. The demand for organ transplantation has increased over time. Although organ donation can save the lives of many needy patients, the problem is, there simply are not enough organ donors to meet demand^[8,9]. Hospitals and health centers

with organ transplant facilities have been facing critical shortage of donor organs and the scale of the problem is getting worse every year all over the world. There has been a tremendous increase in the number of patients on organ transplant waiting list as well as a sharp rise in the number of patients dying while awaiting lifesaving organ transplant operations for years. As a result, one in 10 on transplant waiting list dies before organ is found and millions of people die due to the lack of available organs for transplant^[10-13].

The fundamental concepts and the recorded use of the term tissue engineering, as it is applied today in biomedical field, was originally published by Langer and Vacanti (1993) in *Science*^[14]. Since then, tissue engineering research has grown exponentially due to the recognition that tissue engineering base strategies have the potential to replace, repair, and regenerate tissue/organs for a variety of biomedical applications including transplantation, therapeutic investigation, bioassay, disease modeling, drug development, and delivery. The rapidly evolving cross-disciplinary field of tissue engineering along with its intimately intertwined field of regenerative medicine continues to develop and advance.

The most intrinsic purpose, mission, and goal of tissue engineering and regenerative medicine (TERM) is to provide alternate solutions for restoring, replacing, and maintaining of organ functions of the problematic tissue/organ of interest using applied science and engineering approaches^[14,15]. The crucial difference between TERM and general science is that TERM is not a traditional way of doing science or to simply elucidate the origins and mechanisms of natural phenomenon based on intellectual curiosity but has a practical purposes of producing bioartificial organs that make possible medical treatments for patients with serious tissue/organ diseases/injuries and offers new hope to many patients who are suffering from end-stage organ failure.

During recent years, a large number of scientists, researchers, clinicians, and biomedical engineering companies have been actively engaged in tissue engineering and regenerative medicine research. Simple tissues (e.g., skin and engineered cartilage) have already been developed and being used clinically^[16-20]. Several other less complex tissues manufactured from a variety of biomaterials using a plethora of engineering approaches are at different stages of development. Despite scientific progress in tissue engineering, there are still several big obstacles in producing complex, functional, and large-sized three-dimensional (3D) tissues/organs, especially the tissues of the vital organs that are urgently required for experimental and clinical transplantation applications.

2. Final Mission of Biofabrication - Bioprinting

The challenging fields of biofabrication-bioprinting have emerged as revolutionary approaches to break the limitations of conventional TERM methods by offering potential technological solution. In brief, bioprinting can be defined as the manufacturing process by employing computer-aided two-dimensional (2D)/3D printing techniques to create 2D/3D patterns and to construct complex 3D structures with living and non-living biological raw materials to produce 2D or 3D tissues and organs of interest^[21,22]. The official definition of the

term “biofabrication” for TERM was recently proposed by the International Society for Biofabrication (ISBF)^[23]. According to ISBF, biofabrication can be defined as “the automated generation of biologically functional products with structural organization from living cells, bioactive molecules, biomaterials, cell aggregates such as microtissues, or hybrid cell material constructs, through bioprinting or bioassembly and subsequent tissue maturation processes.” In a more narrow sense, emerging field of biofabrication basically enables the researchers to use or to combine advanced fabrication technologies including 2D/3D printing, biomanufacturing, and bioassembly of living 3D functional biological products using smart and cytocompatible biomaterials.

Recently, there has been a big boom in 3D printing or additive manufacturing (AM) research, and various 3D printers have been developed, commercialized, and distributed worldwide. Today, many researchers from different backgrounds (science, engineering, and medical) have joined the interdisciplinary research field of bioprinting and biofabrication to open the doors to previously unimaginable possibilities in medicine or to search possible application of 3D printing in the biomedical field. 3D fabricated plastic organ models have become very popular for medical education. In TERM research field, biological 3D tissue models are one of the most attractive topics of application of the bioprinting and biofabrication, such as 3D tissue models for drug screening, disease models, tissue or organ on a chip, medical sensors, and biological actuators^[24-26]. Such research studies are certainly very useful and effective for drug discovery, drug development, and pharmaceutical industry.

Although drug administration is the first choice for treating patients with diseased/injured organs or organ failure, drugs are effective only in the early stages of the disease or minor injuries. Moreover, for the patients who require organ replacement or artificial organs for transplantation, generally, no significant effect can be expected for drug treatment.

Thus, the biomanufacturing of complex tissue/organ substitutes that fully mimic the natural physiological conditions of particular tissues/organs could help to alleviate organ failure/replacement issue. In the recent past, there has been a substantial and commendable progress in the field of TERM^[27-35]. Although successful fabrication of various tissue models has been reported, taking the engineered complex tissues/organ constructs from the bench to the bedside still needs focused efforts on scientific as well as potential technological fronts^[2,31,36-50].

3. Biofabrication Bioprinting Solving Various Challenges

As mentioned above, the main focus of bioprinting and biofabrication research is to overcome various challenges

of tissue engineering and future studies could prove “whether human beings can produce organs using printing and manufacturing techniques.” In this mini review paper, we have focused on various challenges which need to be overcome by bioprinting and biofabrication to produce organ or organ substitutes.

3.1. Challenge of Producing Organs by AM

In bioprinting and biofabrication, 3D tissues and organs are constructed by aligning living cells and biomaterials and by stacking or laminating them in 3D^[51,52]. Such bottom-up fabrication method of building 3D structures is called AM. Most of the conventional manufacturing techniques used to build 3D objects are based on molding and subtractive methods. By traditional manufacturing, 3D shapes can be made by starting from an object having an initial size or shape, materials are often molded, carved out, or removed by a sharp cutting tool until product of the desired shape is formed. However, there are still challenging issues associated with these methods, especially the inability to create or control the internal structure of the 3D objects. When it comes to manufacturing of 3D structures that specifically mimic human tissue- and organ-specific microarchitecture, these conventional approaches certainly are not useful because all vital organs are highly complex in nature, and each individual organ possesses its own specialized micro-sized histological, anatomical, and morphological structures which are very essential to perform all organ-specific physiological activities^[53].

Therefore, the technologies to construct 3D structure both internal and external structures simultaneously are highly needed for TERM research. As such, any structure we fabricate needs to exactly match that level of complex structural heterogeneity. Thus, the only hope for generating such structures is AM technology. For this reason, bioprinting and biofabrication have ever being challenged to produce organs or their spare bioparts by AM approach. As mentioned in Groll *et al.* (2016), bioprinting is a complimentary strategy within biofabrication. With bio-AM, this term can be used for describing a holistic approach that combines both bioprinting and biofabrication technologies for constructing engineered tissues/organs. Indeed, bio-AM has the potential to transform global health care and medicine, but bio-AM is still in its infancy and there are several obvious challenges that need to be overcome. Since such AM techniques have been only recently proposed and developed, only limited research is reported on their application for realizing truly biologically inspired new engineering solutions for clinical health care and bioindustry.

Although biological materials, especially living cells, can be regarded as the key materials for bio-AM, several other well-controlled and biocompatible biomaterials,

and adequate integration of biological tissue components with their application contexts are also needed to obtain biologically active 2D/3D tissues and organs or related bioproducts, but as yet, such materials have never been utilized in usual AM purposes^[54-57]. There is no doubt that all of the AM procedures required to be carried out in biologically safe environment. Although bio-AM can be helpful for the biomanufacturing, there are practically no established 3D biofabrication machines which can realize the manufacturing of arbitrary 3D structures. Therefore, it is necessary to develop advanced biofabrication machines to achieve the goal of arranging several different materials (including cells) in 3D space for engineering of multicellular constructs (tissues/organs) or bioproducts on demand. Apparently, a growing number of 3D printers have entered into the mainstream biomanufacturing technology, but only extrusion-based bioprinting (EBB) is rapidly growing^[21,22,24,37,43,45]. Even though EBB is considered to be the most accepted technique in tissue engineering field to date, this technique also suffers from several limitations. For example, the resolution of extrusion type printers is still very poor (more than 500 μm)^[41,44,58-78]. On the other hand, an average human cells range in size from about 10 to 30 μm , while very fine capillary vessels, which are the essential tissue components are on the order of 10 μm in diameter. Therefore, 3D bioprinters with sufficient resolution ability are needed, ultimately.

In addition, diverse bioink formulation is also one of the challenging aspects of this field. Typical adult human body consists of myriad of cell populations, tissue components, and microstructures that work together to perform particular body functions. At present, effective materials for bioink of 3D bioprinting or biofabrication are extremely limited, the reader is referred to more specialized reports^[54,56,57,79]. Therefore, the development and formulation of effective bioinks are necessary for the clinical application of bioprinting technology. More importantly, novel kind of hydrogel materials exhibiting remarkably favorable properties, including compatibility with different bioprinting methods, rapid or instantaneous gelling properties, flexibility and stability in medium, cytocompatibility, or biofunctionality, are still needed for proper cell growth, differentiation, and tissue formation or regeneration during bioprinting processes (pre-bioprinting, bioprinting, and post-bioprinting). The formulation of wide range of the biomaterials to design a variety of bioinks exhibiting the above-mentioned properties remains an important research direction for biofabrication research. Nowadays, bioink is one of the emerging and hot topics in bioprinting and biofabrication^[80,81]. Expanding biofabrication technology and fostering the invention of new biomanufacturing machines and development of novel bioink materials

could advance the reliability of bio-AM, and only bio-AM can potentially deal with the increasing demand for replacing/regenerating tissues/organs. In the end, such developments could be promising in on-demand tissue engineering or bioindustrial applications using bio-AM technologies.

3.2. Challenge of the Digitalization of TERM

The rate at which biofabrication research is being carried out worldwide reflects remarkable technological achievements. Printers (including 3D printers) are considered as excellent output devices^[21,23,61,77,80-82]. Recent trends in implementation of bioprinters in biofabrication technology within the field of TERM have shown that the number of research papers has significantly grown, while bioprinting and biofabrication have become more popular research areas^[23,81].

Broadly speaking, biofabrication is complex technology comprised several techniques including computer-aided design (CAD), computer-aided engineering (CAE), and biological processes and subprocesses. CAD, CAM, and CAE terminologies for biofabrication have been proposed to parallel similar strategies in other engineering fields. However, there are several big and important distinctions between the disciplines. To avoid confusion, we propose that for biofabrication (bioengineering of complex tissues/organs), these terms should be used as bio-CAD, bio-CAM, and bio-CAE (Figure 1).

Bio-CAD, bio-CAM, and bio-CAE approaches are generally regarded as promising future technology for the biomanufacturing of complex and heterogeneous bioconstructs. Bio-CAD process can be used to design tissue and organ blueprints, bio-CAM process can

be to manufacture biological products, and bio-CAE process can be used to create complex bioarchitectures, and validate and optimize biomanufacturing tools and bioproducts. Effective implementation of bio-CAD, bio-CAM, and bio-CAE processes (biofabrication) depends on the combination of several interrelated components/parameters, such as biomaterials, biomolecules, cells, and tissues, integrated with computational approaches (e.g., digital designing, information mapping, sophisticated virtual product modeling, 3D simulation, and data mining).

Digitization of bio-CAD, bio-CAM, and bio-CAE processes are the key issues of the day for biofabrication scientists and researchers. Indeed, the current biofabrication initiatives are possible, but computer programmers and technologists will undoubtedly have to invent highly inclusive digital technologies that involve integrating imaging, database, and computer numerically controlled bioprinting machines and artificial intelligence^[83-91]. If all these parameters will function in a coordinated way, then we can achieve the final objective of digital fabrication in TERM or automated production of biologically functional human tissues and organs for transplantation and commercialization purposes^[92-105].

4. Challenge to Rapid Production of Organs beyond the Biological Natural Course

The human body with its interconnected organ systems is the most advanced and complex living structure in the known universe. In the natural process inside our bodies, organ developmental phenomena are strictly controlled by natural laws of embryology and anatomy.

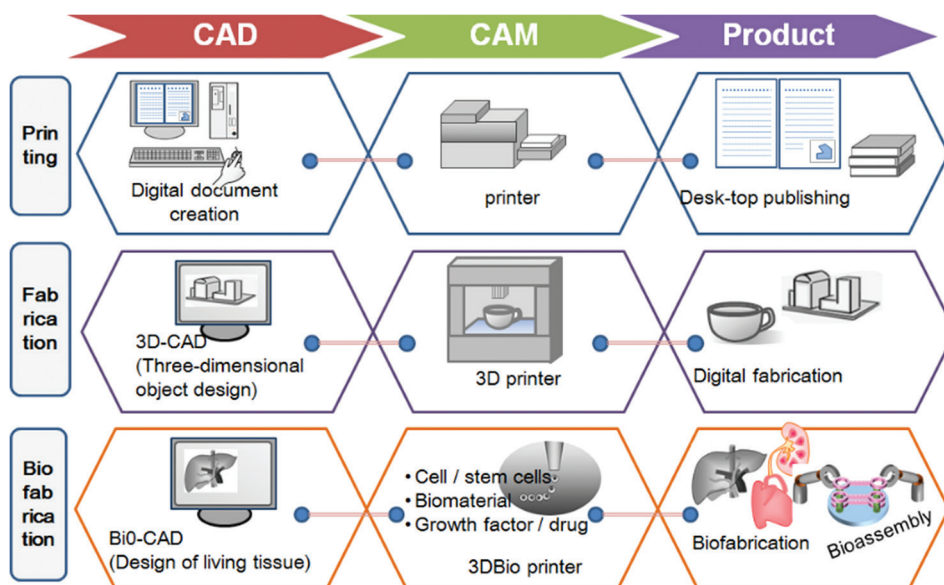


Figure 1. The sketch shows a schematic drawing of the potential strategy for digitalization of tissue engineering and regenerative medicine.

All of organs arise through a process that begins with the fertilized egg which undergoes through cell divisions, differentiation processes resulting in the formation of organs and ends with a new individual living entity. From a pragmatic point of view, there are several factors which directly or indirectly influence the continued growth and development of organs (differentiation of the cells into two or more histologically specialized structures that organize to form specific organs and organs systems) to the time of delivery. The rate of organ growth and development varies individually and is dependent on multiple sequential and reciprocal interactive influences mediated by genetic structure, genetic information and genetic diversity traits, maternal traits, and internal environmental control mechanisms of the body. Morphology, histology, and functional anatomy are largely limited to the hierarchical level of organs. Thus, the anatomical and histomorphologic features build up continually according to the growth of the organ and whole body through many complicated processes across respective assembly locations in the body. For example, if you see the complex embryology of heart, it forms initially in the embryonic disc as a simple paired tube inside the forming pericardial cavity. However, as it develops four chambers with four valves, the complexity creates even more complex dynamics of the physiological systems^[106-109].

In the natural process inside our bodies, tissue and structures originate and mature as an individual grows. Therefore, it is not so easy to establish feasible organs substitute through natural processes because it takes many years for an individual and its organs to grow, develop, and generate sufficient functions. In contrasts, patients who require organ transplants cannot wait for organs for such a long time. For this reason, organs or organ substitutes for transplantation should be provided as soon as possible, ideally within a few months. There

are many excellent scientific researches on generation of organs based on the embryological phenomena^[110-113]; however, it seems difficult to provide enough organs for patients with organ failure/disease during the waiting period. For these reasons, natural process is generally considered as unsuitable option for complex tissue and organ regeneration (Figure 2).

Although several tissue engineering approaches have been implemented for various biomedical research strategies, differences in the natural formation of living human tissues/organs with their unique shapes and material properties, hierarchical, morphological, anatomical, and physiological biochemical features limit the ability of conventional tissue engineering approaches to regenerate complex metabolic organs. In other words, tissues/organs require more sophisticated biomimetic 3D microenvironments capable of providing multilayer information to differentiating cells. Novel biomanufacturing methodologies and biomimetic materials still need to be developed and be used to assemble microscale building blocks capable of accurately mimicking/replacing 3D complex Humanscale living vascularized tissue/organ analogs with optimal physiological activity.

Therefore, bio-AM is the only hope to overcome these challenges and to simultaneously construct fully functional organ analogs with great complexity (including histologically essential structures) by applying 2D/3D printing and related AM strategies. Although available biofabrication methods have several features and capabilities, still there are limitations to exploit the exact structure/function of the native tissue organ. More complex material distribution and construction of anatomically and physiologically relevant tissues/organs may not be easily achieved without advanced biofabrication approaches. To produce a substitute for natural organ or autologous tissue graft that is ready to be transplanted, improvements in biofabrication

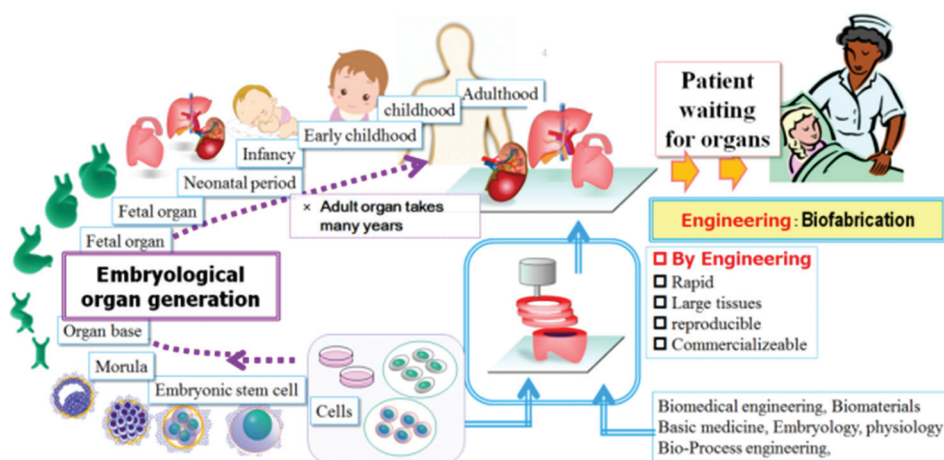


Figure 2. The sketch shows a schematic drawing of the strategy of rapid production of the organs beyond the biological natural course.

technologies for facilitating the precise programmable designing, computer-controlled efficient biofabrication, and post-fabrication incubation are urgently needed. There is still a long way to go in fabricating bioartificial organs on demand, but these technologies are achievable. If biofabrication approaches will work out, it will become possible to fabricate not only Humanscale patient-specific tissue grafts/organs at a rapid pace (irrespective of organ size) but it will also shorten the time required of growth performance and development of organs. Then, no patient will ever have to wait on lengthy transplant lists for donor organs, and no patient will ever have to take powerful, debilitating and potentially fatal drugs to treat chronic pains and to prevent their immune systems from rejecting new body parts or from attacking the transplanted organ when the organ is not closely matched.

1.5. Challenge to Extracorporeal Organ Engineering

In conventional TERM methods including cell transplantation and the scaffold-based tissue repair strategies, morphogenesis of tissue architectures and regulation of essential biochemical and physiological processes all depends on the regenerative ability of cells and the recipient *in vivo*. While modern tissue engineering approaches are good at making tissues in the laboratory, we still cannot control the important processes of complex tissue and organ after implantation. Here comes the role of bio-AM filed to make extracorporeal systems having the capability to repair/return organ function permanently or for implantation. Broadly speaking, bio-AM is aiming at challenges of producing extracorporeal organ reconstruction systems, in which the important tissue structures can be printed, fabricated, assembled, cultured, and matured before implantation. In addition, ideally, the functions of engineered organs should also be established before implantation.

It is often thought that the *in vivo* approaches are physiologically better and look realistic enough. However, this is not necessarily true and *in vivo* approaches are not always better for organ engineering. One of the main limitations of *in vivo* approaches for tissue engineering research is the regeneration of only fibrous tissues. It has been demonstrated that fibroblasts and inflammatory cells are the most strongly proliferating cells in the living body consuming large amount of nutrients and oxygen to provide energy for proliferation of metabolically active cells^[114-116]. Therefore, the transplanted cells cannot survive without a blood supply, sufficient supply of essential nutrients, or adequate means for waste product removal. Instead, outside the body, the desired tissues or organs can be grown without pro-fibrogenic environment. Therefore, better *in vitro* strategies are really needed to support, instruct, and maintain cells inside immune-privileged 3D microenvironments, where cultured cells do not lose their unique intrinsic characteristics (Figure 3). As mentioned above, bioprinting is the only hope for fabricating appropriate environments for cells to be able to effectively produce spare parts of the human tissue/organs beyond the limits attained *in vivo*. For instance, biofabricated organs can be incubated in the environment under the high or low oxygen tension, high or low growth factors, and exposure of drugs which have high pharmacological activity. Control of such incubating parameters is impossible *in vivo* situations due to harmful effects on the recipients. Therefore, the concept of extracorporeal system is clinically appealing and advanced extracorporeal systems are necessary for biofabrication research. Such systems may enable precise spatial and temporal control arbitrarily without affecting the recipients. Building on this momentum, the next regenerative medicine frontier lies in how biofabrication researchers can better develop novel extracorporeal

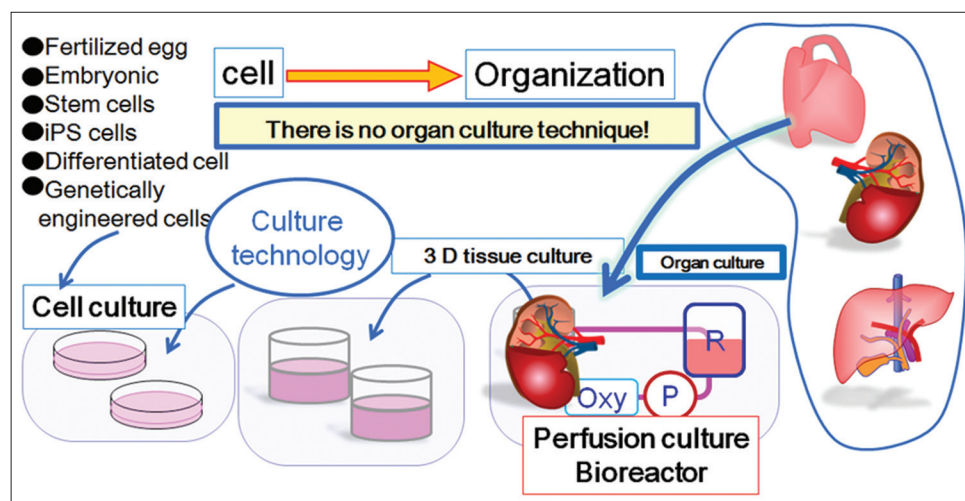


Figure 3. The sketch shows a schematic drawing of the general culture strategy and organ perfusion bioreactor technology.

systems and bioprocess engineering aims. However, no one has ever succeeded to produce functional engineered organs. One of the reasons is because we cannot do anything with natural process at all after implantation. Thus, biofabrication is aiming at extracorporeal organ engineering, in which tissue formation can be controlled and facilitated under human control.

2.6. Conclusion

In this mini review, four major challenges by bioprinting and biofabrication are focused and explained from the views from the final mission. These challenges were started just from the beginning of this research field but are still important issues urgently needed to be addressed. Based on our more than 15 years of research endeavors in this field, we anticipate that biofabrication (bioprinting) holds great promise for the development of artificial tissue/organs. There are indeed still many technological issues; however, such problems are also essential to better understand potential limitations of conventional methods, overcome challenges, and produce organs or organ substitutes. Even though we cannot foresee the use of available biofabrication approaches for fabricating fully functional organs in the near future, there is great potential and promise for the applications of biofabrication approaches in the research area of TERM. We conclude with the quote of Theodor von Karman who once said “Scientists study the world as it is; engineers create the world that has never been.” Tissue engineers’ worldwide have been doing amazing research and putting their efforts on the development and implementation of advance biofabrication technologies (e.g., bioprinting) to create the world of biomedicine that has never been.

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Additive manufacturing of bone scaffolds

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Abstract: Additive manufacturing (AM) can obtain not only customized external shape but also porous internal structure for scaffolds, both of which are of great importance for repairing large segmental bone defects. The scaffold fabrication process generally involves scaffold design, AM, and post-treatments. Thus, this article firstly reviews the state-of-the-art of scaffold design, including computer-aided design, reverse modeling, topology optimization, and mathematical modeling. In addition, the current characteristics of several typical AM techniques, including selective laser sintering, fused deposition modeling (FDM), and electron beam melting (EBM), especially their advantages and limitations are presented. In particular, selective laser sintering is able to obtain scaffolds with nanoscale grains, due to its high heating rate and a short holding time. However, this character usually results in insufficient densification. FDM can fabricate scaffolds with a relative high accuracy of pore structure but with a relative low mechanical strength. EBM with a high beam-material coupling efficiency can process high melting point metals, but it exhibits a low-resolution and poor surface quality. Furthermore, the common post-treatments, with main focus on heat and surface treatments, which are applied to improve the comprehensive performance are also discussed. Finally, this review also discusses the future directions for AM scaffolds for bone tissue engineering.

Keywords: Additive manufacturing; bone scaffolds; scaffolds design; post-treatments

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1. Introduction

Bone tissue is able to subject to biological remodeling through a dynamic process of the absorption of mature bone tissue by osteoclasts and subsequent generation of new bone induced by osteoblasts^[1,2]. Nevertheless, the body usually cannot fulfill the self-repairing as a large segmental bone defect occurs, in which the bone defect exceeds a critical size of about 10 mm^[3]. In this condition, an external intervention is required to aid in the self-repairing by means of building bridges on the bone defect site^[4]. Therefore, bone grafts are extensively required

in clinical surgeries to aid in the healing of these large segmental bone defects. Data from the center for disease control show that bone is the second most commonly graft tissue, with more than 2 million surgical operations utilizing bone grafts annually^[5]. Autografts taken from the patient-self are considered as the gold standard for bone repair^[6]. However, the size of the autograft is very limited. Moreover, harvesting the autograft inevitably causes an additional surgical trauma associated with a serious risk of morbidity at the donor site. Allografts taken from other persons are an alternative and are in larger supply as compared with the autografts. However, they usually lead

to disease transmission and immune rejection^[7]. Thus, idea bone substitutes are urgently demanded for bone tissue repair in surgical application.

Desired bone substitutes should have a customized external shape, aiming to avoid the excess removal of bone tissue at defect sites. More importantly, they also need to have porous and interconnected pore structure, so as to create a microenvironment, which is conducive to cell activity and reproduction^[8,9]. To obtain scaffolds with the porous structure for bone repair, several approaches, such as pore-forming agent method^[10,11], gas foaming method^[12,13], sol-gel method^[14,15], and freezing drying method^[16,17], have been proposed. Although these methods exhibit a certain ability to fabricate porous structure, they are also with some limitations, such as inaccurate control of the pore structure and poor ability to customize for specific defect sites^[18]. Moreover, some of these approaches inevitably leave some organic residues of pore-forming agent, which will reduce the biological properties of the scaffolds and sacrifice the quality of bone repair. Therefore, exploring a fabrication technique that is not only limited to obtain the individual external shape but also accurately control the pore structure for scaffolds is of great significance for their further orthopedic application.

Additive manufacturing (AM) can produce a porous scaffold with individual external shape and porous internal structure^[19]. Before AM process, a three-dimensional (3D) scaffold model is generally designed with desired architecture using computer-aided design (CAD) software. The 3D scaffold model is sliced into a series of two-dimensional (2D) slices before converting to typical Stereolithography (STL) files, which contain detailed 2D slice information. Based on these STL files, an AM machine performs the necessary toolpath along the 2D directions for direct building of 2D layers. Each layer is just built on top of the other to construct a 3D part. Due to the fabrication process of adding one layer on the previous one, this manufacturing technique is described as AM. Currently, researchers around the world are committed to apply AM techniques to produce porous implants for bone repair. For instance, Poukens^[20] successfully applied AM to fabricate porous mandible implant, which was subsequently implanted in a patient. Brazilian Jardim *et al.*^[21] used an AM processed customized porous scaffold to repair a large cranial defect. Australian Peter^[22] implanted the AM-derived porous titanium implants into a 71-year-old patient who faced an amputation of the heel bone. All these successes positively render the AM techniques a promising future for bone tissue repair.

A typical application of porous scaffolds fabricated by AM includes the scaffolds design, AM, and post-treatments, as schematically illustrated in Figure 1. Therefore, this work reviews the overall process for AM of bone

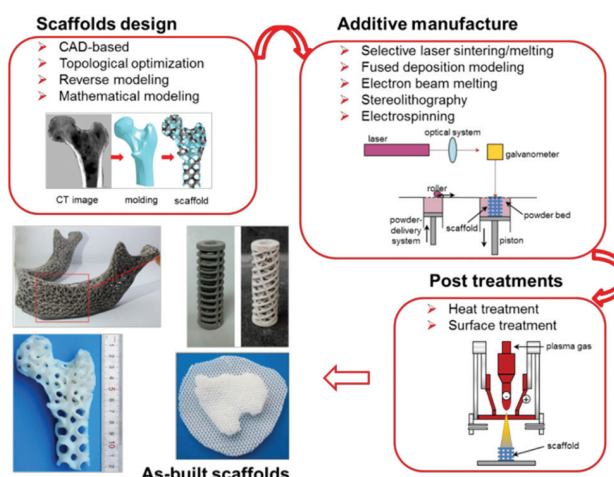


Figure 1. A schematic diagram for the design, additive manufacturing (AM), post-treatments of bone scaffolds, and several typical AM-derived scaffolds. Images adapted from references^[29-32].

scaffolds. Several typical structure design methods are first examined, especially the mathematical modeling method, which can achieve with both bionic design and topological optimization of scaffolds. Following on, the most relevant AM techniques using for scaffolds fabrication, with their advantages and disadvantages, are highlighted. The common post-treatments related to AM-derived scaffolds are also discussed. Finally, the future trends for AM scaffolds for bone repair are addressed.

2. Scaffold Design

Scaffold design is an important step in AM bone scaffolds. This is because the features of the porous structure, including the porosity, pore size, and pore interconnectivity, have great influence on their biological performance and mechanical properties^[23-25]. In particular, an appropriate pore size and high porosity facilitate the absorption of nutrients and the excretion of metabolic waste, thus providing a suitable environment for the growth of bone tissue^[26]. Meanwhile, the internal pore structure and the distribution of materials directly influence the plasticity and stiffness, thus determining the stress environment of the surrounding bone tissue as implanted *in vivo*^[27]. Besides, the mechanical function of the scaffolds dynamically changes after implantation, which should be taken into consideration in scaffolds design. On the other hand, the external shape features of the scaffolds should conform to the morphological characteristics of the defect site to obtain the desired shape. It should be noted that the designed scaffolds are required to easily build using specific AM techniques. For instance, the overhanging structure commonly causes some undesirable defects, if a corresponding supporting structure is absent when it is building^[28].

2.1. CAD-Based Method

CAD-based method, the most common method for scaffolds design, is realized by utilizing various CAD tools, such as UG, CATIA, and Pro/E.^[33,34] These CAD tools construct models based on constructive solid geometry (CSG) or boundary representation (B-Rep) principle. In the CSG method, specific models are constructed using a series of Boolean operations with standard solid primitives, such as the cubic, cylinder, ball, and prism, whereas, in B-Rep method, the solid models are depicted using their boundaries. The typical boundaries include vertices, edges, and loops, with no direct relation among them^[35]. Models obtained by B-Rep require more storage space, as compared to CSG. Therefore, as the model becomes larger or has a more detailed internal structure, the size of the file containing B-Rep-derived model will increase dramatically, causing great difficulty in further operation.

Based on the above-mentioned modeling principles, CAD tools construct various porous unit cells and assemble them to build the whole scaffolds. A series of unit cells have been designed to characterize the architecture of bone scaffolds^[36,37]. Melchels *et al.*^[38] designed three sophisticated unit cells, including a cube, a diamond, and a gyroid. While space-filling computer models with distinct architectures were generated from assemblies of

these unit cells, as shown in Figure 2A. Finite element simulations of the compression behavior indicated that gyroid architecture presented more homogeneous distribution of stress and strain, as compared to cube architecture (Figure 2B). Thus, scaffolds with gyroid architecture are believed to expose adherent cells with relatively more equal mechanical stimuli, which might be more beneficial for bone regeneration. Sercombe *et al.*^[39] designed a unit cell with octahedral geometry. Finite element analysis of the failure mechanisms revealed that this structure could bear high tensile stress.

To simplify the CAD-based design process, some dedicated design software has been successfully developed. For instance, Belgium Materialise Company has developed a 3D printing pre-processing software named Magics, in which designers can directly instruct various integrated unit cells. Murr *et al.*^[40] reported a case of quickly building scaffolds using MATERAILAS software elements, including cross 1, G6, G7, and code thin, as shown in Figure 2C. Researchers also developed a computer-aided system for tissue scaffolds (CASTS), which included a parametric library of scaffold structures and an algorithm to define the pore size, porosity, and surface-to-volume ratio^[33,41]. With CASTS, designers can obtain desired scaffold models with proper porosity and pore size by setting a series of parameters. Although CAD-based design shows powerful design ability, it

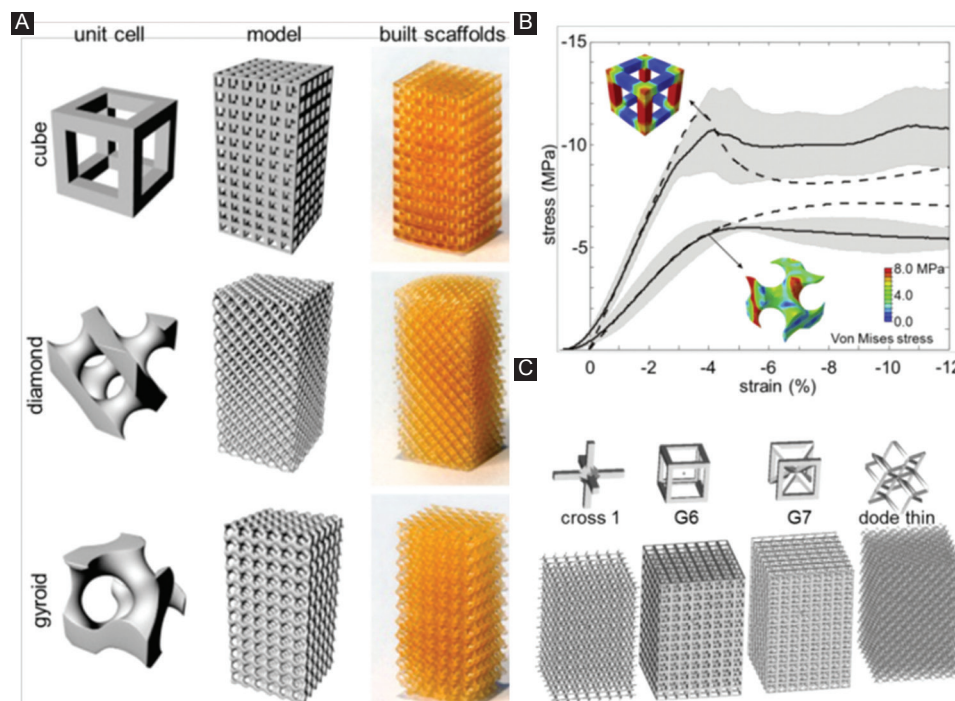


Figure 2. (A) Computer-aided design-based unit cells with cube, diamond, and gyroid architecture, as well as their assemblies and as-built scaffolds. (B) Stress-strain curves of the scaffolds with cube and gyroid architecture^[38]. The obtained data are described as the averages in solid lines with standard deviation in shaded areas, and finite element results are depicted in dashed lines. (C) Materialise Software elements and designed scaffold models^[40].

exhibits poor controllability on the structural performance and mechanical properties of designed scaffolds. Moreover, there is an inescapable staircase phenomenon on the external contour of models caused by Boolean operation, leading to geometric distortion and mechanical instability^[42]. Besides, the CAD-based method can only design the scaffolds with periodic and regular structure.

2.2. Topology Optimization

Ideal bone scaffolds should not only have highly porous structure to facilitate cell in-growth and nutrient transport but should also possess enough mechanical properties to provide stable structure support^[30,43-45]. Paradoxically, increasing the porosity of the scaffolds enhances the material transport capacity but inevitably impairs the mechanical properties. Thus, the scaffold designer should balance these two conflicting properties to obtain an optimal comprehensive performance. Topology optimization is a method to optimize the distribution of materials in a given region based on the given load condition, constraint condition, and performance index^[46]. Designing scaffolds with topology optimization is expected to achieve an optimized comprehensive performance with certain constraints, for example, the maximum mechanical properties with certain porosity or maximum permeability. In topology optimization of scaffolds, the optimization problem is generally solved indirectly through optimizing a unit cell with specific optimization algorithms. After obtaining the optimized unit cell architecture, the whole scaffold is formed by repeating it periodically. The classic optimization algorithms applied in topology optimization of scaffolds include Solid Isotropic Material with Penalization (SIMP) method and evolutionary structural optimization (ESO) method, which describe the structure point-by-point in topology optimization^[47]. Guest *et al.*

^[48] optimized a scaffold based on the SIMP optimization algorithm, in which two competing properties, including the modulus and permeability, were tailored using a single objective function^[49]. SIMP method is also applied to optimize the elastic tensor of scaffolds^[50]. *In vivo* tests showed that the optimized scaffolds with similar elastic properties to that of human bone exhibited an accelerated bone remodeling rate. Besides, a series of unit cells with maximal shear and bulk modulus, predefined stiffness ratios and functionally graded structure, were obtained through the ESO-based topology optimization^[51]. Some scaffolds optimized by bidirectional evolutionary ESO method, which obtain the maximum bulk or shear modulus under various prescribed volume fractions, are depicted in Figure 3^[52].

Another typical topology optimization method is level-set *al.* orithm, which centers on tracing the phase boundaries, thus effectively describing smooth boundaries to control the topology changes^[53]. A level-set based method for scaffolds design was proposed to obtain material with maximal permeability^[54]. Level-set based topology optimization made it possible that the no-slip boundary condition of fluids in Stokes flow could be naturally satisfied^[55]. Topology optimization was also reported to tailor the thermal expansion of porous multimaterials^[56]. However, as mentioned before, the topology optimization of the scaffolds begins with the optimization of the unit cells and proceeds with the subsequent periodic arrangement. Therefore, it can only achieve regular porous architecture, which is considerably different from the irregular structure of natural bone.

2.3. Reverse Modeling

Reverse modeling design, also known as image-based design, reconstructs bone tissue microstructure directly based on object's computed tomography (CT) or magnetic resonance imaging (MRI)^[57]. In this method, the CT/MRI slice images undergo a series of analysis, with an aim to extract the key features for reconstruction. Binary value method is commonly used to analyze the slice information, in which element "1" represents the solid, whereas "0" represents the void^[58]. Then, the pre-defined unit cell is mapped according to the extracted slice information to construct the 2D model. This 2D model will directly be transformed into STL files and transmitted to an AM equipment to construct 2D layer. After this, a 3D part is obtained using the layer-by-layer method. Obviously, reverse modeling design combines advanced medical imaging system, powerful image analysis software as well as rapid AM technique, which guarantees a more mimic architecture for bone tissue engineering.

Reverse modeling has appealed to a large number of researchers for constructing customized scaffolds. Sun *et al.*^[59] systematically investigated the modeling principles and

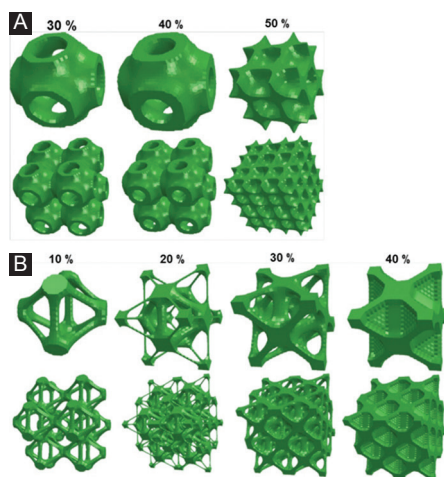


Figure 3. Topology optimized unit cells and scaffolds with (A) maximum bulk modulus and (B) maximum shear modulus under various predefined volume fractions^[52].

the unit library approach to biomimetically create porous structures. Similar method approach was also researched by Hollister *et al.*^[60,61] by creating the internal architecture by altering the density in the voxel dataset. Based on these early studies, Podshivalov *et al.*^[62] constructed microscale structure scaffolds based on the processing of actual micro-CT images followed by reconstructing a highly accurate geometrical model, which resembles the actual trabecular bone structure. Related studies showed that the porous structure obtained by reverse modeling was more conducive to the growth of cells, which also proved the superiority of irregular porous structure^[63,64].

Although reverse modeling design can obtain the porous structure that perfectly coincides with the natural bone, the accuracy of the model highly depends on the resolution of the image acquisition device. On top of that, it needs a lot of computing resources and storage space and proposes higher requirements for software developers and users.

2.4. Mathematical Modeling

Mathematical modeling mainly utilizes shape functions to construct porous scaffolds with implicit function surfaces or irregular polygonal models, which breaks through the geometry limitations of the traditional porous element. Among them, triply periodic minimal surface (TPMS) method uses trigonometric functions to derive a complex porous structure with a minimal surface, in which the curvature at any point is zero^[58]. The TPMS structure also presents a periodicity in three independent directions due to the periodic characteristic of the trigonometric function, with no sealed cavities exist in the geometry^[65]. In fact, the examples of the minimal surface geometry exist in nature, including beetle shells, butterfly wings, and crustacean bones^[66].

Rajagopalan *et al.* first proposed a TPMS-based method for designing tissue scaffolds and a simple primitive (P-type) unit^[67]. Other types of TPMS units including diamond (D type) and gyroid (G type) were also proposed for bone scaffolds design^[68], as shown in Figure 4A. Studies on the influence of these morphologies on cell migration revealed that the scaffolds with

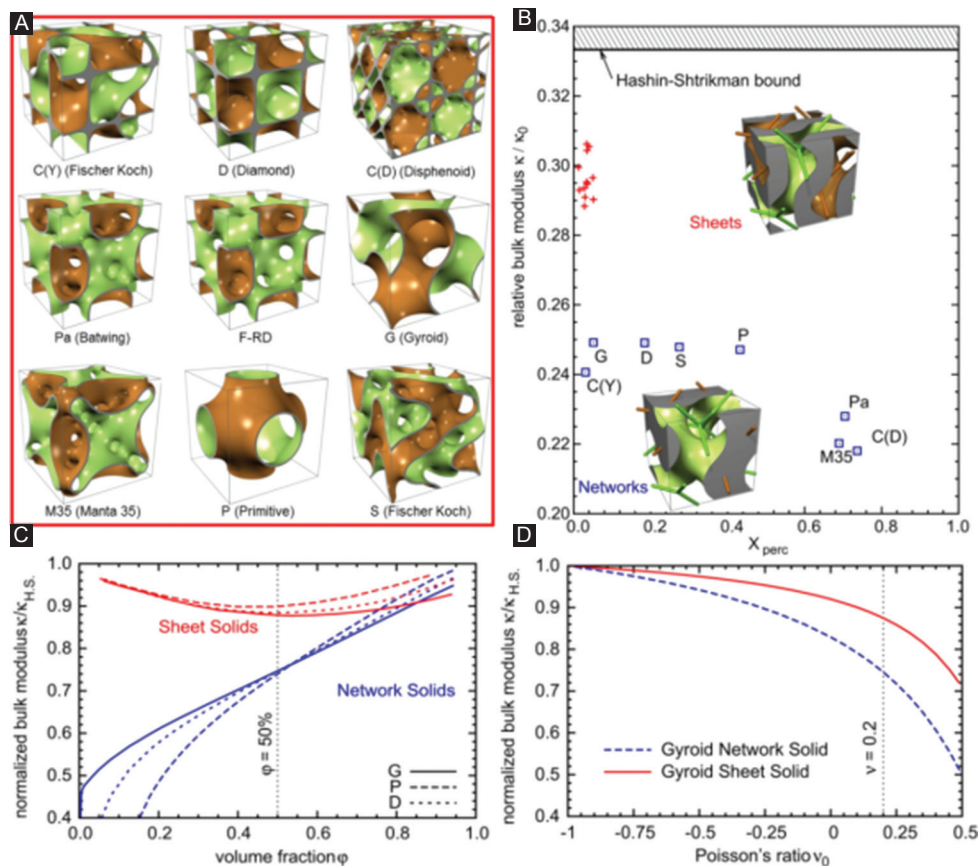


Figure 4. (A) Various triply periodic minimal surface (TPMS) unit cells for bone scaffolds design^[68]. (B) The relative bulk modulus of TPMS scaffolds with a volume fraction of 50% and Poisson's ratio 0.2. The blue boxes indicate the network solids, whereas the red crosses indicate the sheet solids. X_{perc} represents the dimensionless non-percolation ratio. Insets are two kinds of scaffold structure based on the gyroid-type TPMS with the volume fraction of 50%. The curves showed the relationships between the stiffness and volume fraction (C) and Poisson's ratio (D). With identical volume fraction or Poisson's ratio, sheet solid scaffolds exhibited higher stiffness as compared with network solid scaffolds.

minimal surfaces were more easily wetted with higher permeability, leading to deeper cells into growth as well as more uniform cell distribution, as compared with a salt-leached scaffold with a random-pore architecture^[69]. Kapfer *et al.*^[68] investigated two kinds of TPMS-based structure, including network solids and sheet solids. In the network solids, the minimal surface constructs the solid/void interface, whereas in sheet solids, porous solids are constructed by inflating the minimal surfaces to sheets with a predefined thickness. Finite element analysis confirmed that the sheet solids possessed considerably higher mechanical stiffness than that of network solids for identical volume fractions (Figure 4B and C) and Poisson's ratio (Figure 4D). Moreover, the sheet solids also exhibited higher material utilization and provided relatively more surface area and pore space for cell migration and activity.

TPMS has been explored for its possibility to construct gradient, heterogeneous, hybrid, and irregular porous structures. Melchels *et al.*^[38] reported that the design of a gradient porous structure can be constructed based on TPMS by adding a linear function. Feng *et al.*^[31] designed heterogeneous porous scaffolds with non-uniform threshold, period, and unit by combining TPMS and solid T-splines. In addition, Yang *et al.*^[70] proposed two CAD methods to prepare hybrid porous structures for biomimetic design purposes that combine different TPMS-based structures with given transition boundaries. Using his approach, it is able to place TPMS-based substructures on given 3D subspaces with perfect transitions to their adjacent substructures within a scaffold domain. A series of multiscale and multimorphology porous scaffolds were presented in their further studies^[71-74]. For similar goals, Yoo *et al.*^[75-77] combined a kind of distance field with TPMS-based functions to effectively construct pseudorandom porous scaffolds. Yang *et al.*^[78] utilized coordinate transformation based on TPMS to construct an gradient and full irregular porous structure, extending TPMS to irregular design areas. However, the stochastic porous structure constructed by this method had porosities

lower than 74%. Beyond this critical point, the solid phase turned disconnected.

Another typical mathematical modeling is Voronoi-Tessellation method, which constructs porous models using a Voronoi diagram. A typical scaffold design principle based on Voronoi-Tessellation method is depicted in Figure 5. In particular, a set of points (or seeds) are positioned randomly inside the design volume to fulfill a partition of the space in regions. Then, a thickness is assigned to the edges of the partitioned regions to obtain a porous scaffold^[79,80]. The design of porous structure based on Voronoi diagram could be traced back to Kou and Tan^[81,82], where they first proposed to use Voronoi vertices as the control points of a closed B-spline curve to create a convex-shaped cell. Besides, a porous structure was obtained by merging the adjacent cells. However, they only studied the 2D pore structure. While Chow *et al.*^[83] organized Voronoi seeds in concentric circles and formed a 2D shape region. Subsequently, a 3D porous structure was constructed by expanding the time dimension of the dynamic pattern in the third dimension of the 2D shape region.

In recent years, researchers have conducted a more in-depth study on Voronoi-Tessellation-based scaffold design and have achieved great progress. For example, Fantini *et al.*^[84] combined CAD 3D software Rhinoceros with its Plug-in Grasshopper to design bone scaffolds based on Voronoi-Tessellation method. This work successfully correlated to the input parameters, including the number of seeds, porosity, and the pore size of the structure^[85]. Gómez *et al.*^[86] proposed a bone-like trabecular structure design based on Voronoi-Tessellation principle. The seeds of Voronoi diagram were extracted from the micro-CT images of the trabecular bone. The obtained isotropic porous scaffolds were then perfectly matched the main histomorphometric indices of nature bone. More importantly, the final properties could be tailored during the design stage by changing the trabecular separation and thickness. Wang *et al.*^[87] put forward a probability sphere method to generate random seeds based on the Voronoi-Tessellation. In this study, a scale coefficient K was introduced to control the pore size and strut thickness, which successfully achieved the balance between “irregularity” and “controllability.” As a result, highly mimic scaffolds with porosities ranging from 60% to 95% and pore size ranging from 200 to 1200 μm were designed precisely. In addition, a porosity gradient ranging from 0.03 to 0.54 was also obtained. In this regard, it is clear that Voronoi-Tessellation method combines the advantages of reverse modeling method and topology optimization method. It cannot only realize the bionic structure design of bone scaffold but also optimize the structure to achieve desirable properties, such as porosity, permeability, and mechanical strength.

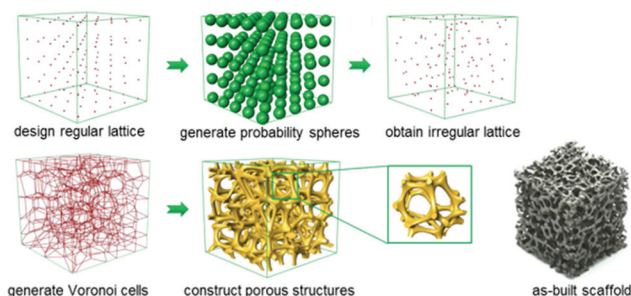


Figure 5. A schematic diagram showing the scaffold design principle based on Voronoi-Tessellation method and as-built scaffolds^[87].

Nevertheless, further investigations using *in vitro* and *in vivo* studies are needed to confirm their biological properties.

3. AM of Bone Scaffolds

The AM technique, which emerged in the 1980s, can rapidly produce scaffolds with external complex contour and internal porous structure. Combining with CT data obtained from the injury sites, AM can produce customized implants in a very short period, thus showing great prospects in orthopedic application. Up to now, many AM techniques exhibit their powerful ability to fabricate complex bone implants. This includes well-developed methods, such as selective laser sintering (SLS), selective laser melting (SLM), fused deposition modeling (FDM), electron beam melting (EBM), stereolithography (SLA), and electrospinning. Meanwhile, the developing AM techniques, including continuous liquid interface polymerization^[88] and two-

photon polymerization^[89], also demonstrate great potential for scaffold fabrication. In this chapter, we only focus on the several most relevant AM techniques for the fabrication of bone scaffolds. Scaffold-based AM method can process a wide range of biomaterials, including metals, polymer, and ceramics. On top of that, the prepared scaffold can provide appropriate biomechanical and biochemical conditions for cell proliferation and ultimate tissue formation. In comparison, scaffold-free AM method mainly utilizes multicellular bio-ink to construct 3D tissue and organ, which focuses on preparing soft tissue^[90,91].

3.1. SLS

The principle of SLS was first proposed in 1986^[92]. In brief, an SLS system mainly consists of a laser, powder bed, a piston to move down in the vertical direction, and a roller to spread a new powder layer. The computer-controlled laser beam sinters the powder, while the untreated powder serves as a structural support for the

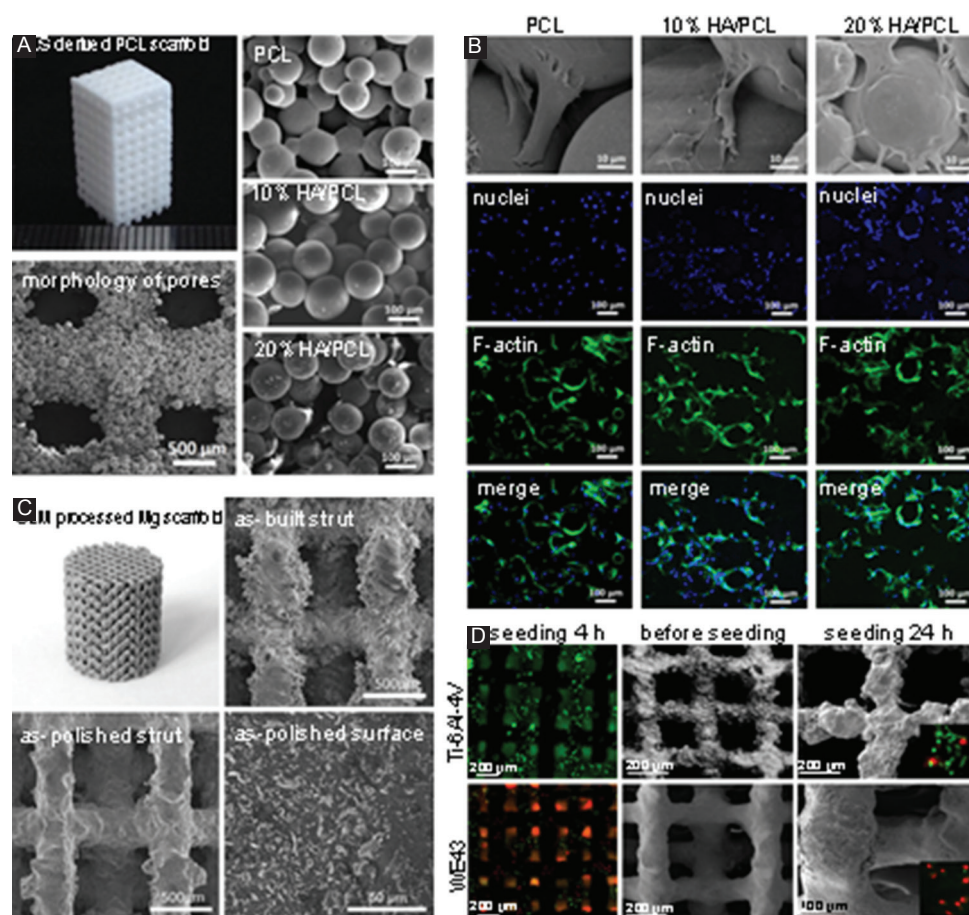


Figure 6. (A) Selective laser sintering (SLS)-derived poly(ϵ -caprolactone) scaffold, with scanning electron microscope (SEM) images showing the morphology of pores and well-connected microspheres^[99]. (B) *In vitro* evaluation of SLS-derived scaffolds, with SEM and confocal images showing the morphologies of adherent mesenchymal stem cells on the scaffolds after culturing for 12 h. (C) Selective laser melting (SLM) processed Mg-based scaffold (WE43), with SEM images showing the surface morphology and microstructure^[124]. (D) *In vitro* evaluation of SLM-derived WE43 and Ti-6Al-4V scaffolds. Fluorescent optical images showed the morphologies of MG 63 cells on scaffolds, in which live cells were stained in green, whereas dead cells were stained in red.

scaffold being built.

SLS usually refers to solid or semisolid consolidation mechanisms at a sintering temperature lower than melting point^[93]. In semisolid sintering, powder particles are partially melted and form a small amount of liquid phase, which bonds other solid particles to become solid parts. SLS with semisolid consolidation mechanisms is suitable for processing low melting point polymer, such as polylactic acid (PLA)^[94], polyglycolide^[95], poly(L-lactide) (PLLA)^[96], and poly(ϵ -caprolactone) (PCL)^[97]. Researchers also used SLS to process the polymer and ceramic composites. Du *et al.* reported that a microsphere-based hydroxyapatite (HA)/PCL composite scaffolds constructed by SLS^[98,99], which shows a highly ordered porous structure, as shown in Figure 6A. Scanning electron microscope (SEM) images confirmed that the microspheres were well connected through laser sintering in the composite scaffolds. *In vitro* assays showed that the scaffolds promoted cell adhesion, cell proliferation, as well as cell differentiation (Figure 6B). In addition, *in vivo* assay demonstrated an excellent histocompatibility and promotion of new vascularization tissue. Kumaresan *et al.*^[100] successfully applied SLS to fabricate polyamide/HA composite scaffolds with porosities ranging from 40% to 70%. With an optimal HA content of 15 wt.%, optimal mechanical properties with a maximum tensile strength of 21.4 MPa and a compression stress of 25.2 MPa were obtained.

SLS can also process bioceramic scaffolds, such as HA^[101], β -tricalcium phosphate (β -TCP)^[102], and bioglass^[103]. It is well known that bioceramics commonly have higher melting point as compared to polymers. Thus, SLS of bioceramic is generally based on the solid consolidation mechanism^[104]. Specifically, high-energy laser beam acts on the ceramic particles, increases the surface temperature, and promotes the particles approach to each other before sintering together. Meanwhile, the material on the grain boundary continues to diffuse to the pores, which promotes the densification behaviors. Nevertheless, SLS has a very short sintering period, due to its high heating rate and short holding time^[105]. Such a short sintering period can effectively restrain the diffuse of grain boundaries, which enables SLS to obtain ceramic scaffolds with nanoscale grains. Moreover, SLS exhibits great potential in fabricating scaffolds reinforced by low-dimensional nanomaterials (LDNs), such as carbon nanotubes, graphene, and boron nitride nanotubes^[106]. This is because the short sintering period combined with low sintering temperature can avoid destructing the structure of LDNs^[107]. Mechanical tests and *in vitro* cell culture confirmed their enhanced mechanical properties and improved biological properties, respectively^[105,108].

Special care should be taken with regard to the limited liquid formation in SLS, which causes insufficient densification and heterogeneous microstructures. To solve this problem,

an introduction of low melting point infiltration into SLS is an alternative method to promote the liquid phase formation and the crystallite rearrangement, thus enhancing the densification behavior. Duan *et al.*^[109] introduced CaO-Al₂O₃-SiO₂ as a liquid phase into HA scaffolds in SLS. The improved densification in sintering led to enhanced mechanical properties, with the compression strength, fracture toughness, and hardness increased by 105%, 63%, and 11%, respectively. Liu *et al.*^[110] also reported an increase of 18.18% in fracture toughness after introducing PLLA as a liquid phase into β -TCP scaffolds.

3.2. SLM

SLM is initially designed for 3D freeform fabrication of metals^[104]. Compared with SLS, SLM applied higher energy density laser to fulfill a complete melting/solidification mechanism^[111]. Thus, SLM-derived parts normally have an improved surface quality, density, and resultant superior mechanical strength. In consideration of the high-energy density, SLM is mainly applied to process metal scaffolds. Čapek *et al.*^[112] prepared a highly porous (87 vol.%) 316 L stainless steel scaffold as joint replacement by SLM. The scaffolds exhibited similar mechanical properties to those of trabecular bone with a compressive modulus of elasticity 0.15 GPa and compressive yield strength of 3 MPa. Weißmann *et al.*^[113] reported that Ti-6Al-4V porous scaffolds are formed with 3.4 and 26.3 GPa and porosity ranging from 54% to 60%. Besides, it also revealed a clear influence of the unit cell orientation on elastic modulus, compressive strength, and strain. Wang *et al.*^[114] evaluated the effects of parametrical variations on the mechanical properties of SLM-derived scaffolds. Results revealed that porous design could reduce the effective modulus of scaffolds by 75–80%. Shah *et al.*^[115] used SLM to produce Ti-6Al-4V scaffolds for load-bearing orthopedic applications. The bone/scaffold interface was studied after implantation in an adult sheep for 6 months. The compressive strength ranged from 35 to 120 MPa as the porosity ranged from 55% to 75%.

Recently, biodegradable metals, mainly Mg and Zn alloys, have drawn an increasing interest of researchers, due to their inherent degradability as well as close mechanical properties to that of natural bone^[116–119]. At present, the main problem of Mg alloys as bone implants is their poor corrosion resistance. Surprisingly, recent studies revealed that SLM-processed Mg alloys exhibited improved corrosion resistance. This could be ascribed to that SLM involved a rapid solidification, resulting in grains refinement and reduced composition segregation, both of which made contribution to the enhanced corrosion resistance of biodegradable Mg alloys^[4,120–122]. However, SLM of Mg alloys is still technically challenging. This is because Mg possesses a very active chemical property and is flammable even at

bulk state. In addition, the melting point of Mg is very close to the boiling point. In spite of those, our research group explored the application of SLM to prepare porous Mg with a home-made SLM system^[123]. Under the protection of Ar gas, an Mg scaffold was successfully formed at optimized process parameters. Li *et al.*^[124] also successfully prepared porous Mg alloy (WE43) scaffolds by SLM, as shown in Figure 6C. Mechanical tests revealed that the obtained Mg scaffolds exhibited sufficient Young's modulus of 700–800 MPa, which was comparable to trabecular bone after biodegradation for 4 weeks. The Mg scaffolds showed a proper degradation rate (20% volume loss after immersion for 4 weeks) and good compatibility (level 0 cytotoxicity) (Figure 6D). As for Zn and its alloys, the low melting and boiling points also challenge their process stability in SLM. SLM of

Zn powder easily causes a large amount of plume due to the metallic vapor. The formed plume will change the optical properties of the laser beam, such as the beam profile and the energy density. These reduce the process stability and cause poor part quality. To address these issues, Grasso *et al.*^[125] applied an *in situ* monitoring approach to detect the unstable process behaviors and anticipated severe defects in SLM of pure Zn. Besides, some researchers explored the use of SLM to prepare bulk Zn for bone tissue repair^[126–128]. However, to our best knowledge, there are few reports regarding SLM of Zn alloys scaffolds. Instead, a technique merging both gravity casting and 3D printing achieved success in producing porous Zn scaffolds^[129].

3.3. FDM

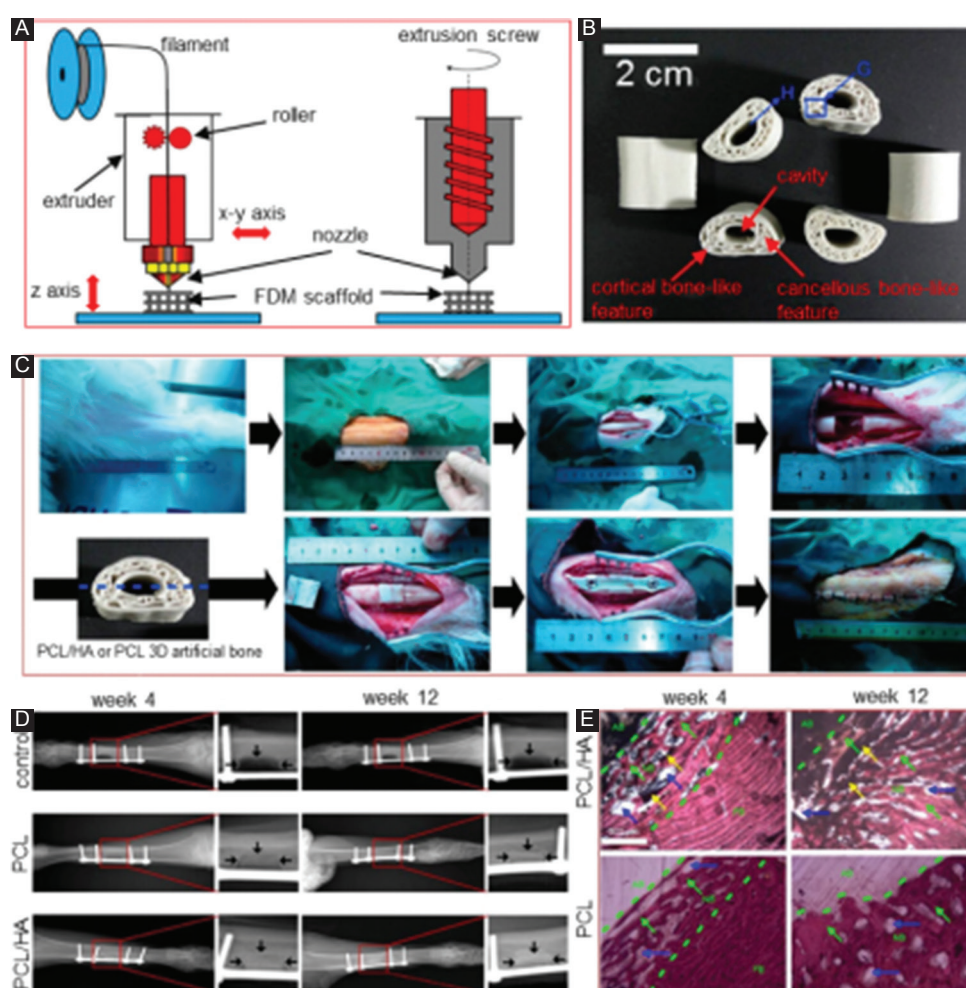


Figure 7. (A) A diagram for fused deposition modeling (FDM) process. (B) The FDM-derived poly(ϵ -caprolactone)/hydroxyapatite (PCL/HA) scaffolds^[139]. (C) The implantation of FDM-derived PCL/HA scaffolds. (D) X-ray images of the goat legs after implantation for 4 and 12 weeks, in which the arrows mark the bone defect edges. (E) Histological images showing the interfaces between the scaffolds and the surrounding tissue. AB represents artificial bone, FB represents natural goat femur bone, and NB represents new bone. The scaffolds were filled with new bone after 12 weeks' implantation.

FDM, also known as extrusion-based processes, was first put forward by Crump in 1988. In FDM, the materials are heated up until flowing before extruding or squeezing out of a nozzle. The extruded fluid subsequently deposited on the substrate with a layer-wise pattern based on the motion of the nozzle in each layer; then, a 3D scaffold is built layer by layer. A diagram for the FDM process is depicted in Figure 7A. The accuracy of extruded scaffolds greatly depends on the printing nozzle.

FDM technology is mainly applied to process low-fusing temperature polymer. Hutmacher *et al.*^[130] reported a use of FDM to fabricate porous scaffolds with PCL, which presented $0^\circ/60^\circ/120^\circ$ orientation patterns with the porosity more than 56% and pore sizes ranging from 380 to 590 μm . Zhou *et al.*^[131] fabricated hierarchical polymer scaffolds with macropores between 100 and 800 μm through the FDM. It was demonstrated that porosity printing errors between the obtained scaffolds and the designed model were $<5\%$, indicating that FDM is an efficient technology to obtain scaffolds with a relative high accuracy of pore structure. Tellis *et al.*^[132] combined micro-CT and FDM to produce polybutylene terephthalate scaffolds before applying for trabecular repair. Kosorn *et al.*^[133] reported that PCL/poly(hydroxybutyrate-co-valerate) (PHBV) blended porous scaffolds fabricated by FDM, founding that the compressive strength increased with incorporated PHBV increasing. Composite scaffolds based on PCL and poly(ethylene glycol) (PEG) were also fabricated by FDM^[134].

Recently, polymers with a relative high melting point have also been utilized in FDM. For example, polyether ether ketone (PEEK) with superior melting point between 330°C and 340°C was developed into scaffolds with a self-developed FDM system^[135]. In this system, the syringe consists of two different metal tubes, including a brass tube with an internal diameter of 17 mm attached to a 500 μm nozzle and a stainless steel tube. The brass tube with a good thermal conductivity was able to help PEEK absorbed sufficient energy to get fully melted. Controlling the nozzle temperature between 400°C and 430°C and the extrusion rate of 2.2 mg/s, PEEK scaffolds with 38% porosity were successfully obtained, which showed a compressive yield strength of 29.34 MPa and a compressive yield strain of 4.4%. Furthermore, Rinaldi *et al.*^[136] also reported a potential usage of FDM in fabricating PEEK scaffolds. However, with high melting point polymers in FDM, severe shrinkage, warpage, and delamination normally occur due to the sharp temperature gradient caused by the relative high extrusion temperature. Therefore, it is necessary to control the cooling process in FDM.

FDM has also been reported for the preparation of polymer and ceramic composite scaffolds^[137,138]. Xu *et al.*^[139] used CT-guided FDM to fabricate PCL/HA bones scaffolds with cortical bonelike features, as shown in

Figure 7B. The scaffolds exhibited close mechanics to that of natural bone in regard to structure feature and chemical composition. *In vivo* assays confirmed their enchanted biodegradability and improved new bone formability (Figure 7C, 7D and 7E), as compared to pure PCL scaffolds. Besides, Kim *et al.*^[140] produced a scaffold composed of polylactic-co-glycolic acid (PLGA) and β -TCP by FDM. After 12 weeks' implantation, the scaffolds integrated tightly with the surrounding bone tissue, indicating their good biocompatibility. Poh *et al.*^[141] fabricated composite scaffolds containing PCL and bioglass by FDM. Interestingly, *in vitro* tests revealed that the composite scaffold showed an upregulation of osteogenic gene expression. In addition, it was found that the host tissue infiltrated well into the scaffolds after 8 weeks' implantation into the nude rats. Though introducing bioactive ceramics can improve the biological properties of polymer scaffolds but also brings other concerns. During FDM of composites, the incorporated bioceramics with higher melting point exist in solid phase, which will increase the viscosity and reduce the fluidity of the slurry and ultimately reduce the accuracy and efficiency of the molding. On the other hand, due to the different shrinkage characteristics, a large number of pores will form between the ceramic particles and matrix, which greatly reduces its mechanical properties. Therefore, a further process is required to compensate for mechanical properties loss.

3.4. EBM

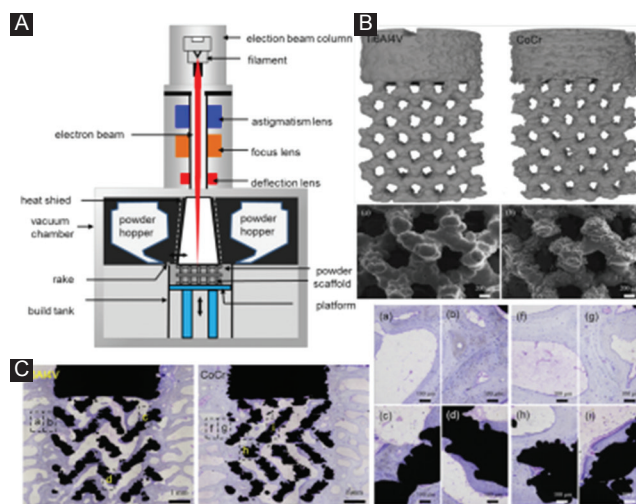


Figure 8. (A) A schematic diagram for electron beam melting (EBM) equipment. (B) Micro-computed tomography images showing the geometry of EBM-processed scaffolds, and scanning electron microscope images showing the responding roughness surface^[147]. (C) Undecalcified toluidine blue stained images showing the pattern of bone formation after the implantation of EBM-derived Ti6Al4V and CoCr scaffolds.

EBM was first developed and patented by Swedish Arcam Company^[142]. The EBM equipment is mainly composed of an electron beam gun compartment and a specimen-fabrication compartment, both of which are kept in a high vacuum (Figure 8A). Unlike SLS or SLM, EBM technology applies high-energy electron beam to melt the metal powder. The electron beam commonly scans the powder layer quickly before EBM, with an aim to preheat the powder bed and reach to a slight-sintering state. Following on, the electron beam selectively scans the powder layer based on 3D hierarchical data, enabling the preheated powder to melt and solidify together.

Compared to SLS/SLM, a primary advantage of EBM is that it has high beam-material coupling efficiency, which makes it easily process metals with an extreme high melting point^[143]. Thus, extensive researches are focused on utilizing EBM to produce porous metal scaffolds. Yan *et al.*^[29] reported a case that a 3D Ti scaffold was designed based on a volunteer with whole mandible defect and fabricated through EBM. After implantation, the grafted mandibular recovered well, showing a great potential of EBM in the bone graft. Ataei *et al.* produced Ti-6Al-4V gyroid scaffolds by EBM, which exhibited extreme high porosities ranging from 82% to 85%. In addition, the obtained yield strength and elastic modulus were in the range of 13.1–15.0 MPa and 637–1084 MPa, respectively, which were comparable to those of trabecular bone^[144]. Surmeneva *et al.*^[145] fabricated triple- and double-layered Ti-based scaffolds by EBM. Mechanical tests revealed that these scaffolds with gradient porosities of 21–65% had a compressive strength of 31–212 MPa and elastic modulus of 0.9–3.6 GPa, respectively. The compressive strength, elastic modulus, and deformation behavior of EBM-processed Ti-6Al-4V scaffolds could be optimized by controlling the cell shape^[146]. Shah *et al.*^[147] obtained Ti-6Al-4V and CoCr scaffolds with similar architecture using EBM, as shown in Figure 8B. *In vivo* tests were performed to investigate their effects on bone tissue growth. Although similar bone formation patterns presented in the porous network, higher osteocyte density was observed at the periphery of the CoCr scaffolds, due to its more favorable biomechanical environment. These results confirmed the great potential of osseointegrated CoCr scaffolds for load-bearing applications.

Zhao *et al.*^[148] fabricated Ti-6Al-4V scaffolds with cubic, G7, and rhombic dodecahedron unit cells using EBM before investigating their fatigue behavior. It was revealed that the fatigue mechanism for these scaffolds is the interaction of cyclic ratcheting and fatigue crack growth on the struts, which is closely related to the cumulative effect of buckling and bending deformation of the strut. Zhao *et al.*^[149] studied the corrosion behavior of EBM-processed scaffolds, revealing a better corrosion resistance as compared to wrought scaffolds.

As a permanent implant, the Ti-6Al-4V scaffolds with high anti-corrosion ability led to reduced precipitate of harmful metallic ion, such as Al and V ions, which might avoid serious complication. The cytocompatibility and osteogenesis of EBM-processed Ti-based scaffolds were also investigated^[150]. Results revealed that the scaffolds supported the cell attachment and proliferation with a minimal inflammatory cytokine secretion. In addition, the scaffolds with a pore size of 640 μm exhibited better biocompatibility than those with a pore size of 1200 μm because of their larger specific surface area.

It should be noted that the electron beam utilized in EBM normally has a low resolution because the electron beam is difficult to focus. Thus, the scaffolds prepared from EBM have large surface roughness^[151]. The accuracy of EBM is limited within a range of 0.3–0.4 mm, which makes it difficult to fabricate scaffolds with a small pore size. Eldesoukya *et al.*^[152] evaluated the geometric deviation between the EBM processed scaffolds and the initial CAD model utilizing a digital optical microscope. It was found that the struts designed with a smaller thickness would be produced oversized, leading to a corresponding pore size reduction and higher relative density. On top of that, strut thicknesses below 0.5 mm were under the threshold of processing with EBM. Besides, the cooling process during EBM takes a long period, which significantly reduces the efficiency^[153]. In comparison, EBM is limited to process conductive metal materials, whereas SLS/SLM is able to process a wide range of biomaterials, including metals, ceramics, and polymers.

3.5. SLA

SLA, also known as vat polymerization, fabricates products through selectively curing photoreactive resin^[154]. Specifically, it initiates with the formulation of the photopolymer liquid in a vat. Then, an ultraviolet light radiates on the surface with designed pattern and initiates the polymerization of the photoreactive liquid, while the platform moves the parts being built downward after each new layer is cured. This step will be repeated as the entire object is constructed. After draining the excessive resin, the object with desired structure is finally obtained. In general, two kinds of polymerization reaction, including free-radical polymerization and cationic polymerization, are utilized in SLA^[155,156].

In terms of irradiation type, SLA can be further divided into vector scan approach and mask projection approach, as presented in Figure 9A. In the first approach, one ultraviolet beam serves as the radiation source and projects on the liquid surface for polymerization through optics and a scanning galvanometer. However, in the second approach, the radiation source creates a large-area pattern with the aiding of a digital micromirror device, thus hardening one layer at a time. Comparatively,

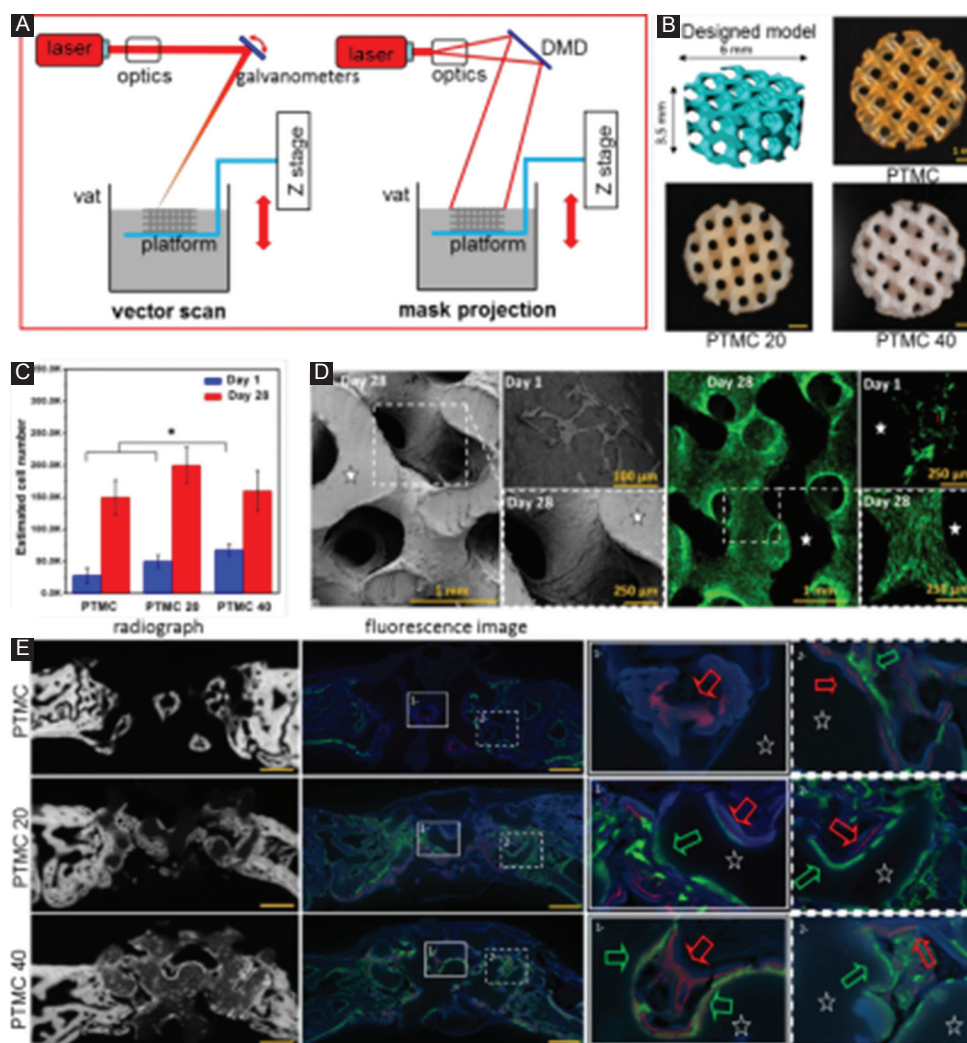


Figure 9. (A) A diagram for two irradiation types for stereolithography (SLA), including vector scan and mask projection. (B) Poly(trimethylene carbonate) (PTMC) scaffolds fabricated by SLA with various hydroxyapatite (HA) contents, with PTMC20 and PTMC40 containing 20 and 40 wt.% HA, respectively^[160]. (C) *In vitro* cell culture on scaffolds, with (D) scanning electron microscope and fluorescence images showing the different cell morphologies on the scaffold. (E) Contact radiographs of the defects combined with fluorescence images showing the newly formed bone after implantation for 2 weeks (in green) and 4 weeks (in red).

the second method can obtain an improved building efficiency, whereas the first method can achieve a higher accuracy.

Some synthetic polymers combined with photoreactive features, good biocompatibility, and suitable mechanical properties are used in SLA. For instance, PCL was used to fabricate scaffolds by SLA^[157]. Mechanical tests showed that the SLA-processed PCL scaffolds had elastic mechanical properties with Young's modulus ranging from 6.7 to 15.4 MPa. In addition, cell culture experiments confirmed its good biocompatibility. Poly(tetrahydrofuran) was also used for printed scaffolds with Young's modulus ranging from 5.7 to 27.5 MPa, bending strength ranging from 1.1 to 3.5 MPa^[158], where no cytotoxicity was also showed^[159]. Besides, PEG

was also used in SLA to produce scaffolds with a large elastic modulus range (5.3 ± 0.9 – 74.6 ± 1.5 kPa). There are also a small number of reports on the use of SLA to build composite scaffolds for bone tissue engineering. For example, Guillaume *et al.*^[160] successfully applied SLA to fabricate poly(trimethylene carbonate) (PTMC)/HA composite scaffolds, as shown in Figure 9B. The incorporated HA was enriched on the surface of scaffolds, forming a microscale structured. *In vitro* and *in vivo* experiments revealed an improved marrow stem cell differentiation and accelerated kinetic of bone healing for the microscale-structured PTMC/HA scaffolds (Figure 9C-E).

Ceramic scaffolds can also be fabricated by SLA. In general, ceramic particles are homogeneously suspended

in resin and photopolymerized in the SLA before burning up the resin and sintering the ceramic particles together. Thavornnyutikarn *et al.*^[161] produced bioceramic scaffolds using suspensions, which contain 41 vol.% of bioglass and 49 vol.% of an acrylate-based photopolymer resin together with 10 vol.% of a dispersing agent. The binder was removed after heating to 550°C for 3 h, and the scaffolds were then sintered at 950°C for 2 h. Du *et al.*^[162] also successfully fabricated customized ceramic scaffolds using SLA based on a rabbit femoral segment model. After culturing in oscillatory perfusion for 5 days, the cells attached and proliferated homogeneously on the scaffolds. Similarly, Levy *et al.*^[163] successfully produced HA ceramic scaffolds for orbital floor prosthesis by SLA. Of particular note is that SLA of such a composite resin is extremely difficult, due to its significantly increased viscosity. Moreover, the particle size of the added ceramic should be less than the curing thickness, so as to avoid a damage of the processing accuracy.

Indirect method has also been reported to produce scaffolds through SLA. For example, Sabree *et al.*^[164] used SLA to create an epoxy mold designed from the negative images of implants. Then, a highly loaded HA-acrylate suspension was filled into the mold. Subsequently, both the mold and the acrylic binder were removed by pyrolysis. The remaining HA scaffolds were then sintered to improve the densification rate. The obtained scaffolds possessed a porosity of approximately 42% and pore sizes of 300–600 μm . They presented an average crushing strength of 10–25 MPa, which were close to other ceramic scaffolds with similar porosity produced by different fabrication approaches. Kim *et al.* also used such an indirect method to produce HA scaffolds^[165].

Compare to other AM techniques, the main advantage of SLA is that it has high accuracy and resolution. SLA using two-photon curing method can build parts with an accuracy of 200 nm^[166]. Therefore, SLA is also reported to be used to prepare vascular scaffolds with a smaller pore structure. Nevertheless, the limited material use of photo resins and time-consuming post-processing remain challenges for the further application of SLA in biomedical application.

3.6. Electrospinning

In decades, electrospinning has gained intensive attention from the researchers in tissue engineering field, due to its powerful ability to fabricate scaffolds with micro- and nano-scale structure^[167–169]. A typical electrospinning apparatus generally includes a capillary tube with a spinneret, a high-voltage power supply, and a collector, as shown in Figure 10A. During electrospinning, the polymer solution is extruded from the electrically conductive spinneret to obtain droplets. The high voltage is imposed between the spinneret and the grounded

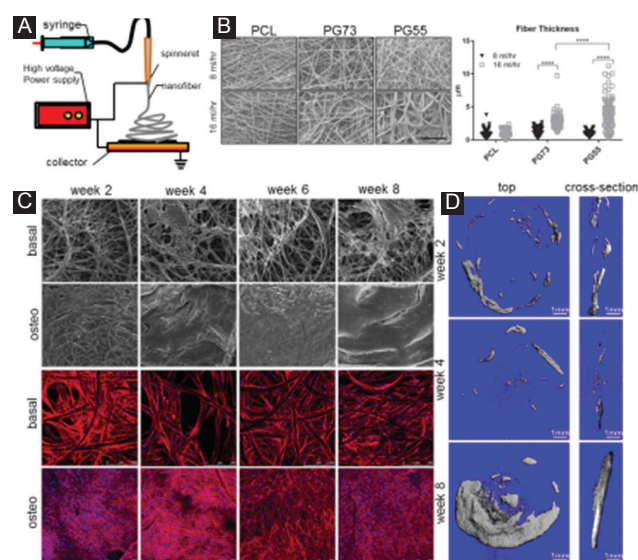


Figure 10. (A) A diagram showing an electrospinning setup. (B) Electrospun PCL/gelatin-blended scaffolds, and the measured fiber thickness at various parameters^[179]. PG73 means the PCL/gelatin ratio of 70:30, and PG55 means the PCL/gelatin ratio of 50:50. (C) Confocal laser microscopy of scaffolds under basal or osteogenic conditions^[177]. (D) Representative 3D reconstructions for the implants at 2, 4, and 8 weeks' post-implantation.

collector. Once the potential within the solution breaks through the surface tension of the obtained droplets, the polymer solution ejects from the spinneret. After the solvent evaporates, the polymer fibers are subsequently collected onto the grounded collector. As a result, a fibrous polymer scaffold with fiber diameter ranging from a 100 nm to several micrometers is constructed. Similar to FDM, electrospinning uses a nozzle to deliver the molten polymer for 3D structure construction. Thus, electrospinning is also described as “electrostatically assisted FDM”^[138]. Significantly, electrospinning produces much thinner filaments by applying high voltage supply. During electrospinning, it is of great importance to form a stable fiber, which in turn demands the polymer chains extensively entangling in the original solution. Otherwise, the solution will be ejected into a series of small droplets or aggregates into large bead-shaped fibers. From this view, low molecular weight polymers with a relative small molecular size are usually difficult to electrospin, due to their poor ability to entangle with each other. On the other hand, polymers with an extremely high molecular weight usually have a considerably increased solution viscosity, which inevitably increases the surface tension of the formed droplet—thus reducing the ability to form a jet fluid^[170]. Based on the above consideration, researchers commonly introduce a second polymer to enhance the chain entanglement without an increase of the viscosity or use of amphiphilic molecules to reduce the surface tension^[171].

Several thermoplastic polymers were used to fabricate electrospun scaffolds for bone tissue engineering, such as PCL^[172], PLGA^[173,174], and PLA^[175]. For instance, Shim *et al.*^[176] reported electrospun PLLA fibrous scaffolds, which were proved to be desirable substrates for cell growth and bone construction, while Vaquette *et al.*^[177] produced electrospun PCL scaffolds and investigated the cell adhesion. It was observed that a dense cell sheet formed on the top and bottom of the samples cultured in osteogenic media (Figure 10C). However, microcomputed tomography analysis revealed the slow bone regeneration until implantation for 8 weeks (Figure 10D). Yao *et al.*^[178] prepared 3D electrospun PCL and PCL/PLA nanofibrous scaffolds, which exhibited a high porosity of ~95.8% and interconnected and multiscale structure with pores sizes ranging from submicrometers to 300 μm . Compared to PCL scaffolds, PCL/PLA scaffolds exhibited enhanced mechanical properties and bioactivities. In fact, the synthetic nanofibrous scaffolds with relative low mechanical strength and poor ability to interact with cells have difficulty in meeting the requirements of bone repair. Thus, researchers attempted to prepare biphasic composite nanofibrous scaffolds with an improved comprehensive performance. Tan *et al.*^[179] obtained PCL and gelatin-blended scaffolds by electrospun, as shown in Figure 10B. It was found that PG73 scaffold (PCL: gelatin ratio of 70:30) spun at high flow rates was more favorable for cell growth and retention. Besides, Li *et al.*^[180] reported electrospun composite nanofibers composed of mesoporous silica nanoparticles and chitosan. The incorporation of mesoporous silica nanoparticles was proved to enhance the mechanical properties and promote biomineralization ability of the scaffolds. In addition, Lin *et al.*^[181] reported electrospun PLGA/HAp/Zein scaffolds, which exhibited excellent ability to promote *in vivo* cartilage formation. Bagchi *et al.*^[182] incorporated three different perovskite ceramic nanoparticles into PCL nanofiber, resulting in an enhanced expression of osteogenic genes. It is noted that an excess addition of nanoparticles would disrupt the formation of polymer fiber, leading to deteriorated mechanical behavior.

The electrospun fiber scaffolds also show great loading and encapsulation capacity of various drugs or small molecules because of their characteristic nanoscale morphological structure^[171,183]. For electrospun nanofiber drug delivery system, it possesses greater permeability to allow shorter response time and more precise control over the release rate. Thus, a large amount of bone scaffolds containing various drugs or small molecules, such as anticancer drugs, antibiotics, polysaccharide, and proteins, was constructed to achieve a controllable drug delivery into defective bone tissues^[184,185]. For example, electrospun PCL fibers were used as carriers

of ketoprofen, a non-steroidal anti-inflammatory drug for local chemotherapy^[186]. Kolambkar *et al.*^[187] fabricated the electrospun PCL nanofibrous scaffolds, which contained a growth factor delivery system for stable release of recombinant bone morphogenetic protein-2 (rhBMP-2). It was found that the delivery system provided a consistent release of rhBMP-2 in the fibrous structure, which effectively induced the bone formation. More comprehensive review regarding the area of using electrospun nanofiber scaffolds as a drug delivery system can be seen in Wang *et al.*^[171] and Bagchi *et al.*^[183].

4. Post-treatments

Although the porous scaffolds fabricated by AM can achieve the analogous porous architecture of natural bone, their various properties, especially mechanical properties and biological characteristics, are commonly lower than expectation. Therefore, post-treatments are commonly needed to enhance the comprehensive performance of AM processed scaffolds so that they can reach the requirements of bone tissue repair. Summarizing the previous literature, the post-treatment technologies applied in AM-processed scaffolds can be classified into two categories, including heat treatment and surface treatment, which are fully reviewed in this chapter.

4.1. Heat Treatment

Heat treatment is a way to improve the performance by modifying the microstructure. In extrusion process, solid ceramic particles are generally mixed with a solvent to form slurry, then extruded through the nozzle, and directly built into scaffolds. Such green scaffolds inevitably have extremely loose structure and resultant poor mechanical property. Thus, the heat treatment is essential to sinter and consolidate the solid particles together, with an aim to improve the mechanical properties. Aleni *et al.*^[188] used extrusion method to fabricate TiO_2 scaffolds with bentonite powder (2 wt.%) as the binder and water (35 wt.%) as the solvent. To harden the scaffolds, they were sintered at 1200–1300°C for 4 h with heating and cooling rates of 10°C/min. Mechanical tests revealed that the elastic modulus of TiO_2 scaffolds after post-heat treatment ranged from 2.08 to 5.90 GPa, which was close to that of high-density cancellous bone. Huang *et al.*^[189] used HA/TCP composite ceramic slurry to fabricate scaffolds by extrusion method. HA and TCP powders were dissolved in the solvent, which consisted of 30 vol.% glycerol and 70 vol.% deionized water. After extrusion, the composite scaffolds were sintered at 400°C to burn out the glycerol before sintering at 1200°C to increase the densification rate. Results showed that scaffolds with good chemical stability had no new phase formed during

sintering. Furthermore, post-treated scaffolds with 70% porosity possessed a comparable compressive strength ~ 12.5 MPa to porous trabecular bone. Similar post-heat treatment was also reported to treat FDM-derived HA-based composite scaffolds^[190,191].

Heat treatment has also been reported to be applied in SLS-produced scaffolds to promote the densification of the structure. It is believed that SLS-produced parts usually have a relatively low densification rate and resultant low strength due to its solid or semisolid consolidation mechanism. Therefore, a post-heat treatment is necessary for SLS-produced scaffolds, especially for high melting point ceramic scaffolds. Feng *et al.*^[192] applied an isothermal heating to secondly sinter the porous HA scaffolds achieved by SLS. It was found that the isothermal heating increased the density by activating grain-boundary diffusion and grain-boundary migration. As a result, the compressive strength of scaffolds was significantly increased from 6.45 to 18.68 Mpa. Other than that, Liu *et al.*^[193] used SLS to construct composites scaffolds, which exhibited a loose structure and high surface roughness. The post-heat treatment was then carried out at various temperatures ranging from 1200°C to 1400°C. After that, the compressive strength was significantly enhanced. Moreover, the surface roughness was decreased with pores shrinking.

Heat treatment is also an effective way to reduce the microstructural defects as well as the residual thermal stress. In SLM or EBM, the powders fuse and form a molten pool under the scanning of high-energy laser or electron beam, thus completing the fully melting/solidification mechanism. Nevertheless, the temperature distribution at the bottom, inner, and upper region of the molten pool is considerably different, which results in various temperature gradients in different directions. In this condition, the cooling rate in each direction varies, causing the anisotropic microstructure in the built parts. Moreover, in SLM or EBM, there is a certain remelting area at each layer, which also presents differed microstructures as compared to the region without remelting. On the other hand, SLM and EBM also involve an extremely high cooling rate, which usually leads to great residual stresses in the as-built parts. It is well known that the residual stresses considerably reduce the ductility of scaffolds. Therefore, a heat treatment is demanded to reduce the anisotropy and relieve the residual stress existing in the SLM- or EBM-deposited materials. Thone *et al.*^[194] investigated the microstructure and ductility of SLM-produced Ti6Al4V after heat treatment. It was revealed that α -martensite decomposed to uniform lamellar α and β structure after heat treatment. Besides, the elongation at failure increased significantly from 1.6% to 11.6%. Similarly, Wauthle *et al.* also confirmed the considerable enhancement in ductility and homogenized

microstructures of SLM-fabricated Ti6Al4V after heat treatment^[195]. However, although the post-heat treatment can reduce thermal stress, it will damage its mechanical strength. The reduced strength is mainly due to the grains growth during the long period heat treatment.

4.2. Surface Treatment

Many surface treatment methods have been explored to improve the biological properties of AM-fabricated porous scaffolds. Among these, coating bioactive ceramics on scaffolds is an effective way to improve the surface bioactivity of scaffolds. Zhao *et al.*^[196] achieved a uniform coating of calcium phosphate on electrospun keratin-PCL scaffolds by immersing the scaffolds into Ca^{2+} and $(\text{PO}_4)^{3-}$ solutions. Results showed that the incorporated keratin provided nucleation sites for the homogeneous deposition of calcium phosphate, which significantly facilitated the cell/matrix interactions. Luo *et al.*^[197] fabricated alginate/nano-HAP composite scaffolds with a nano-HAP layer homogeneously and completely coating the surface. The surface mineralization improved the cell attachment and spreading, as well as supported a sustaining protein release compared to scaffolds without nano-HAP layer. Cell viability studies also demonstrated that polycrystalline diamond coating on Ti6Al4V scaffolds promoted the attachment and proliferation of normal CHO mammalian cells and improved osseointegration^[198]. Another surface coating method is electrolytic deposition. It can prepare a uniform HA layer on porous Ti scaffolds^[199]. The morphology of the HA deposits could be controlled from plate-like to nanorod-like structure by altering the pulse current density. HA coating allowed more adsorption of serum proteins and further enhanced the ALP activity of MC3T3-E1 cells. Intriguingly, Chai *et al.*^[200] successfully prepared CaP coating on Ti scaffolds using electrolytic deposition. However, the surface modification with brittle ceramic coating usually has an adverse effect on the elastic modulus^[141]. Researchers also explored the use of a collagen layer coating on the scaffolds. It was reported that collagen coating not only improved its surface bioactivity but also improved the mechanical properties of the scaffold^[201,202].

Another popular surface treatment is chemical etching, which is an effective method for modifying surface microstructures by various corrosion methods. It is well known that natural bone has a hierarchical pore structure, including macroscale, microscale, submicroscale, and nanoscale pores. Diverse scale pores act different biological functions^[203]. Nevertheless, the present AM technologies are usually applied to prepare the macropore structure of bone scaffolds but come across a difficulty in fabricating microscale and submicroscale pores due to their limited resolution. To maximally mimic the hierarchical pore structure, the post-chemical etching is

proposed to construct micro- or nano-scale pore structure on AM-produced scaffolds. Amin *et al.*^[204] applied both acid-alkali and alkali-acid heat treatment to functionalize the SLM-processed porous Ti scaffolds. The modified scaffolds exhibited irregular etching nano-scale pits with the size ranging from 100 to 200 nm. *In vivo* tests revealed that such features improved the apatite-forming ability, resulting in significantly larger volumes of newly formed bone within the pores of the scaffolds. Cheng *et al.*^[205] treated the SLS-processed Ti-6Al-4V scaffolds through a combination of sandblasting, acid etching, and pickling. Then, a desirable multiscale micro-/nano-roughness was obtained on the surface, which was proved to enhance the osseointegration. Besides, Shuai *et al.*^[206] also used chemical etching method to treat SLS-processed PLLA scaffolds. In sodium hydroxide solution, PLLA was etched into soluble polar groups through a hydrolysis reaction. Thus, well-ordered pores (1–3 μm) and smaller penetrated pores with a pore size $<1 \mu\text{m}$ left on the surface. The chemical-treated scaffolds exhibited surprising bioactivity due to the formed polar groups on the surfaces. In addition, the degradation was adjustable through controlling the size and quantity of the surface pores. Ramier *et al.* reported^[207] an introduction of epoxy groups on the surface of electrospun poly(3-hydroxyalkanoate) scaffolds using chemical etching. It was found that human mesenchymal stromal cells exhibited a better adhesion on the modified scaffolds as compared to the control cells. It should be taken that chemical corrosion inevitably damages the strut of scaffolds to some extent, which is possibly resulting in a negative effect on the mechanical properties of the scaffolds. It was reported that alkali treatment caused a deterioration of the mechanical strength of porous PLLA scaffold, with the compressive strength decreased by 30.1%^[206]. Similar mechanical loss also occurred to porous Ti scaffolds after chemical etching^[208]. Besides, researchers also applied oxygen plasma treatment to increase the surface hydrophilicity to enhance the biocompatibility^[209-212].

5. Conclusions and Challenges

Currently, the demand for bone scaffolds for clinical operations is increasing rapidly. AM techniques offer unique advantages for bone scaffolds fabrication with respect to its ability to produce customized external shape and interconnected pore structure. Combining with scaffolds design and specific post-treatments, they can produce customized scaffolds with desired comprehensive performance, including suitable mechanical properties and good biological behaviors, for bone repair in a short development period. Nevertheless, the current state of AM of scaffolds for clinical application is still behind expectation. AM of bone scaffolds belongs to multidisciplinary, including manufacturing engineering,

materials science, and biomedical engineering, which requires the coordination and cooperation of researchers in different fields.

In terms of porous structure, hierarchical and gradient pore structure similar to that of natural bone is the most conducive to the growth of bone tissue. The current CAD design software enables the designer to easily complete scaffolds design, but it is still not able to support the complex geometric features for scaffold modeling. In contrary, reverse modeling and mathematical modeling methods are time-consuming but exhibit powerful ability to construct complex geometric gradient features. Therefore, combining CAD design with CT imaging or mathematical modeling may help to achieve more rapid and mimic scaffold model. Bone scaffolds are also expected to meet the requirements of suitable mechanical and biological properties, including elastic modulus, stiffness, porosity, and permeability. However, there is still no definite design standard for bone scaffold design. Therefore, the application of computer-aided engineering technology in multiobjective optimization analysis of scaffold structure will become the focus of bone scaffolds design in the further investigation. Using topology optimization, the mechanical and biological performance of the scaffold can be optimized to achieve the optimal comprehensive performance. Nonetheless, the macro- and micro-integration design of porous scaffolds needs further study through topology optimization.

In terms of AM process, the AM techniques applied in bone tissue engineering are far behind the industrial application standards. Improving the accuracy and efficiency of processing should be one of the research directions in the future. The accuracy of AM built scaffolds is influenced by many factors, including models files, equipment system, and process parameters^[36,213,214]. Although the post-treatment process can improve the surface accuracy, it is time-consuming and tends to reduce the efficiency. To overcome this issue, one primary task is to achieve a fundamental understanding of the affecting mechanism of the processing parameters on the formation quality. Take SLM or EBM as an example, solving the key technical issues, such as the interaction between laser beam (electron beam) and powder, the control of residual stress, and the processing stability, will unquestionably make a positive influence on improving the processing accuracy. On the other hand, nano- and micro-technology have been developing rapidly in recent years. The combination of nano- and micro-technology with AM technology may offer a great chance for improving the processing accuracy of AM-derived bone scaffolds in the near future.

From the view of material system, developing more kinds of functional material for bone tissue repair is another future research direction for AM techniques. This is because that there are very limited materials can

be used in AM techniques. The material used in AM of scaffolds should not only meet the requirements of medical applications but also need to meet the technical requirements of AM. For instance, the resins used in SLA are very limited because the resin should be a liquid that rapidly solidifies on illumination with light. What is more serious is the current SLA technique is still limited to the use of a single resin at a time. As for materials used in SLS or SLM, it should have good fluidity and thermal conductivity and the powder should have a suitable shape for powder application. In fact, powders used for SLM equipment require spherical shape and a uniform particle size distribution. To satisfy these specific requirements, it is necessary to develop the dedicated material system for AM. On the other hand, hybrid materials integrate the advantages of several materials, which may exhibit better properties as used in AM scaffolds for bone regeneration. Previous researches have confirmed that AM technology could fabricate parts with multiple materials. These compositional variations are not only expected to change their processability but also are expected to achieve better mechanical or biological properties.

There is no doubt that the application of AM scaffolds for bone repair has a bright future. Thus, multidisciplinary research will be necessary to face those challenges and fully realize the potential of AM in bone repair applications in the coming days.

Conflicts of Interest

There are no conflict of interest.

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New microorganism isolation techniques with emphasis on laser printing

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Abstract: The study of biodiversity, growth, development, and metabolism of cultivated microorganisms is an integral part of modern microbiological, biotechnological, and medical research. Such studies require the development of new methods of isolation, cultivation, manipulation, and study of individual bacterial cells and their consortia. To this end, in recent years, there has been an active development of different isolation and three-dimensional cell positioning methods. In this review, the optical tweezers, surface heterogeneous functionalization, multiphoton lithography, microfluidic techniques, and laser printing are reviewed. Laser printing is considered as one of the most promising techniques and is discussed in detail.

Keywords: Laser printing; bacteria isolation; soil; unculturable microorganisms; biodiversity

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1. Introduction

According to current evaluations, more than 99% of the prokaryotes biodiversity remain unculturable when using traditional cultivation methods^[1-4]. To investigate the biodiversity and functions of unculturable microorganisms *in situ*, different molecular biological methods are used^[5-7]. At the same time, cultivation is still necessary for a detailed investigation of the growth and metabolism of microorganisms, genetic manipulations, and introduction of promising strains in biotechnological processes^[8-11]. The study of processes carried out by microorganisms in nature is necessary for understanding of functioning principles of ecosystems, assessment of biogeochemical flows of matter, modeling, and forecasting of various biospheric processes, which are associated with the problems of climate change and anthropogenic influence on the biosphere^[12,13]. It is equally important that new species and strains of microorganisms can be valuable producers of bioactive substances, such as enzymes and antibiotics^[14,15]. The impossibility of complete reproduction of the conditions of the natural habitat of microorganisms in

the laboratory is considered the main reason for their unculturability^[4,8]. In natural heterogeneous environments, such as lands, soils, and bottom sediments, physical-chemical conditions of the existence of microorganisms change on a microscale^[16-18]. Such natural environments are combinations of microzones, different in concentration and composition of organic matter (including microbial metabolites), salts, gases, mineral composition, and other parameters. It is obvious that the reproduction of the entire set of environmental parameters *in vitro* is impossible. Intra- and inter-population interactions play an important role in the functioning of microorganisms within natural ecosystems^[19-23]. In particular, such interactions include the formation of biofilms, production of growth regulators and signal metabolites, and production of antibiotics. Sample preparation procedures for traditional cultivation methods, including desorption of cells from mineral particles, disrupt communications between the microorganisms, which is especially important for strongly associated groups such as symbionts and pathogens^[24].

2. New Isolation Methods

Nowadays, a wide variety of methods is used to expose new, previously uncultivated microorganisms^[8]. In particular, new nutrient media containing specific substances are used; cultivation is carried out under different physical and chemical conditions of the environment, such as atmospheric composition, temperature, and pH.^[8,25] Furthermore, applications of diluted nutrient media in combination with long incubation periods^[25,26], as well as, joint cultivation of microorganisms of different species, have been studied^[1,27]. In recent years, some fundamentally new methods of cultivation based on the placement of microorganisms in the natural environment without the usage of nutrient media have been developed. Such methods utilize diffusion chambers^[28,29] and polymer coatings^[30] in which microbial cells are placed. In this case, microorganisms receive all necessary nutrients from the natural environment, remaining isolated from it. The allocation of a new antibiotic producer (teixobactin) using such methods is considered as an important achievement^[14]. Another approach includes simultaneous cultivation and screening of tens and hundreds of thousands of bacterial microcolonies on porous polymer or ceramic isolation chips^[31,32]. For the cultivation of “unculturable” microorganisms, methods allowing isolation of single cells from natural environments can also be used. Among these methods, the most popular is based on the dilution of microorganism suspension, flow cytometry and cell sorting, laser microdissection, compartmentalization, and application of micromanipulators^[33,34]. In addition to the cultivation of previously uncultivated species of microorganisms, the isolation of single microbial cells is necessary for the study of cell physiology, interactions between cells, as well as for the search of new metabolites, such as antibiotics and enzymes^[34].

Modern scientific and technological progress provides many opportunities in terms of the development of novel methods for cultivation, isolation, manipulation, and study of individual bacterial cells and their consortia. New approaches may speed up the process of working with microorganisms significantly and allow carrying out their more complete and comprehensive studies. It is necessary to note that so far very few methods have been proposed for positioning of bacteria arrays with micrometer accuracy. In Akselrod *et al.*^[35], three-dimensional (3D) networks of living cells in hydrogel were formed without loss of their viability using arrays of multiplexed holographic optical traps (tweezers) with unprecedented accuracy (<400 nm). To form optical traps, two lasers were used: Ar⁺ laser (20 W, 514 nm wavelength) and continuous wave Ti: Sapphire laser, tunable in the range of $\lambda = 850\text{--}900$ nm, as well as a combination of two diffraction elements, combined with different lenses in an inverted optical microscope. Networks of 3T3 fibroblasts

surrounded by a ring of bacteria were formed. The ability to manipulate hundreds of *Pseudomonas aeruginosa* bacteria simultaneously in two-dimensional (2D) and 3D arrays was also demonstrated. The method of holographic optical trapping is very accurate but technically difficult to perform.

In the study of Rowan *et al.*^[36], the method of heterogeneous functionalization of surfaces is proposed, which is a four-step lithographic process based on microcontact printing of organic monolayers, implantation of hyperbranched polymer, and its further functionalization. As a result, structures, on which the directed inoculation of bacterial cells is carried out, are obtained. The investigations of cell survival have shown that cells remain viable on the obtained structured surfaces. Large isolates of bacteria containing 18 ± 5 bacteria and small isolates containing 2 ± 1 bacteria were obtained. According to this paper, the demonstrated method can be used for high-throughput screening and biosensing. However, it is difficult to combine the heterogeneous functionalization of surfaces using this method^[36] with routine biological research and conditions of microorganism cultivation (temperature, pH, nutrients, etc.). It is necessary to note that the task of finding simple ways to provide high resolution of living bacteria arrays with the opportunity of various biological studies was solved in some publications. The approaches proposed in these works are, in fact, the harbingers of 3D printing.

In a study of Weibel *et al.*^[37], the technique of living bacteria stamping on agarose plates was proposed. Bacteria arrays were printed (the size of a single spot with bacteria >200 μm) in the area of up to 50 cm^2 . Polydimethylsiloxane (PDMS) stamps were produced with the help of photolithographic technique. The achieved minimum size of the print protrusion was 190 μm at the height of 140 μm , which, however, is far from the size required to separate bacteria. This method is fast, reproducible, and convenient and can be used to control the pattern, spacing, and orientation between colonies of different bacteria. In Xu *et al.* study^[38], living bacteria arrays with cellular resolution were printed on agarose substrate using elastomeric (PDMS) stamps with a high aspect ratio, obtained by the reverse *in situ* lithography (RISL) method. Figure 1 shows the advantages of the RISL method over the standard ultraviolet photolithography. The only limitation of the RISL technology is the protrusion diameter, which can hardly be <1 μm due to the optical diffraction limit.

The method of microcontact printing of bacteria arrays works as follows: The drop of *Escherichia coli* in the culture medium LB is deposited on agarose gel (3 weight [wt]% in LB) and, on the agarose substrate, a monolayer of bacteria is formed (the liquid is absorbed by agarose gel). Then, the PDMS stamp contacts with a monolayer of

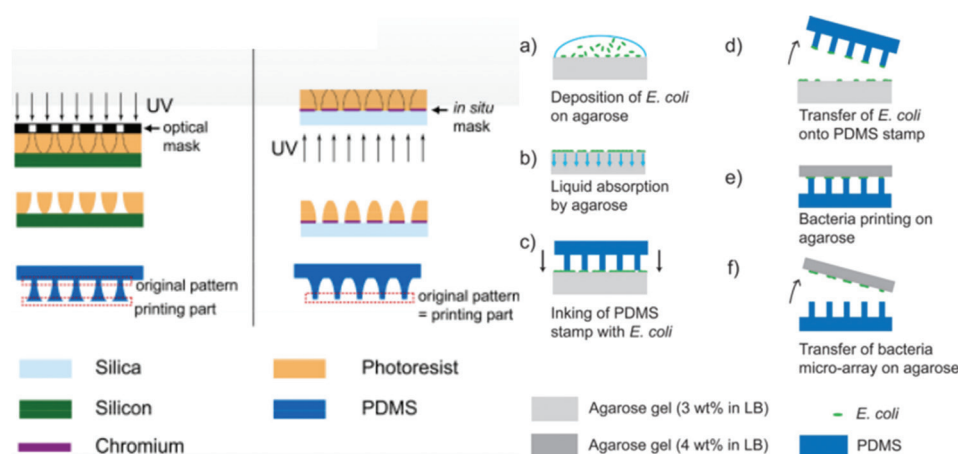


Figure 1. The advantages of the RISL method over the standard ultraviolet photolithography. “Reprinted with permission from (Xu L, Robert L., Ouyang Q., Taddei F., Chen Y., Lindner A. B., Baigl D. Microcontact printing of living bacteria arrays with cellular resolution// Nano Lett. -2007. Vol. 7 - № 7. - P. 2068–2072). Copyright (2007) American Chemical Society.”

bacteria covering the agarose gel. The stamp is removed, and the part of bacteria remains on it. Then, on the contact of the stamp with the layer of agarose gel (4 wt% in LB, thickness 200 μm), bacteria are transferred to the agarose layer. Thus, in a few seconds, arrays of *E. coli* bacteria can be printed directly on the agarose substrate with micron resolution, up to single bacteria, on a large area (cm^2). It was shown that, after the pattern is printed, bacteria continue to grow and divide, as in the conditions of mass culture, i.e., the bacteria retain their normal physiological behavior after printing. It was also shown that the agarose concentration is crucial for good printing performance. Too little concentration leads to a distortion of the printed pattern, while too high concentration is not suitable for bacteria cultivation. To obtain arrays of single bacteria, the effect of reduction of the initial bacteria concentration in a drop of the culture medium LB was studied. For the initial concentrations of 10^9 and 10^8 cells/ml, the average number of bacteria per spot was measured at 12.1 and 1.4, respectively. A very narrow distribution was obtained at the low concentration of bacteria: 44.6% of spots had only one *E. coli* cell and 40.1% of spots had 0 or 2 cells. These results demonstrated that the microcontact printing allows the production of regular arrays of single bacteria. Moreover, it was shown that this approach to the separation of bacterial arrays allows analyzing the growth rates of individual lines of bacteria. This methodology provides a simple way for any desired spatial 2D distribution of bacteria and can be used for both screening and studies of bacterial phenotypic variation, population dynamics, and evolution of ecosystems.

A fast-developing area - sociomicrobiology - has identified the mechanisms with the help of which bacteria participate in collaborative and competitive relationships by influencing nearby neighbors through physical contact

and modification of the chemical composition of their common microenvironment. To explore the behavior of small microbial aggregates, different technologies of microprocessing that limit bacteria in microfluidic devices, microresonators, and ultra-low volume liquid droplets were developed^[39-45]. The ability to integrate analytical systems with microfluidics has made these isolation platforms attractive for high-performance screening for antibiotic resistance and enzymatic activity analysis. For example, in Eun *et al.*^[39], a high-performance analysis and isolation of bacterial cells encapsulated in agarose microparticles with the use of fluorescent-activated cell sorting (FACS) are described. Flow-focusing microfluidic systems were used to create monodisperse microparticles with a diameter of $\approx 30 \mu\text{m}$. The sizes of these particles made them compatible with flow cytometry and FACS, and the sensitivity of these methods reduced the incubation time for cell replication before carrying out of the analyses. The small volume of microparticles ($\approx 1-50$ picoliter [pL]) minimized the number of reagents required for bacterial studies. This platform made it possible to allocate and isolate bacteria effectively, as well as to use the combination of methods for quick identification of targets for biologically active small molecules. As an experimental demonstration of this method, *E. coli* cells were encapsulated in agarose microparticles, incubated in the presence of different concentrations of rifampicin, and analyzed with the use of FACS. The minimum inhibitory concentration of rifampicin was determined, and spontaneous mutants that had antibiotic resistance were isolated with the help of FACS and characterized by DNA sequencing. Using this approach, the time and amount of antibiotics needed to isolate mutants were reduced by 8 and 150 times, respectively, compared to traditional microbiological methods using nutrient agar

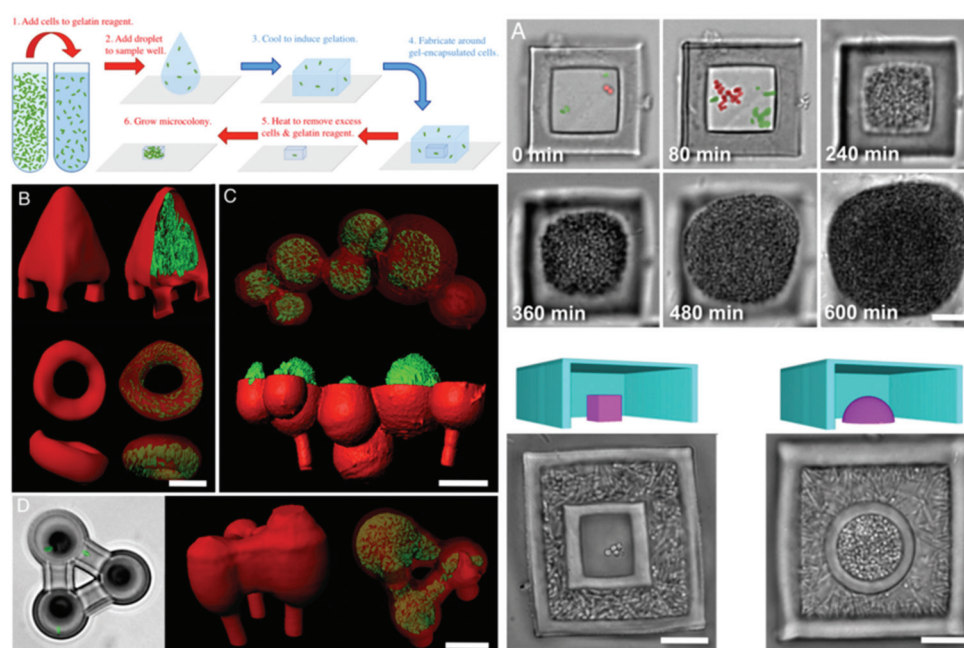


Figure 2. Gelatin-based micro-three-dimensional printing in the presence of bacteria. (Left) engineering polymicrobial communities (right) “Reprinted from (Connell J.L., Ritschdorff E.T., Whiteley M., Shear J.B. 3D printing of microscopic bacterial communities // Proc. Natl. Acad. Sci. - 2013. - Vol. 110 - № 46. - P. 18380–18385).”

plates. Thus, this method is important in the fields of chemical biology, chemistry of natural products, as well as for the discovery and characterization of biologically active secondary metabolites.

The approaches mentioned above have been useful for limiting the size, shape, and physical attributes (microhabitat); however, none of them have provided the opportunity to determine the 3D geometry of bacterial aggregates or the orientation of multiple populations arbitrarily. In addition, the process of cell encapsulation in ultra-low volume cavities often limits mass transport, leading to conditions that are incompatible with growth and signaling between physically isolated populations. A growing number of proofs highlight the importance of microcolonies in bacterial reproduction^[27]; however, the lack of tools for systematic assessment of cell behavior in such communities is observed. New strategies for the creation of a 3D cultural environment on a microscopic scale can play a crucial role in identifying how bacteria manage antibiotic resistance and other social behaviors in small dense aggregates.

In Connell *et al.* and Connell *et al.*^[46,47], the method of laser formation of microscopic 3D chambers based on multiphoton lithography (MPL)^[48-51] is described. The MPL method has high-throughput capacity and the ability to produce arbitrary patterns. It offers opportunities for the industrial production of 3D microdevices such as micro-optical components, scaffolds for tissue engineering, and microfluidic chips. In Connell *et al.* study^[52], bovine serum albumin (BSA), a highly soluble protein that can

be crosslinked into porous, durable, and biocompatible hydrogels, was used to create microscopic 3D bacterial chambers. Some separate bacteria were enclosed in the BSA chambers with the volume of 1 pl and were then grown in clonal populations. Due to the diffusion of biologically significant molecules and antibiotics through the walls of BSA, as well as the exchange of quorum-sensitive signals, the social behavior of bacterial communities was studied in relation to the size and density of the population, the shape of the container, and the flow rate of the environment.

Within the human body, bacteria usually exist in structured 3D communities consisting of several bacterial species. To get detailed information about the effect of geometry on pathogenicity, a 3D printing strategy is described in Connell *et al.*^[52] for bacterial communities, in which physically distinct but chemically interactive populations of a certain size, shape, and density can be organized in any form essentially (Figure 2). Using this approach, it was shown that the stability of a single pathogenic species of bacteria to the antibiotic might enhance the resistance of the second species because of their 3D relations. With the help of laser lithographic technique, microscopic containers of up to 1 pl volume with a container wall thickness of up to 2 µm around selected bacteria suspended in gelatin were formed by cross-linking of the polypeptide molecules in the focal region due to non-linear absorption of laser radiation by photosensitizer molecules. The result of this multiphoton absorption is the formation of singlet oxygen, which

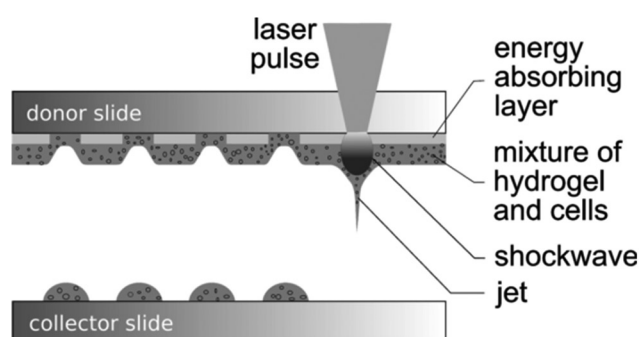


Figure 3. Schematic sketch of the laser-assisted bioprinting.

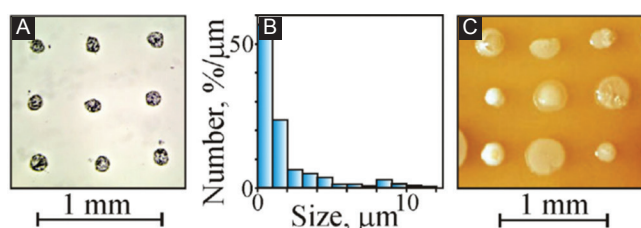


Figure 4. Gel/soil microdroplets on an acceptor plate (A), soil microparticles distribution in microdroplets (B), and colonies as the result of microbial growth after gel/soil printing of gel/soil microdroplets onto agar plates (C) with $E = 20 \mu\text{J}$.

stimulates intramolecular and intermolecular covalent cross-linking reactions between BSA and gelatin. The unique physical and chemical properties of gelatin have motivated the interest in its use for a variety of applications, including storage, immobilization, and 3D cultivation of bacteria^[53-55]. After removal of the excess reagent, the bacteria are localized in sealed cavities formed by cross-linked gelatin, which is a highly porous material and supports a rapid growth of fully enclosed cell populations. It is easily permeable for polypeptides, antibiotics, and to the physical and chemical signals with the help of which interaction between bacteria occurs. The isolation of cells in microcontainers provides the opportunities for embedding of different types/densities of containers into each other, as well as for dynamic changes in the orientation of the entire populations of bacteria within the community.

The authors have shown that spatially localized interactions of the Gram-positive *Staphylococcus aureus* and the Gram-negative *P. aeruginosa* bacteria (two human pathogens that often form persistent coinfections inside wounds, catheters, and lung of patients with mucoviscidosis) may increase *Staphylococcus* survival in the treatment with the β -lactam antibiotics.

Micro-3D cell printing fundamentally expands the possibilities for probing of antibiotic resistance when a single bacterial microgroup can affect the antibiotic susceptibility of adjacent surrounding or embedded populations - a matter of particular relevance for *in vivo*

infections (e.g., wounds, oral cavity, and cystic fibrosis of the lungs) where tissues are often colonized by several species of bacteria simultaneously. The true power of 3D cell printing lies in the ability to organize the microbial communities in the unlimited range of geometries. Micro-3D cell printing can also be a valuable tool for the investigations of mechanisms and dynamics of the adaptive responses to environmental conditions.

3. Laser Bioprinting

Over the past two decades, bioprinting, including the printing of mammalian and bacterial cells, has become an extensive field of research. Printers, starting with modified inkjet printers, extrusion pens, electrospinning, and laser systems, have demonstrated the ability to create submillimeter resolution samples of biomaterials. Tests for viability, genetic damages, cell differentiation, and stress tests were performed after printing to demonstrate that each of these tools can form patterns and 3D structures of intact living cells directly without the aid of surface functionalization or patterns (lithography, masks, etc.)^[56,57]. Currently, bioprinting is used in laboratories all over the world to print living cells ranging from stem cells, bacteria, and viruses to create microchips and 3D tissue engineering constructs *in vitro*^[57-60].

Most methods, such as inkjet printers and extrusion pens, require a nozzle or print head to print microdrops of “bio-ink”^[60]. These nozzles are unable to print solid particles without clogging up. The modified method of laser-induced forward transfer (LIFT), such as biological laser printing, does not require the nozzles or holes of any type because it is based on a focused laser beam. Laser bioprinting based on LIFT (Figure 3) is a relatively new bioprocessing technique^[61] for placing biological materials or living cells in well-defined positions on samples (Figure 4). This method allows fast transfer of ultra-small amounts of biological material to different substrates with spatial accuracy better than $5 \mu\text{m}$ at the deposition rate up to 100 pixels of biological material per second. With the help of laser bioprinting, one can successfully create print arrays and samples of biological materials from liquid and solid-phase “bio-inks,” including proteins, viruses, mammalian cells, and bacteria^[56,57,59,61,62].

Laser bioprinting of microorganisms opens the door for the development of new and accurate methods that could be used for the study of: (i) The development of microorganisms in solid matrices in the presence of nutrient gradients, (ii) interactions of the same and different organic colonies next to each other, (iii) response to the stress and resistance to inhibitors, and (iv) cellular communication or quorum determination. This method provides a relatively simple way to perform experiments with a large number of replicas and can even be used for the selection of strains in the future. Laser printing can also serve as a means for carrying out multifactor experiments^[63].

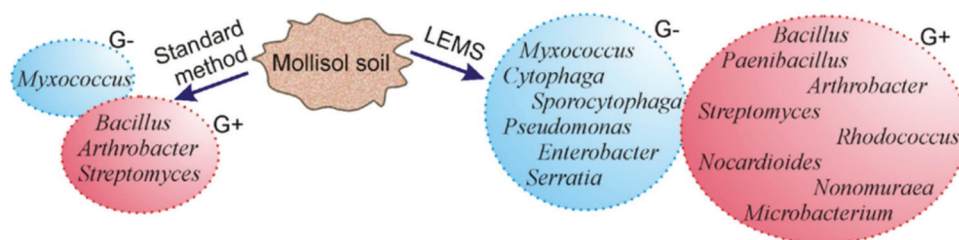


Figure 5. Cultivated and identified groups of G+ and G- bacteria from the mollisol soil using the standard method and laser engineering of microbial systems technology.

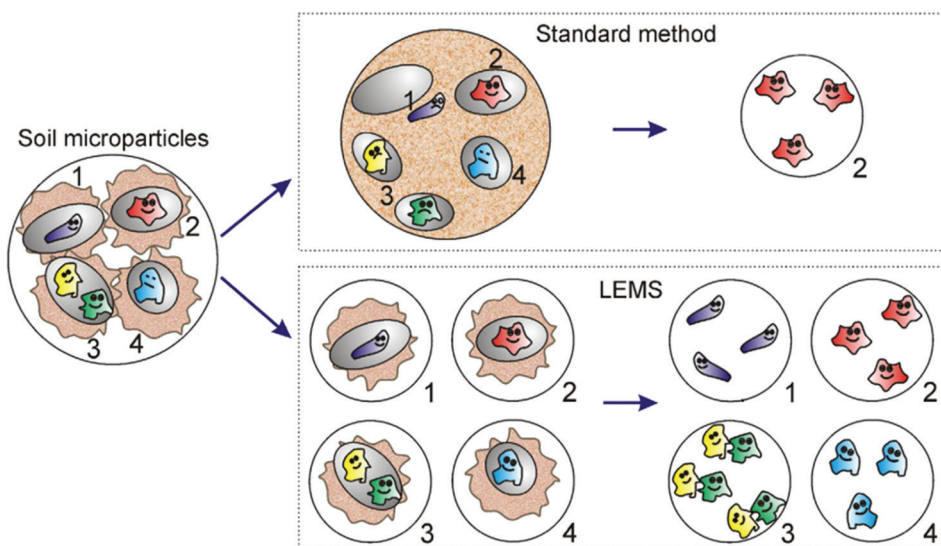


Figure 6. A diagram illustrating the main differences between the laser engineering of microbial systems and standard method, leading to an increase in biodiversity in the isolation of microorganisms from soil. The numbers indicate microbes that, with the standard cultivation method: 1 - easy to flush out of their microenvironment, 2 - most actively multiply, 3 - separate from those with which they exist in symbiosis, and 4 - remain in the "sleeping" state.

The printing process provides an unprecedented level of accuracy. Using traditional methods, cell suspensions can be diluted in sterile environments and manually placed as droplets in certain positions on the growth matrix; realistically, the volume of the droplet cannot be $<1 \mu\text{l}$, and the accuracy of the human hand will require that the droplets were not located closer than 2–3 mm from each other. Laser printing of microdroplets of cell suspensions is carried out with micron accuracy and drop volumes of $<10 \text{ pL}$.

The principle of laser printing is as follows: The donor slide is covered with a layer that absorbs laser radiation and a layer of biomaterial that needs to be transferred; usually, it is hydrogel with cells. Laser pulses are focused through the upper glass slide in the absorbing layer (Figure 3). The evaporation of this layer creates a high gas pressure that transfers the biomaterial to the bottom slide. The vapor bubble reaches its maximum volume in a few microseconds and collapses when its internal pressure drops below the atmospheric pressure^[64]. However, accelerated biomaterial continues to move by inertia to

the receiving slide and forms a thin jet at the front of the bubble, which lasts several hundred milliseconds. As a result, the volume from several pL to several nanoliters (nL) is transferred to the surface of the receiving slide (a collector) in the form of a drop (Figure 4). Biomaterial droplets can be arranged in 2D models by moving the donor and collector slides relatively to each other. The volume of printed droplets depends on the laser pulse energy, the thickness of the biomaterial layer, as well as the viscosity of the biomaterial layer on the donor slide^[65]. The number of cells in each droplet usually depends on the initial cell density in the biomaterial layer and the volume of the printed droplet.

In a study of Taidi *et al.*^[63], laser bioprinting was used for the precise placement of eukaryotic microorganisms in certain patterns. *Saccharomyces cerevisiae* var. *bayanus* and *Chlorella vulgaris* were the first used as the model organisms for this purpose. The authors used laser pulses with a wavelength of 1064 nm, pulse duration of 10 ns, and pulse energy of about 20 μJ corresponding to laser energy density from 1 up to 2 J/cm^2 at the focal point, which was

focused through the donor slide on the absorbent surface (60 nm of gold). The conditions used for printing of *S. bayanus* and *C. vulgaris* were droplet volume (180 pl) and the cell concentration of 200 cells per a droplet. The growth and development of microcolonies were studied by confocal microscopy, and the growth rates of colonies were determined by the image analysis. The developed protocols for printing of microorganisms and determining the growth rate of microcolonies are very promising for future studies of the growth and development of colonies. In Koch *et al.* study^[66], skin cell lines (fibroblasts/keratinocytes) and human mesenchymal stem cells (hMSCs) were selected for laser printing experiments because of their high potential in human skin regeneration and new applications of stem cell therapy. The effect of laser printing on cell survival, proliferation, apoptotic activity, and DNA damage has been investigated. Approximately 98% of skin cells and about 90% of hMSC cells survived after the laser printing procedure. All used cell types kept their ability to reproduce after laser printing. In addition, skin cells and hMSC showed no increase in apoptosis or the DNA fragmentation. The hMSCs also have maintained their phenotype, as confirmed by the analysis of sorting with the help of FACS. This study declares laser printing as a suitable method for computer positioning of different cell types and a promising tool for future applications in *ex vivo* tissue generation.

In Deng *et al.* study^[67], the influences of laser pulse energy, laser spot size, distance to the acceptor substrate on the number, size, and proliferation of laser printed HELA cells are analyzed. It is shown that the laser power and the thickness of the titanium film are the main factors affecting the survival of the isolated cells. To provide a sufficient working distance and increase the viscosity of the culture medium, glycerin was used. To soften the landing of the cell on the acceptor plate a layer of alginate was used. It was found that the optimal parameters to obtain a viable cell are pulse energy - 9 μ J, spot size - 60 μ m, the thickness of the titanium film - 12 nm, working distance - 700 μ m, the concentration of glycerin in the culture medium 2–4%, and the thickness of the alginate is more than 1 μ m. To avoid contamination and increase humidity, the process of cell shooting was carried out in a special chamber made from PDMS.

It should be noted that very few works devoted to the isolation of microorganisms from complex heterogeneous systems with the use of laser printing are existing in the literature. Nowadays, two modifications of the laser printing method - biological laser printing^[68,69] and laser engineering of microbial systems (LEMS) - have been proposed for the cultivation of microorganisms from natural environments^[70-72].

As it was noted above, traditional methods to isolate microorganisms from environmental samples, such as

soil or sediment, require pretreatment to remove living cells from their solid-phase carrier, creating a liquid phase sample. This process destroys close relationships that can be crucial for the cultivation and study of the isolated microorganisms. In Ringeisen *et al.* study^[68], a high-performance automated method based on laser printing that isolates pure microbial cultures and spatially bound microbial consortia directly from solid-phase complex environmental samples is described. A mixture of soil with water or water and glycerol was applied to a quartz tape coated with titanium dioxide, 85 nm thick, producing a donor slide. Adjustable amounts of soil were transferred to different substrates using a pulsed excimer laser (wavelength 248 nm, pulse duration in the range of 2–10 ns, and pulse energy varied from 7 to 23 μ J), including 96-well plates filled with broth at the rate exceeding 20 microparticles per second or more than a thousand microparticles per minute. After printing, the viability of microbial cultures, culture value, and significant morphological diversity have been demonstrated. However, it is not clear whether it exceeds the diversity obtained by cultivation with the use of traditional methods. Nevertheless, the results showed that single-stage soil printing could be used to (a) produce pure microbial cultures (isolates) and (b) isolate consortia from the micro-ecological system. The study, described here, is the first extension of bioprinting to solid-phase environmental samples for the isolation and cultivation of individual microorganisms or consortia.

The LEMS method uses 8 ns, 24 μ J, 1.06 μ m laser pulses^[70-72]. The donor plate is a glass coated with a 50–100 nm layer of gold, titanium, or chromium. To print biological objects, bacterial cells or soil are mixed with a gel (2% hyaluronic acid), which prevents rapid drying of the sample and spraying of microdroplets during laser printing. This technology allows obtaining a large number of separate bacterial colonies. It was also demonstrated that the LEMS technology allows cultivating a significantly higher bacterial diversity in comparison with traditional methods of cultivation (Figures 5 and 6). In particular, with the use of this method, a strain of a rare *Nonomuraea*^[70,71] genus was isolated from the soil (Figure 5), while the isolation of bacteria of this kind by traditional methods requires the use of a number of special techniques, for example, the addition of antibiotics and vitamins to the nutrient media^[73,74]. When using the LEMS method, in addition to increase of the microorganisms diversity, an increase in the number of cultivated microorganisms was observed^[70,72]. This effect was demonstrated both for natural samples and for pure cultures of bacteria.

The reasons for the high efficiency of laser printing methods in the isolation of bacteria from heterogeneous environments are not yet sufficiently investigated. Probably, one of the reasons is that, unlike traditional cultivation methods, these methods do not use

the desorption of microorganisms from mineral particles^[68,70,71]. The direct printing techniques are likely to isolate neighboring microorganisms while they are still attached to soil particles (Figure 6). In this case, the natural micro-ecological environment and relationships between microorganisms in the process of isolation and screening are maintained. In the case of preserving the relationships between microorganisms, it is possible to cultivate a wider microbial diversity^[1,24,68]. Changes in microbial diversity and activation of bacterial growth may also be caused by laser radiation. In the LEMS method, even though most of the radiation is absorbed by the metal film, a small part of the radiation reaches the gel-containing microbial cells^[75]. At the same time, it is known that laser radiation can activate the growth and metabolism of bacteria^[76-78]. Another factor that can affect the state of microorganisms during laser printing is the formation of nanoparticles, generated from the absorbing metal film on the donor glass slide^[71,72,75]. Nanoparticles, in relation to their shape, size, their material and some other factors, can be both toxic to bacteria or stimulate their growth^[79-82]. However, in general, the processes that cause changes in microbial diversity and growth during and after laser printing are practically not studied and require further investigations.

In addition to the isolation of microorganisms from complex substrates, it is assumed to use laser printing methods to create biochips and bioelectronic interfaces. It is shown that various modifications of laser printing allow transferring cells of pure microorganism cultures to various acceptor surfaces with high accuracy (in the zone $<50 \times 50 \mu\text{m}$)^[61,83-85]. At the same time, very high survival of microorganisms (up to 100%) is observed. As it was mentioned before, laser printing can also be used to isolate single microorganism cells^[67,68], which is necessary for the study of growth, metabolism, the genome of individual cells, as well as for the study of interactions between microbial cells^[27,68,86,87]. One of the relevant tasks of modern biotechnology is the creation of multilayer structures consisting of microorganisms to produce some new materials, in particular, bioplastics, adhesives, bio-based electrical switches, etc.^[88-91] Nowadays, the use of 3D printers can help to solve these biotechnology problems^[91]. Application of laser printing in this field is very promising due to the high accuracy of microbial transfer and high cell survival.

In conclusion, at present, there are only a few publications on the use of laser printing for the transfer of microorganisms. However, it has been shown that laser printing is effective for isolating microorganisms from complex natural substrates, as well as for transferring microbial cells to various acceptor surfaces with high

speed and accuracy. Due to this fact, it is expected that this technology will be actively used for the studies of microbial diversity of different ecosystems, for the creation of biochips and bioelectronic interfaces, isolation of single cells of microorganisms, and printing of multilayer cell structures and biofilm studies.

Acknowledgments

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Mechanisms and modeling of electrohydrodynamic phenomena

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Abstract: The purpose of this paper is to review the mechanisms of electrohydrodynamic (EHD) phenomenon. From this review, researchers and students can learn principles and development history of EHD. Significant progress has been identified in research and development of EHD high-resolution deposition as a direct additive manufacturing method, and more effort will be driven to this direction soon. An introduction is given about current trend of additive manufacturing and advantages of EHD inkjet printing. Both theoretical models and experiment approaches about the formation of cone, development of cone-jet transition and stability of jet are presented. The formation of a stable cone-jet is the key factor for precision EHD printing which will be discussed. Different scaling laws can be used to predict the diameter of jet and emitted current in different parametrical ranges. The information available in this review builds a bridge between EHD phenomenon and three-dimensional high-resolution inkjet printing.

Keywords: Electrohydrodynamic; cone-jet; jet stability; inkjet printing; additive manufacturing

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1 Introduction

Due to the current trend toward the miniaturization of devices, traditional manufacturing methods such as casting, injection molding, and computer numerical control machining cannot satisfy the developing needs of industries. The lithography-based microfabrication experiences inherent limitations in processing diverse materials, and extending fabrication into the third dimension^[1]. In addition, it is a time-consuming process to use masks and photo-resistant chemicals, and also expensive when the manufacturing process requires a sophisticated, clean room and state-of-the-art lithographic equipment^[2]. Additive manufacturing, based on localized deposition of material and a layer-by-layer printing process, is able to build customized products in a short time-frame and offers significant advantages over traditional manufacturing processes in the area of design

freedom and reduction of assembly time and cost. Inkjet printing, which is a low cost, high speed and large area additive manufacturing process^[3], relies on the generation of droplets at or near a nozzle aperture, followed by non-contact deposition onto a substrate with high spatial control. Unfortunately, inkjet printing systems share the same drawback, namely that the droplet size is solely dependent on the nozzle diameter, with droplet diameter about twice the nozzle diameter^[4], and the nozzle diameter cannot be scaled down continuously.

To overcome the above limitations, electrohydrodynamic (EHD) inkjet printing (also called “EHD jet printing”) has been proposed as a solution to the limited resolution of the conventional inkjet printer system, because EHD printing can generate small droplets without the need to miniaturize the nozzle. The process involved, called “electrospray in the cone-jet mode,” uses electrical

("Maxwell") forces to *pull* the liquids from the nozzle tip, rather than apply thermal or acoustic energy to *push* liquid from a fine capillary. The EHD inkjet printing can be a high-resolution inkjet printing technology because there is a large "neck-down ratio" between the inner diameter of the nozzle and the jet: The jet diameter is about two orders of magnitude smaller than the nozzle diameter. Thus, in EHD jet printing the nozzle inner diameter can be much larger than that used in thermal or piezoelectric inkjet printing (about 20 μm); this makes blockages much less likely and makes it easy to employ a highly viscous liquid^[5]. EHD ink-jet printing is a mask-less, non-contact, direct-write, and additive process, and it is used in the field of micro or nanomanufacturing for patterning of a large class of materials on a variety of substrates without adversely affecting the chemical properties of the deposited materials. Park^[6] developed an EHD inkjet printing system, and they used a microcapillary with diameter from 0.3 to 30 μm to produce single droplet with sub-micrometer size. Followed by their seminal work, a series of applications appear in recent years, and applications include varied electronics, biotechnology, and three-dimensional (3D) printing. Liang^[7] used the coaxial nozzle to print microscale 3D cell-laden constructs, and Liu^[8] investigated that influence of EHD jetting parameters on the morphology of PCL scaffolds. Designs of EHD printing system and recent applications of high-resolution printing will be introduced in the following paper due to the limitation of length. In the remaining sections of this paper, we provide a brief account of the history of EHDs and related technology. Then, we review the known theories and principles related to EHD printing, especially the theory of Taylor cone formation, mechanism of cone-jet transition, and criteria for jet stability.

2 History of EHDs and Jet Formation Technology

The phenomenon of a liquid drop subject to sufficiently strong electric forces adopting a roughly conical shape has been reported by Gilbert in 1600^[9,10]. The electrically induced formation of a cone-jet and its breakup into droplets were first reported by Grey in the 1700s^[11]. In 1882, Rayleigh conducted research on the theoretical analysis of the breakup of a spherical liquid droplet under electrical stress and derived an instability condition for an electrically charged spherical droplets^[12]. His result implies that (for a spherical droplet) the stability criterion is equivalent to the condition that the electric stress outward is equal to the surface tension stress inwards. The rapid EHD pulsation of the electrified liquid surface, now known as the cone-jet transition that a thin liquid jet is ejected from the tip of an electrified cone shape, is discovered first time by Zeleny in 1915^[13]. Zeleny also characterized the resulting spray as

a function of the nature of the solvent, high voltage and pressure of the liquid at the tip of the tube (which is linked to the flow rate in modern electrospray experiments)^[12,14]. Before 1960s most work focused on the behavior of perfect conductors (mercury or water) or perfect dielectrics (a polar liquid such as benzene), but Allan and Mason (1962) started to study on poorly conducting liquids - and established the leaky dielectrics model or ohmic model^[15,16]. In 1969 Taylor derived a pioneering theory to adequately explain the peculiar form of meniscus cones, referred as Taylor cone, and the related concept is only recently extended for use in micro patterning^[17]. Then, Cloupeau and Prunet proposed a classification of EHD jetting modes on the basis of their observations in the air at atmospheric pressure^[18]. Specially, Cloupeau and Prunet-Foch organized EHD jetting modes according to liquid flows from the meniscus in a continuous or pulsating manner into: (1) Continuous manner including cone-jet, multi-jet, simple jet, and ramified jet modes and (2) pulsating manner including dripping, micro dripping produced at the end of the capillary or meniscus, pulsed cone-jet produced by breakup of a jet, and spindle mode^[18]. In terms of location where drops are formed, spraying modes can also be divided into two different groups: Electrified droplets pinched off either from a protruding meniscus or from a jet emerging from a conical meniscus^[18,19]. It should be noted that the release of charged droplets directly from the apex of a cone is not possible in competition with the formation of a cone-jet^[20]. Fenn *et al.* found that flowing solution of large polymers and proteins can be formed by electrostatic atomization of their solutions from a Taylor cone in a bath gas, and this led to the 2002 Nobel Prize in Chemistry received by Fenn *et al.*^[21,22]. Early reports on the use of this phenomenon for the controlled deposition of materials drew inspiration from electrospray techniques for the generation of charged droplets^[23-25]. De la Mora and Loscertales explored the relationship between the various parameter and emitted current and jet diameter for high conducting liquid. Ganan-Calvo proposed six different scaling laws to define cone-jet transition^[26]. Chen performed a series of studies on the generation and placement of droplets with a high positioning accuracy about $4 \pm 2 \mu\text{m}$ ^[27,28]. Park *et al.* used the EHD method to eject different types of ink from microcapillary nozzles of different sizes and obtained printing resolution up to 1 μm ^[6,29]. Maginean *et al.*^[30] and Chen^[28] separately investigated pulsating cone-jet formation and summarized scaling law for oscillation frequency in the two different scenarios.

3 Theory and Mechanism of EHD Inkjet Printing

The mechanisms of EHD jetting are complicated, and the physics behind this phenomenon is not completely known. This paper summarizes some of the contributions

in this area and gives readers a basic introduction to some important theories related to EHD inkjet printing. The following section specially discusses liquid cone formation, the cone-jet transition, and jet stability.

3.1 Formation of the Liquid Cone Shape

Figure 1A shows a common setup for EHD printing. The pressure head of the liquid in the cylindrical nozzle is initially adjusted by the pressure regulator until the meniscus “A” in Figure 1A is almost flat at the nozzle exit. Then, a high voltage is applied between the metal nozzle and a grounded electrode to create an electrical potential difference. The electric field strength near the apex of an operating jet can be close to the threshold for electrical breakdown of air (3×10^6 V/m), but a cone-jet only forms in the absence of gas discharge^[31]. As the voltage is increased, the increasing Maxwell stress causes liquid deformation, from a hemispherical droplet in Figure 1B to a roughly conical meniscus in Figure 1C. The process of surface deformation is triggered by small perturbations of the surface, which, in turn, enhance the local electric field through charge concentration, and further increase the electrostatic pull in a cascading effect^[32]. The conical meniscus is maintained over a certain critical limit of electric potential^[33], and a limiting case is reached when the apex of the cone approaches a point, i.e., a singularity^[32]. Inside the conducting nozzle, the liquid is electrically neutral before emerging from the nozzle outlet, and there is no charge separation in the nozzle regardless of liquid conductivity because the electric field inside the nozzle is zero.

In 1964, Taylor obtained the first analytical model for the cone structure and constructed a modal analysis of a conducting liquid cone at equilibrium (without a jet from the apex)^[32,35]. In 1969, Taylor used the two plate experimental setup to explore the criteria for the deformation from a sphere to a cone. At the critical potential difference, the spherical cap above the tip of the tube is taken to be in an equilibrium defined by electrostatic force, hydrodynamic force, the surface

tension force, and weight of the spherical cap. The force balance is given in equation (1):

$$F + \Delta p \pi r^2 = 2\pi r \gamma \cos \theta + W \quad (1)$$

In this two plate experimental geometry, the value of the force, F , exerted on a cylinder of length, L , is^[17]:

$$F = \frac{V^2 L^2}{4H^2} \frac{1}{\ln\left(\frac{2L}{R}\right) - \frac{3}{2}} \quad (2)$$

Above, F is the attractive force due to the imposed field; Δp is the pressure difference between inside the liquid cone at its base and ambient pressure; γ is the surface tension; R is the nozzle radius; L is the distance between the end of nozzle and plate E ; W is the hydrodynamic force including the weight of liquid in the spherical cap above the orifice; and θ is the semi-cone angle. The hydrodynamic force Δp continuously supplies fluid to the emerging droplet^[33].

In the classical theory of dielectrics, the charge is situated at the interface between two substances, which have different dielectric constants^[36]. Thus, electro forces, which stem from two isotropic media which have different electrical conductivity and dielectric constant within or across the phase boundaries^[37], induces electrical charges which exist in the fluid along the fluid interface^[38]. While surface tension tends to keep the pendant droplet in spherical shape which has the smallest surface-to-volume ratio, and also to reduce interfacial energy^[39], the repulsion between the surface charges acts against the surface tension force to deform the sphere into cone^[38].

If pressure (Δp) at the top of the fluid equal to zero, the cone semi-vertical angle (θ) is 49.3° . If the weight of the fluid is also neglected, the critical voltage (static equilibrium would no longer be maintained) for the appearance of cone with a straight generatrix^[40] before the jet forms can be predicted by equation (3) which is derived by substituting equation (2) into equation (1)^[17]:

$$V = \frac{2H}{L} \sqrt{\left(\ln \frac{2L}{R} - \frac{3}{2}\right) (2\pi r \gamma \cos 49.3^\circ)} \quad (3)$$

Above the critical voltage, the static equilibrium value would no longer be maintained^[17]. For a liquid meniscus formed at the exit of a conducting needle, like electrodes of the needle to plate setup, the critical voltage can be approximately calculated as^[41,42]:

$$V_{\text{critical}} \approx \sqrt{\frac{2\gamma R \cos \theta}{\epsilon_0}} \ln\left(\frac{4l}{R}\right) \quad (4)$$

Where l is the distance between the electrode of nozzle and electrode of substrate. This expression is valid when the electrode separation l is much larger than the radius of nozzle r ^[5]. This formula only gives an approximation to the required starting voltage (experimentally verified

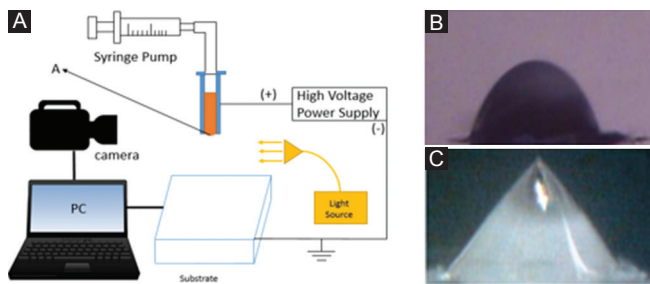


Figure 1. (A) A schematic of electrohydrodynamic printing setup. Ethylene glycol liquid meniscus (B) at 0 volts; (C) at 2.5kV^[34]. Adapted by permission from Lozano *et al.* (2004) under the Elsevier license.

within 10%) for each simulation^[32].

The Taylor angle ($\theta_T=49.3^\circ$) is theoretically calculated from the balance of surface-tension stress and electric stress under no fluid motion (hydrostatic pressure P , as shown above, equal to zero)^[33]. Thus, the critical voltage, which Taylor predicted, is only valid in the limit of no liquid jet emission and it does not account for any EHD effect caused by subsequent jet emission^[38]. The semi-vertical angle, which Taylor predicted above, is not always 49.3° and Fernandez de la Mora showed that several factors, including liquid loss through the cone, space charge, flow rate, and ambient pressure, might result in cones established at different semi-vertical angles^[19,43-45]. Cloupeau and Prunet-Foch also showed that there are stable conical menisci for certain range of variation in the hydrostatic pressure and applied potential difference, but the value of the angle at the apex is variable, and the generatrix of the cone may be either concave or convex^[18,40]. In addition, the actual electrode setup in experiments may not replicate the geometry of Taylor's model^[32].

In a word, it is important to obtain starting electric potential for initiation of EHD printing, and equations (3, 4) are used to estimate the critical value of the voltage. The semi-vertical angle from experiments can be different from Taylor angle applied to these equations, and this is a reason to cause a discrepancy between theoretical and experimental critical voltage. Another reason arises from factors which are not counted in equations, such as space charge and shape of nozzle.

3.2 The Cone-jet Transition Stage

As shown in Figure 2A, when the electric field is absent or small, the liquid flow out from a nozzle by the assistance of gravitational force occurs by the dripping mode at a small flow rate or the jetting mode at high flow rate^[1,46,47]. At a critical potential difference the Taylor

cone is formed, and with further increase of field strength, the cone becomes unstable and then a small droplet or a very thin jet (small and thin compared with the capillary diameter) is emitted from the conical apex^[17]. Juraschek and Rollgen classified spray of axisymmetric regimes into three modes, based on current measurements^[19,20]. With the increase of voltage, a pulsating jet (axial mode I) appears, with pulsations frequencies existing in the low kilohertz range or less^[20]. Marginean considered axial mode I, which exhibits two periods, as burst regimen^[19]. As the voltage grows to the axial mode II, only high-frequency pulsation at the low kilohertz is observed^[20]. The intrinsic or natural pulsating modes are caused by an imbalance between the supply and loss of liquid in the entire cone volume (low frequencies) or the cone's apex (high frequencies)^[20,28,48]. Juraschek and Rollgen claimed that capillary current is linearly dependent on the pulsation frequency and independent of the capillary potential applied in the high pulsation mode^[20]. Chen *et al.* observed steady-state results from a long exposure time (0.4 ms), but the intrinsic pulsating mode in the kilohertz range was observed when the exposure time is reduced to 0.1 ms or less^[28]. Marginean *et al.*, first, used a set of sequential images (Figure 2B) to show spontaneous Taylor cone deformation and a similar intrinsic pulsation at 1 μ s per frame^[14]. Figure 2B clearly shows periodic formation, relaxation of meniscus, and cone-jet, and they provide a direct connection between Taylor cone pulsation and electrospray current oscillations^[14,28]. Yogi *et al.* used an external stimuli method to generate picoliter droplets by applying a 1000V, and 10ms pulse voltage to the tip of a capillary tube and drop-on-demand (D-O-D) deposition can be achieved using external stimuli^[24]. In contrast to deposition in a small area by electrospray, pulsating jet caused by either intrinsic or external stimuli approaches are able to generate single micro-scale droplet on the substrate. In addition, D-O-D

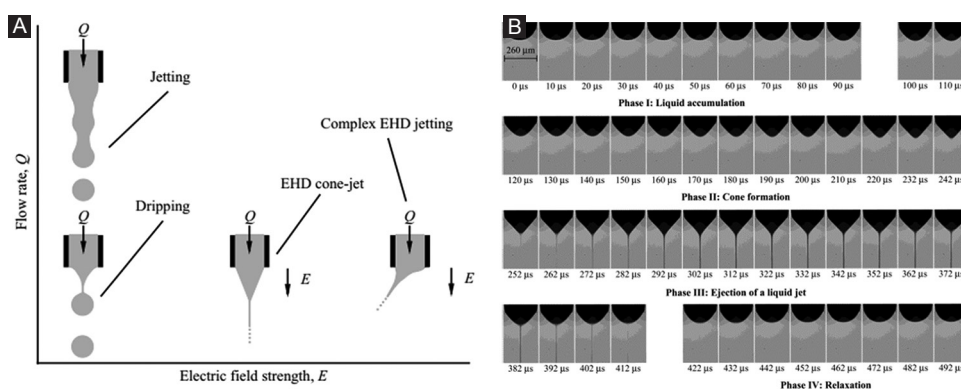


Figure 2. (A) Phase diagram depicting flow transitions that occur as flow rate and/or electric field strength is varied^[46]. Adapted by permission from Robert T. Collins *et al.* (2007) under the Cambridge University Press. (B) Time images of the pulsating Taylor cone with the four phases of the cycle. Delay time values measured from the most retracted meniscus (Δt) are shown under the individual images. Each frame is an average of 100 exposures with the same delay^[14]. Adapted by permission from Marginean *et al.* (2004) under the ACS Publications.

pulsating jet deposition can achieve higher sensitivity and generate the customized volume of droplets by changing pulse duration. In general, pulsation frequency depends on flow rate, properties of liquid and nozzle diameter. Several models were proposed to express the relationship between frequency and parameters, and these models will be discussed in section 3.2.3.

When the applied potential difference is further increased, continuous emission of liquid through cone jet is developed. This mode is known as axial mode III^[20]. In addition, a concavely shape cone observed in the two pulsation mode is changed to a nearly straight cone in the continuous spray mode^[20]. Marginean *et al.* revealed that a complex transition into an astable regime may exist between pulsating and continuous cone-jet regime^[19]. In Figure 2A a complex jetting behavior, like tilted jets, is shown at higher electric field strength, but these phenomena are not considered to be controllable in a precision deposition. When the applied voltage is slightly lower than the voltage required to obtain a single permanent jet, the jet may be emitted only intermittently, which means the apex of meniscus alternately takes on a pointed or rounded form, and emission phases may occur at perfectly regular time intervals^[18]. This intermittent or pulsed cone-jet is similar to the axial mode I and II. The diameter of the jet varies during these emission phases, and thus the distribution of droplet size is never narrow^[18]. These studies suggest that pulsating and continuous cone-jet can be obtained by adjusting the voltage and flow rate. However, there are noteworthy exceptions, and polycaprolactone is one of them. A stream of pulsating droplets has not been observed since it is hard to accumulate enough charges to overcome the surface tension and high viscous force^[49]. For paraffin wax, pulsating cone-jet was only formed^[50], and this result may attribute to its low viscosity and electrical conductivity. In one word, generation of pulsating or continuous cone-jet is controlled not only by changes of voltage and flow rate but also material properties, such as surface tension, electrical conductivity, and viscosity.

Zeleny first investigated the continuous production of drops by the breakup of a permanent jet extending from a meniscus in the conical form^[13,51], and then Vonnegut and Neubauer acquired the same mode during their experiments on liquids^[52]. Cloupeau and Prunet used the compound word “cone-jet” to describe the phenomenon that a permanent jet extending from an axisymmetric conical volume of liquid and jet is stretched along the capillary axis^[18]. The creation of a jet requires penetration of the field lines into the liquid with a dielectric liquid, and only penetration will allow the appearance of a component of the electric field tangent to the surface; this acts on the surface charges and creates a force that drives the liquid and an acceleration of the jet

downstream^[18]. Melcher and Taylor also pointed out that for an interface to be subjected to shear stress, the surface must simultaneously support the surface charge and be subjected to a tangential electric field^[53,54]. In addition, Melcher *et al.* mentioned two limiting cases and the first one is the perfectly conducting interface^[53]. In this case, electrical surface forces always act perpendicularly to the surface and thus interface supports no tangential electric stress^[53]. The second limiting case is a perfectly insulated surface with no free charge density while the polarization force density is operative at the interface. Therefore, the electric surface force density acts in the direction of permittivity gradient ($-\nabla\epsilon$), which is perpendicular to an interface^[53]. Thus, the liquid must not be perfectly conducting or insulating^[18], and for a leaky dielectric liquid, the tangential component of the electric field can develop a shear force along the conical surface.

When a steady flow rate is issuing from the cone's tip in the form of a microjet, an electric field toward liquid surface must appear in the cone to supply the electric current, which is emitted by the microjet in the form of charged droplets^[55]. The tangential electrical stress pulls the charged surface toward the tip, and tangential electrical stress provokes sufficient axial momentum to transfer onto the liquid to begin a progressive deformation of the cone into a jet^[55]. Hayati *et al.* showed an axisymmetric circulation pattern where the particles travel downward along the surface of the cone and reverse upward along its axis in the conical base^[54]. The electric field drives free charges to the surface of the cone; hence, the surface is charged and is subjected to a normal electric field E_n . A potential difference will exist between the base of the cone at the end of the capillary and the tip of the cone due to the semiconducting nature of the liquid^[54]. This potential difference ensures that the interface is subjected to a tangential electric field E in the direction of flow^[54]. Barrero *et al.* observed a similar recirculating meridional motion and an additional vigorous swirl coupled to the meridional motion, and they attributed the appearance of different motion to a distinct value of liquid conductivity and viscosity^[56]. The meridional motion can be considered as a proof of the existence of tangential electrical stress on the cone surface. De la Mora considered the domain of cone as hydrostatic since recirculating flow is slow and have little influence on jet formation^[45].

Even though the phenomenon of cone-jet transition has been known for over 200 years, the actual process is not understood completely. It is complex to analyze theoretical multi-physical free surface flow by mathematical methods, and there are also limitations in simplifying assumptions in models^[5]. In addition, based on the complexity of EHD phenomenon controlled by multi-parameters, it is not always possible to know beforehand the mode obtained when the values of main

parameters are given^[18]. According to data obtained from experiments, Cloupeau and Prunet found that the cone-jet mode only appears at a certain range of voltage and flow rate, and instabilities including skewed and multiple-jet regime appear in a larger electric field^[57]. At specific conditions, operating diagram of cone-jet can be described as changes of flow rate and electric field strength^[5,57]. Domains of cone-jet may become different by small changes in the selection of parameters mentioned above (such as working fluid, setup of the experiment, and geometry of nozzle). Furthermore, experimental measurements are also difficult for the flow fields in a free jet whose diameter is often on the verge of optical resolution^[5].

3.2.1 Dimensional Analysis

In this section, the effect of parameters on the transition process is reviewed. The cone-jet transition is confined to the region near the conical apex. First, the dimensional analysis is used to give a qualitative description of the jet diameter, D_j , and emitted current, I_j ^[38]. The jet diameter and current are related to the operating parameters (flow rate Q and electric field strength E), liquid material properties (density ρ , viscosity μ , electrical conductivity K , gas-liquid surface tension γ , and fluid relative permittivity ϵ_r), and geometrical parameters (nozzle diameter D_n and distance between two electrodes H). If parameters ρ , γ , K , and the vacuum permittivity, ϵ_0 , are selected as dimensionally independent variables in this functional relation for the jet diameter and current, dimensions of these related variable are $[\rho] = \text{ML}^{-3}$, $[\gamma] = \text{MT}^{-2}$, $[K] = \text{MLT}^{-3}\text{V}^{-2}$, and . Next, the Buckingham's Π theorem can be applied to perform a dimensional analysis^[38,55]:

$$\frac{D_j}{D_0} = f \left(\frac{Q}{Q_0}, \frac{V}{\left[\frac{\gamma D_0}{\epsilon_0} \right]^{0.5}}, \epsilon_r, \frac{\rho Q_0}{\mu D_0}, \frac{D_n}{D_0}, \frac{1}{D_0} \right) \quad (5)$$

$$\frac{I_j}{I_0} = g \left(\frac{Q}{Q_0}, \frac{V}{\left[\frac{\gamma D_0}{\epsilon_0} \right]^{0.5}}, \epsilon_r, \frac{\rho Q_0}{\mu D_0}, \frac{D_n}{D_0}, \frac{1}{D_0} \right) \quad (6)$$

Where $f()$ and $g()$ are dimensionless functions. Lee *et al.* add a ratio of two characteristic times, t_e/t_h , where t_e is charge relaxation time, which is the characteristic time of charge transport determined by the electrical properties of the fluid, and t_h is hydrodynamic time, which is the characteristic time of the fluid supply^[33,55]. The charge relaxation time is $t_e = \frac{\epsilon_0 \epsilon_r}{K}$ and

hydrodynamic time is $t_h = \frac{ID_n^2}{Q}$. The characteristic flow rate is $Q_0 = \frac{\gamma \epsilon_0 \epsilon_r}{\rho K}$, characteristic distance $\frac{V}{\left[\frac{\gamma D_0}{\epsilon_0} \right]^{0.5}}$ and characteristic current $I_0 = \gamma \left(\frac{\epsilon_0}{\rho} \right)^{0.5}$.

These dimensionless groups have a certain influence on jet diameter and current, qualitatively. In the classical EHD jetting system, the charge relaxation time is smaller than the hydrodynamic time ($t_e < t_h$); most of EHD printing, electrospray, and electrospinning belong to this category^[33]. Charges are induced toward the surface of the liquid to form a thin layer of charge under the liquid-gas interface^[33], and both the shape of the liquid cone and the jet stability are affected by the amount of electric charge on the liquid surface^[38]. For liquids with relatively high conductivities (above 10^{-4} S/m), the electrical relaxation time is short and sufficient charge can accumulate on the surface to counteract the surface tension force^[38]. The jet formation zone is limited to the apex of the conical meniscus^[57]. The remaining surface is practically equipotential, and an almost static equilibrium of forces exists at each point^[57]. The shape of the cone may have a practically straight generatrix with a very fine jet (Figure 3A) or exhibit a shape as in Figure 3B^[18]. In Figure 3C and D, the acceleration zone extends further toward the base of the cone and the profile of liquid has a similar shape of cone-jet with an open cone for decreasing conductivities^[57]. Flow rate required for stable cone-jet mode moves toward a lower threshold with an increase in conductivity when all other parameters are kept same^[57]. In the non-classical EHD ($t_e > t_h$), there is insufficient time to develop an appreciable surface charge, and electrically forced jet may appear, like a ball cone-jet^[33,58,59]. Although it is not possible to obtain very fine jets with liquid of low conductivity unless special methods are used^[57], it is still possible to produce a jet for liquids with low electrical conductivities (10^{-13} S/m)^[58]. The conical shape ultimately disappears when the liquid has a very low conductivity^[57]. Electrical stress cannot counteract the surface tension to deform the meniscus into a cone, and the pendant droplet grows in volume and finally drips off.

In the experiment of Juraschek and Rollgen, both

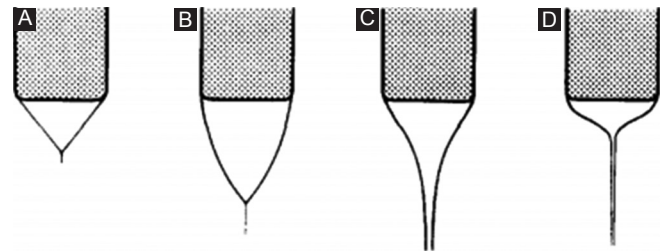


Figure 3. (A-D) Different forms of the meniscus in cone-jet mode^[57]. Adapted by permission from Michel Cloupeau *et al.* (1989) under the Elsevier.

high and low pulsation frequencies weakly increase with the increase of electrical conductivities, since the dielectric relaxation time of liquid without the addition of electrolyte concentration is already shorter than the time scale of a single pulse period^[20]. The addition of the electrolyte causes decreases of capillary potential, and as a result the measured average capillary current increases due to an increase of emitted electric charges during a single pulse^[20].

The first one (Q/Q_0) in the dimensionless group is the dimensionless flow rate ($\frac{\rho K Q}{\gamma \epsilon_0}$) and the jet diameter can be varied by two orders of magnitude by changing the flow rate^[55]. The potential required for the formation of steady cone-jet transition is affected by flow rate and the resulting shape of the liquid cone^[38]. The minimum flow rate and its associated electric field are considered as a stability boundary of cone-jet^[5], and Q_0 is of the order of the minimum flow rate for a given liquid^[60]. The minimum flow rate is not, simply, the flow rate due to the upstream pressure, but rather the specific flow rate that causes electrical stress to strip off (or shear) the surface charge layer of the fluid^[33,61]. The cone-jet mode only appears within a limited range of values of flow rate for a given conductivity^[18]. The value of the current and diameter of jet grow with the increase of flow rate^[57]. Chen provided operating modes of steady cone-jet in the E-Q diagram for the conductivity of liquids above 10^{-4} S/m when other materials and geometry parameters are kept fixed^[5]. In the experiment of Juraschek and Rollgen, the “low” frequency of the appearance of current pulse sequence increases with the increase of liquid flow rate, since a shorter time is needed to build up the required large cone volume with a critical radius for the onset of a liquid emission process^[20]. The number of pulses in a sequence also slightly increases due to the extension of emission time by raised liquid supply^[20]. The “high” pulsation frequency is weakly changed by liquid flow rate, but the amplitude and width of the pulse increase with flow rate accordingly. In conclusion, the duty cycle of the “high” frequency pulsations is affected by liquid supply to the apex region of the cone and weakly affected by liquid supply to the bulk of the cone.

The second is dimensionless electrical voltage related to Taylor’s critical voltage $\frac{V}{\left[\frac{\gamma D_0}{\epsilon_0}\right]^{0.5}}$ ^[55]. The

electric field promotes the charge migration away from the electrode partly through the solution bulk after the charge is produced electrochemically at the emitter liquid interface, partly along the liquid surface^[19]. The value of an electric field on the liquid is not only depends on value of voltage but also varied with a dimension of the capillary, the shape, and dimensions of the bottom

electrode and distance between two electrodes^[57]. With the increase of electric field strength, there are many different forms of cone-jet transition^[18]. Within the regime of the steady cone-jet transition, the liquid meniscus of a Taylor cone recedes toward the nozzle base as the electric field strength is increased^[44]. Within the operating envelope, the range of applied voltage for steady cone-jet transition is $<7\%$ for a given flow rate^[55], which is small in comparison with the changes due to the liquid flow rate. Since the range of electric field strengths for the steady cone-jet transition is fairly narrow, this group can be considered as an invariant^[55].

The third is the relative permittivity of fluid ϵ_r , which is determined by the amount of the alignment of dipoles^[33]. When two liquids have a similar value of the fourth dimensionless group, the liquid with higher relative permittivity has a smaller charge relaxation length, and thus a larger potential is required to achieve lower threshold for cone-jet formation^[33].

The fourth dimensionless group is $\left(\frac{\rho \epsilon_0 \gamma^2}{K \mu^3}\right)^{\frac{1}{3}}$, which only depends on the liquid properties. It can be considered as the ratio of the propagation velocity of a perturbation across the jet by the velocity of liquid $\frac{Q_0}{D_0^2}$ and viscous diffusion $\frac{\mu}{\rho D_0}$ ^[55]. The axial velocity profile of the liquid

jet depends on the viscous dimensionless parameter. Viscosity does not explicitly affect the jet diameter. The viscosity of the liquid mainly affects the stability of the jet, especially with polymer jets where the huge viscoelastic effects and elongational viscosity associated with polymers prevent the capillary instability^[62,63]. Thus, higher viscosity liquids form thicker jets under the cone-jet transition^[38]. In the cone-jet mode, the length of the jets increases with the viscosity, the resistivity, and the flow rate of the liquid^[18].

The following dimensionless group relates to the nozzle diameter D_n , which has a small influence on the diameter of jet D_j , particularly for conductive liquids. For high conductivity liquids (10^{-4} S/m and above), De la Mora *et al.* demonstrated that the current is independent of the needle voltage and geometry of the electrodes as long as a stable cone is formed^[31]. This conclusion is consistent with the result that current becomes decreasingly dependent on needle voltage at increasing conductivities in I-Q curves^[57]. The jet diameter becomes irrelevant to nozzle diameter when the ratio of the two diameters approaches to zero^[60]. Under these conditions, the dynamic effect due to the jet emission is negligible and limited to the vicinity of the conical apex (e.g., $d_n/d_0 \gg 1$ in the derivation). However, this simple behavior does not hold for liquids with small electrical conductivities and area of stable conical meniscus depends on electrode geometry^[31]. Choi *et al.*

claimed the dependence of jet diameter on nozzle diameter from their study, and this different result from above can be attributed to the lower electrical conductivity of ink^[64]. At this circumstance, smaller droplets can be the outcome of using a thinner nozzle. In a stable cone-jet transition, smaller nozzle diameters will extend both the low and high flow rate limit for a given liquid conductivity^[57]. The electric field strength near the nozzle depends on both the applied voltage and the nozzle diameter. Thus, with decreasing outer diameter, the field strength increases and the resulting electrostatic forces, as well as the entrance velocity, raise up^[20]. Furthermore, a smaller nozzle diameter or distance between two electrodes, the required onset voltage is lower^[44,65]. It is favorable for high-resolution printing and precise placement to keep a short distance “l” (usually smaller than 5 mm) between nozzle and ground electrode due to reduction of lateral variations^[6]. As printing complex feature with sharp turns or small corners, a small distance “l” enables the process to response promptly to the abrupt path change. Another key factor for high-resolution printing is plotting speed, and it is also important to consider compatibility between plotting speed and jetting speed. As a stable jet emits from the tip of the cone, the speed of jetting depends on flow rate and electrical potential. The plotting speed is the movement speed of the substrate. If two speeds match each other, a straight jet appears, and a uniform continuous plotted line is deposited on the substrate. In the pulsating mode, it shows an equal space between droplets. Material properties also need to be added into consideration in regard to the matching process.

Finally, the effect of surface tension needs to be considered. In Taylor’s theory, the formation of a jet in the cone-jet transition only occurs when the stress induced by the electrical field ($\epsilon_0 E^2/2$) is larger than the stress induced by surface tension ($2\gamma/r$), and a square dependence exists between the onset voltage and the surface tension. If the surface tension of the liquid is too high, triggering of corona discharges prevents electrostatic atomization in the air^[57]. The effect of lowering surface tension stress at the liquid apex by adding surfactants is the same as an increase of the Maxwell stress^[20]. The suppression of low-frequency spray and the reduction of cone length at low surfactant concentrations is probably related to a weak reduction of the surface tension of the liquid cone^[20]. The phenomenon of corona discharge usually occurs with liquids having a high surface tension and thus requiring high fields to overcome the surface tension stress^[57]. The corona can be suppressed by increasing the dielectric strength of the surrounding medium, such as Freon 12, CO₂, and SF₆^[41] or lowering the temperature of the ambient air^[31,57]. The hysteresis phenomenon that the minimum voltage needed to maintain the cone-jet mode is lower than the voltage needed to initiate the cone-jet mode needs to be considered^[57].

Wettability of capillary may cause a liquid ridge over the entire periphery of the nozzle outlet or only on the small part of it. During the growth of the ridge, the flow rate of the atomized liquid is lower than the total rate, causing the electric field to decrease due to the increase in the section of the capillary^[57]. Wettability can be reduced by coating non-wettable substance around nozzle except on the outlet section^[57].

3.2.2 Characteristic Dimensions and Scaling Law Related to Cone-jet Transition

3.2.2.1 Ganon-Calvo’s Scaling Law for Four Different Conditions

In the model of Ganon-Calvo^[66], EHD spraying in steady cone-jet mode is a quasi-electrostatic quasi-one-dimensional (1D) condition based on two assumptions: (1) The inner electric displacement $\epsilon_0 \epsilon_r E_n^i$, is small compared to the displacement, $\epsilon_0 E_n^o$, where E_n^o and E_n^i are the normal outer and inner electric fields on the jet’s surface, respectively; (2) the radial variations of the liquid velocity are small compared to the average velocity for most liquids and EHD spraying conditions. Since the transverse section of the cone region is very large compared to that of the emitted microjet, and the liquid velocity is small compared to that in the microjet, the bulk ohmic electric conduction is dominant in the cone with very small inner electric fields^[55,60,67]. Thus, the cone region is considered as perfectly electrostatic^[35,60]. The axial electric field E_z should be of the order of the normal electric field at the cone:

$$E_z \sim \left(\frac{\gamma}{\epsilon_0 L_o}\right)^{0.5} \quad (7)$$

In the ohmic model or “leaky dielectric” model^[5,16], an ohmic constitutive law for the current in the cone-jet transition region is assumed^[53,66].

$$I = I_{scv} + I_{ocd} = \frac{2Q\epsilon_0 E_n}{\xi} + \pi \xi^2 K E_z \quad (8)$$

where I_{scv} is surface convection current, I_{ocd} is ohmic bulk conduction current, Q is emitted flow rate, ξ is radius of jet, and K is liquid electric conductivity^[66,68]. The point where the surface convection current equals the ohmic bulk conduction current is located close to the jet’s origin^[67].

$$\frac{Q\epsilon_0 E_{no}}{R_o} \sim R_o^2 K E_{zo} \quad (9)$$

The pressure difference across the jet’s surface is balanced by the normal electrostatic stress. The polarization stress $(\epsilon_0 \epsilon_r - \epsilon_0) E_z^2 / 2$ is negligible^[66]. Thus,

$$\epsilon_0 (E_{no})^2 \sim \frac{\rho Q^2}{R_o^4} \quad (10)$$

The kinetic energy per unit volume of the liquid becomes of the order of the electrostatic stress once the jet is developed and the gradient of the kinetic energy in the axial direction is mostly balanced by the tangential electric stress resultant on the jet's surface^[66]:

$$\frac{\rho Q^2}{R_o^4 L_o} \sim \frac{\epsilon_0 E_{no} E_{zo}}{R_o} \quad (11)$$

Here $R_o = d_0 \left(\frac{Q}{Q_0}\right)^{0.5}$, $L_o = d_0 \frac{Q}{Q_0}$, $E_{no} = \left(\frac{2\gamma}{\epsilon_0 d_0}\right)^{0.5}$, and $E_{zo} = E_{no} \left(\frac{Q}{Q_0}\right)^{-0.5}$ are the characteristic values of the jet's radius, axial length, outer normal electric field, and tangential electric field. The characteristic flow rate $Q_0' = \frac{\gamma \epsilon_0}{\rho K}$, characteristic distance $D_0' = \left[\frac{\gamma (\epsilon_0)^2}{\rho K^2}\right]^{\frac{1}{3}}$, and characteristic current $I_0' = \gamma \left(\frac{\epsilon_0}{\rho}\right)^{0.5}$ are shown in the dimensionless analysis above^[66].

Ganan-Calvo defined two dimensionless expressions for flow rate^[26]:

$$\alpha_\rho = \frac{\rho K Q}{\gamma \epsilon_0}, \alpha_\mu = \frac{K^2 \mu^3 Q}{\epsilon_0^2 \gamma^3} \quad (12)$$

When the inertia stress is large, the dimensionless flow rate is the ratio of flow rate, Q , and dimensional variable sets $(\gamma \epsilon_0 / \rho K)$. When viscous stress is large, the dimensionless flow rate is the ratio of flow rate, Q , and dimensional variable sets $(\gamma^3 \epsilon_0^2 / \mu^3 K^2)$. The dominance of surface tension is a marginal situation^[26].

For six different situations, Ganan-Calvo *et al.* identified different scaling laws for the jet diameter D_j and the electric current I ^[26]. Three of them have been found in published experimental data, and they are presented below:

- *IE-scaling*: Dominance of inertia and electrostatic suction in the limits of $\alpha_\mu^{\frac{1}{4}} \ll \alpha_\rho, 1 \ll \frac{\alpha_\rho}{\epsilon_r - 1}$. Where, D_j is the same as the characteristic value (R_o) ^[26]:

$$I = (\gamma K Q)^{0.5}, D_j = \left(\frac{\rho \epsilon_0 Q^3}{\gamma K}\right)^{1/6} \quad (13)$$

The IE scaling law is the most common regime. It has been widely verified in numerous experimental works^[26,66,69-71], and data for octanol were accord with this scaling law^[26,31].

- *IP-scaling*: Dominance of inertia and polarization forces in the limit of $\frac{\alpha_\mu}{(\epsilon_r - 1)^4} \ll \frac{\alpha_\rho}{\epsilon_r - 1} \ll$ ^[26]:

$$I = \left(\frac{\rho K^2 Q^2}{(\epsilon_r - 1) \epsilon_0}\right)^{0.5}, D_j = \left(\frac{\rho \epsilon_0 Q^3}{\gamma K}\right)^{1/6} \quad (14)$$

The IP scaling law is used for polar liquids, and the scaling of jet diameter is the same as for IE scaling, no matter whether the dominant factor is electrostatic or

polarization force^[26]. Data for water and formamide tend to match with the IP scaling solution^[26,31].

- *VE-scaling*: Dominance of viscous force and electrostatic suction in the limit of

$$\alpha_\rho \ll \alpha_\mu^{\frac{1}{4}}, 1 \ll \frac{\alpha_\mu}{(\epsilon_r - 1)^4} \quad (26):$$

$$I = (\gamma K Q)^{0.5}, D_j = \left(\frac{\mu \epsilon_0^2 Q^3}{\gamma K^2}\right)^{1/8} \quad (15)$$

The IP scaling law is applicable to high viscosity liquids with a sufficiently large electric conductivity. The trend of this scaling law has been confirmed by experiments that use glycerol with different electrical conductivities^[26].

3.2.2.2 De la Mora's Scaling Law

In the model of De la Mora and Loscertales, the conductivities of the tested liquids are all in the range of 10^{-4} S/m and above; hence, these are considered as high conductivity liquids. They concluded that the current and jet structure for liquids with high conductivities are insensitive to changes of needle voltage, diameter, the distance between two electrodes, the meniscus shape, and the spray structure^[31]. The static Taylor equilibrium is maintained to the point where the flow time becomes shorter than the electrical relaxation time. Beyond this point, there is a rapid transition to a cone-jet. After the same point, the ohmic bulk conduction current (I_{ocd}) that is dominated in the cone also fast transfer to surface free charge convection current (I_{scv}) by the accelerating liquid stream, which is dominant close to jet's end where is very small cross-section and large liquid velocity^[31,32,55]. In the cone-jet transition area between these two regions, both conduction and convection are of the same order^[55].

A non-dimensional factor, η^2 , can be defined as the ratio of inertia pressure ($\rho Q^2 / r^4$) where ρ is liquid density, Q is the flow rate, and r is local radius, and capillary pressure is (γ / r) ^[31,32].

$$\eta^2 = \frac{\rho Q^2}{\gamma r^3}$$

The formation of the jet may be determined either by inertia or by charge relaxation, depending on which of these two phenomena acts first as the cone apex is approached^[31]. When η is smaller than unity, the diameter of jet scale as characteristic length r^* . In the opposite limit, where η is much larger than one, the diameter of jet scales as characteristic length R^* ^[31]. A characteristic distance λ from the cone apex is closely related to the jet radius^[31], and it also relates to the thickness of surface charge layer, which is built up by the bulk conduction^[72]. When the jet scaling length r^* , which is the same magnitude of charge relaxation length, λ , where hydrodynamic time, t_h is of the order of the electrical relaxation time, t_e , the characteristic distance, r^* , is expressed as the following equation^[31,32].

$$D_j \sim r^* = \left(Q \frac{\epsilon_r \epsilon_0}{K}\right)^{1/3} \quad (17)$$

When η equals to one and r equals to r^* , the minimum flow rate for the stable cone-jet structure is found, and it is also known as the characteristic flow rate^[31,32]. The minimum flow rate is expressed as:

$$Q_0 \sim Q_{\min} \sim \frac{\gamma \epsilon_r \epsilon_0}{\rho K} \quad (18)$$

The characteristic distance, d_0 , can be obtained from the characteristic flow rate, Q_0 , through the equation below:

$$Q_0 \sim Q_{\min} \sim \frac{\gamma \epsilon_r \epsilon_0}{\rho K} \quad (19)$$

When the dynamic pressure $\frac{1}{2} \frac{\rho Q^2}{r^4}$ becomes comparable with surface tension, γ/r , the diameter of jet scale as characteristic length, R^* ^[31].

$$D_j \sim R^* = \left(\frac{\rho Q^2}{\gamma} \right)^{1/3} \quad (20)$$

The intermediate region which is analyzed in the model of Ganan-Calvo provides consistent for matching the cone to transition region^[26]. Interestingly, when two characteristic distances (r^* and R^*) are multiplied together, the product of these two characteristic distances is similar with the expression of jet diameter for IE or IP scaling. In the liquid meniscus, charges are transmitted by convection and conduction at the charged interface and only by conduction through the bulk^[31]. In this model, the surface conduction term is ignored since it is small compared to surface convective term, and the surface convection current is then given by^[31]:

$$I_{scv} = 2\pi r u_s \sigma \quad (21)$$

Where u_s is the liquid speed at the interface and the free surface charge density, σ , equals to $\epsilon_0 E_n^o$. At the point where charge relaxation become non-negligible and the current I which jet carries is given^[31]:

$$I \sim f(\epsilon) \left(\frac{\gamma K Q}{\epsilon_r} \right)^{0.5} \sim \eta \gamma \left(\frac{\epsilon_0}{\rho} \right)^{0.5} \quad (22)$$

Where $f(\epsilon)$, as obtained from experiment, is a function of ϵ . Scaling law expressed in equation (22) is similar with scaling law of current in IE regime of Ganan-Calvo model, and actually $f(\epsilon)$ become very small when the dielectric constant is small. When η is equal to one, characteristic current, I_0 , is closely related to current, I , and can be obtained from:

$$I_0 \sim \gamma \left(\frac{\epsilon_0}{\rho} \right)^{0.5} \quad (23)$$

The parameter Z which equals to $\left(\frac{\gamma t_e}{\mu Q^{1/3}} \right)^{2/3}$ is defined to measure the radial variation in the axial velocity profile of the jet^[31]. The electrical shear stresses which tend to make the liquid velocity larger at the surface than at the core, while viscosity tends to make the axial velocity radially uniform.

In conclusion, the scaling model presented above has been confirmed by asymptotically self-similar solutions,

and it shows that the emitted current and diameter of jet mainly depend on flow rate and liquid properties. They argue that the jet radius is independent of the boundary condition and applied potential since the typical jet radius is sufficiently smaller than the local size of the tip region. Table 1 summarizes scaling laws that describe the dependence of jet diameter and magnitude of current on working parameters.

However, Hohman *et al.* obtained a different scaling law from electrospinning experiments, and they found current not only depends on flow rate and material properties but also applied voltage, shape, and material of nozzle^[73]. Discrepancies in scaling laws of electrospray and electrospinning may be attributed to the following reasons. Solutions from steady-state equations for electrospray are a function of imposed flow rate, current, and voltage drop between two electrodes. Boundary condition on a surface charge is that jet shape is matched to a perfectly conducting nozzle. However, the current is determined dynamically in electrospinning experiment, and flow rate and field strength are only independent parameters. Hohman *et al.* found that the radius of the jet has already decreased substantially before entering the asymptotic regime. Thus, current is determined more than just the behavior in the asymptotic regime. Numerical steady solutions have only been obtained for low conductivity material (10^{-6} S/m) at the high electric field after counting fringe fields of the nozzle and considering small surface charge density as a boundary condition at the nozzle^[73]. The electric field around nozzle and boundary condition become key factors to influence shape and charge densities on the liquid jet. It is helpful for understanding this influence to explore what the predominant charge transport mechanism near the nozzle is. Differences in geometry setup are also a possible reason to lead different scaling law.

3.2.3 Pulsating Cone-jet Regime

Although most attention is focused on the “continuous regime” where a jet is continuously ejected from its apex, a continuous regime only happens for certain ranges of the operating parameters. Changes in liquid properties can also cause instabilities^[30]. The D-O-D pulsate printing as produced by an external voltage pulse has become more popular due to low reagent consumption and high ion transmission efficiency at small flow rate^[28]. The frequency of the lowest excitation mode for a negligible amount of charge is expressed as^[30] and can be calculated by equation (24) which is derived from the frequency spectrum for the capillary waves on the surface of a charged droplet proposed by Rayleigh^[12].

$$f_{2,z=0}^2 = \frac{2}{\pi^2} \frac{\gamma}{\rho r^3} \quad (24)$$

Table 1. A summary of scaling laws that outline the relationship between processing parameters and diameter of jet or emitting current

Name	Dominant conditions	Scaling of jet diameter	Scaling of current	Material	Reference
Ganon-Calvo scaling law for high electric conductivity (Above 10^{-4} S/m)	IE-scaling (inertia stress and electrostatic suction)	$D_j = (\frac{\rho \epsilon_0 Q^3}{\gamma K})^{1/6}$	$I = (\gamma K Q)^{0.5}$	Non-polar liquid with low viscosity, such as Octanol	
	IP-scaling (inertia stress and polarization force)	$D_j = (\frac{\rho \epsilon_0 Q^3}{\gamma K})^{1/6}$	$I = (\frac{\rho K^2 Q^2}{(\epsilon_r - 1) \epsilon_0})^{0.5}$	Polar liquid with low viscosity, such as water, and formamide	
	VE-scaling (viscous force and electrostatic suction)	$D_j = (\frac{\mu \epsilon_0^2 Q^3}{\gamma K})^{1/8}$	$I = (\gamma K Q)^1$	Ink material with high electric conductivity, such as glycerol	
	VP-scaling (Viscous force and polarization force)	$D_j = (\frac{\mu Q}{(\epsilon_r - 1) \gamma})^{0.5}$	$I = (\frac{\mu^3 K^3 Q^2}{(\epsilon_r - 1)^4 \gamma^2 \epsilon_0^2})^{0.5}$	Not found in published paper	
De la Mora scaling law for high electronic conductivity (Above 10^{-4} S/m)	Hydrodynamic time and electrical relaxation time	$D_j \sim (Q \frac{\epsilon_r \epsilon_0}{K})^{1/3}$	$I = f(\epsilon) \frac{\gamma K Q}{(\epsilon_r)}^{0.5}$	Ethylene glycol	
	Inertia stress and surface tension	$D_j \sim (\frac{\rho Q^2}{\gamma})^{1/3}$	$I = f(\epsilon) \frac{\gamma K Q}{(\epsilon_r)}^{0.5}$	Glycerine solution	
Choi scaling law	Electrical field force and surface tension	$D_j \sim \sqrt{\frac{\gamma}{\epsilon_0} \frac{\sqrt{d_N}}{E}}$	Not mentioned in the reference paper	Water (0.1 mM KBr added), and Glycerine	

Where r is the droplet radius. Marginean *et al.* found^[30] that equation (24) can also express the relationship between the square of the pulsation frequency and the cube of the anchoring radius of the menisci (the position of the contact line at the tip of the capillary during the liquid recoil phase) for low-conductivity liquids and relatively large nozzle radius. They also found that the effect of charge in this region is negligible.

Another finding is that the capillary waves not only governs transitions associated with the meniscus (dripping mode to spindle mode) but also influences the breakup of the liquid filament (varicose to kink)^[30]. Choi *et al.* proposed a scaling law for jet diameter in the pulsating cone-jet regime, based on data from their experiments and literature^[64]. They defined the ratio between the surface tension and the electric field forces as the “electrical capillary number (Ca),” and considered D_j and D_n as characteristic length scales associated with the surface tension force and the electrical field force separately^[64]. The scaling law for jet diameter is:

$$d \propto \sqrt{\frac{\gamma}{\epsilon_0} \frac{\sqrt{d_N}}{E}} \quad (25)$$

This scaling law is different from those in section 3.2.2; a simple reason is that Choi *et al.* obtained diameter of the jet at initiation condition where the jet is the widest. Choi *et al.* substituted equation (25) into equation (24) to derive a scaling law without droplet radius. The pulsation frequency read as:

$$\beta = \epsilon / \epsilon_0 \quad (26)$$

Chen *et al.* proposed a different model for the intrinsic pulsations frequency; this is related to the drop formation rate, Q , in equation (27), (28).

$$f_{pj} \sim \frac{Q}{V_{pj}} \sim \frac{Q}{Q_0 \Delta t_{pj}} \sim \frac{Q_c}{(d_n d_0)^{3/2}} \sim \frac{d_n}{\mu L} \cdot \epsilon_0 E^2 \cdot \frac{K}{\epsilon_r \epsilon_0} \cdot (\frac{\rho d_n^3}{\gamma})^{0.5} \quad (27)$$

In conclusion, as upstream flow rates are low, the cone formation rate is affected by stress at the liquid-to-air interface and by viscous drag in the thin nozzle, and Taylor cone only deforms at the tip^[28]. Equation (27) is used to obtain frequency at a low flow rate, and the drop formation rate scales as $d^4 E^2 L^{-1}$ where d is inner diameter of nozzle, E is the nominal electric field, and L is the nozzle length^[28]. However, as supplied flow rate is not limited by upstream conditions, the equation (24) is applied to acquire frequency (Chen, 2011). Since equation (25) reveals a 0.5 power dependence between the diameter of jet and nozzle, it implies this equation may not be employed for high conductivity liquid ($\geq 10^{-4}$ S/m).

3.3 Theory and Analysis of Jet Stability

In EHD inkjet printing, a continuous fine jet is generated at the apex of a liquid cone in the cone-jet mode and the length of jet increases with the viscosity, the resistivity and flow rate of liquid^[18]. The field strength at the tip of the jet increases with the length of the jet, due to a decrease

of the electrical field shielding by the large outer diameter of the capillary and also a decrease of the diameter of the accelerated jet, which behaves as a vena contracta^[74]. This corresponds to increasing field stress at the tip of the jet in the direction toward the counter electrode^[20]. It is difficult to stabilize and control the trajectory of submicron jets under an electric field and jet may have different instable status. When the charge of the jet is not too high, liquid jet is broken up into drops by varicose instabilities as shown in Figure 4 and satellite droplets may be created at the moment of the breakups^[18]. The process of a breakup may degrade resolution on the substrate. The smaller droplets move away from the axis of the jet, which is faster than the larger ones^[18]. For slightly higher voltages, lateral kink-type instabilities appear, and the jet stretches out into fine droplets of different sizes^[18]. Thus, a big challenge for utilizing the electrically driven cone-jet transition as a writing device still exist in overcoming the rapid disintegration in electrospray and controlling the whipping of the jet in electrospinning^[38]. The common feature of them is no boundary jetting methods, but axisymmetric instability is applied on breaking up the jet into numerous tiny droplets in electrospraying, and non-axisymmetric, known as whipping action, is used to thin the liquid jet and deposit it as a fiber filament in electrospinning. However, both of instabilities need to be prevented from high resolution printing process. In the following part, the theory of the jet stabilization is discussed, and then the stabilization of jet under an electric field is considered.

When a liquid jet is either neutral or lower surface charging under the condition of lower field strengths, the breakup of a jet into droplets is attributed to the effect of surface waves^[75]. Surface waves propagating along the surface of a jet are generated by disturbances of the jet^[61]. As the amplitude of surface waves of an appropriate wavelength ("varicose waves") is enhanced by surface tension forces, they lead to the breakup of a cylindrical jet into droplets with radius $r=1.89a$ ^[20]. This mechanism is well established for uncharged jets and was first described by Rayleigh^[76]. Rayleigh reveals that disturbances on a jet with wavelengths greater than the circumference of the jet will grow, and rapid growing disturbance will control the breakup of jet^[77].

3.3.1 Three Instability Modes of a Charged Jet

Saville performed qualitative analysis for the stability of an uncharged liquid cylinder in the longitudinal electric field^[62], and this analysis ignores the presence of surface charge on the jet and thinning of the radius^[78,79]. Another analysis of stability is for a charged cylinder in a radial electric field (without tangential field) and a constant radius jet^[80]. Hohman *et al.* developed a 1D model for a long and slender object made of a leaky dielectric material, and this model is asymptotically valid in the assumption

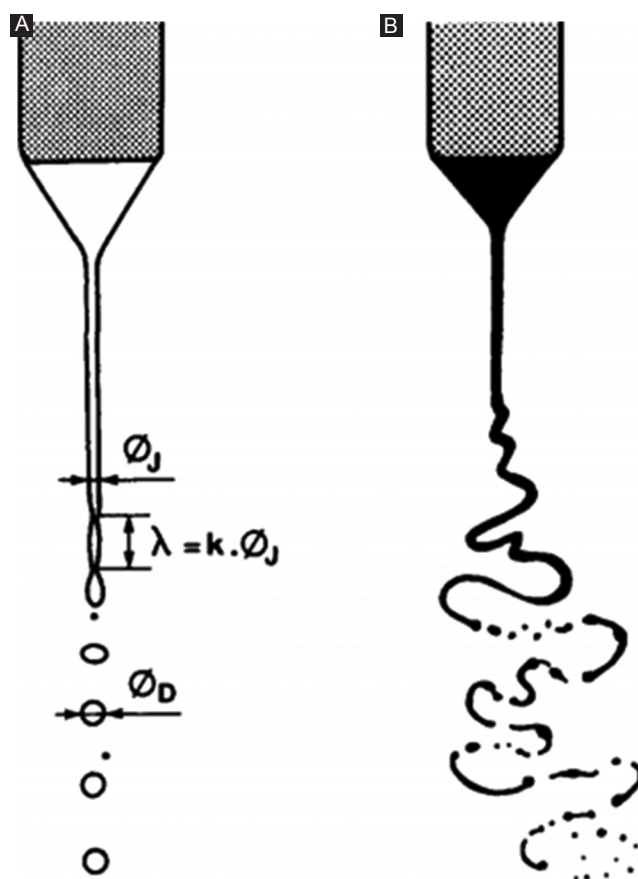


Figure 4. Cone-jet mode: (A) Varicose instabilities; (B) kink instabilities^[18]. Adapted by permission from Michel Cloupeau *et al.* (1994) under the Elsevier.

that tangential stress caused by the electric field must be much smaller than the radial viscous stress^[79]. They performed a local linear stability analysis by considering axisymmetric perturbation to a charged cylinder of constant radius under an assumption that the wavelength of the perturbation is much smaller than the characteristic decay length of jet^[79]. They identified three different instability modes that are axisymmetric extensions of the classical Rayleigh instability, and two conducting modes, which only exist when the conductivity of fluid is finite, including the axisymmetric conducting mode motivated by different time scale for fluid response and axial surface charge arrangement, and a whipping conducting mode caused by static charge density of jet, which dominated in local electric field. Rayleigh instability is suppressed when the electrical pressure per unit length of the jet exceeds the surface tension pressure per unit length with an increase of electric field and surface charge density, and conducting mode which is raised from the interaction of electric field with a surface charge on the jet makes jet unstable^[79]. Both the axisymmetric modes are stronger than the whipping mode at low fields by adding the effect of viscosity and surface charge, but the whipping mode

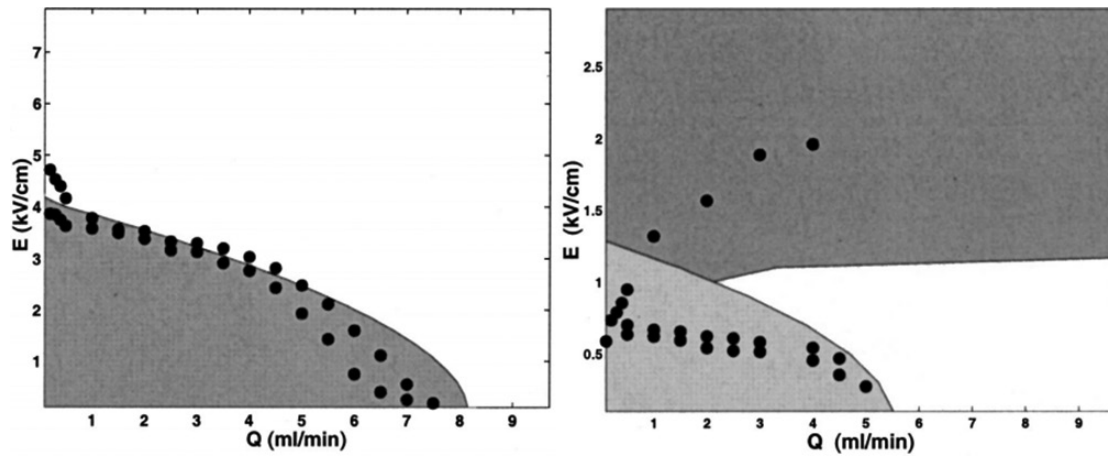


Figure 5. Operating diagram for (a) glycerol jet ($K = 0.1 \times 10^{-5} \text{ S/m}$, $\rho = 1.26 \times 10^3 \text{ Kg/m}^3$, $\mu = 1.87 \text{ Pa}\cdot\text{s}$). The shaded region is varicose perturbations, and no whipping instability is present. (b) PEO jet ($K = 1.2 \times 10^{-2} \text{ S/m}$, $\rho = 1.2 \times 10^3 \text{ Kg/m}^3$, $\mu = 2 \text{ Pa}\cdot\text{s}$). The lower shaded region is varicose perturbation, and the upper shaded region is whipping instability. The points represent experimental measurements are consistent with theoretical prediction^[73]. Adapted by permission from Moses Hohman *et al.* (2001) under the AIP Publishing LLC.

is stronger than both of the axisymmetric modes at high fields^[79]. Figure 5 compares the theoretical operating diagrams for glycerol and PEO jet with experimental measurements of the instability thresholds^[73]. The shape of operating diagram for a less viscous and highly conducting fluids is similar to the range of cone-jet mode in electrospray^[73]. The lower threshold of onset of cone-jet is identified as cessation of varicose instability, and upper threshold is considered as onset of whipping. With increase of electrical conductivity, the critical flow rate of cone-jet mode decreases. A higher surface charge density in higher conductivity fluids leads a stable jet at a lower flow rate due to suppression of Rayleigh mode^[18,57]. In other words, steady cone-jet which is most used for EHD high-resolution printing should locate in the area between varicose and whipping instability in E-Q operating diagram.

In Table 2, the main models mentioned in this review are summarized by the phenomenon, the name of models, assumptions, advantages, and disadvantages of the model.

3.3.2 Stability Analysis in Finite Conductivity and Permittivity Jets

Herrera *et al.* studied influence of arbitrary liquid viscosity, permittivity, and conductivity in the presence of a DC radial electric field, and found 1D average models are inaccurate in low viscosity imperfect conductor jet since an interfacial boundary layer may exist in the axial velocity profile^[81]. These results show that the accuracy of 1D approximation increases with the increase of liquids' electrical conductivity and/or viscosity by comparing results obtained from 1D approximation and two-dimensional leaky dielectric model^[82]. They focused on axisymmetric capillary jet instability modes that are dominant in the moderate range of Weber number that is

the ratio of average axial velocity, U , to capillary velocity (v_c).

$$We = \frac{\rho U^2}{\gamma} = \left(\frac{U}{v_c}\right)^2 \quad (28)$$

In the developed EHD jet, the axial electric field can be neglected since it is much less than radial electric field that is created by surface charge density located at the interface^[60,69]. According to cubic roots of dispersion relation, Hohman *et al.* found three unstable modes in the presence of an axial electric field^[79], but Herrera obtained four-order dispersion relation (four modes) in the presence of radial electric field^[81]. Artana *et al.* indicated that the intact length of the jet is proportional to the inverse of growth rate σ_m in the fastest growing mode under the assumption of electrified inviscid limit and perfect conductor limit^[83]. Effects of relaxation parameter (α) that is the ratio of the capillary time (t_c) to the electrical relaxation time (t_e), liquid's relative permittivity (β) and electric bond number (χ), which is the ratio of the electric pressure to capillary pressure are explored in this study.

$$\alpha = [\rho r^3 K^2 / (\gamma e^2)]^{0.5} = t_c / t_e \quad \beta = \epsilon / \epsilon_0 \quad \chi = r \epsilon_0 E_0^2 / \gamma \quad (29)$$

Where $\epsilon_0 E_0$ is surface charge density and E_0 is a radial electric field. With the increase of conductivity, the maximum growth rate σ_m increases and the wave number k_m decreases in the fastest growing mode in Figure 6A. Growing relative permittivity leads to a similar trend^[81]. This figure shows that instability increases with the rise of breakup wavelength. The conductivity and permittivity do not influence a range of stability, but in Figure 6C, the instability lobe expands with an increase of electrical bond number^[81]. Herrera *et al.* pointed out that tangential electric stress will attenuate the growth of perturbations

Table 2. A summary of theoretical models for three phenomena under different assumptions, and their advantages and disadvantages

Phenomenon	Theories or models	Assumption and conditions	Advantages	Disadvantages
Conical meniscus	Taylor electrohydrostatic model (Taylor cone)	Semi-vertical angle of cone is 49.3°; Pressure is zero; Neglected weight of the liquid cone	Obtaining critical voltage value for jet emission from the tip of the cone	Semi-angle is not always 49.3°; Approximate 10% difference from the critical voltage of experiment
Cone-jet transition	Leaky dielectric model; Surface couple model	Penetration of electric field lines into liquid; Appearance of surface electric charges	Described operating diagram of cone-jet for inks with partial electrical conductivity	Only for leaky dielectric material and stable cone-jet mode; Complexity in a mathematical method
Stability of a long and slender jet	One-dimensional model	Tangential stress caused by electric field must be much smaller than the radial viscous stress; Wavelength of the perturbation is much smaller than the characteristic decay length of the jet	Identified three instability modes based on electric field strength; Represented operating diagram of varicose and whipping instability thresholds	Inaccurate in low viscosity and/or imperfect conductor jet

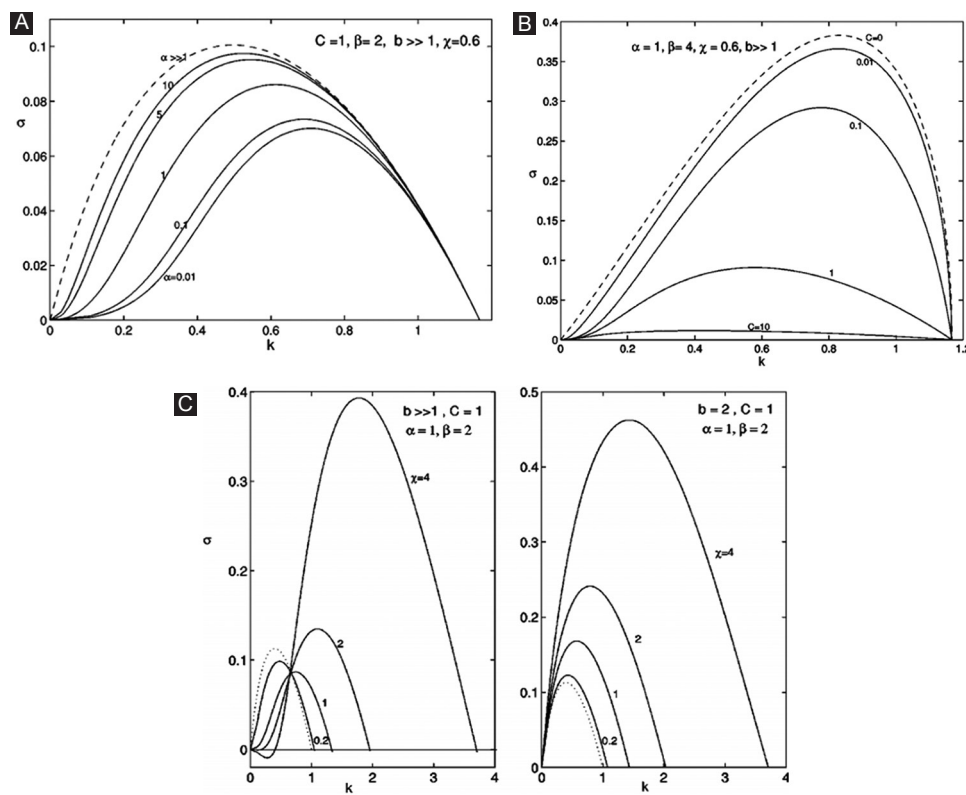


Figure 6. (A) Growth rate σ versus the wavenumber k for a jet with b (distance between two radial electrodes) $\gg 1$, $\chi = 0.6$, C (Ohnesorge number) = 1, and β (liquid relative permittivity) = 2 and several values of the relaxation parameter α ; (B) Growth rate σ versus the wavenumber k for a jet with different values of the Ohnesorge number C ($b \gg 1$, $\alpha = 1$, $\beta = 1$, $\chi = 0.6$). (C) Growth rate σ versus the wave number k for a jet with $b \gg 1$ and $b = 2$. The other parameters are fixed at $C = 1$, $\alpha = 1$, and $\beta = 2$. Several values of the electrification number χ are plotted^[81]. Adapted by permission from Jose Lopez-Herrera *et al.* (2005) under the AIP Publishing LLC.

and may promote longer liquid jets with moderate viscosity in the non-whipping jet regime for an imperfect conductor^[81].

The effects of the viscosity can be reflected by comparing growth curves having different Ohnesorge numbers, C , defined as a ratio of viscous forces to capillary forces,

$$C = \mu / (\rho \gamma r)^{0.5} \quad (30)$$

Viscosity does not change the stability limits, but it lowers the growth rate of any perturbation. The most unstable wavelength becomes longer with an increase of Ohnesorge number in Figure 6B, whereas the growth rate decreases^[81]. A narrow interfacial boundary layer with

The meniscus may globally be either stable or unstable up to values of the operational parameters, and the global stability of the liquid meniscus is a prerequisite for steady jetting^[84]. The liquid flow accelerates from a subcritical to a supercritical regime at a critical point near the exit of feeding needle by considering self-induction effects and jets issued from Taylor cone are always supercritical at region before breakup point^[67]. A point of instability is defined as a boundary line to separate the supercritical region and the breakup regions and supercritical region prevents upstream propagation of disturbance as long as the point of instability is far away from the critical point^[67]. Transition between jetting and dripping is related to transition between convective and absolute instability transitions^[85]. Convective instability is defined as the surface energy that is sufficiently smaller than the kinetic energy per unit jet length, and the disturbance can only propagate and increase downstream^[85]. The disturbance can propagate and increase both upstream and downstream for absolute instability. The critical convective velocity in the developed jet that relates to critical Weber number mainly depends on the viscosity of fluid that scale as the Reynolds number during the transition between the absolute and convective instability. Herrera *et al.* introduced different electric parameters (relaxation parameter α , liquid relative permittivity β , and electric bond number γ) to the classical Leib and Goldstein curve

This paper reviews the initiation condition of cone-jet. The critical voltage predicted by Taylor does not account for EHD effect caused by subsequent jet emission, and the semi-vertical angle is not always Taylor angle. The following section study effects of processing parameters and materials properties on the operating diagram of cone-jet. Domain of cone-jet may become different by small changes in the selection of parameters. The minimum flow rate in operating diagram is not, simply, the flow rate due to the upstream pressure, but rather the specific flow rate that causes electrical stress to strip off (or shear) the surface charge layer of the fluid. Several scaling laws have been proposed to predict jet diameter and emitted current, and each of them is applied to different parametric ranges according to material attribute and working condition. Communities have not reached an agreement on the charge transport mechanism. In the future, it is helpful to study what the predominant transport mechanism is, especially near the nozzle. It is difficult to measure a thin free jet whose diameter is usually at the verge of optical

resolution experimentally. It is known that a stable jet is a prerequisite condition for acquiring high printing resolution, but the theoretical prediction of jet stability still remains to be a difficulty. Understanding of this physical process requires more systematical studies on the formation of a stable cone-jet.

3D high-resolution printing as one of an important branch of EHD family is rising rapidly in recent ten years. In contrast to deposition in a small area by electrospray or thinning and bending process by electrospinning, high-resolution EHD printing can generate single droplet or continuous line at single micro-scale. Designs and recent applications of EHD 3D printing will be discussed in the next paper due to length limitation.

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Of balls, inks and cages: Hybrid biofabrication of 3D tissue analogs

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Abstract: The overarching principle of three-dimensional (3D) bioprinting is the placing of cells or cell clusters in the 3D space to generate a cohesive tissue microarchitecture that comes close to *in vivo* characteristics. To achieve this goal, several technical solutions are available, generating considerable combinatorial bandwidth: (i) Support structures are generated first, and cells are seeded subsequently; (ii) alternatively, cells are delivered in a printing medium, so-called “bioink,” that contains them during the printing process and ensures shape fidelity of the generated structure; and (iii) a “scaffold-free” version of bioprinting, where only cells are used and the extracellular matrix is produced by the cells themselves, also recently entered a phase of accelerated development and successful applications. However, the scaffold-free approaches may still benefit from secondary incorporation of scaffolding materials, thus expanding their versatility. Reversibly, the bioink-based bioprinting could also be improved by adopting some of the principles and practices of scaffold-free biofabrication. Collectively, we anticipate that combinations of these complementary methods in a “hybrid” approach, rather than their development in separate technological niches, will largely increase their efficiency and applicability in tissue engineering.

Keywords: Tissue engineering, scaffolds, scaffold-free, bioprinting

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1. Biofabrication of Tissue Analogs - The Main Approaches

Biofabrication, the three-dimensional (3D) assembly of living cells in structured systems ^[1], has evolved in the past decades as the biological facet of additive manufacturing ^[2]. Technically, biofabrication can be roughly divided into “bioprinting,” a technology akin to the classical use of ink and paper ^[3], and “tissue assembly” ^[4], where other techniques such as “tissue strands” ^[5], lamination of “cell sheets” ^[6] or cell spheroid piercing-and-stringing ^[7], have recently evolved. The purpose is to assemble cells into a living, functional system that serves for *in vitro* drug development and substance testing, or as an *in vivo* implantable tissue ^[1]. However, the boundaries between approaches are blurring, because they will be increasingly

shared to create synergies with an eclectic mix of techniques for reaching specific goals. In future, the combined use of the main approaches in biofabrication will likely lead to more “hybrid” systems and subsequent expectations on appropriate instrumentation ^[8]. For this reason, in our overview, we will discuss the strengths and weaknesses of these approaches, according to their main features.

Moroni *et al.*, Groll *et al.* ^[1,4] and Moldovan ^[9] have noticed some confusion regarding the terminology in this field. Therefore, here we need to specify again what - in our opinion - 3D Bioprinting is not: namely, it is not the mere additive manufacturing (a.k.a. 3D printing) of implantable materials which, by all intents and purposes, would be “biomaterials” ^[10]. The implantation of such a material aims either at total immunological inertia (sometimes

also misnamed in this context as “biocompatibility”), or alternatively seeks histo-integration, and therefore the implant’s colonization by cells that migrate onto and into its structure [11]. As an example, the osteoinductive structures made of various materials can be considered [12]. This approach is common in regenerative medicine, constituting a form of “*in vivo* tissue engineering” with the host tissue acting as a bioreactor [13]. In this case, a tissue-repairing activity requires merely shape and mechanical function of interest; therefore, the used materials are polymeric or natural/decellularized fibrillar matrices. Hydrogels, another major medium for tissue engineering, are also occasionally injected directly in the recipients to elicit a repairing response either by themselves or often as cell carriers [14].

Many *bona fide* 3D biofabrication tasks primarily target the *in vitro* applications, and either include the spatial arrangement of living cells during the printing process or make them adhere in a directed manner on specifically preprinted structures. As a future development, pre-existing structures of living host tissues might be considered as the equivalent of preprinted structures and therefore might invite direct *in vivo* printing approaches onto wound grounds [15].

Whatever the approach, biofabrication is done with the understanding that besides a variety of cells, animal tissues contain various proportions of extracellular matrix (ECM) in the form of fibrils, fibrillary networks, sheaths, and hydrogels, as well as a fluid phase representing a complex mixture of molecules with metabolic or signaling functions. Correspondingly, a re-creation of tissue analogs largely recapitulates this blueprint (Figure 1).

1.1 Bioprinting

1.1.1 The Print and Populate Approach

In this approach, support structures are printed first, and cells are positioned subsequently on them in a targeted way. Often a layer-by-layer deployment method is pursued, such that lines of the hydrogel are printed

first, and then covered by jetted cells [16]. The system works by printing bioinks in contact mode followed by polymerization (e.g., by ultraviolet light) of the printed lines, and subsequent jetting of cells onto printed lines in nanodroplets. One such cycle can be repeated several times resulting in a layered structure [16]. Since hydrogels are materials with an absorptive surface, they could take up the excess fluid from the jetted droplets.

The print and populate method also works with hard materials, such as polycaprolactone. In tissue engineering, another version of this method is the colonization with cells of fibrillar and/or porous scaffolds prepared by electrospinning. This is usually performed after the shape of the scaffold is pre-determined, such as layer and tube [17]. However, for cell placement, simple cell sedimentation usually does not suffice, because the superficial pores are quickly clogged, preventing their further penetration in the scaffold. To force them inside of the structure, the cells may need to be exposed to negative pressure [18], to gravitational (centrifugation) [19] or magnetic [20] forces.

1.1.2 Direct Cells Printing - the Role of Bioinks

A typical commercial bioprinter offers the extrusion mode, by which a viscous medium is continuously expelled from ready-to-use cartridges through printing needles or nozzles. The extrusion can be achieved through microvalves propelling the extrudate downward, a plunger system, or in the case of very viscous materials, a screw pump [21]. The cells are delivered in a printing medium, the so-called “bioink,” that contains them during the printing process, ensures shape fidelity of the printed structure, and protects cells against shear forces [22]. Alternatively, the bioinks might be deployed by inkjet methods [23], one of which is laser-assisted droplet generation [24,25].

Technically, the bioinks represent *scaffolds*, which is particularly obvious after chemical, photo or enzymatic crosslinking [26]. Therefore, to avoid confusion, occasional claims (e.g., [27]) that bioink-based printing starting with more fluid solutions, or because these are further

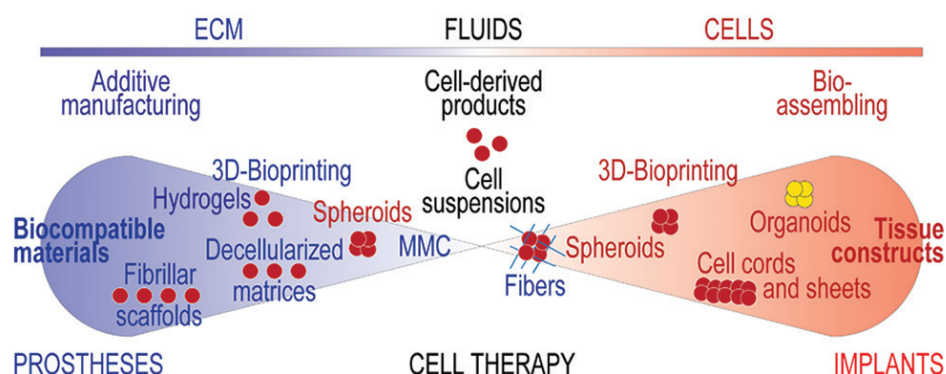


Figure 1. Graphic overview of the biofabrication methods.

combined with fibrillar meshes, would be “scaffold-free,” should not be entertained.

1.1.3 Scaffold-in-scaffold: Integrated or Composite 3D Bioprinting

When the hydrogels alone do not attain enough strength by polymerization, another option is to print harder or stiffer scaffolds as cage-like system first and then to fill them with cell-laden bioinks on hydrogel-basis [28]. For this, the terms of “composite” [29], “integrated” [30] or even “hybrid” [31] bioprinting have been coined.

1.1.4 Advantages and Limitations of Hydrogels-based Bioprinting

Although only a few decades old, the concept of bioprinting and the instruments operated on this principle inspired from additive manufacturing have progressed impressively and already generated convincing proofs of concept. Responding to a large societal interest, a market emerged for commercialization of a variety of bioprinting materials and instruments. However, with bioink-based bioprinting, it is not trivial to satisfy simultaneously the requirement of both printability and biocompatibility [22]. Essentially, bioinks are soft biomaterials, which need to permit extrusion or jetting, yet are required to maintain a printed shape. This is achieved by employing thixotropic gels that show shear-thinning during an extrusion process and regain larger stiffness immediately after deposition or a by the post-printing chemical crosslinking process. These bioink modifications have consequences for cellular viability and proliferative and migratory behavior. Cell damage and post-printing dysfunctionality may occur for a variety of reasons, such as high shear stress, lack of growth factors or suitable ECM, or limitation of intercellular communication [9]. Despite promising results in this direction, an ideal bioink is yet to be found. Taking into consideration that there are so many different cell types in the body, all with their refined needs regarding the microenvironment, it appears that a variety of suitable bioinks still have to be developed to address the needs of different cell types. In addition, the constructs obtained by the current bioprinting techniques display a simplistic cellular architecture. For example, although pre-vascular tubes could be embedded in the structural bioink as “sacrificial” hydrogels [28], their patterns lack the fractal organization of natural microvascular networks.

In summary, this approach relies on hydrogels as primary shape generators and cell carriers. While it remains promising for large, cell-homogenous, matrix-rich tissues, representing mostly the skeletal-muscular system, it is still struggling to solve its hard to conciliate requirements, related to the hydrogels printability on one side, and cellular needs on the other. More work will need to be invested into self-organizing systems,

where main (yet small) blood vessels can be printed into tissue constructs, to further allow sprouting from and to capillary systems. Clearly, the fourth dimension comes into play, namely the time-component of maturation.

2. Tissue Assembly

In this section, we describe 3D tissue engineering methods that do not make overt use of the ink and paper analogy. In most cases, the methods of this type could be called “scaffold-free,” indicating a version of biofabrication where only cells are used, and the needed matrix is produced by the cells themselves. With regard to the configuration of the cells in the initial assembly step, this approach comes in two variants: A planar and a spherical mode.

2.1 Planar Biofabrication

This mode works with cell sheets that have a preferred two-dimensional distribution. In essence, this biofabrication method is a lamination approach and hinges on the adhesion of cells to a substrate and the ECM produced by them. Thanks to a thermosensitive polymer (NiPAAM and pNiPAAM) as a support structure, a reduction of temperature below standard cultivation at 37°C will lead to a repulsion of the cell layer from the polymer, leading to the detachment of the cell culture as a contiguous cell sheet [32]. Several cell sheets can be stacked, thus resulting in a multilayered tissue [33]. The approach, originally proposed by Michael Sefton for drug delivery purposes, later developed as a tissue engineering mode [34] and was driven forward by Owaki *et al.* [35].

2.2 The Spheroidal Mode of Cell Assembling

2.2.1 Make and Cast

The 3D nature of tissues’ architecture is well captured by employing cell spheroids. Frequently referred to also as “microtissues” [36] they are increasingly preferred to isolated cells to improve the efficacy of cell therapy [37], or particularly as tumor models (oncospheres) [38]. When these spheroids are grown from stem cells, they are called “organoids” (or “embryoid bodies” in the case of embryonic or induced pluripotent stem cells), representing a promising new development in tissue engineering [39]. Alternatively, cell aggregates can be also prepared as cell cords, for example, for further processing into spheroids [40].

A completely different bioprinting approach is based on the notion that the cell clusters forming spheroids merge when brought into contact with each other [41]. The fusion of spheroids is based on the same principles that govern their formation in the first place: Minimization of a system’s potential energy, generated by the adhesive interactions between cells, through intercellular adhesion

molecules, mainly the cadherins and connexins [42]. The cell cytoskeleton [43] and metabolism may also have indirect roles but probably are less important than direct cell-cell attachment. A good argument that spheroid fusion is relatively well understood is the ability to simulate it by computer modeling on several platforms, for example, CompuCell3D [44], initially developed around this type of applications [45]. We recently expanded a similar model to capture the role of oxygen during spheroid fusion in larger structures [46].

Spheroid generation is best achieved by placing the cells on non-binding surfaces either in large flat or small, round wells [47]. Cells sedimenting on a surface that offers no anchorage will adhere to other cells thus forming spherical clusters, a tendency that can be promoted by small inverse pyramidal depressions [48]. Depending on geometry and coating, the latter allows for homodisperse spheroid sizes, while larger non-binding wells might give rise to numerous differently sized spheroids. The spheroids so formed then have to be repositioned to induce them to fuse into 3D structures. To this end, the spheroids can be placed in molds where they form 3D structures by fusing [41]. Tissue models of cartilage [49], or cardiac patches prepared from spheroids fused by flotation on culture medium [50], as well as vascular rings [51] and tumor models [52] assembled by magnetic force, have been described. As a variant, thicker honeycomb-shaped cell toroids prepared in molds, then stacked in register, and fused in larger constructs were proposed for improved distribution of nutrients, even in the absence of a bona fide vascularization [53].

2.2.2 Pick and Place

Alternatively, spheroids can be individually manipulated by dedicated instruments (such as the “Fabion” bioprinter [54]), and placed in a pattern on support where they fuse and form hollow or mixed massive structures. Interestingly, the targeted placement of spheroids on support (which some authors address as “biopaper” [55-57]) refers back to the printing analogy.

To facilitate the formation of 3D constructs from spheroids, a method was needed to keep them in contact long enough to effect fusion, and at the same time to allow the cells to produce their own ECM [58]. The companies Organovo [59] and 3D Bioprinting Solutions [54] are performing this step on their bioprinters using “fugitive” hydrogels as supports, which are removed after the spheroid fusion process.

2.2.3 Pick and Skewer

An ingenious solution to spheroids assembling problem has been developed in Japan [60]. Essentially, this is based on using a spheroids-assembling robot, which skewers them on a rectangular array of stainless steel micro-needles (“Kenzan”) [7]. Although technologically

different, this method has common goals with regular bioprinting, such as the use of a nozzle to place the cells in a layer-by-layer formed 3D pattern, as well as the computer-assisted design and operation.

Recently, several pre-clinical examples of “Kenzan” scaffold-free biofabrication have been reported: Surgically-robust small diameter vascular grafts [61], tracheal [62] and urethral [63] tubes, neural bridges [64], beating cardiac patches [65], liver buds [66], and gastric diaphragm [63]. However, the Kenzan method is not without its own limitations, as we commented previously [7]. Among the more significant ones are: (i) Inability to import anatomically-correct 3D images; (ii) dependence of this method on the cells’ propensity to make spheroids of required size (commensurate with the inter-needle distance); (iii) secretion of a matrix strong enough to keep the construct compact; and (iv) length of the constructs limited to that of the microneedles.

3. Hybrid Biofabrication

Since the inception of bioprinting, those involved in its development rightly appreciated the difficulties derived from the use of a biomaterial and contemplated alternatives (for instance, [64]). These consist of using cell spheroids as building blocks for direct assembling the 3D construct, even if the spheroids themselves may require temporary support of some sort (such as “fugitive” hydrogels, or mechanical assistance).

Thus, some of the properties of scaffold-free biofabrication could be complemented, at least in part, by including biomaterials into the “scaffold-free” constructs. For example, these biomaterials could compensate for the slower intrinsic secretion of an ECM by some cell types, when prepared as spheroids. Alternatively, “classical” bioprinting may also benefit from several principles of the scaffold-free approach. Combined, this technological inter-breeding establishes the field of “hybrid” biofabrication.

The use of sacrificial hydrogels for holding spheroids in place, until fusion and during a “post-printing maturation” phase [54,65], is a typical example of hybrid bioprinting. Combination of spheroids with hydrogels could be profitable for regular bioprinting as well if instead of single-cell suspensions pre-formed spheroids are mixed within the bioink. In practice, this solution has been used to increase the human adipose-derived stromal cells survival and promoted their ability to differentiate after bioprinting [66]. The bioprinter can be also used to prepare cells mixed in bioinks of alginate [67] or collagen [68] in droplet form, as an efficient method of cell encapsulation, for subsequent *in vitro* or *in vivo* deployment. Moreover, the addition of fibrillary materials to hydrogels has been shown to improve their mechanical properties, thus generating stronger tissue-like constructs [30,69,70].

Direct encapsulation of spheroids in polymeric cages [71], or incorporation of fibrillary matrix fragments into spheroids [72] are other examples of hybrid biofabrication, with the potential to improve spheroid-based tissue engineering. For example, polycaprolactone was prepared as a fibrillary mesh by electrospinning, then fragmented by limited hydrolysis. The resulting material was added to mesenchymal stromal cells during centrifugation-assisted spheroid preparation, becoming uniformly distributed within their volume. Consequently, the resulting spheroids were less compact, and thus better aerated and nurtured, as compared to their scaffold-free counter-parts, which positively impacted on cell survival and osteogenic differentiation [72].

4. Post-printing Considerations

One of the big questions for all modes of biofabrication is what happens after the initial assembly of cells and spheroids, namely how will the cells captured in scaffolds survive, differentiate and proliferate? And how will spheroids hold together: Will there be sufficient intrinsic ECM produced to generate tissue-specific and tissue-preserving connective tissue? In fact, the post-printing ECM formation and remodeling is one of the biggest current challenges in biofabrication. ECM components are made by quite a variety of cells, but the immobilization and their firm deposition around the cells is a challenge in standard aqueous culture conditions. One way to enhance ECM accumulation is the application of macromolecular crowding (MMC). Of relevance to this discussion is the use of polydisperse additives, some akin to “particulate” hydrogels, known MMC agents [73].

A special class of polymers with hydrogel-like properties, consisting of smaller molecular weight compounds than those commonly used for tissue engineering and bioprinting (e.g. Ficol 400), are increasingly used to bring the molecular concentration of the extracellular milieu to values close to those encountered in natural tissues [74]. These molecules produce the biophysical effect of MMC and are referred to simply as “crowders.” By size, charge, and hydration shell they exclude volume and collectively generate a fractional volume occupancy [75]. This has important consequences on the codissolved materials. The effects of MMC are mediated through an increase in the chance of direct interaction between the active molecules, thus accelerating the speed of their aggregation, or increasing their stability or the rate of their processing by codissolved enzymes. Collectively, these effects lead to a denser, more structured ECM, a phenomenon studied mostly in terms of the properties of collagen I microfibrils [75-77], but also of other types of collagen, as well as laminin, fibronectin, etc. [78]. Addition of MMC to the medium used for spheroids preparation enhances matrix deposited and thus may facilitate their aggregation, shape,

and stability [79]. This concept has been recently applied to bioprinting, as a means to manipulate the porosity within multilayered collagen-based hydrogels, by altering the collagen fibrillogenesis process through controlling the number of macromolecule-based bioink droplets printed on each collagen layer [80].

In summary, “hybrid biofabrication” is the cross-pollination between approaches, methods, and materials already common in this field as elsewhere in technology, which could be further expanded and exploited as a proven method to programmatically increase the “vitality” (i.e., applicability and value generation) of the resulting products.

Following the basic structure of tissues which contain cells, extracellular matrix, and fluids, the composition of biofabricated tissue analogs may vary from biomaterials-only, to cell suspensions, or cells-only constructs, with numerous intermediaries or “hybrid” situations (e.g., spheroids in hydrogels or fibers in spheroids as illustrated here). Correspondingly, these constructs can be used in a spectrum of applications ranging from inorganic biocompatible prostheses to live cell-based implants. Macromolecular crowding agents can be also added to cell preparations to bring their environment closer to the natural density. The methods of bioprinting may also come in these two versions, namely biomaterial-based or cell-based (“scaffold-free”).

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Revealing emerging science and technology research for dentistry applications of 3D bioprinting

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Abstract: Science and technology (S&T) on three-dimensional (3D) bioprinting is growing at an increasingly accelerated pace; one major challenge represents how to develop new solutions for frequent oral diseases such as periodontal problems and loss of alveolar bone. 3D bioprinting is expected to revolutionize the health industry in the upcoming years. In dentistry, this technology can become a significant contributor. This study applies a Competitive Technology Intelligence methodology to uncover the main S&T drivers in this domain. Looking at a 6-year period from 2012 to 2018 an analysis of scientific and technology production was made. Three principal S&T drivers were identified: Scaffolds development, analysis of natural and synthetic materials, and the study of scaffold characteristics. Innovative hybrid and multiphasic scaffolds are being developed to regenerate periodontal tissue and alveolar bone by combining them with stem cells from the pulp or periodontal ligament. To improve scaffolds performance, biodegradable synthetic polymers are often used in combination with bioceramics. The characteristics of scaffolds such as fiber orientation, porosity, and geometry, were also investigated. This research contributes to people interested in bringing innovative solutions to the health industry, particularly by applying state-of-the-art technologies such as 3D bioprinting, in this case for dental tissues and dental bone diseases.

Keywords: Competitive technology intelligence; three-dimensional bioprinting; dentistry; dental; Science and technology trends.

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1. Background

Over the past few years, three-dimensional (3D) printing has attracted the attention of the science and technology (S&T) community. Since Chuck Hull's invention of stereolithography, which was patented in 1984, new technologies and applications in a variety of industries have been emerging; and since the early 2000s, their applications have sky-rocketed. Although conventional use of 3D printing technology is becoming increasingly accessible to people, applications in the health industry are still in a nascent stage, particularly those that involve bioprocesses to prevent, maintain, or heal body tissue.

3D bioprinting technology opens a large innovative window, giving the potential to bring in new solutions

that could have a major impact in many fields including complex organs study, ophthalmology, drug delivery, and so forth. In the realm of dentistry, one of the most frequent illness concerns is periodontal diseases and the loss of alveolar bone. In this context, the current research applies a Competitive Technology Intelligence methodology to uncover principal S&T trends.

1.1 Competitive Technology Intelligence

Competitive intelligence is based on the systematic and ethical process of gathering, analyzing and transforming information into actionable knowledge in the context of the competitive environment of an organization^[1]; it contributes to making decisions in organizations of all sizes and across different disciplines. Specifically,

Competitive Technology Intelligence involves the analysis of S&T environment. Moreover, it has been applied for years in different industries; however, in the field of 3D printing and, moreover, for bio-applications, the studies are still scarce. In this sense, Rodríguez-Salvador *et al.* (2017)^[1] combined competitive technology intelligence with scientometrics tools to analyze scientific and patent literature from 2000 to mid-2016, determining the knowledge landscape on 3D bioprinting. On the other hand, Trappey *et al.*^[2] evaluated the development of 3D printing technology for biomedical applications through a US patent analysis where a search time frame was set from 1980 to August 2014. While both studies analyze 3D printing from a general perspective, this research pursues to fill the gap associated to the lack of competitive technology studies on specific applications of 3D bioprinting, in this case by analyzing its innovative presence on dentistry.

1.2 3D Bioprinting

3D printing is a revolutionary technology that is also known as additive manufacturing. The American Society for Testing and Materials (ASTM International) define additive manufacturing as a group of techniques which apply the additive shaping principle and thereby build physical 3D geometries by successive addition of material. Hence, it is important to clarify that according to the ASTM the terminology of 3D printing can also be used as synonymous of additive manufacturing in a non-technical context (Cerneels, 2015; ISO/ASTM 52900, 2015). The application of this technology is growing because it offers unique characteristics, such as customization, the production of complex geometries and waste reduction. The principal processes of 3D printing involve the following: Fused deposition modeling^[3], selective laser sintering^[4], electron beam melting^[5], inkjet 3D printing^[6], extrusion 3D printing^[7], and laser-assisted printing^[8]. As a diverse number of industries, ranging from automotive and aerospace to health, have a growing interest in the development and implementation of this technology; knowing scientific and technology trends will play a fundamental role to identify and manage opportunities to innovate.

In recent years, the use of these technologies for medical applications has increased^[9], and as a result, the term 3D bioprinting has born as a specialized class of 3D printing. 3D bioprinting is a layer by layer precise positioning of biological materials and living cells^[10]. Some applications of 3D bioprinting include stem cell research^[11], cancer model^[12], drug testing^[13], and tissue engineering^[14]. In tissue engineering, for manufacturing scaffolds, these technologies are able to control pore size, shape, distribution, and interconnectivity of pores. In addition to this, combined with the ability CAD and

3D medical imaging such as computed tomography, 3D printing permits the fabrication of personalized constructs (patient-specific)^[15].

3D bioprinting was first introduced by Thomas Boland in 2003; he patented the use of inkjet 3D printing for cells^[16]. According to a recent publication by the founder of one of the biggest 3D bioprinting centers, Antony Atala, 3D bioprinting is based on three central approaches: Biomimicry, autonomous-self-assembly, and mini-tissue building blocks^[10]. Biomimicry consists of the creation of exact replicas of the cellular and extracellular parts of a tissue and organ^[17]. Self-assembly is the scaffold-free method that mimics the behavior of embryonic stem cells. Finally, mini-tissues can be defined as the smallest structural and functional component of a tissue^[18]. In the review of the field by Murphy and Atala, they suggest that the combination of these three mentioned approaches are needed in order to print complex 3D biological structures with multiple functionality, structure, and mechanical properties^[10].

The main technologies used for 3D bioprinting living and biological materials are inkjet, laser-assisted printing, and micro-extrusion. Different specifications and features of them have to be contemplated based on the most important factors that affect bioprinting which are a resolution, cell viability, and the materials used for printing. Inkjet 3D printing is a non-contact (nozzle away from the substrate) printing technology where 2D and 3D structures are generated using picoliter ink droplets jetted onto a substrate following a digital pattern^[19]. The usual amount of material dispensed is between 1 and 100 picoliters allowing very high resolution. All drops are spherical in flight and identical to their neighbors^[20]. Several mechanisms can be used to generate the bioink droplets, the most frequently used for cells are thermal and piezoelectric. In the thermal method, a heat generator increases the temperature up to 300°C within the chamber. Then, the heating produces a bubble which expels the droplet^[21]. With the piezo-electric method, a direct mechanical pulse is applied to the bioink which results in the ejection of the droplet^[20].

A standard laser assisted bioprinting (LAB) set-up is usually composed of three elements: A pulsed laser source, a target coated with the material to be printed (the ribbon) and a receiving substrate. Depending on the bioink optical absorption and the laser wavelength, a laser absorbing interlayer may be necessary to induce transfer and is placed between the support and the bioink^[8]. LAB functions using focused laser pulses on the absorbing layer of the ribbon to generate a high-pressure bubble that propels cell-containing materials toward the collector substrate. This technology allows for the precise deposition of materials and high densities of cells in relatively small 3D structures without affecting cell viability^[22].

Extrusion 3D printing is a contact dispensing system (nozzle in contact with the substrate) where continuous strands of material are forced through a micro-nozzle from a movable head onto a platform. The print head can move in three axes xyz ^[23]. This printing method can be used for creating scaffolds with defined architectures from biocompatible materials and cell-laden hydrogels. Different extrusion systems have been used for 3D printing such as pneumatic pressure, piston, and screw driven. For the pneumatic pressure and piston systems, the material is usually loaded into a syringe and dispensed with the respective methods^[24]. The screw-driven method has a separate reservoir with or without temperature control. This method is usually used for highly viscous materials. The material is transported from the reservoir to the printhead by pressure then the screw assists the deposition process^[25].

During the bioprinting process, biocompatible materials (bioinks) are used to facilitate the printing and act as matrices for printed cells^[26]. The bioink should act as a cell carrier during the printing process and allow the cells to grow and secrete their own emulate the extracellular matrix (ECM) post-printing. The bioinks can be natural, synthetic materials or combinations of both. Polymeric hydrogels, highly hydrated 3D polymeric networks, are one of the most viable classes of bioinks due to their structural similarities to natural tissue and can offer a synthetic surrogate of ECM^[27]. Hydrogels can facilitate matrix remodeling, cell migration, and cell-cell interactions necessary for the normal development of functional tissue^[28]. The bioink properties that need to be considered for deciding if it is suitable for printing to include viscosity, shear-thinning, viscoelasticity, cytocompatibility, gelation kinetics, and biodegradation. These properties will determine the fidelity, stability, and functionality of the final cell-laden construct^[29]. Important efforts of S&T are being devoted to the development of methods that enable printing and to the production of bioinks and biomaterial inks that can produce scaffolds that can mimic the functions of the human body^[30].

1.2.1 Importance of Scaffolds in Dental Applications

Scaffolds play a key role in obtaining functional tissues. They are designed to ECM by providing structural support that stimulates attachment, proliferation, and differentiation^[31]. Dentistry applications of scaffolds are recently being investigated with the aim of enhancing the regeneration of tissue and alveolar bone.

Recently, Sharma *et al.*^[32] identified a variety of biomaterials that can be used to develop either natural or synthetic scaffolds. As they indicate, natural scaffolds offer good cellular compatibility but have some disadvantages, such as limited range of mechanical properties and a lack of control over pore size. In addition, they specify that

synthetic biomaterials are cheaper and can be customized regarding their shape, mechanical and chemical properties (strength, pore characteristics, and degradation rate) for specific applications. Nevertheless, they require chemical modifications to improve cell adhesion. Given this, it is necessary to combine different types of materials to meet the tissue requirements in terms of porosity, surface area, and mechanical strength. Asa'ad *et al.*^[33] established biodegradable synthetic polymers that hold potential in bone tissue engineering applications due to their low cost and ability to be produced in large quantities with a long shelf life, especially when compared with natural biomaterials.

2. Methodology

The methodology applied in the current study was adapted from the competitive technology intelligence approach of Rodríguez-Salvador *et al.*^[1], and it consists of a hybrid model that combines a virtuous knowledge cycle with expert feedback, comprising of the following:

- **Planning process:** The main goals, activities, and participants are stated. In this research, the principal purpose was to identify S&T trends in 3D bioprinting for dental tissue and bone applications.
- **Selection of primary and secondary information sources:** Primary information mainly consists of expert participation. In this case, distinguished dentists, periodontists, and experts in 3D printing and 3D bioprinting were consulted. They kindly asked to remain anonymous. Secondary sources encompass explicit and documented knowledge through papers, patents, reports, and websites. In this case, Web of Science, Scopus, EBSCO Health and Science Direct were analyzed.
- **Information collection:** To gather information from databases, it is important to establish a proper search strategy, including the terminology that defines the field under analysis and the query for information retrieval. The terms that define 3D bioprinting and dental domains were identified from a literature review and the experts consulted. Different queries were designed and tested according to each database consulted. Boolean operators and inclusion and exclusion terms were used for this task. The time frame for gathering the information was a 6-year period from 2012 to 2018 (when this research concluded).
- **Analysis:** It consists of the transformation of information into intelligence. In this case, a statistical analysis combined with manual examination was developed. Given the novelty of this domain, and after a cleaning a deduplication process, only a few documents were identified, coming out to <100.

Experts participated throughout the entire process to validate collection up to the final analysis.

3. 3D Bioprinting Global Trends for Dental Applications

The main findings from the current research show three principal S&T drivers for knowledge management (Table 1).

Specific research efforts were identified for each of these trends. The following tables (Tables 2-4) correspond

to the most recent and representative studies from the documents analysed for each global trend:

4. Discussion and Conclusions

To face current global changes, it is important to keep abreast of breakthrough technologies including detecting

Table 1. 3D bioprinting global trends for dental applications

S&T driver	Description
Scaffolds development	Creation of hybrid and biphasic scaffolds to regenerate periodontal tissue and alveolar bone
Analysis of natural and synthetic materials	Emphasis on biodegradable synthetic polymers such as PCL, PLA, PGA, and PLGA that are combined with bioceramics such as HA
Study of the scaffold functional characteristics	Assessment and improvement of properties such as fiber orientation, porosity, and geometry

S&T: Science and technology, PCL: Polycaprolactone, PLA: Polylactic acid, PGA: Polyglycolic acid, PLGA: Polylactide-co-glycolic acid, HA: Hydroxyapatite

Table 2. Global trend: Scaffolds development

Article	Institution/country	Description
Rasperini <i>et al.</i> ^[34] “3D-printed bioresorbable scaffold for periodontal repair”	University of Milan Italy University of Michigan USA Dankook University South Korea	The first reported human case of treating a large periodontal osseous defect with a 3D-printed bioresorbable patient-specific polymer scaffold and signaling growth factor
Costa <i>et al.</i> ^[35] “advanced tissue engineering scaffold design for regeneration of the complex hierarchical periodontal structure”	University of Minho headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine Portugal Queensland University of Technology Australia Sichuan University China Griffith University Australia	Construction of biphasic scaffolds by attaching a fused deposition-modeled bone compartment to a melt electrospun periodontal compartment. The main purpose is to simultaneously regenerate alveolar bone, periodontal ligament, and cementum
Lee <i>et al.</i> ^[36] “3D printed multiphase scaffolds for regeneration of periodontium complex”	Columbia University Medical Center US	Development of multiphase region-specific micro scaffolds with spatiotemporal delivery of bioactive cues for integrated periodontium regeneration. It is demonstrated that by seeding these scaffolds with DPSCs, PDLSCs, or ABSCs, distinctive tissue phenotypes can be formed with collagen I-rich fibers especially by PDLSCs and mineralized tissues.

3D: Three-dimensional, PDLSCs: Periodontal ligament stem cells, DPSCs: Dental pulp stem/progenitor cells, ABSCs: Alveolar bone stem/progenitor cells

Table 3. Global trend: Analysis of natural and synthetic materials

Article	Institution/Country	Description
Asa'ad <i>et al.</i> ^[33] “3D-printed scaffolds and biomaterials: Review of alveolar bone augmentation and periodontal regeneration applications”	University of Milan Italy University of Michigan USA	PCL is the most used biomaterial for periodontal applications due to its biocompatibility, suitability for various scaffold fabrication techniques, remarkably slow degradation rate and mechanical stability. It might enhance the maintenance of produced bone volume and the bone contour over time. Similar to PCL, PLA, and PLGA are hydrophobic while PGA is hydrophilic. They are usually combined with bioceramics such as calcium phosphates for alveolar bone regeneration. The predominant calcium phosphate ceramic in BTE is HA because it has the same chemical composition as native bone minerals
Ma <i>et al.</i> ^[37] “bioprinted microarray for screening the response of periodontal ligament stem cells response to GelMA/PEG hydrogels”	Xi'an Jiaotong University China	PDLSCs have been found to promote formation of new bone, cementum and functional periodontal ligament in diseased periodontium when properly stimulated. A high throughput method for testing the response of PDLSCs to the different gradient of biomaterials was developed. This method exhibits that bioprinting can be utilized as a tool to screen cell-biomaterial interactions in a more efficient way.
Sharma <i>et al.</i> ^[32] “biomaterials in tooth tissue Engineering: A review”	ESIC Dental College and Hospital India	The biomaterials for tooth regeneration are categorized as natural or synthetic. Natural biomaterials are proteins such as collagen, fibrin, and silk and polysaccharides such as chitosan, hyaluronic acid, alginate, and agarose. Synthetic biomaterials can be organic like organic polymers such as PLA, PGA, PLGA, and PCL. Moreover, they can also be inorganic, as with calcium phosphate materials such as HA or β TCP and compositions of silicate and phosphate glasses. PLA, PGA, PLGA, and PCL are the few polymers that are commonly used for forming porous scaffolds. Synthetic polymers are the most frequent materials employed for teeth regeneration

BTE: Bone tissue engineering, PCL: Polycaprolactone, PLA: Polylactic acid, PGA: Polyglycolic acid, PLGA: Polylactide-co-glycolic acid, HA: Hydroxyapatite, 3D: Three-dimensional, PDLSCs: Periodontal ligament stem cells, β TCP: Beta-tricalcium phosphate

Table 4. Global trend: The study of scaffolds' functional characteristics

Article	Institution/Country	Description
Kim <i>et al.</i> (2018) ^[38] “ <i>In vivo</i> evaluation of 3D Printed PCL scaffold combined with β -TCP for alveolar bone augmentation.”	Korea Institute of Machinery and Materials Korea Seoul National University Bundang Hospital Seoul	<i>In vivo</i> studies are primordial for studying the performance of scaffolds inside the body. In this study, 3D printed PCL scaffolds are implanted to evaluate the effect in bone augmentation of two different lattice designs and the addition of β -TCP
Do <i>et al.</i> (2015) ^[31] “3D Printing of Scaffolds for Tissue Regeneration Applications”	University of Iowa USA	3D printing can mimic the ECM by producing scaffolds with a high degree of complexity, where fine details can be included at a micro level. The criteria for printing viable and functional scaffolds, scaffolding materials and 3D printing technologies are assessed. Scaffolds should mimic ECM characteristics in terms of biological activity, mechanical strength, processability, and controllable degradation rates. Moreover, it is important to determine the inflammatory effect of the biomaterial(s) used and the scaffold structure designed to produce the desired tissue. Porosity, layer configuration, mechanical properties, and morphology are also characteristics to consider.
Bencherif <i>et al.</i> ^[39] “advances in the design of macroporous polymer scaffolds for potential applications in dentistry”	Harvard University USA École Polytechnique Fédérale de Lausanne Switzerland	Pore size and porosity are crucial when designing scaffolds in the tissue engineering domain as they influence tissue production and function. This includes cell distribution, interconnection throughout engineered tissues, and diffusion of nutrients and oxygen, specifically in the absence of a functional vascular system. 3D nano-fibrous gelatine/silica bioactive glass hybrid scaffolds that mimic the nanostructured architecture and chemical composition of a dental ECM are applied to improve odontogenic differentiation and biomineralization of human dental pulp stem cells

ECM: Extracellular matrix, PCL: Polycaprolactone, PLA: Polylactic acid, β TCP: Beta-tricalcium phosphate, 3D: Three-dimensional

new applications. 3D printing is an emerging field that has gained the attention of the academic community and industries such as automotive, aerospace, and more recently health. Although the initial efforts of 3D printing were focused on prototyping, new applications are being investigated, specifically those that deal with the human body, where there are challenges extremely complex. Oral diseases and tooth loss represent one of the most prevalent health problems. Overcoming the drawbacks that conventional procedures have, 3D bioprinting brings new solutions that could help restore and regenerate tissue and alveolar bone. In this research, a Competitive Technology Intelligence methodology was applied; insights revealed that recent S&T efforts in 3D bioprinting in dentistry are focused on developing scaffolds, the analysis of natural and synthetic biomaterials needed for their creation and the improvement of their characteristics. In addition, it was also found that a large part of the research in the field involves the assessment of the interaction and behavior of the cellular component with the materials and scaffolds microstructure.

Most of the studies agreed that controlling the biophysical properties and microstructure of the scaffolds is necessary to reproduce the periodontium complex which is formed by soft (periodontal ligament) and hard tissues (alveolar bone and cementum). As well it was determined that bioceramics such as β -TCP and thermoplastics such as polycaprolactone are the preferred type of biomaterial ink for bone, enamel, and cementum regeneration. In addition, it was found that the most used cell type for these applications is dental pulp stem cells and periodontal ligament stem cells due to their ability to differentiate into the different lineages of the periodontium

complex. Finally, it was also determined that biphasic and multiphasic structures are able to mimic closely the microenvironment of cells of the periodontium complex and promote regeneration of the different tissues.

The current research adds value to the understanding of the emerging incursion of 3D bioprinting on dental applications. Moreover, the insights obtained can contribute to those who are involved in R&D and who are interested in finding opportunities to innovate through radical technologies such as 3D bioprinting.

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Designs and applications of electrohydrodynamic 3D printing

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Abstract: This paper mainly reviews the designs of electrohydrodynamic (EHD) inkjet printing machine and related applications. The review introduces the features of EHD printing and its possible research directions. Significant progress has been identified in research and development of EHD high-resolution printing as a direct additive manufacturing method, and more effort will be driven to this direction soon. An introduction is given about current trend of additive manufacturing and advantages of EHD inkjet printing. Designs of EHD printing platform and applications of different technologies are discussed. Currently, EHD jet printing is in its infancy stage with several inherent problems to be overcome, such as low yielding rate and limitation of stand-off height. Some potential modifications are proposed to improve printing performance. EHD high-resolution printing has already been applied to precision components for electronics and biotechnology applications. This paper gives a review about the latest research regarding EHD used for high-resolution inkjet printing. A starting base is given to help researchers and students to get a quick overview on the recent development of EHD printing technology.

Keywords: Electrohydrodynamic printing; inkjet printing devices; additive manufacturing

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1. Introduction

With the rapid development of modern microelectronic industry and biotechnology, traditional manufacturing methods cannot satisfy the developing needs of these industries due to inherent restrictions. Additive manufacturing, based on localized deposition of material and a layer-by-layer printing process, is able to build customized products in a short time frame and offers significant advantages over traditional manufacturing processes in the area of design freedom and reduction of assembly time and cost. Electrohydrodynamic (EHD) inkjet, also called “e-jet,” printing as a mask-less, non-contact, direct-write, and additive manufacturing process has attracted the remarkable attention. EHD inkjet printing is first proposed as a solution to the limited resolution of the conventional inkjet printer system since EHD printing can produce a thin and intact jet without the need to miniaturize the nozzle. The process involved,

called “electrospray in the cone-jet mode,” uses electrical (“Maxwell”) forces to *pull* the liquids from the nozzle tip, rather than apply thermal or acoustic energy to *push* liquid from a fine capillary. In the liquid cone, the electric field causes electric charges to accumulate at the liquid surface. The electrical forces counteract surface tension, and the resultant force leads the meniscus at the nozzle end to change from a hemispherical to a conical shape. With the increase of electric field, the electric stress at the tip of cone exceeds the surface tension and a droplet or jet of liquid is emitted toward ground substrate. The EHD inkjet printing can be a high-resolution inkjet printing technology because there is a large “neck-down ratio” between the diameters of the nozzle and the jet: The jet diameter is about two orders of magnitude smaller than the nozzle diameter. Thus, in EHD jet printing, the nozzle diameter ($>100\ \mu\text{m}$) can be much larger than that used in thermal or piezoelectric inkjet printing (about $20\ \mu\text{m}$); this makes blockages much less likely and makes it easy

to employ a highly viscous liquid^[1]. It is used in the field of micro- or nano-manufacturing for patterning of a large class of materials on a variety of substrates without adversely affecting the chemical properties of the deposited materials. This paper is organized as follows. We describe different types of EHD printing machines designed on the basis of fundamental EHD mechanisms. The ensuing section discusses about the application of EHD inkjet printing in different fields of research.

2. EHD Inkjet Printing Machines and Control

An EHD inkjet printing system usually consists of a fluid supply system, linear/rotary motorized or manual positioners, a high voltage power supply, and visualizing and imaging devices^[2]. The fluid supply system delivers liquid ink through a pipeline to the tip of the nozzle at the desired flow rate. The supply system can be a syringe or vacuum pump^[3] or a liquid reservoir with a constant liquid height^[4,5]. The dispensing nozzle (usually made from stainless steel) is compatible with interchangeable syringe and can be purchased from commercial companies. The tiny size of nozzle (smaller than 50 μm) can be fabricated by pulling glass pipettes or borosilicate capillaries and the tip of nozzle is coated by a thin layer of metal. To prevent wetting of the ink and obtain a large contacting angle at the nozzle outlet, metal nozzles are usually processed by chemical agents. The nozzle is grounded while a high negative potential is applied to the substrate and this prevents charge accumulation and potential shock hazards during the operation of the pumping system^[6]. The gap between the nozzle tip and the substrate, known as standoff height, is critical for stable printing. Thus, it is necessary to use a high precision motorized stage to adjust standoff height. A high DC voltage power source is employed to produce a strong electric field to deform the liquid meniscus, and a function or pulse generator is used to facilitate drop-on-demand printing. To supply voltage and ink material to multiple nozzles separately, a voltage distributor and a multichannel syringe pump are necessary^[7]. Since the decay rate of residual charges on

droplets is slow on the insulating substrates, the residual charge of droplets deposited onto a substrate will change the electrostatic field distribution and may interrupt the subsequent printing behavior^[8]. Park *et al.* used a sinusoidal ac voltage to switch the charge polarity of droplets^[8] and charges in the subsequent droplet to neutralize charges in the previous droplet. A visualization system includes a high-speed charge-coupled device camera, a microscopic zoom lens system, and an illumination source. Since the timescale for the most relevant EHD phenomena is very short (less than a microsecond), a conventional continuous-illumination source will cause blurring images by overexposure of jet motion. Thus, a high-speed shutter system or a short duration flash with an open shutter is suitable for high-speed events. The external disturbances, such as vibration coming from the building, may be damped by vibrational isolation platform. These attributes often lead to demands for careful control and optimization for each ink material. Table 1 summarizes the optional devices for each subsystem of EHD print.

Park demonstrated that a nozzle with a diameter of 50 μm can create dot diameter not $<20 \mu\text{m}$ in graphic arts. In addition, they developed an EHD inkjet printing system as shown in Figure 1a and a droplet with submicrometer size was able to be produced by a microcapillary with a diameter from 0.3 to 30 μm ^[9]. The resulting high resolution is partially attributed to a fine nozzle with sharp tips. Another reason is that small distance between nozzle and substrate shown in Figure 1b can minimize lateral variations in the placement of droplets. Furthermore, breakup occurring in electrospray can be avoided at such short distance^[5], and a sharp corner is also benefited from a small gap. Fluid is propelled through a glass capillary by a syringe pump. A high-speed camera with 66,000 frames per second was used to observe the dynamics of EHD jetting, and both pulsating and stable jet modes are captured by imaging devices. Park *et al.* used a solution of a conducting polymer ink to electrohydrodynamically print high-resolution patterns with 10 μm droplet diameter^[9], as shown in Figure 1c. Figure 1d shows EHD printing results of silicon nanoparticles and single crystal Si rods dispersed in 1-octanol^[9].

Table 1. Functions of each working device in different EHD printing platforms

Names of EHD printing subsystems	Optional devices	Function
Fluid supply	Syringe pump/vacuum pump/liquid reservoir, pipeline and connector	Delivering ink to the tip of printing nozzle
Positioner	Linear/rotary motorized positioner/manual positioner	Transmitting nozzle to printing position accurately; adjusting distance between two electrodes
Power supply	High voltage DC/AC power supply (up to 10 kV)	Providing strong electric field to produce stable cone-jet
Visualizing and imaging device	High-speed CCD camera, Microscopic zoom lens, LED light	Observing deformation of cone and high-speed emission of droplet
Vibration isolation platform	Mechanical vibration isolation platform/optical tables	Preventing vibration from effecting printing results

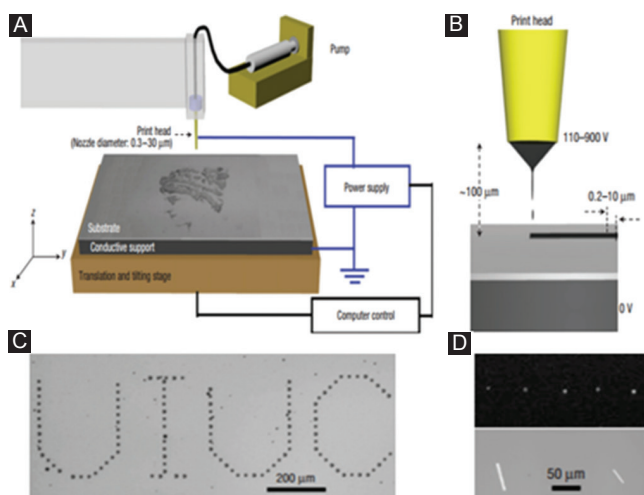


Figure 1. (A) A layout of e-jet printing system. (B) Configuration of the nozzle electrode and substrate electrode. A small diameter of gold-coated nozzle with sharp tip, and a short distance between two electrodes. (C) The average dot diameter of letters printed with the conducting polymer is about 10 μm . (D) The average diameter of Si nanoparticles printed from a suspension in 1-octanol is 3 nm (Adapted from Park *et al.*^[9]).

2.1. Multiple Printing Heads EHD Printing System

Barton built up a desktop EHD inkjet printing system of advantages including compactness, high resolution, and low cost (<50 k USD). Most components of this desktop printer were purchased from vendors. A 5- μm nozzle tip is employed to produce average 2.8 μm diameter droplets; slight discrepancies in droplet placement were observed, caused by natural frequency of the pulsating cone-jet model^[10]. Figure 2A shows the overall setup. The flow rate of liquid ink is controlled by a pneumatic pressure regulator, and the value of pressure can be read out from pressure gauge. Similarly, there are X- and Y-axis electronic positioning stages, as well as a manual z-axis and a manual rotary axis included in the moving system. The substrate sits on the substrate mount, and they are held in place by a vacuum chuck. To print different inks, a multiple nozzle holder was designed as shown in Figure 2B and it consists of several individual nozzle holders and a manual rotation mount^[10]. The LabVIEW software is used to read encoder position, monitor voltage output repeatedly, and control as well as coordinate voltage and position commands.

Sutanto *et al.* designed a multiprint head to print heterogeneous materials, and a tilted rotary mechanism was designed to switch between the active and inactive printing nozzle, as shown in Figure 2C^[11]. A pressure distributor was used to control back pressure of each nozzle, and a circuit board is employed to assign voltage signal to active printing unit installed in the print head

holder. Since the weight of print heads and their distance from the axis of rotation produce a large rotational inertia, a transmission with a high gear ratio is required to couple with the motor to provide a sufficient large torque^[11]. However, the stick-slip resistance coming from the gear reduces the accuracy of overlay in drop-on-demand. To offset this undesired effect, a two-step macro/micro-positioning method is employed to achieve micropositioning accuracy. The function of the macropositioning algorithm is to bring the nozzle into the camera view field, and duty of the micropositioning controller is to apply precise servo level control to locate nozzle position with respect to the substrate^[11].

A low flow rate is favorable to the generation of a small jet since the diameter of charged liquid jet depends on flow rate. A multiplexed EHD printing head that can deposit multiple jets simultaneously has been investigated to obtain a high yielding rate for high-resolution printing. In the configuration of the linear array of multinozzles, the meniscus and jets at the end of nozzle are often deflected due to asymmetric electric field and repulsive forces between adjacent jets^[12]. Figure 2D shows a jetting angle between the axis of the nozzle and the tip of the cone^[12]. Tran compared two methods to alleviate undesired end effects^[13]. In the first method, a non-conductive nozzle made of polymethyl methacrylate was fabricated, and in the second method, two dummy capillary nozzles located at both sides of the array were used. Both methods can dramatically minimize end effects, supported both by simulation of electric field and by experiments. Lee used MEMS method to fabricate three single nozzles in a base that is made of silicon and applied a conductive material on the surface of silicon base at the side of nozzle outlet^[14]. The asymmetric cones are observed because of end effect in the pin-shaped nozzle array. However, the end effect is not detected in the silicon-based multinozzle structure. Si *et al.*^[15] used two dummy nozzles at the left- and right-most side in an array of five capillaries to reduce end effects. Figure 2E shows that two nozzles next to middle nozzle still experienced asymmetric emission, and this is caused by the effect of the meniscus tip. Khan *et al.* used a numerical method to investigate the magnitude of the electric field strength around the tip of multinozzles to optimize the distance between nozzles^[16]. Axisymmetric cone-jets were formed at the tip of each nozzle in Figure 2F. As multiple pin-shaped nozzles are arrayed, the onset voltage is changed with the distance of operating nozzles due to interference and distortion in the electric field^[17].

Choi *et al.* used three glass nozzles with independent voltage connection and ink supply source to explore that interaction (cross-talking) between the charged neighboring jets in the electric field^[12]. As a result, the interaction of electric fields among multiple nozzles is

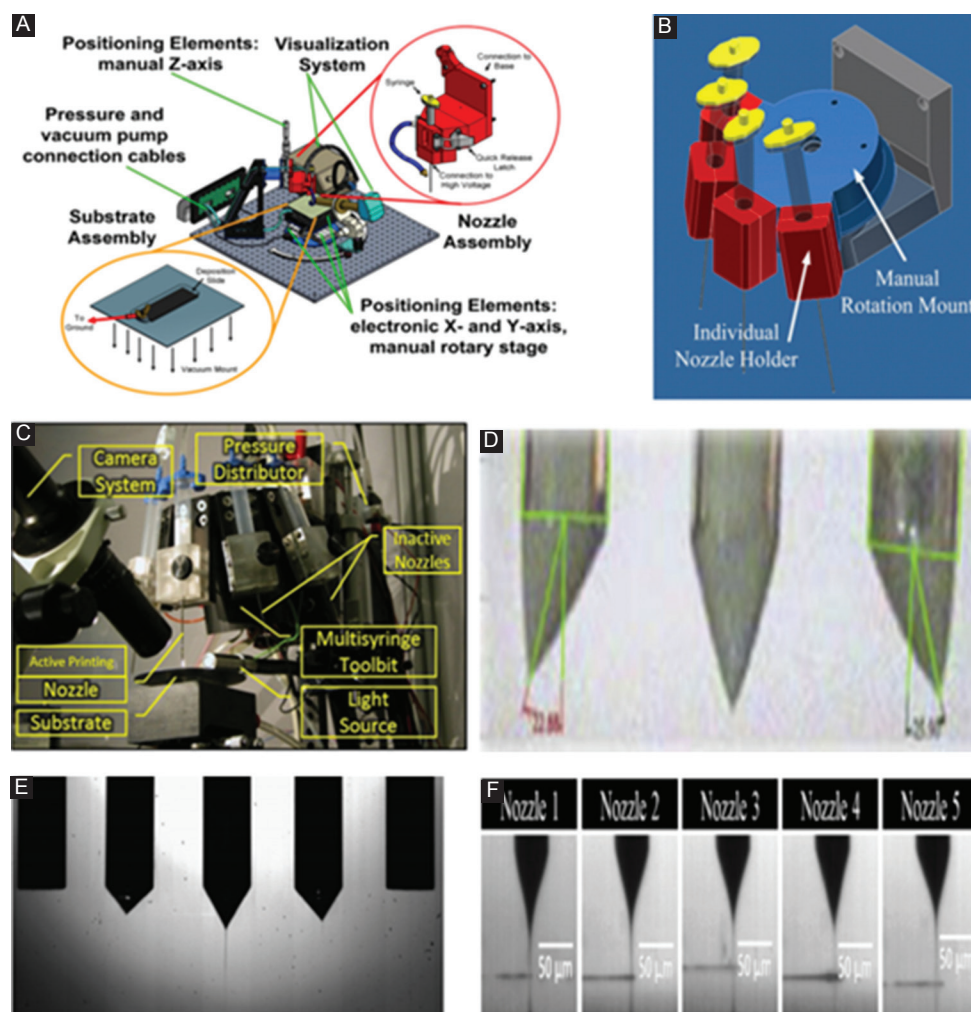


Figure 2. (A) Layout of desktop e-jet system and related hardware. (B) A multinozzle holder (from^[10]). (C) An EHD printing system with multiprinting head (adapted from Sutanto *et al.*^[11]). (D) The jetting angle in linear array multinozzle EHD inkjet printing head (Adapted from Choi *et al.*^[12]). (E) End effects in an array of multiple emitters (adapted from Tran *et al.*^[15]). (F) Axisymmetric cone-jets formed at apex of each nozzle (adapted from Khan *et al.*^[7]).

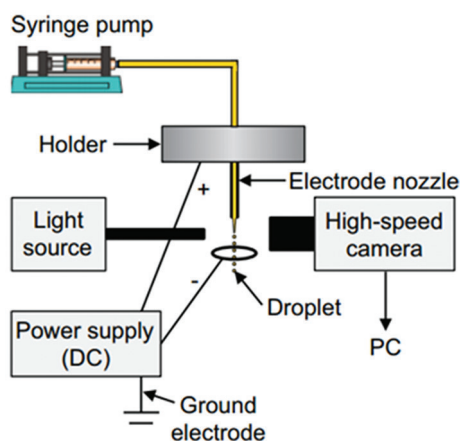


Figure 3. Schematic of nozzle-ring electrode configuration (adapted from Zhang *et al.*^[18]).

reduced by taking a triangular array of nozzles instead of a traditional linear array of nozzles^[12]. The end effect between the neighboring jets reduces with a decrease of nozzle diameter. However, the smaller diameter of the nozzle may cause a risk of nozzle blockage^[7].

2.2. Nozzle-ring Electrode Configuration

A nozzle-ring electrode configuration is designed to integrate the nozzle and ground electrode to eliminate the disturbance due to the surface irregularity of the substrate, to enable printing on a non-conductive surface, and to overcome restriction of standoff distance. As shown in Figure 3, A single nozzle-ring electrode was employed to investigate the formation of organic solvent jet^[18]. Kim compared onset voltage of two types of electrodes (plane- and hole-type) by adjusting different processing

parameters, including diameter of glass capillary nozzle, hydrostatic back pressure head in the reservoir, and standoff height^[19]. When other processing parameters are maintained the same, onset jetting voltage for hole-type electrode is larger than that for plane-type electrode since the less surface area of electrode in the previous type. However, when the gap distance was larger than 200 μm , the onset jetting voltage was insensitive to the change of hole diameter^[19]. The assumption that onset radius of curvature is not affected by the whole distribution of electric field for the same nozzle seems reasonable from simulation results^[19]. Thus, value of onset electric field at the meniscus did not change with either diameter of hole or gap distance^[19].

Lee *et al.* used a ring-shaped gate electrode to suppress intact jet from generating satellite droplets or spraying^[20]. A maximal suppression was obtained as the inner diameter of ring electrode is 5 times bigger than the outer diameter of nozzle, and the gap distance between nozzle tip and gate electrode is $2\frac{1}{2}$ times as big as the outer diameter of nozzle^[20]. Tse developed a double layer electric field shaping print head to obtain smaller size of droplets compared to results from single layer field shaping print head^[21]. To maintain constant operating conditions, Barton *et al.* investigated a two degree of freedom control algorithm^[21]. They used an iterative learning control algorithm and a feedback controller to obtain feedforward control signal. The feedforward voltage signal is applied to compensate for repeatable changes, such as standoff height, and feedback component is employed for uncertainty in jetting operating conditions^[22]. Table 2 lists some optional solutions for problems that usually appear in EHD printing process.

4. Applications of EHD Inkjet Printing

EHD printing is developed as a cost-effective inkjet printing process with high resolution. With EHD printing, functional materials can be selectively and precisely printed on desired substrates. Applications of EHD in different industries have been summarized by

other authors^[23,24]. This review only introduces three-dimensional (3D) printing that we are interested, and applications of two-dimensional (2D) printing are reviewed for comparison reasons. Most EHD printing applications have been focused on 2D printing, and only a few research groups have explored 3D printing techniques that are restricted by several issues discussed in previous sections. The following sections review the representative results of both 2D and 3D printings.

3.1. Applications of EHD 2D Printing

3.1.1 Electronics Applications

EHD inkjet printing is an attractive fabrication method for microscale electronic devices since this method can obtain features with fine resolution and it is compatible with a series of functional inks. The rest of this section introduces several applications of EHD printing in fabrication of electronic components. As an example, Park *et al.* used EHD method to print photocurable polyurethane (PU) precursor as a resist layer for patterning metal electrodes by chemical etching^[9]. The printing results are shown in Figure 4A and B.

Metallo-organic compound solutions and inks formulated from metal nanoparticle suspension are two main kinds of ink for printing metallic patterns. The former ink is a true solution where the metal salt is fully dissolved in the solvent and this property decreases sediments and clogging issues arising from nanoparticles in the suspension^[25]. An uniform silver microtracks with a 35- μm line width was printed by Wang *et al.*, and its resistivity was measured to be within a range of $(2-4) \times 10^{-8} \Omega\text{m}$, which is 2.4 times of the theoretical value of bulk silver ($1.6 \times 10^{-8} \Omega\text{m}$)^[25,26]. As shown in Figure 4C, the edge of micro track is more porous compared to central part, since repulsive interaction between the charged droplets leads to an incompletely suppressed jet breakup. Sutanto *et al.* successfully fabricated a crossover intersecting structure using a multimaterial e-jet printing system to deposit conductive lines, which was made of an organic silver

Table 2. Some corresponding solutions for possible problems in EHD 3D printing

Existed problems	Alternative solutions
Reducing the size of droplet	For large conductivity ($\geq 10^{-4} \text{ S/m}$)
	Reducing flow rate, increasing voltage
	For small conductivity ($< 10^{-4} \text{ S/m}$)
	Reducing flow rate, minimizing diameter of nozzle outlet
Increasing precision of placement	Keeping a small distance between nozzle and substrate
Increasing yielding rate	Increasing numbers of nozzle
Alleviating end effects from neighboring nozzle	Inserting non-conductive/dummy nozzles; inserting metal nozzles into non-conductive material-based multinozzle structure; increasing distance between neighboring nozzle; changing the shape of nozzle array
Eliminating disturbance due to surface irregularity of the substrate, such as uneven surface, restriction of standoff distance, non-conductive material	Changing configuration of electrodes, such as nozzle-ring type; applying sinusoidal AC voltage and print droplet whose charge alternates between positive and negative charges

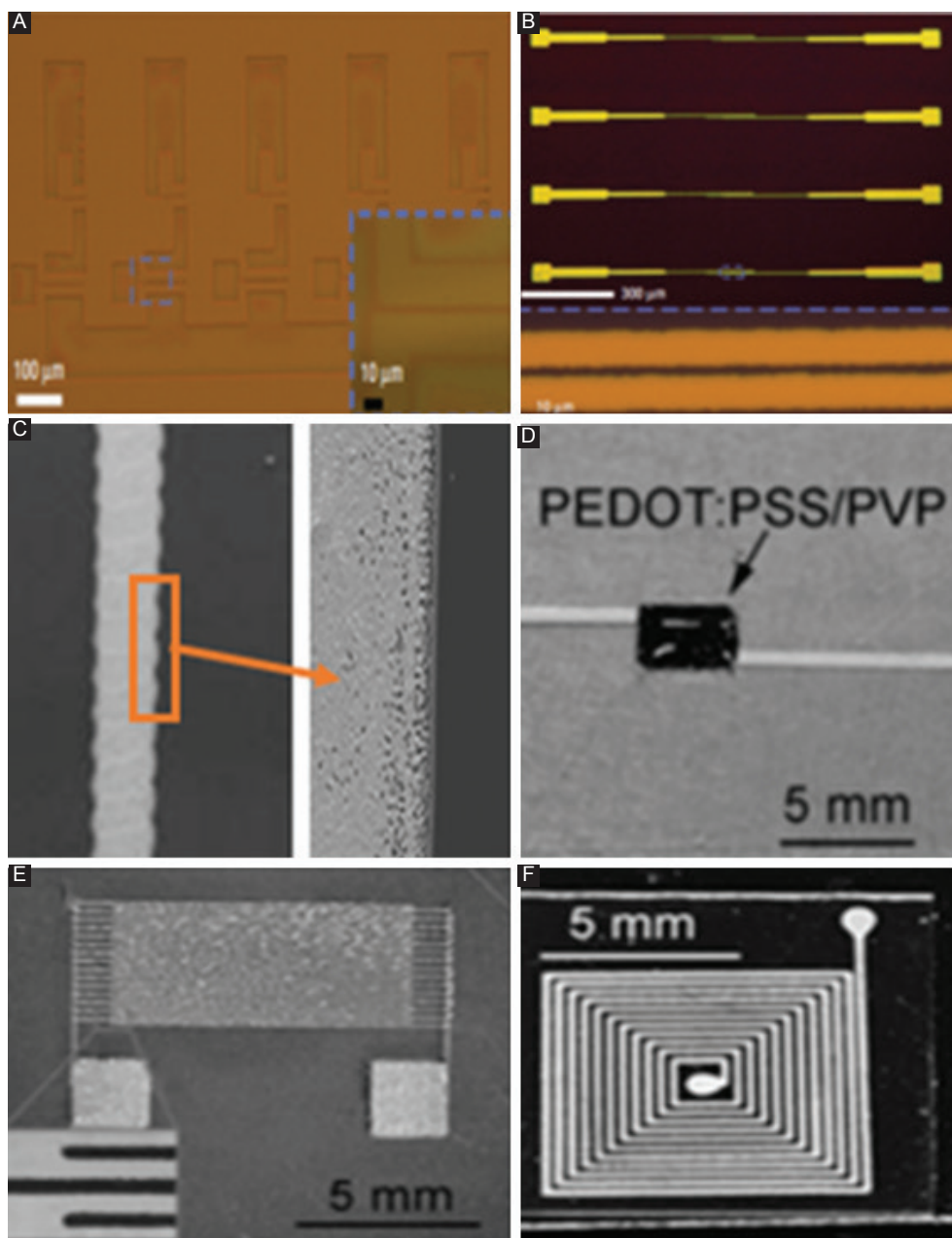


Figure 4. (A) Polyurethane etch resist for a circuit is printed by electrohydrodynamic (EHD) before etching the metal layer. (B) EHD printed array of source or drain electrode pairs after deposition of resist layer, etching of metal, and stripping the resist (adapted from Park *et al.*^[9]). (C) Surface morphology of the edge of the silver tracks scanned by scanning electron microscope at a magnification of $\times 5000$ (adapted from Wang *et al.*). (D) A printed coated resistor. (E) A printed interdigitated capacitor. (F) A printed spiral inductor (adapted from Wang *et al.*^[30]).

ink without any particle structure, on the substrate, and a photo-curable polymer droplet was printed at two intersecting conductive lines as isolating points^[11]. The latter material, which is a liquid ink containing metal nanoparticles, is usually directly deposited on the substrate; then, heating the metal nanoparticles makes them connect to each other. Heating process helps those particles to remove cover and form a continuous dense metal structures. EHD inkjet printing is able to print conductive lines for metallization in printed circuit

boards and backplanes of printable transistors^[27]. Since noble nanoparticles, such as gold and silver, have good electrical conductivity and non-oxidizing properties, their colloidal solution is firstly applied in the application of electronics. Khan *et al.* used a multinozzle to print three parallel conductive lines made out of colloidal solution containing silver particles and the width of line is about $140\ \mu\text{m}$. After sintering at 250°C for 1 h at a constant heating rate of $2^\circ\text{C}/\text{min}$, the resistivity of silver line is $5.05 \times 10^{-8}\ \Omega\ \text{m}$, 3 times higher than the resistivity of

bulk silver^[16]. Prasetyo *et al.* found that diameters of Ag dots can be reduced on a hydrophobic surface coated with octadecyltrichlorosilane (OTS), due to lower surface energy. A hydrophobic substrate with OTS coating can also overcome the existence of the coffee-stain effect^[28]. Youn showed that the width of minimum silver line is about 5.8 μm using a tilted nozzle^[29]. Wang succeeded in printing several passive electrical components, such as coated resistors, interdigitated capacitors, and spiral inductors as shown in Figure 4D-F and the minimum line width about 60 μm is achieved using a 110 μm nozzle^[30]. Khalid deposited silver patterns on thick glass substrates and found that the diameter of droplet after the sintering process is around 3.6 μm , which is 2.78 times smaller than the internal diameter of nozzle^[31]. Kang used EHD to print silver lines within 10 μm wide on a large flexible graphene substrate and the sheet resistance of hybrid electrode is as low as 4 ohm per unit square with 78% optical transmittance^[32].

To lower cost and obtain less electromigration effect, colloidal solutions containing copper nanoparticles were tested^[7]. As a result, the resistivity of deposited copper track was 5 times as high as resistivity of bulk copper and the difference of resistivity is attributed to relatively porous microstructure of copper track^[7]. Rahman *et al.* applied EHD methods to print a colloidal solution containing copper nanoparticles on a silicon substrate; the range of resistivity of the printed copper tracks was from $5.98 \times 10^{-8} \Omega\text{m}$ to $2.42 \times 10^{-7} \Omega\text{m}$.^[33] Han *et al.* used a nozzle with an inner diameter of 160 μm to print molten-metal by an EHD method, and a straight metal line with a diameter of 50 μm was obtained at a plotting speed of 1 mm/s^[34].

3.1.2 Biotechnology Applications

Inkjet printing had been previously employed to process living cells^[35], but the processing of concentrated biosuspensions has been severely limited by cellular loading and this has resulted in the formation of rather coarse structures^[36]. Jayasinghe *et al.* for the first time used electric field-driven technology to process and deposit living cells, like Jurkat cell suspension, and human peripheral blood monocytes in suspension. A strong electric field was created between nozzle and ring-shaped ground electrode and polydisperse distribution of droplets, indicating that an unstable mode of jetting was observed. This can be attributed to high electric conductivity and relatively low viscosity of solution^[36]. As a result, no adverse effects on either cellular structure or basic activities of living cells, such as cellular damage and irregular rate of division, had been found^[36]. To generate a near-mono distribution of cell-bearing droplets in stable jetting conditions, Kwok *et al.* reported a hybrid processing approach by combining bio-electrosprays

and aerodynamically assisted bio-jetting and jetted cells exhibited all expected cellular behavior^[37].

Although electrospray is able to produce relatively smaller droplets, electrospray cannot emit single microscale droplet on substrate, and sometimes, patterns have broad and non-uniform size distribution. EHD high-resolution technique allows to print a controlled droplet size and deposit at desired locations. Park *et al.* patterned large areas of DNA inks with complex configuration and feature size with resolutions on the order of 100 nm^[38]. Properties of DNA were not adversely affected by this printing process. As shown in Figure 5A, a pattern of butterfly was deposited at an inherent frequency when the nozzle scan over the substrate and the positions of individual droplets are not well controlled^[38]. In Figure 5B, a 14×14 arrays of the DNA dots were deposited by drop-on-demand e-jet printing method; the driving voltage was turned on and off on demand and it offered excellent control over the placement of individual droplets at expense of printing speed^[38].

Sutanto *et al.* used two biological materials to print droplets whose size is about 2 μm on average, and yellow color dots at right bottom corner are formed by overlaying two ink dots^[11]. It can be considered as a proof to demonstrate registration accuracy of the droplet diameter is within 2 μm ^[11]. Shigeta *et al.* used multinozzle EHD inkjet printing system to create micro- and nano-scale patterns of proteins on flat silica substrates. A structured plasmonic analysis is shown in Figure 5C and the study showed that the printing process does not adversely alter the protein structure or function^[39].

3.2. EHD 3D Printing

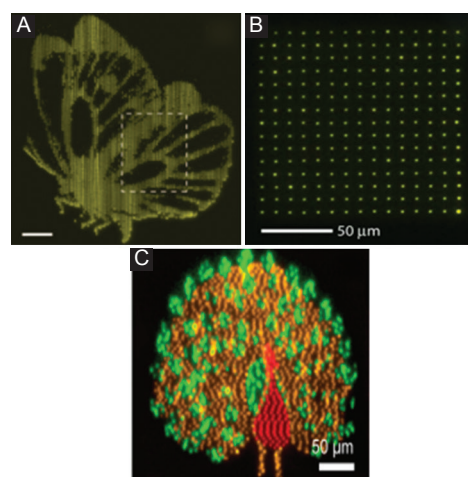


Figure 5. Dot diameter is 2 μm (a) butterfly pattern in fluorescence micrographs. (b) 14×14 DNA arrays in fluorescence micrograph printed in drop-on-demand mode (adapted from Park *et al.*^[38]). (c) Fluorescence microscope images of a peacock pattern formed using different fluorescently proteins (adapted from Shigeta *et al.*^[39]).

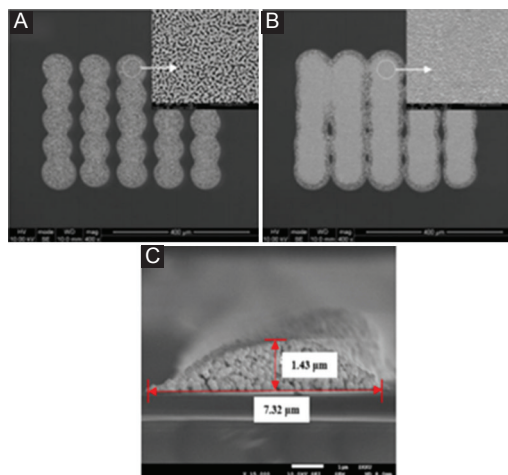


Figure 6. (A) Five stripes and each stripe built by single-layered printing. (B) Each stripe built by three-layered printing (adapted from Wang and Stark^[40]). (C) Field emission scanning electron microscope cross-sectional image of electrohydrodynamic jet-printed Ag mesh on the surface (adapted from Seong *et al.*^[33]).

3.2.1 Electronics Applications

In Figure 6A, Wang *et al.* used silver ink to deposit five overlapping drops of 88 μm size by a single-layered printing^[40]. Figure 6B indicates that droplets were mainly accumulated vertically rather than spreading laterally by three-layered printing and a denser morphology was obtained compared to porous structures built by single-layered printing^[40]. Previously, EHD jet printing has been applied to the fabrication of devices on a 2D flat substrate. Seong *et al.* succeeded in printing lines with a width of 7 μm and height of 1.43 μm on a 3D-curved surface with curvature, diameter, and height of 5.5 m^{-1} , 60 mm, and 10 mm, respectively, as shown in Figure 6C. The resulting sheet resistance is below 1.5 Ω ^[41].

3.2.2 Biotechnology Applications

Sullivan *et al.* presented three ground electrode geometries and they were the ring-shaped electrode, a plate shaped electrode and a point-shaped electrode. The ring-shaped and a plate shaped electrode produced a cone-shaped spray diverging droplets, while the point-shaped electrode focus the spray on a point. They employed the point-shaped electrode to create a self-supporting arch between two adjacent vertical wall or pillar structures^[42]. In addition, they were able to build a three-dimensional structure by using the same electrode geometry and this structure is biocompatible^[42].

Ahmad *et al.* utilized a controlled 3D writing method by decreasing deposition distance to build ordered and complex structures^[43]. In Figure 7A and b, an overlapped and orientated 3D grid pattern was printed using a pure PU polymer solution which is widely used as a biomaterial for blood contact applications^[43].

The control of layer-by-layer scaffold structure mentioned above was poor, and thus, researchers explored a precision controlled deposition method to fabricate 3D structures with controlled fiber diameter, orientation, and pore size. Particularly, Wei *et al.* applied a hybrid hierarchical approach to fabricate 3D scaffolds using polycaprolactone (PCL), a phase-change, biocompatible, and biodegradable printing material. Melting extrusion was employed under pneumatic pressure to produce a filament of hundreds of microns in diameter, and EHD jetting was used to deposit small feature with single micron resolution. Both 2D and 3D patterns were fabricated as shown in Figure 7C-E^[44,45]. Cai *et al.* used a similar EHD direct writing technique to build a 3D PCL scaffold with controlled fiber diameter and orientation [Figure 7F and G], and collagen was grafted to scaffold to improve regeneration of cartilage^[46]. Collagen-grafted scaffold helps chondrocytes to maintain healthy phenotypes, and it also increases production of cartilage-like extracellular matrices, when compared with conventional PCL scaffolds^[46].

Ahn *et al.* used the EHD printing method to fabricate a 3D porous structure, and the result showed that biological capabilities of scaffolds benefit from roughened surface and enhanced water absorptivity^[47]. Instead of traditional substrates, a polyethylene oxide (PEO) solution bath was used as a ground target to provide an elastically cushion to prevent plotted struts crumbling, as shown in Figure 7H and i. In addition, a grounded copper plate was immersed in the PEO bath, and previously deposited layers of struts can be dissolved in the remnant solvents to eliminate residue charges^[47].

4. Conclusion

EHD phenomenon has been known for more than a century, while EHD technology was just applied as a high-resolution additive manufacturing method a decade ago. EHD inkjet printing utilizes a unique cone-jet phenomenon to obtain a thin liquid jet without nozzle blockage as commonly experienced by conventional inkjet printing. It is necessary to keep cone-jet in stable operating regime to achieve the goal of high resolution. Many functional materials have been directly deposited on a variety of substrates by EHD deposition in different industrial areas, such as rapid prototyping, electronics, and biotechnology. However, low production rate of EHD inkjet printing is a main drawback that impedes widespread applications. This is, especially, a concern when small diameter jets are required for high-resolution printing. Although a multinozzle printing head is considered as a remedy to this problem, unstable jets may appear in this circumstance because of an asymmetric electric field arising from neighboring nozzles. Researchers developed different methods to alleviate the interaction of adjacent

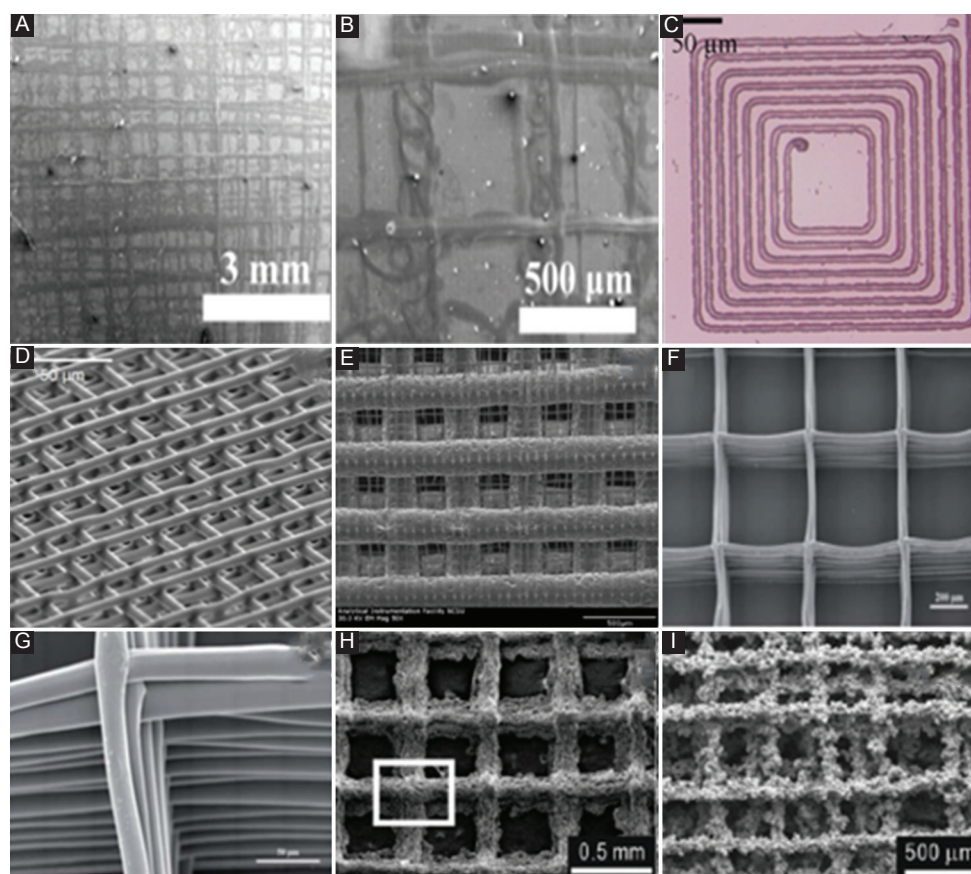


Figure 7. (A) Multilayered ordered complex structure of polyurethane. (B) Close-up view of (c) (adapted from Ahmad *et al.*[1]). (C) Spiral-in circle two-dimensional (2D) pattern fabricated by electrohydrodynamic (EHD) jet printing of melted polycaprolactone (PCL). (D) 3D scaffold structures fabricated by EHD jet plotting of melted PCL. (E) Scanning electron microscope (SEM) images of the hierarchical 3D scaffold structures (adapted from Wei and Dong^[44]) and (G) Surface topography of PCL structure built by EHD printing method (adapted from Cai *et al.*^[46]). (H) 3D scaffold fabricated by a normal EHD-printing without cushioning bath in SEM micrographs. (I) The highly roughened surface morphology of the EHD-plotted 2D scaffold on the PEO solution bath (adapted from Ahn *et al.*^[47]).

jets. In addition, a nozzle-ring electrode is proposed to improve the performance of EHD printing in 3D printing applications.

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RESEARCH ARTICLE

Near-field electrospinning of a polymer/bioactive glass composite to fabricate 3D biomimetic structures

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Abstract: Bioactive glasses have recently gained attention in tissue engineering and three-dimensional (3D) bioprinting because of their ability to enhance angiogenesis. Some challenges for developing biological tissues with bioactive glasses include incorporation of glass particles and achieving a 3D architecture mimicking natural tissues. In this study, we investigate the fabrication of scaffolds with a polymer/bioactive glass composite using near-field electrospinning (NFES). An overall controlled 3D scaffold with pores, containing random fibers, is created and aimed to provide superior cell proliferation. Highly angiogenic borate bioactive glass (13-93B3) in 20 wt.% is added to polycaprolactone (PCL) to fabricate scaffolds using the NFES technique. Scaffolds measuring 5 mm × 5 mm × 0.2 mm³ in overall dimensions were seeded with human adipose-derived mesenchymal stem cells to investigate the cell viability. The cell viability on PCL and PCL+glass scaffolds fabricated using NFES technique and 3D printing is compared and discussed. The results indicated higher cell proliferation on 3D biomimetic scaffolds fabricated by NFES technique.

Keywords: Near-field electrospinning; three-dimensional biomimetic scaffold; polycaprolactone; polymer/bioactive glass composite; borate bioactive glass; human adipose-derived stem cells

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1. Introduction

Porosity, pore geometry, and pore size distribution are the most important parameters in scaffold fabrication in the field of tissue engineering. Different cell types require different pore sizes for optimal growth and proliferation^[1]. Previous investigations have shown that pore geometry in three-dimensional (3D) scaffolds mimicking natural tissue architecture could offer a superior environment for cell proliferation^[2]. While powder or resin bed-based additive manufacturing (AM) techniques offer flexibility to fabricate scaffolds mimicking natural tissue architecture, fabricating scaffolds with complex pore

geometries are limited with extrusion-based 3D printing methods^[3-5]. Extrusion-based 3D printing is the most versatile and widely adopted AM technique in bioprinting because of a wide range of hydrogels that are suitable for cell suspension and extrusion^[6]. However, creating macrostructures that mimic natural tissue architecture with extrusion 3D printing has been a challenge.

On the other hand, electrospinning is a mature technology for fabricating aligned and randomly oriented fiber mats for different tissue engineering applications^[7]. In recent years, a most common approach adopted by researchers to achieve the 3D biomimetic structures has

been to combine the electrospinning and 3D printing techniques to provide a nanofiber mesh in between the 3D printed macroporous layers^[8]. An alternative approach to the traditional electrospinning is called near-field electrospinning (NFES), where the substrate distance from nozzle tip is decreased to control the fiber deposition^[9,10]. In NFES technique, fiber instability is restricted because of the shorter substrate distance and deposition is precisely controlled to obtain the desired part shape. Figure 1 illustrates the type of fiber deposition obtained at different substrate distances. In this work, the substrate distance was maintained such that the fabricated scaffold would have an overall defined shape with directional filaments. Simultaneously, it was also made sure that the deposited fibers would have a certain degree of randomness to create the biomimetic architecture that resembles the cancellous bone.

Researchers have investigated electrospinning of polycaprolactone (PCL) + glass composites (typically, 5–10 wt.% silicate-based glass) to improve the scaffold bioactivity^[11,12]. In this study, we use borate glass (13-93B3 or B3) that is biocompatible, osteoconductive, and angiogenic and has a higher reaction rate (5–10 times faster than silicate glasses) and is antimicrobial^[13]. The glass can heal even difficult-to-heal wounds by generating a healthy scar-free tissue with improved vascularization. In the current work, we investigate the feasibility of fabricating a bioactive 3D scaffold mimicking the native bone architecture using NFES of PCL+B3 glass composite.

2. Materials and Methods

2.1. Preparation of PCL+B3 Glass Paste

0.25 g of B3 glass (Mo-Sci Corporation, Rolla, MO) particles of $< \sim 20 \mu\text{m}$ were ultrasonicated for 2 min in 3 ml chloroform (CF) (Sigma-Aldrich, St. Louis, MO). B3 glass composition can be found in literature^[14], 1 g of PCL (Polysciences, Warrington, PA; M.W. = 50,000 g/mol) was added immediately after

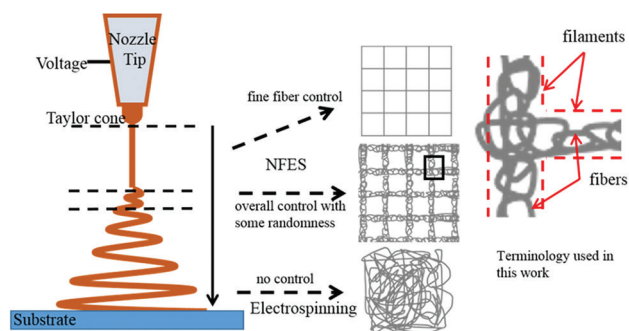


Figure 1. Illustration of the fiber and filament control with increasing substrate distance from nozzle tip during electrospinning.

ultrasonication and materials were uniformly mixed for 5 min at 2000 RPM in a planetary mixer (SpeedMixer™, FlakTek Inc., Landrum, SC). The weight ratio of materials was selected in such a way to provide a 20% glass in weight in the fabricated composite scaffolds after eventual CF evaporation.

2.2. Scaffold Fabrication and Characterization

For NFES, a home-built, three-axis gantry system with pressurized air extrusion and a power source was utilized. Scaffolds were fabricated on an aluminum foil placed on a metal substrate and a custom-made software interface was used to control the printing parameters including applied voltage, air pressure, and printing speed. A metal tip (0.25 mm internal diameter) was used to fabricate scaffolds measuring $20 \text{ mm} \times 20 \text{ mm} \times 0.2 \text{ mm}^3$, which were later cut into $(5 \text{ mm} \times 5 \text{ cm} \times 0.2 \text{ mm}^3)$ dimensions for *in vitro* assessment. For comparison, scaffolds were also 3D printed using the same paste composition as described in our previous work^[14]. Optical microscopic images were used to measure the filament width and pore sizes with at least 10 measurements and the results were reported as average $\pm$ standard deviation. Scaffolds were soaked in 2 ml of the complete culture media (CCM) to simulate the *in vitro* conditions and evaluate the surface morphology and formation of hydroxyapatite-like material on the surface. After soaking for up to 7 days in CCM, scaffolds were dried overnight, coated with Au-Pd, and observed under a scanning electron microscope (S-4700, Hitachi, Japan).

2.3. Cell Culture

Frozen vials of approximately 1×10^6 adipose-derived human mesenchymal stem cells (ASCs) were obtained from three separate donors (LaCell, New Orleans, LA). Vials were thawed, plated on 150 cm² culture dishes (Nunc, Rochester, NY) in 25 mL CCM consisting of 10% fetal bovine serum (FBS, Corning, Manassas, VA), alpha-minimum essential media (α -MEM, Sigma-Aldrich, St. Louis, MO), 1% $\times 100$ L-glutamine (GE Life Sciences, Logan, UT), 1% $\times 100$ antibiotic/antimycotic (GE Life Sciences, Logan, UT), and incubated at 37.5°C with 5% humidified CO₂. After 24 h, the media were removed and adherent, viable cells were washed twice with phosphate-buffered saline (PBS), harvested with 0.25% trypsin/1 mM ethylenediaminetetraacetic acid (Gibco), and replated at 100 cells/cm² in CCM. Media were changed every 3–4 days. Subconfluent cells ($\leq 70\%$ confluent) between passage 2 and passage 6 were used for all experiments as subsequent passages could affect pluripotent properties of ASCs.

2.4. Cell Viability

Scaffolds were seeded with 30,000 ASCs suspended in 30 μl of CCM. After a 2 h incubation to allow cell

attachment, scaffolds were transferred to 35 mm Petri dishes with 2 ml of CCM. A Live/Dead cell imaging kit (ref. R37601, Eugene, OR) was used for qualitative assessment of cell viability. Briefly, after a 24 h incubation period, scaffolds were washed with PBS, stained for 30 min at room temperature, and examined under a fluorescent microscope (Olympus IX51, Melville, NY). Five scaffolds were examined per experimental group, with at least five pictures taken per scaffold.

To quantify cell viability, scaffolds were analyzed for total DNA using CyQuant cell proliferation assay (Invitrogen), using the manufacturer's protocol to normalize all results to cell number. Briefly, 24 h after seeding cells, cellularized scaffolds were gently washed with PBS and frozen at -80°C overnight. Scaffolds were thawed the next day and analyzed with CyQuant. A sample size of $n = 5$ was used for all experiments except for CyQuant assay for 3D printed PCL scaffolds ($n = 4$). Scaffolds without cells were used for background controls. One-way ANOVA was performed in Minitab to analyze the results and difference was considered significant if $P < 0.05$.

3. Results and Discussion

3.1. Scaffold Fabrication

The effect of fabrication parameters including applied electric field, printing speed, and extrusion pressure on the filament deposition was investigated. The above parameters control the porosity of the fabricated part by changing the filament size, fiber size, and the amount of deposited material as shown in Figure 2a. The filament and fiber sizes at different parameters are shown in Table 1. A 10 kV/cm electric field with 5 mm/s printing speed and 30 psi extrusion pressure (test #8) provided a suitable filament with randomly distributed small fibers to create a biomimetic 3D structure. The printing schema and fabricated 3D scaffold with cancellous bone microstructure similarities can be observed in Figures 2f and 2g. Furthermore, the pore size distribution in NFES scaffolds varied from 20 μm to 250 μm that are desirable for bone tissue growth.

In this study, one of our aims was also to compare and contrast the NFES scaffolds with 3D printing scaffolds in terms of bioactivity and cell proliferation. Therefore, we have used the solvent-based 3D printing process to fabricate scaffolds with the same compositions of PCL+B3 glass and PCL pastes used in NFES technique. More details about the solvent-based 3D printing and scaffold fabrication with PCL+B3 glass composite (up to 50 wt.% glass) can be found in our previous work^[14,15]. 3D printed scaffolds were designed to have pore sizes not exceeding the pore sizes obtained using NFES technique (i.e., 250 μm) and to reflect an average pore size. A printing speed of 20 mm/s, air pressure of 30 psi, and filament-to-filament spacing of 0.35 mm were used to fabricate the scaffold. The average filament width of $167 \pm 38 \mu\text{m}$ and pore size of $188 \pm 28 \mu\text{m}$ were obtained for the 3D printed scaffold.

3.2. Scaffold Bioactivity

The scaffold porosity was calculated based on 2D optical images after the printing of the first two layers of the scaffold with both processes. The obtained porosity for NFES scaffold was ~50% compared to ~30% for 3D printed scaffold. The higher porosity and wide range of pore sizes of NFES scaffold are beneficial for B3 glass dissolution because of the larger surface area. Scaffolds were soaked in CCM for up to 7 days to evaluate the formation of hydroxyapatite (HA)-like layer on the scaffold surface (Figure 3). The results indicated nanosized HA-like crystal formations on scaffolds made by both processes. The crystals were uniformly spread out on NFES scaffold surface and were observed in patches on 3D printed scaffolds. This indicates a faster B3 glass dissolution from NFES scaffolds compared to 3D printed scaffolds. The X-ray diffraction results showed peaks indicating the formation of non-stoichiometric HA which is consistent with our previous studies where PCL+B3 glass scaffolds showed similar conversion^[14].

3.3. Cell Viability and Proliferation

Viability of ASCs was studied by seeding cells on scaffolds and performing live/dead assay after 1 day and

Table 1. Effects of fabrication parameter on filament and fiber sizes in NFES

Test	Electric field (kV/cm)	Printing speed (mm/s)	Extrusion pressure (psi)	Filament size (μm)	Fiber size (μm)
#1	12	7.5	50	565 ± 37.4	80.2 ± 12.1
#2	10	7.5	50	567.5 ± 37.9	46.8 ± 6.9
#3	8	7.5	50	562.5 ± 52.6	87.2 ± 20.8
#4	6	7.5	50	N/A	103 ± 10.9
#5	10	5	50	578.75 ± 41.9	47.8 ± 18.5
#6	10	2.5	50	501 ± 28.6	99.8 ± 28.8
#7	10	5	40	350.25 ± 87.2	42.2 ± 18.5
#8	10	5	30	517.5 ± 27.9	33.4 ± 6.3
#9	10	5	20	N/A	48 ± 12.0

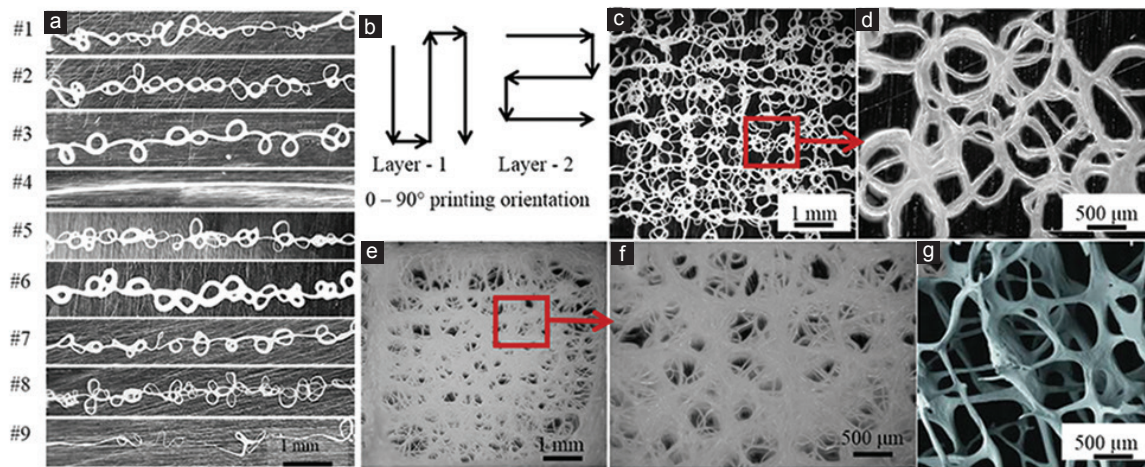


Figure 2. Near-field electrospinning of polycaprolactone (NFES) +B3 glass composite. (a) Fiber deposition control with different NFES fabrication parameters, (b) printing schema, (c) after printing one raster in both 0° and 90° orientation, (d) magnified image showing the fiber dimensions, (e) 10-layer scaffold, (f) magnified image of the 10-layer scaffold, and (g) cancellous bone microstructure (reproduced with permission^[16]).

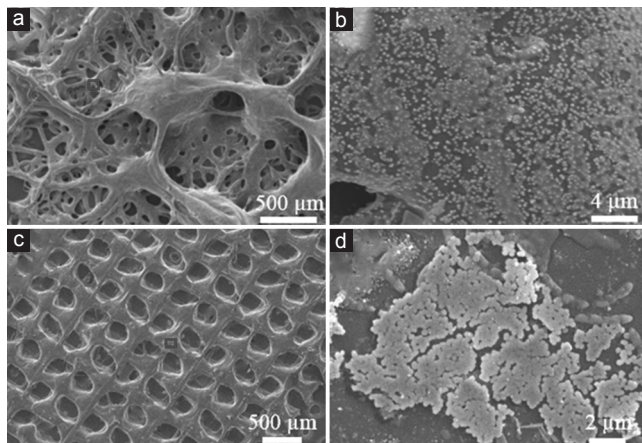


Figure 3. Scanning electron microscope images of near-field electrospinning of polycaprolactone (NFES) and three-dimensional (3D) printed scaffolds soaked in complete culture media for 7 days. Scaffolds showed HA-like crystal formations on the scaffold surface. (a-b) NFES scaffold and its magnified image, (c-d) 3D printed scaffold and its magnified image.

7 days. The assay reagents consist of a non-fluorescent calcein dye which is converted to green fluorescent calcein after intracellular esterases in live cells causing the live cells to stain green. In dying or dead cells, a bright red fluorescence is generated on binding to DNA. Overall, in all scaffold types, the results indicated more live cells in comparison to dead cells (Figure 4). Specifically, more live cells were observed in all NFES scaffolds with or without glass in comparison to 3D printed scaffolds after 7 days incubation. This can be clearly seen in Figures 4g-h. However, the live/dead assay images were not used to quantify the cell viability results because of the high background staining of B3 glass particles, which

can be observed in PCL + B3 glass composite scaffolds (Figures 4b-f and 4d-h). In normal conditions, the non-fluorescent calcein acetoxymethyl compound present in live/dead assay transports into cells, and intracellular esterases remove the acetoxymethyl group, thereby producing strong green fluorescence. It is believed that the borate ions present in the B3 glass interfere with the acetoxymethyl group, thereby producing strong green fluorescence even without cells. The PCL only scaffolds do not exhibit strong fluorescence in the absence of glass particles, which can be clearly observed in Figures 4a-e and 4c-g (strong green fluorescence in Figure 4g is because of more cells and not material). The background staining makes it difficult to contrast and quantify cells on PCL+B3 glass scaffolds using the ImageJ software. The green fluorescence and staining of the 3D printed PCL+B3 glass scaffold filament can be clearly observed in Figures 4b-f. Overall, the live/dead assay results qualitatively indicated more cell ASC proliferation in NFES scaffolds compared to 3D printed scaffolds.

To quantify cell proliferation, CyQuant assay was performed on the ASC-seeded scaffolds (Figure 5). The CyQuant GR is a green fluorescence dye that intensifies after it binds to the nucleic acid of DNA to provide a reading that is then converted to cell number based on the standard curve. The results indicated increased cell proliferation in NFES PCL scaffolds compared to 3D printed PCL scaffolds after 1 day and 7 days incubation. However, the results are not statistically significant with $P = 0.4$. A wide pore size distribution in NFES scaffolds could have provided uniform cell distribution and proliferation, whereas cells were mainly observed on filaments in 3D printed scaffolds. The Live/Dead

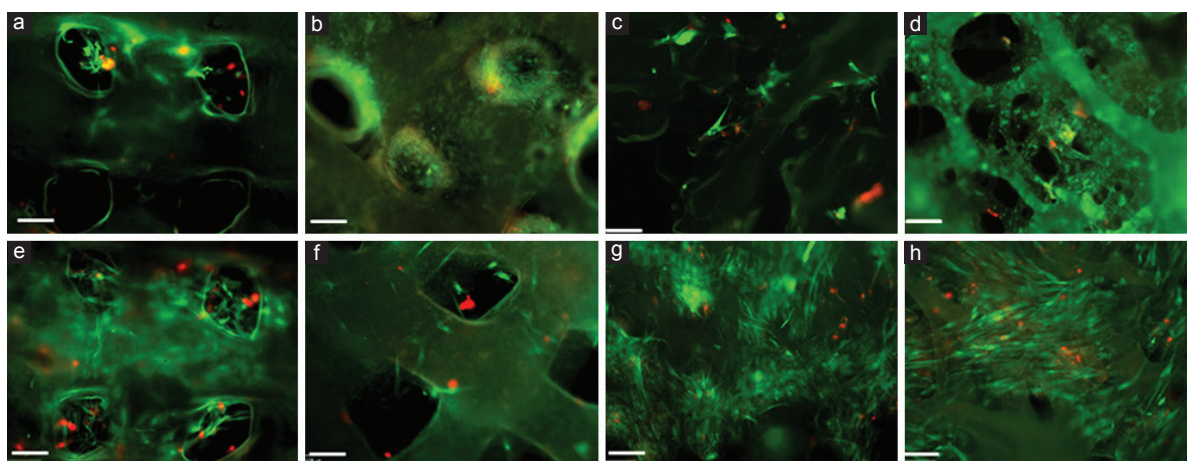


Figure 4. Live/dead images showing the viability of adipose-derived human mesenchymal stem cells seeded on scaffolds (scale bar: 100 μ m). (a-d) after 1 day and (e-h) after 7-day incubation, (a and e) three-dimensional (3D) printed polycaprolactone (PCL), (b and f) 3D printed PCL+B3 glass, (c and g) near-field electrospinning of polycaprolactone (NFES) PCL, (d and h) NFES PCL+B3 glass. NFES scaffolds show high cell proliferation after 7 days compared to 3D printed scaffolds

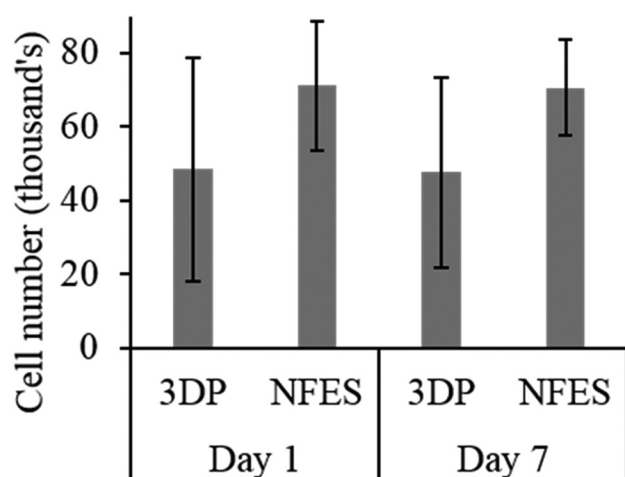


Figure 5. Cell proliferation measured by CyQuant. Near-field electrospinning of polycaprolactone scaffolds showed increased cell proliferation in polycaprolactone scaffolds. All scaffolds were seeded with 30,000 adipose-derived human mesenchymal stem cells

assay results consistently showed more number of cells in NFES scaffolds, and the CyQuant results are in consistent for PCL scaffolds as shown in Figure 5. In addition, more dead cells (red spots) were observed in PCL+B3 scaffolds (both NFES and 3D printed) compared to PCL only scaffolds (e.g., Figure 4g vs. 4h). One possible reason for a relatively higher cell death in PCL+B3 glass scaffolds compared to PCL only scaffolds could be because of the pH change due to B3 glass dissolution and the released ionic products which could harm cells, especially, in static culture conditions. Poor cell viability was previously reported on cell-seeded B3 glass scaffolds in static conditions that improved under dynamic conditions^[2,17]. Regardless, with respect to PCL

only scaffolds, the *in vitro* assessment performed in this study definitively indicates improved ASC proliferation on NFES scaffolds in comparison to 3D printed scaffolds showing the potential of the NFES technique and significance of the biomimetic 3D structure compared to the 3D printed lattice structure. The additional advantage of NFES technique is that the process can be easily integrated for bioprinting applications with simultaneous bio-ink extrusion in a 3D architecture mimicking the extracellular matrix.

4. Conclusion

This study investigated the feasibility of fabricating a biomimetic 3D scaffold with PCL and PCL/bioactive glass composite (20 wt.% glass) using the NFES technique. NFES scaffolds had a microstructure similar to the cancellous bone, ~50% porosity, and a wide pore distribution (20–250 μ m). In comparison with 3D printed scaffolds, NFES scaffolds were highly bioactive providing a faster glass dissolution and more uniform formation of hydroxyapatite-like crystalline formations throughout the scaffold surface after 7 days. Live/dead assessment with human adipose-derived mesenchymal stem cells indicated high cell proliferation and uniform cell distribution in NFES scaffolds compared to 3D printed scaffolds. Overall, the NFES technique showed the process potential for tissue engineering and bioprinting applications.

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Conflicts of Interest and Funding

No conflicts of interest were reported by the authors.

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RESEARCH ARTICLE

Electrohydrodynamic printing process monitoring by microscopic image identification

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Abstract: Electrohydrodynamic printing (EHDP) is able to precisely manipulate the position, size, and morphology of micro-/nano-fibers and fabricate high-resolution scaffolds using viscous biopolymer solutions. However, less attention has been paid to the influence of EHDP jet characteristics and key process parameters on deposited fiber patterns. To ensure the printing quality, it is very necessary to establish the relationship between the cone shapes and the stability of scaffold fabrication process. In this work, we used a digital microscopic imaging technique to monitor EHDP cones during printing, with subsequent image processing algorithms to extract related features, and a recognition algorithm to determine the suitability of Taylor cones for EHDP scaffold fabrication. Based on the experimental data, it has been concluded that the images of EHDP cone modes and the extracted features (centroid, jet diameter) are affected by their process parameters such as nozzle-substrate distance, the applied voltage, and stage moving speed. A convolutional neural network is then developed to classify these EHDP cone modes with the consideration of training time consumption and testing accuracy. A control algorithm will be developed to regulate the process parameters at the next stage for effective scaffold fabrication.

Keywords: electrohydrodynamic jetting; convolutional neural network; image processing, scaffold fabrication

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1. Introduction

With the rapid development of micro-/nano-manufacturing, electrohydrodynamic printing (EHDP) has recently drawn great attention for its capability to print micron to nanometer-scale fibers. EHDP technology uses a pneumatic or syringe pump to supply viscous solution to a nozzle with constant flow rate and applies a high voltage between the nozzle and substrate to generate electric field. The solution is charged and distorted into a Taylor cone shape at the nozzle tip. When the electrostatic force exerted on the Taylor cone overcomes its surface tension, a microjet ejects downward, quickly solidifies due to solvent evaporation. The solidified fibers are deposited on the substrate with a nearly circular cross

section^[1]. The printing resolution of EHDP is improved by about two orders of magnitude in comparison to the conventional inkjet printing systems^[2]. The deposited fibers on the substrate can be orientated by a computer-controlled precision stage and stacked into scaffolds with tailored microstructure. With the advantages of low cost, simple setup, and high printing resolution, EHDP has attracted a wide range of innovative explorations. Most importantly, EHDP enables the fabrication of biomimetic fibrous scaffolds with the feature size close to the scale of extracellular matrix, which can facilitate the cell attachment, proliferation, and tissue regeneration^[3]. Even though the EHDP mechanism is conceptually simple, the actual formation process strongly depends

on the properties of the inks (viscosity, surface tension, and electrical conductivity), environmental factors (temperature and humidity), and other process parameters (nozzle-to-substrate distance, solution feeding rate, and dimensions of nozzle). Due to the process complexity and the variation of electric field strength in fiber layer stacking process, EHDP lacks an accurate process model and easily becomes unstable in large-scale fabrication (a few hours). Therefore, it becomes an urgent and, at the same time, challenging task to characterize the EHDP process and control the morphologies of deposited fibers and constructed scaffolds by fiber layer stacking. In the past, we could only verify the scaffold's morphology by taking scanning electron microscopic image after the printing process.

Ideally, we like to monitor the EHDP jet formation and regulate its process adaptively to achieve the desired scaffold morphology. This is quite similar to what have been done in electrospinning process monitoring^[4]. Researchers usually installed high-speed cameras to visualize the ejection of droplets from the nozzle in a safety distance and investigated the relation between the geometry of Taylor cone, environmental factors, and produced fibers^[5,6]. However, such monitoring systems are too expensive, compared with the setup cost of EHDP system. Besides, the EHDP process can generate high-velocity jet which is around a few meters per second with the use of low viscous solutions^[7]. It is difficult to regulate such a process in a timely manner with control. Thus, most of the current low-viscosity EHDP systems are open loop (i.e., without feedback or feedforward control).

The EHDP jet formation time would be significantly prolonged with the use of high viscous biopolymer solutions for scaffold fabrication. Thus, it is possible to detect any abnormal modes using a digital microscope device. This imaging device should be able to observe comprehensive details in a cost-effective way so as to observe the jet triggering voltage and the lowest voltage for stable EHDP process. Researchers have studied Taylor cone modes with different viscous solutions^[8]. When high viscous solutions are fed into the EHDP system, the jet ejection velocity can be comparable with that of the computer-controlled precision stage in the range of 50–400 mm/s^[9].

This paper presents an EHDP monitoring approach in scaffold fabrication process. More specifically, this paper will focus on (1) the development of the microscopic image sensing technique which enables real-time monitoring of EHDP and (2) an identification algorithm which can process the captured images, extract features, and classify the EHDP cone modes by convolutional neural network (CNN). The rest of the paper is as follows: Section 2 provides a description of the EHDP process and monitoring system setup, Section 3 introduces image processing methods applied to the captured microscopic

images, applying CNN in EHDP cone modes' classification and validating the performance of the developed CNN models are presented in Section 4, and Section 5 provides concluding remarks and future directions.

2. Experimental setup

2.1. Introduction of EHDP system

Figure 1A illustrates the schematic diagram of our laboratory built EHDP system, which consists of a three-axis motorized stage, a high voltage power supply to trigger and maintain EHDP process, a digital microscope, a solution feeding system, and a substrate. The solution feeding system consists of a syringe pump (New Era Pump System), a 5 ml syringe, a flexible hose, and a stainless steel nozzle with internal diameter of 0.5 mm. The syringe is filled with the solution obtained by dissolving the polycaprolactone (PCL) pellets with an average molecular weight (wt) of 80 kDa in the acetic acid (>99.7% pure). As shown in Figure 1B, a jet ejects at the apex of the Taylor cone when the applied voltage gradually increases to the jet triggering value. The EHDP process characterization is based on the above fundamental phenomenon. Its stability is determined by the ratio of length-to-width of Taylor cones, as shown in Figure 1B. This ratio can be used to determine the lowest voltage for stable EHDP process, and a larger ratio means a longer cone shape^[10].

The three-axis precision stage from Aerotech, Inc. (Pittsburgh, PA, USA) is driven by linear motors. It has a travel range of 150 mm with 3 μ m accuracy on X and Y axes, and a travel range of 50 mm with 5 μ m accuracy on Z axis. The substrate, a polished silicon wafer, is fixed on X-Y plane. The motorized stage where the substrate rests on is digitally controlled and hence is capable to stretch the jetting fiber in a programmable manner. The stage speed (SS) along X and Y axes is set within the same range of 50–300 mm/s. The moving stage along X and Y axes could generate a mechanical drawing force to pull the deposited fiber, where the fiber diameter and morphology are influenced by the SS.

In this study, three EHDP key process parameters are looked into to explore their influence on jetting fibers and printed patterns, namely the applied voltage (V: 2.6–3.4 kV), the nozzle-substrate distance (D: 3–4.5 mm), and the SS: 50–300 mm/s. We obtain the images of EHDP Taylor cones formed by PCL solutions (65 w/V% in acetic acid; 70 w/V% in acetic acid; and 80 w/V% in acetic acid, g/mL), and PCL/zein composite solutions (50 w/V% PCL and 20 w/V% zein in acetic acid; 60 w/V% PCL and 10 w/V% zein in acetic acid), and with the syringe feed rate (FR) at 0.7 μ l/min, temperature at 25°C, and humidity around 50–60%.

2.2 Monitoring System Setup

Our monitoring region focuses on the EHDP Taylor cone and its jet. Since the captured images were strongly influenced by the lighting conditions, an adjustable LED light source is introduced in a reflective way to ensure good image quality. The EHDP process is video recorded by a Supereyes B011 digital microscope with 200 magnification and 30 frames per second. One can view and analyze microscale features of the observation region on a computer screen. The camera position and shooting angle are calibrated by aligning the area of the gray scale nozzle image with a predefined nozzle position.

Figure 2A shows a standard EHDP cone shape with a straight jet. Figure 2B shows a cone jet with helical deformation which consists of two distinct parts: A long, roughly vertical “tail” (with length in millimeters and diameter in micrometers to nanometers) which deforms primarily by severe electrostatic force stretching and a helical “coil” in which the deformation is dominated by bending and twisting. Once these coiled fibers are dispersed along the stage moving direction, versatile structures can be formed on the substrate as shown in Figure 2C.

3. Image Processing and Feature Extraction

Various Taylor cones and jets images are recorded. The next step is to process these images automatically and construct the linkage between the diversity of Taylor cones and deposited fibers. Image quality and size may

vary from one process run to another. Thus, we need to interpret these monitoring images by acquisition, segmentation, and feature extraction.

3.1 Image Processing

Some cones can be distinguished from the background in the captured images. Thus, the image processing would start with binarization, followed by the detection of maximum connected region. For those images, the binarization with a single threshold may not be sufficiently to extract the cone images from the background. A method named “sharpen” will be implemented, followed by edge detection, dilation, and erosion. These processing methods are briefly described as below. Table 1 shows the images before and after each processing method.

3.1.1 Sharpen

In sharpen, a constant (>1) is added to the transfer function of high-pass filters in the frequency domain to recall some low-frequency components. This can improve the contrast of the edge region and keep the smooth region at the same time^[1]. After sharpening, the observed region becomes easier to be detected than that of the original image as shown in the first column of Table 1.

3.1.2 Maximum Connected Region

When color images are transformed into binary images, there are numerous connected regions. As shown in the second column of Table 1, it consists of one major

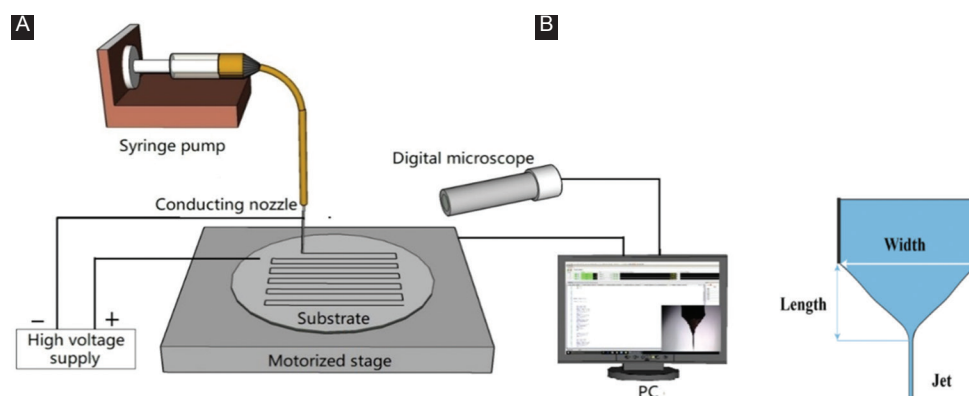


Figure 1. Electrohydrodynamic printing (EHDP) setup and the monitoring system. (A) Schematic diagram of EHDP setup and monitoring system (B) Taylor cone.

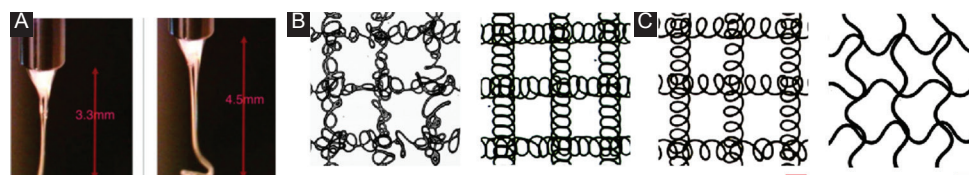
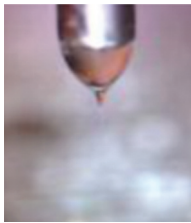
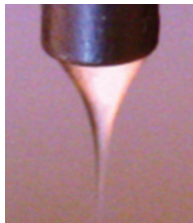
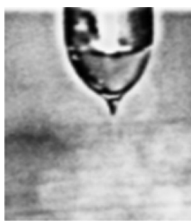
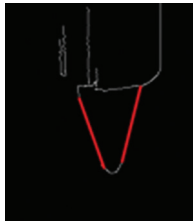


Figure 2. (A) Standard electrohydrodynamic printing cone shape, (B) cone-jet region with helical deformation, (C) diverse deposited non-straight fiber patterns (scale bar: 200 μm).

Table 1. Image processing methods

Images	Sharpen	Maximum connected region	Edge detection	Straight line detection	Erosion and dilation
Before					
After					

connected region (target of interest) and some smaller regions due to noise, grids, or reflected light. Pixels are counted in every labeled region, and then, the maximum connected region is identified, that is, Taylor cone and jet.

3.1.3 Edge Detection

Edge refers to the most obvious change of local intensity within an image. Canny operator with non-maximum suppression method is used for edge detection, since it can detect thinner edges than those of other operators. A quasi-Gauss function is introduced to realize a smoothing operation and reduce noise influence^[11]. To detect the edge of Taylor cone and nozzle more precisely, a threshold (about 0.15–0.3) is introduced to determine the minimum gradient according to the brightness and contrast.

3.1.4 Straight Line Detection

After the edge detection, the image will be further processed by the straight line detection to extract cone features. In the Euclidean space, the points on a straight line correspond to a sinusoidal cluster in the Hough parameter space^[11]. The intersection in the Hough parameter space defines the straight line between the points, which can be applied to evaluate the Taylor cone shapes and their stability.

3.1.5 Erosion and Dilation

The dilation makes the target “grow” in the image, while the erosion can make target “shrink” and get intersection of target and structural elements^[11]. In this study, we apply an open operation (dilation followed by erosion) to remove the noise in the images, followed by a close way (erosion followed by dilation) to close the small gaps on the thin edges. An example is shown in the last column of Table 1.

3.2 Feature Extraction

After the above-mentioned image processing steps, two key features are extracted to describe the Taylor cone and jet.

3.2.1 Centroid

Centroid is the arithmetic mean position of all the points in the cone, that is, center of mass^[12]. Since the inner diameter of the nozzle is fixed, the value of centroid can be used to describe the Taylor cone shape and size. To be more specific, the smaller centroid value indicates a shorter cone.

3.2.2 Jet Diameter

The flying jet diameter is usually measured at the position close to the substrate. Under a proper nozzle-substrate distance, the solvent in micro-/nano-jet can fully evaporate, and the semi-solid jet eventually becomes solidified fiber patterns on the substrate.

3.3. Influences of Process Parameters on the Extracted Features

3.3.1 Effect of the Applied Voltage and Nozzle-substrate Distance

Figure 3A describes the effect of the applied voltage on centroid value under different nozzle-substrate distances. 65 wt/v% PCL is used for this experiment, with the solution FR at 0.7 $\mu\text{l}/\text{min}$ and the SS at 150 mm/s. Under the same applied voltage, the size of Taylor cone described by centroid value is proportional to the nozzle-substrate distance. In other words, a larger nozzle-substrate distance would relate to a larger size of Taylor cone.

Figure 3A also includes Taylor cone images at the nozzle-substrate distance of 3 mm. In general, the size of Taylor

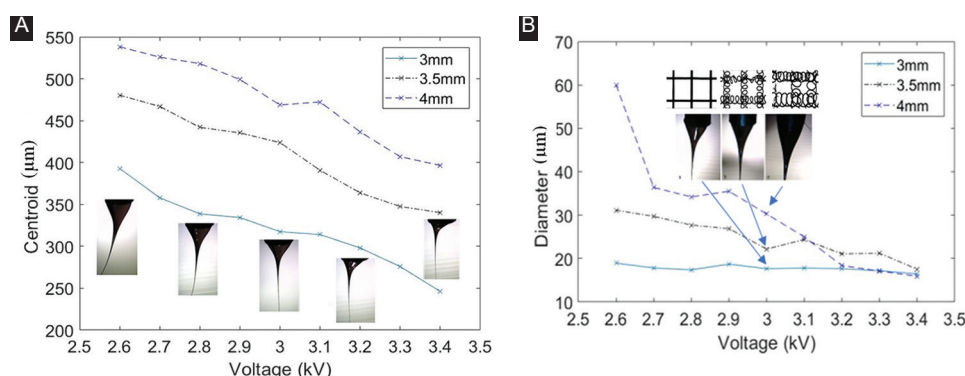


Figure 3. Effect of the applied voltage on centroid and diameter under varied distance (65 wt/v% polycaprolactone, feed rate = 0.7 μ l/min, stage speed = 150 mm/s). (A) Effect of voltage and distance on centroid. (B) Effect of voltage and distance on diameter

cone is inversely proportional to the applied voltage. It is found that a voltage around 2.6 kV can generate a huge size of Taylor cone, and this size reduces with the increase of the voltage. The cone shape becomes excessively tiny when the voltage is around 3.4 kV. In other words, increasing the applied voltage above a threshold will reduce the stability of the jet^[10]. Similar phenomena are also observed for the nozzle-substrate distance at 3.5 mm and 4 mm. Similarly, we also investigate the size and shape of Taylor cones using 70 wt/v% PCL and draw the same conclusion.

About the effect of the applied voltage on the cone-jet diameter in Figure 3B, increasing the applied voltage leads to slightly decrease of the jet diameter when the nozzle-substrate distance is 3 mm. The jet diameter decreases more significantly when the nozzle-substrate distance increases to 3.5 and 4 mm. As the electrostatic force becomes weaker under a larger distance, EHDP process generates a thicker jet which can be further stretched with the increase of the applied voltage^[10].

The captured cone images at 3 kV under different nozzle-substrate distances and their corresponding deposited fiber patterns are shown in Figure 3B. Compared with the Taylor cone shape at 3 mm, the cone deforms significantly at 4 mm. To achieve stable printing, the ratio of the applied voltage to the nozzle-substrate distance should be kept in a reasonable range. At 3 mm, EHDP could avoid the whipping instability and realize the direct writing of straight micro-/nano-fibers. With the increase of this distance to 3.5 mm and above, the serpentine structures are observed on the substrate.

At 3 mm, the deposited fiber patterns on the substrate may vary with the applied voltage as shown in Figure 4. When the applied voltage is about 2.6–3 kV, the straight fiber can be collected on the substrate. When the applied voltage reaches to 3.2 kV, the fiber patterns change to serpentine structure. The amplitude of the serpentine structure increases significantly at 3.4 kV applied voltage. Through adjusting the key process parameters,

we can manipulate the solidified electrospun fiber flying in a stabilized helical manner. Thus, versatile serpentine structures can be direct written on a moving substrate with diverse amplitude and cycles.

3.3.2 Effect of the SS

We conduct several tests to examine the effect of the SS (100–300 mm/s) on Taylor cone and jet. The mechanical drawing force acting on the jetting increases with the SS. This leads to an increasing degree of slant jet and its elongation rate and eventually generates diverse trajectory. When the SS is greater than the downward speed of the jet, the fiber length deposited on the substrate is larger than the jet length fallen on substrate per unit time^[13].

As shown in Figure 5, the EHDP jet falls down almost vertically and buckles in some degree near the substrate at the SS range of 50–150 mm/s. The jet evolves into a compressed “heel” shape (Figure 5A and B) and becomes unstable with periodically meandering. Thus, a lower SS may lead to a series of bifurcations, such as alternating loops, and translated coiling on the substrates as shown in Figure 2. This jet buckling effect is less obvious when the SS is above 200 mm/s, and straight fibers can be observed on the substrate.

4. CNN in EHDP Cone Modes' Classification

Conventional machine learning algorithms such as support vector (v) machines have limited capability to process natural data (such as the pixel values of an image). Constructing a pattern recognition system requires considerable domain expertise to design a feature extractor which transforms these data into suitable features for these learning algorithms. Applying these algorithms in EHDP cone mode classification need to go through both feature extraction and classification processes in a separated way, which inevitably lead to information loss. Furthermore, a feature selection process



Figure 4. Effect of the applied voltage on the deposited fiber patterns (65 wt/v% polycaprolactone, stage speed = 150 mm/s, D = 3 mm). (A) 2.6–3 kV. (B) 3.2 kV. (C) 3.4 kV

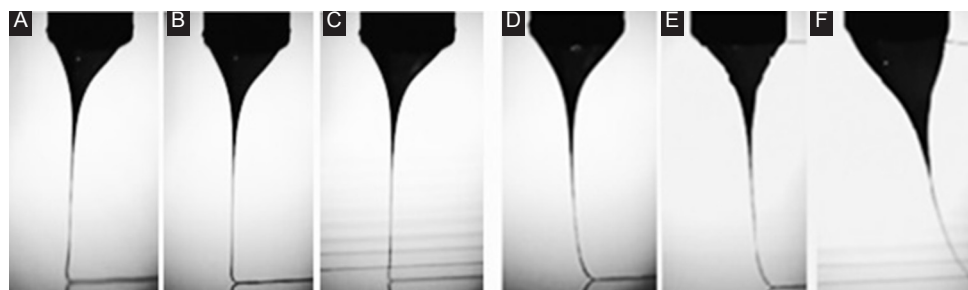


Figure 5. Electrohydrodynamic printing jet under varied stage speed (65 wt/v% polycaprolactone, V = 3 kV) (A) 50 mm/s; (B) 100 mm/s; (C) 150 mm/s; (D) 200 mm/s; (E) 250 mm/s; (F) 300 mm/s

is essential to decide which features should be used as the inputs of the learning algorithms^[14].

In the past decade, CNN is making major advances by allowing a learning algorithm to be fed with natural data and to automatically discover the representations needed for detection or classification^[15]. CNN is widely used in image classifications and considered as a dominant approach for many recognition and detection applications^[16]. A CNN model gets its output by conducting convolutional on an input image data and a wt/v, updating the wt/v iteratively by back propagation, and finally ending up with an optimal network for classification applications^[17,18].

In this study, CNN is used to classify the microscopic images into eight categories as shown in Table 2, including a standard cone mode (stable cone-jet mode) and seven other categories. The EHDP cone categories are usually characterized by the geometrical form of the droplets at the nozzle tip, the breakup mechanisms, and the type of instabilities^[19]. Majority of researchers use the stable cone-jet mode for biopolymer scaffold fabrication. Such mode is known to exist only for a limited window, where the applied voltage is just above the lowest voltage for stable printing and the FR is above a minimum threshold necessary to sustain a steady jet.

It is quite hard to describe the physical conditions for each mode using EHDP process parameters, since they are significantly affected by the properties of biopolymer solutions. The causes for spontaneous mode switch mainly come from the change of the electric field strength in fiber layer stacking process, solution impurities or

precipitation, variation of process parameters, and environmental factors.

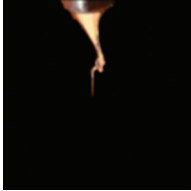
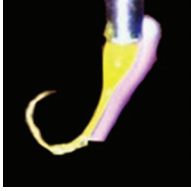
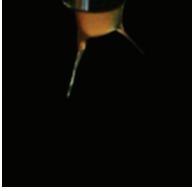
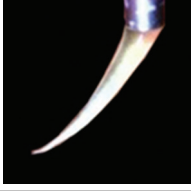
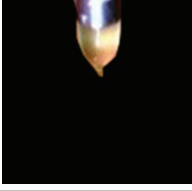
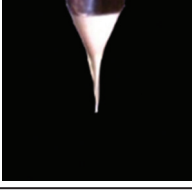
Under the same fabrication process parameters, the cone shape would shift from standard to meniscus if the PCL ink concentration increases from 70 wt/v% to 80 wt/v% and above. The larger cone in the meniscus mode is due to higher surface tension caused by higher PCL concentration. For 70 wt/v% PCL solution, the cone shape can evolve from meniscus, standard, and tiny to multijet when the applied voltage increases from 2 to 5 kV while keeping other EHDP process parameters constant. Besides, the cone shape would be tiny or huge when the solution FR is low (FR < 0.5 $\mu\text{L}/\text{min}$) or high (FR > 1.2 $\mu\text{L}/\text{min}$). Moreover, the cone would break when the solution feeding rate is too low or the SS is too fast (SS > 250 mm/s). Environmental parameters such as temperature and humidity have distinct effects on the cone modes. The cone jet may become dry or continuous discharge when the relative humidity is < 50%, since the lower humidity speedup the evaporation rate of PCL/zein composite solutions^[20].

4.1 Applying CNN for EHDP Cone Modes' Classification

The proposed CNN model can perform feature extraction and classification in a unified framework. Compared with traditional machine learning methods (such as support vector machines), the proposed CNN is a better option for the mentioned application.

We have tried different CNN layer structures in the training and explored a tradeoff between the training

Table 2. Categories of Taylor cone modes

Cone shape	Characteristics	Typical image	Cone shape	Characteristics	Typical image
Broken	Cone broken due to faster SS or jet discharge		Tiny	Cone length/width ratio: 0.5–0.9	
Discharge	Discharge cone due to high conductivity of solution		Multijet	Multiple unstable jet at the end of cone	
Dry	Semi-solidified cone due to low humidity		Meniscus	Meniscus cone shape	
Huge	Cone length/width ratio ≥ 2.0		Standard	Cone length/width ratio: 1.2–1.6	

time consumption and the model accuracy in testing. The classification accuracies of CNN models are reported for varying convolution layer depth from 1 to 8 and increasing the number of convolution layers from 1 to 2 results a performance boost. Thus, the CNN model with two convolutional layers, two fully-connected (FC) layers, and a softmax layer is proposed. Every convolutional layer comes with a max-pooling layer and a normalization layer. The images are processed into the same size by cropping or padding. The output of the CNN model is the eight-category classification. Cross entropy defined as equation (1) is used to evaluate the loss for this CNN model.

$$H_y(y) = -\sum_i y_i' \log(y_i) \quad (1)$$

Where, refers to the i^{th} labeled value and y_i refers to i^{th} output of the softmax layer.

The wt/v is initialized using Gaussian distribution and then optimized by AdamOptimizer using gradient descent method. The Adam optimizer optimizes the wt/v in every layer so as to improve the traditional gradient descent and promote the dynamic adjustment of the wt/v. We use TensorFlow developed by Google^[21], as the framework to build the CNN model for this application.

4.2 Data Set Preparation

In total, 5000 image samples are randomly divided into three sets: A training set (4000 samples), a validation set (500 samples), and a test set (500 samples). Uniformly distributed samples from each category are used in both training and testing dataset. To avoid overfit, both cross-validation and data argumentation are implemented to ensure the high accuracy in both training and testing. The data argumentation can generate more training examples by deformation such as rotation and translation. Besides, a regularization technique called dropout is used at the end of FC layers to randomly drop neurons units with 50% probability during training to avoid overfitting^[22].

4.3 Training and Testing Results

We evaluate the performance of CNN models using both accuracy and training time. In each step of training, a batch of 16 or 32 images from each category is input into the CNN for performance comparison. The training is conducted with a total of 10000 steps. The CNN model trained with the smaller batch size (16) is chosen due to better performance in terms of testing accuracy and training time consumption. We also vary the size of training samples from 500, 1000, 2000 to

Table 3. The accuracy of predicted classes

Classification category	Fine	Broken	Discharge	Dry	Huge	Tiny	Multi	Meniscus
Accuracy	93.7	95.1	90.6	95.1	95.7	93.8	95.3	96.5

4000, and the training time increases significantly with the size of training samples. The corresponding testing accuracy is 88.9%, 91.3%, 92.3%, and 94.7%, respectively. The CNN models with larger size of training data have some improvement on the testing accuracy. With the training data size of 4000 samples, the accuracy of the trained CNN model under each category is shown in Table 3.

To have better classification in EHDP real-time monitoring, continuous 15 frames of the microscope images are used for classification and the cone mode is finalized by the maximum vote. This analysis would facilitate the development of EHDP process control algorithms at the next stage, which could regulate the applied voltage and the printing SS from run to run, for more reliable and viable manufacturing.

5. Conclusion

EHDP has recently drawn great attention for its capability to print micron to nano scaffolds using biopolymer solutions. This great potential motivates researchers to devote intensive efforts on the development of EHDP monitoring and control system. In the paper, we collect digital microscopic images to monitor EHDP scaffold fabrication process. The captured microscopic images are used to evaluate the influence of the key process parameters on the cone shape and jet by features such as centroid and jet diameter. A CNN identification model is developed to classify EHDP cone modes into eight categories with good classification accuracy and low computational time. While, besides the information derived from the microscopic images, material properties such as conductivity or solvent evaporation rate should also be taken into account to identify the EHDP process. With the aid of advanced control techniques, we may regulate the EHDP key process parameters to achieve a more viable manufacturing process in near future. These efforts would eventually translate EHDP fabrication from a research stage to the commercially viable manufacturing process.

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RESEARCH ARTICLE

Optimization of a 3D bioprinting process using ultrashort peptide bioinks

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Abstract: The field of three-dimensional (3D) bioprinting is rapidly emerging as an additive manufacturing method for tissue and organ fabrication. The demand for tissues and organ transplants is ever increasing, although donors are not as readily available. Consequently, tissue engineering is gaining much attention to alleviate this problem. The process of achieving well-structured 3D bioprinted constructs using hydrogel bioinks depends on symmetrical precision, regulated flow rates, and viability of cells. Even with the mentioned parameters optimized, the printed structures need additional refining by removing excessive liquids, as peptide hydrogel bioprints encapsulate water. However, it is challenging to eliminate the confined fluids without compromising the printing process. In this paper, we introduced a vacuum system to our 3D bioprinting robotic arm and thus optimized the printing quality for complex and refined 3D scaffolds. Moreover, the proposed vacuum system supports printing with cells. Our results show improved printing resolution which facilitates the printing of higher and more stable structures.

Keywords: Three-dimensional bioprinting; ultrashort peptides; biomaterials; bioinks; tissue engineering; vacuum system

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1. Introduction

On average, 20 people die every day in the US alone while waiting for organ transplants^[1]. As this number continues to grow and more patients are added to the waiting lists, biologists and biomedical engineers struggle to find a stable solution that can be cost-effective and suitable for tissue and organ fabrication. Research is rapidly growing in the field of tissue engineering as an alternative solution to tissue and organ transplantation, with a particular focus on three-dimensional (3D) bioprinting^[1].

3D bioprinting is gaining much attention because of its potential to resolve issues that occur with classical tissue engineering^[2]. Although tissue engineering has been

effective for the regeneration of several types of organs, tissue-engineered scaffolds do not entirely mimic the native tissue and lack the intricate details of its structure. Furthermore, the use of organic solvents in the process of tissue fabrication may negatively affect cell growth^[3]. Consequently, a valuable advantage of 3D bioprinting is the possibility of whole organ fabrication. With the help of computer-aided design techniques, models can be designed and generated to closely mimic organ structures^[4]. Additional advantages include rapid prototyping, high precision, high resolution, and computer-automated control^[5].

Inkjet bioprinting is quite similar to conventional paper printing. Bioinks are stored in cartridges and acoustic or

thermal waves, generated by air bubbles or piezoelectric actuators, which are used for dispensation^[3,5].

Extrusion-based bioprinting involves a linear moving extruder and stage unit which moves across the X-Y-Z axes. Bioinks are extruded through nozzles using microfluidic pumps, pneumatic pressure, or solenoid control^[5].

Laser-assisted bioprinting uses laser beams to print at a cell resolution^[5]. It is becoming widely popular due to its high precision. An additional advantage is that it does not require a nozzle, which eliminates issues of clogging^[3].

However, for complex structures, recent research indicates the advantages in robotic 3D printing as compared to linear printing. This approach allows for a minimum of six degrees of freedom, providing much more precision, flexibility, and speed. It has the potential to provide scaffold-free printing, precise tissue dispensation, and better scalability of organ fabrication^[6]. Our system adopts the approach of robotic 3D bioprinting to be more compact, versatile, and achieve a higher level of accuracy while being cost-effective.

However, several challenges need to be overcome before this technology will reach full implementation and commercialization. The complexity to merge tissue engineering processes with an automated printing mechanism involves multiple factors including print quality, vascularization, cell viability, mechanical strength of scaffolds, and surface topography^[1,7].

Another area of concern is the durability of bioinks. Biomaterials are assessed based on printability, cell compatibility, and mechanical properties^[7]. Natural and synthetic polymers are commonly used for bioprinting. Some natural polymers include alginate, collagen, and fibrin. Synthetic polymers, such as polyethylene glycol and poly(L-lactic acid), are also used as bioinks^[3].

In our proposed robotic 3D bioprinting system, we investigate ultrashort self-assembling peptides which have proven to be promising biomaterials for tissue engineering applications. These peptides are composed of only four natural amino acids which can easily be synthesized by solid phase peptide synthesis. Due to their amphiphilic character and their innate tendency to self-assemble in water, they form rapidly nanofibrous scaffolds in an aqueous solution in forms of soft solid and transparent hydrogels. The natural but synthetic character of these self-assembling peptides renders them as appealing bioinks for bioprinting^[8]. Peptides are generally known for their biocompatibility, biodegradability, and suitability for cell growth^[9]. However, one challenge of using peptides as bioinks is their low viscosity. As peptide hydrogels retain high amounts of water, the extrusion system tends to accumulate water at the base of the construct while printing which weakens the printed structure and increases the chance of collapse over time^[10].

Aiming to benefit from the biological properties of the ultrashort peptides and to combat its unstable mechanical properties, we propose introducing a vacuum system into the 3D bioprinting process. The vacuum system, placed under the print bed, will allow the excess water to be drained and leave the refined structure intact. Aspect biosystems have implemented a similar technology in their RX1 Bioprinter^[5]. This paper will assess the effect of a vacuum mechanism in optimizing the robotic 3D bioprinter to achieve better printing results.

2. Materials and Methods

The components of the bioprinter system include the 3D bioprinting robotic arm, our custom-designed coaxial nozzle, three syringe pumps, and the vacuum mechanism. The experimental setup is shown in Figure 1a. A vacuum pump with a maximum pressure of -0.35 bar was fitted with tubings and attached to the hose barb of a vacuum flask. A 5-mm suction cup was placed on a rubber stopper. Then, a PET track-etched cell culture membrane with a pore size of $0.4\ \mu\text{m}$ was placed on the suction cup to serve as the printing surface and to allow the excess of water to penetrate through the membrane and into the flask (Figure 1b).

For the bioprinting process, three fresh solutions were prepared which later made up the peptide hydrogel. A solution of $10 \times$ phosphate buffer was loaded into syringe 1. Serum-free medium was loaded into syringe 2. The peptide powder was weighed out in a ratio of $15\ \text{mg/mL}$ and loaded into syringe 3. The bioprinting process has been discussed in more detail in another publication (Rauf, 2018, submitted).

Cellular viability is an essential factor in the tissue engineering process. In this experiment, neonatal human dermal fibroblasts (HDFn) were used. The cells were first cultured to reach the desired cell number. After centrifugation, cell pellets were transferred into a tube of approximately $500\ \mu\text{m}$. The rate of the seeded cells ranged from 1.46×10^7 to 1.6×10^7 cells. The cells were then added into a $1\ \text{mL}$ solution of serum-free medium. Subsequently, the mixture of cells and medium was

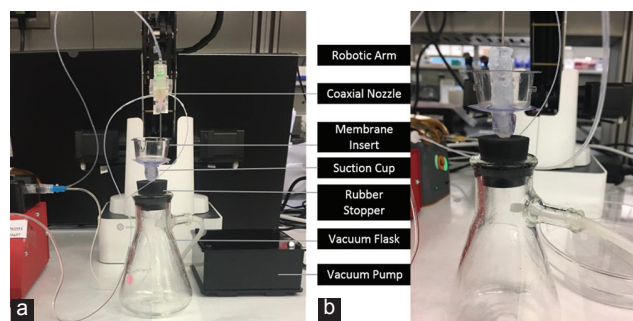


Figure 1. Setup of vacuum mechanism in three-dimensional bioprinting system. Elements of vacuum mechanism (a) A close up of peptide printing with vacuum mechanism (b).

loaded into a microfluidics tube which fed the liquid into the coaxial nozzle. Similarly, another 1 mL of serum-free medium was loaded into a syringe which helped to dispense the preloaded cells from the nozzle.

After completing the bioprinting process, the printed cylinder was submerged with complete medium and then incubated at 37°C, 95% humidity, and 5% CO₂. Consequently, the cylinder was cultured for 2 weeks, to enable the cells to grow and to stretch in all directions (Figure 2a-d).

A cylindrical structure was designed using the CAD software and then transformed into a g-code through a slicing software with dimensions of 10 mm × 40 mm. For comparison reasons, constructs were printed without the vacuum mechanism. Several tests were performed with and without the vacuum mechanism to confirm the observations.

3. Results

Two cylindrical constructs with dimensions of 10 mm × 10 mm × 10 mm were printed. Figure 3a shows how a sample is printed directly onto a Petri dish without applying the vacuum mechanism. Figure 3b shows how a scaffold is printed on a 0.4 µm membrane installed on the flask vacuum mechanism. The images clearly prove that the applied vacuum function significantly reduced the excess water which was pooling at the bottom of the construct (Figure 3a).

The ability to remove excess water while printing with a vacuum system allowed us to print taller scaffold constructs of up to 40 mm, as shown in Figure 4.

The 3D bioprinting system was also tested for cell viability. The HDFn cells were pumped into the nozzle and mixed with the peptide hydrogel on extrusion. Figure 2 shows the results of a 3D bioprinted ring construct after 2 weeks of cell culture. The cells were found to be distributed throughout the ring. They were stretched and were connected in all directions, indicating healthy growth and confirming cell viability during the bioprinting process.

4. Discussion

The experiments which we conducted to test the presence and function of a vacuum mechanism during the 3D bioprinting process were very successful in generating stably printed peptide scaffolds. Adding the vacuum mechanism increased the resolution of printed constructs and made the bioprinting process more facile and stable. Previously, the user would manually remove the excess of water during the printing process using tissue wipes. This step has now been completely eliminated by introducing the vacuum mechanism.

Furthermore, the ability to systematically remove excess water allowed us to print taller constructs. Without the

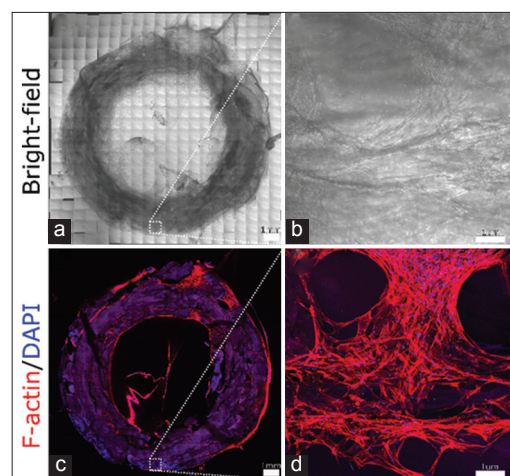


Figure 2. Three-dimensional (3D) bioprinted human dermal fibroblasts (HDFn) after 2 weeks of culture. Whole-mount tile scanning of the 3D bioprinted HDFn ring construct in bright-field (a) and fluorescent F-actin/DAPI staining (c). HDFn cells are distributed throughout the entire ring. Scale bars, 1 mm. Zoom-in of the square in bright-field (b) and fluorescent F-actin/DAPI staining (d). HDFn cells are stretched, interconnected, and grown in all directions. Scale bars are 100 µm.

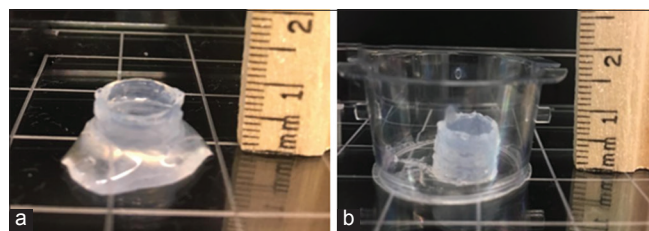


Figure 3. Cylindrical constructs printed with three-dimensional bioprinter. A cylindrical construct of height 10 mm printed without vacuum (a) A cylindrical construct with height 10 mm printed with vacuum (b).

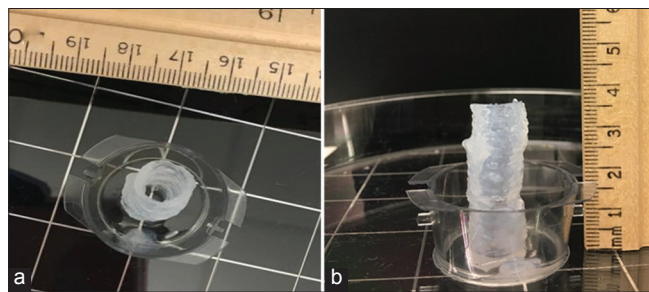


Figure 4. Cylindrical constructs of height 40 mm. Side view (a) Top view (b).

vacuum system, the tallest constructs printed were about 20 mm. The vacuum mechanism allowed us to double the height of the cylindrical structure up to 40 mm, and it is anticipated to fabricate even higher structures. This

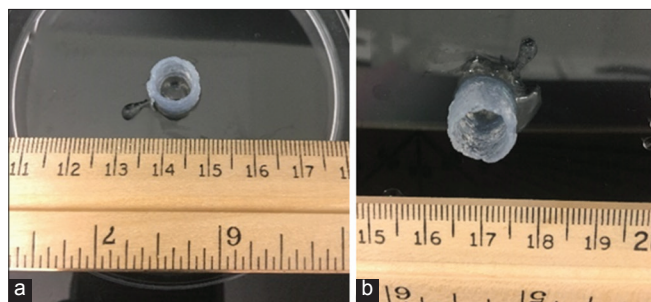


Figure 5. (a) Top view of the printed structure on 1st day, (b) top view of the same print after 7 days.

optimization of the system will be crucial for the printing of more complex structures involving curvatures and structures with finer details.

Prior the newly introduced vacuum system, the printed structures encapsulated liquid between the layers, which created a challenge to remove the water without injuring the print. Over time, the water which had been entrapped by the fiber network would be released, which then weakened the integrity of the printed structure and resulted in an overall decrease in its size Figure 5. Thus, optimizing the process by incorporating the vacuum system allowed the printed construct to keep its shape over a much longer period of time (several months).

5. Conclusion

Our investigation regarding the introduction of an additional vacuum system successfully improved the printability of scaffold when using the robotic 3D bioprinter. By incorporating a vacuum mechanism, the peptide hydrogel produced more refined shapes which is crucial for bioprinting precision. Our experiments were successful in printing 40-mm cylindrical structures with decreased water content, allowing the structure to hold firmly in place. Our results confirm that a vacuum system must be incorporated into the 3D bioprinting system to facilitate printing of more complex structures with a prolonged half-life.

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Authors' Contributions

C.A.E.H guided and supervised the project. S.R. designed and supervised the experiments. K.K. and Z.K. conducted the experiments and wrote the manuscript.

G.R.C did microscopic imaging and N. P. supported the experimental set-up.

Conflicts of Interest

The authors declare that they do not have any competing interests.

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RESEARCH ARTICLE

Hybrid polycaprolactone/hydrogel scaffold fabrication and in-process plasma treatment using PABS

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Abstract: A challenge for tissue engineering is to produce synthetic scaffolds of adequate chemical, physical, and biological cues effectively. Due to the hydrophobicity of the commonly used synthetic polymers, the printed scaffolds are limited in cell-seeding and proliferation efficiency. Furthermore, non-uniform cell distribution along the scaffolds with rare cell attachment in the core region is a common problem. There are no available commercial systems able to produce multi-type material and gradient scaffolds which could mimic the nature tissues. This paper describes a plasma-assisted bio-extrusion system (PABS) to overcome the above limitations and capable of producing functional-gradient scaffolds; it comprises pressure-assisted and screw-assisted extruders and plasma jets. A hybrid scaffold consisting of synthetic biopolymer and natural hybrid hydrogel alginate-gelatin (Alg-Gel) methacrylate anhydride, and full-layer N₂ plasma modification scaffolds were produced using PABS. Water contact angle and *in vitro* biological tests confirm that the plasma modification alters the hydrophilicity properties of synthetic polymers and promotes proliferation of cells, leading to homogeneous cell colonization. The results confirm the printing capability for soft hard material integration of PABS and suggest that it is promising for producing functional gradient scaffolds of biomaterials.

Keywords: Tissue engineering; hybrid scaffold; PABS; in-process plasma modification; functional gradient scaffold

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1. Introduction

Tissue engineering is promising for organ replacement which minimizes the side effects of organ transplantation^[1,2]. Biomanufacturing is the major strategy of tissue engineering aiming at the development of biological substitutes that restore, maintain, or improve tissue function, and it requires the combined use of additive manufacturing (AM), biocompatible and biodegradable materials, cells, and biomolecular signals^[3].

The scaffolds-based strategies (Figure 1) for tissue engineering have been most commonly used, depending

strongly on materials and manufacturing processes. For materials, five types of biomaterials have been used: Acellular tissue matrices, synthetic polymers, natural polymers, ceramics, and polymer/ceramic composites^[4-8]. The most commonly used biomaterial for producing scaffolds are synthetic polymers, such as polycaprolactone (PCL). Polymeric scaffolds play a pivotal role in tissue engineering through cell seeding, proliferation, and new tissue formation in three dimensions (3D), showing great promise in the research of engineering a variety of tissues. Moreover, scaffolds made from collagen are being rapidly replaced with ultraporous

Hybrid polycaprolactone/hydrogel scaffold fabrication and in-process plasma treatment using PABS

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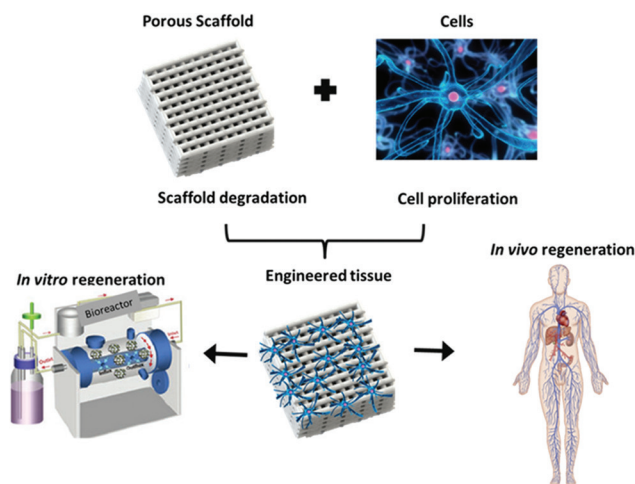


Figure 1. Scaffold-based approach for tissue engineering.

scaffolds from biodegradable polymers. Biodegradable polymers are attractive candidates for scaffolding materials because they degrade as the new tissues are formed, eventually leaving nothing foreign to the body. The major challenges in scaffold manufacturing lie in the design and fabrication of customizable biodegradable constructs with properties that promote cell adhesion and cell porosity, along with sufficient mechanical properties that match the host tissue, with predictable degradation rate and biocompatibility^[9,10].

For the fabrication methods, AM techniques have been commonly applied in scaffold fabrication due to the superior ability in controlling pore size, pore shape, and pore distribution, and thus creating interconnected porous structures^[3,11]. When combined with clinical imaging data, these fabrication techniques can be used to produce constructs that are customized to the shape of the defect or injury^[12]. In terms of tissue and organ manufacturing, the additive nature ensures minimal waste of scarce and expensive building material, namely cells, growth factors, and biomaterials^[13-15]. Among the AM techniques, material extrusion has been mostly applied in the bioengineering field due to the flexibility in material selection based on the use of pneumatic^[16,17], piston^[18], and screw-assisted^[19-21] extrusion systems enabling a wider range of materials to be applied. Some processes operate at room temperature, thus allowing for cell encapsulation and biomolecule incorporation without significantly affecting viability. However, cell-seeding and proliferation efficiency is currently a big challenge due to the following limitations^[22-25]:

- Most AM techniques are limited to single-material fabrication, which is difficult to provide an appropriate environment for cells due to the inadequate chemical, physical, and biological cues provided during AM processes.
- In addition, non-uniform cell distribution, especially

rare cell adhesion in the core region of scaffolds, is often caused by the tortuosity of the constructs.

- Moreover, the synthetic biopolymers, most commonly used, are hydrophobic, and the cell colonization.

Different strategies have been explored to solve the above problems. Multi-material has been developed and utilized to produce multiple-material scaffolds^[16,26]. However, most of these systems can only form one type of biomaterials, either soft hydrogels containing cells or bio-signals in the scale of KPa or rigid biopolymers and composites in the scale of MPa, which fails in the mimicry of natural tissues. In addition, low-temperature plasma modification is capable of improving the hydrophilicity of biopolymers by inducing certain functional groups on the surface to change the chemistry, wettability, and energy without altering the bulk properties^[27,28]. However, most plasma treatment can be conducted after scaffolds printed and the penetration depth is limited, which results in non-uniform cell distribution along the scaffold.

A novel plasma-assisted bioprinting system (PABS) has been developed in the University of Manchester, allowing processing soft hard biomaterial integration and plasma surface modification layer by layer during the fabrication process in the same chamber. This paper utilized the plasma-assisted bio-extrusion system (PABS) to produce PCL/Hydrogel hybrid scaffolds and plasma fully treated scaffolds. The hydrogel is assessed with the preparation process, functionalization process, and rheology properties, while the PCL fully treated scaffolds are both morphologically and biologically assessed.

2. Materials and Methods

2.1. PCL

PCL (CAPATM6500, Mw = 50,000 g/mol), purchased from Perstorp (Cheshire, UK) in the form of 3 mm pellets, was used to produce the scaffolds. PCL is an easy-to-process semi-crystalline polymer with a density of 1.1 g/cm³, a melting temperature between 58 and 60°C, and a glass transition temperature of -60°C.

2.2. Hybrid Hydrogel Methacrylate Anhydride (Alg-Gel) Ma Preparation

Hybrid hydrogel system platform was considered in this paper, which was made of mixing alginate methacrylate and gelatin methacrylate at 50:50% v/v.

Functionalization process for both polymers (alginate and gelatin) is necessary to introduce the carbon-carbon double bond into the polymer chains that eventually convert the polymer into the photopolymerized polymer. According to published protocols^[29], alginate was functionalized with methacrylate groups with minor modifications to be photopolymerized. The 2% wt. powder-type alginate (Sigma-Aldrich, UK) was

dissolved in Dulbecco's phosphate-buffered saline (DPBS) (Sigma-Aldrich, UK), and then mixed with MA (Sigma-Aldrich, UK) at 23% v/v of alginate solution under vigorous stirring. The pH of the solution was kept around 7.4 during the reaction time by the addition of 5M NaOH. The reaction time was 24 h. After the chemical modification, the polymer solution was precipitated with cold ethanol, dried in an oven overnight at 45°C and purified through dialysis for 6 days. The solution was then frozen at -80°C and recovered by lyophilization.

Gelatin was functionalized by dissolving gelatin bovine skin type B (Sigma-Aldrich, UK) at a concentration of 12.5%, in DPBS (Sigma-Aldrich, UK) at a temperature of 45°C. After gelatin dissolution, MA ($\times 10$ molar excess) was added under vigorous. The pH of the solution was kept around 7.0–8.0 during the reaction. The reaction time was 6 h. The solution was purified through dialysis for 7 days, frozen at -80°C and the polymer recovered by lyophilization.

2.3. Plasma-assisted Bio-extrusion System Set Up

Scaffolds were produced using a hybrid AM system called PABS, being developed at the University of Manchester. PABS (Figure 2A) comprises two main units, a multi-extrusion unit and a three-inlet plasma modification unit (Figure 2B). The multi-extrusion unit consists of two pressure-assisted extruders and one screw-assisted extruder, allowing operating a range of biomaterials, such as synthetic biopolymers, hydrogels, and biopolymer/ceramic composites. The extrusion unit has four movements: One rotational movement (C1) for selecting the required extrusion heads, a second rotational movement (C2) for driving and controlling the screw rotational speed of the screw-assisted extruder, and two linear movements (X and Y) in the X-Y plane. All these four movements are controlled by stepper motors and CNC drives. The build platform moves in the Z-direction and constitutes the sixth controllable axis. The build platform was fabricated using 7076 aluminum plate (250 mm $\times$ 200 mm $\times$ 7.5 mm) and is attached to the Z-axis rail guide with L-shape support underneath.

The plasma modification unit is mounted on the X-Y platform which is coplanar with the extrusion platform. Both platforms share common cylindrical guide rails in the X-direction. The Y-direction movement (V axis) for the plasma modification unit is independently driven by a stepper motor parallel to the Y-axis of the extrusion unit. A quartz capillary (outside diameter of 7 mm; inner diameter r of 5 mm; and length of 70 mm) with three gas inlets serves as the reaction jet. A tungsten rod (inner diameter of 2 mm) and one copper film (10 mm of width) wrapped around the quartz tube serve as the high-voltage and ground electrodes, respectively. The electrode is connected to a high-voltage DC power supply (applied

voltage of 10 kV; frequency of 50 kHz). The plasma is generated from the top central electrode, expanding to the surrounding air inside and outside the nozzle.

The control system consists of a motion control system, temperature control system, and gas supply control system. The motion control system utilizes a Geo Brick LV motion controller (Delta Tau Data Systems, Inc) to manipulate all motors. The built-in software allows for complete machine logic control, including G code execution. The temperature control system consists of four digital temperature controllers (P.I.D. Omron E5CN), which is used to precisely control the temperature in the extruder heating chamber with polyimide thermos-foil heating elements (Omega Online/Kapton Heaters) and thermocouples (Omega). The gas supply control system includes an air compressor which works as the main gas supply, regulators, and gauges (SMC, UK), which enables pressure on/off control and manual adjustment. Power supply (230V ac, PHOENIX CONTACT) offers 24V output voltage and 40A current for all the control system. Safety considerations were taken into account regarding the electrical parts, including fuses, main circuit breakers, main filter, push buttons and estops (all supplied by OneCall Electronics Company, UK), and circuits. The control software was developed in MATLAB as previously reported and G code files were generated containing all the instructions for the fabrication process^[30].

This configuration enables multi-material dispensing and plasma modification in a sequential mode. The system is able to achieve a maximum linear velocity of 20 mm/s and a resolution of 0.05 mm.

2.4. Scaffolds Printing Strategies

Scaffolds with a cross-section of 10 mm $\times$ 10 mm and a height of 3 mm were fabricated using the single laydown pattern of 0/90° and a filament distance of 1 mm with a pore size of 1 mm (slice thickness of 0.5 mm; heating temperature of 90°C; extrusion screw rotational speed of 15 rpm; and nozzle tip size of 0.5 mm.). The strategies for printing hybrid scaffolds and plasma treated scaffolds are as follows (Figure 3):

- Hybrid PCL/hydrogel scaffolds fabrication (Figure 3A): After the scaffold being printed with a screw-assisted extruder, the extruder selection unit rotated with 120°, and then the hydrogel solution was printed in the pores using the pressure-assisted extruder.
- Full-layer treated scaffolds fabrication (Figure 3B): The N₂ plasma modification was performed after one layer PCL was deposited, and the process was repeated until the last layer of PCL was complete. The treatment was conducted at a pressure of 0.689 bar and a flow rate of 5 L/min. The deposition speed of the plasma jet was 3 mm/s, and each layer was subjected to the plasma treatment for 1 min. The

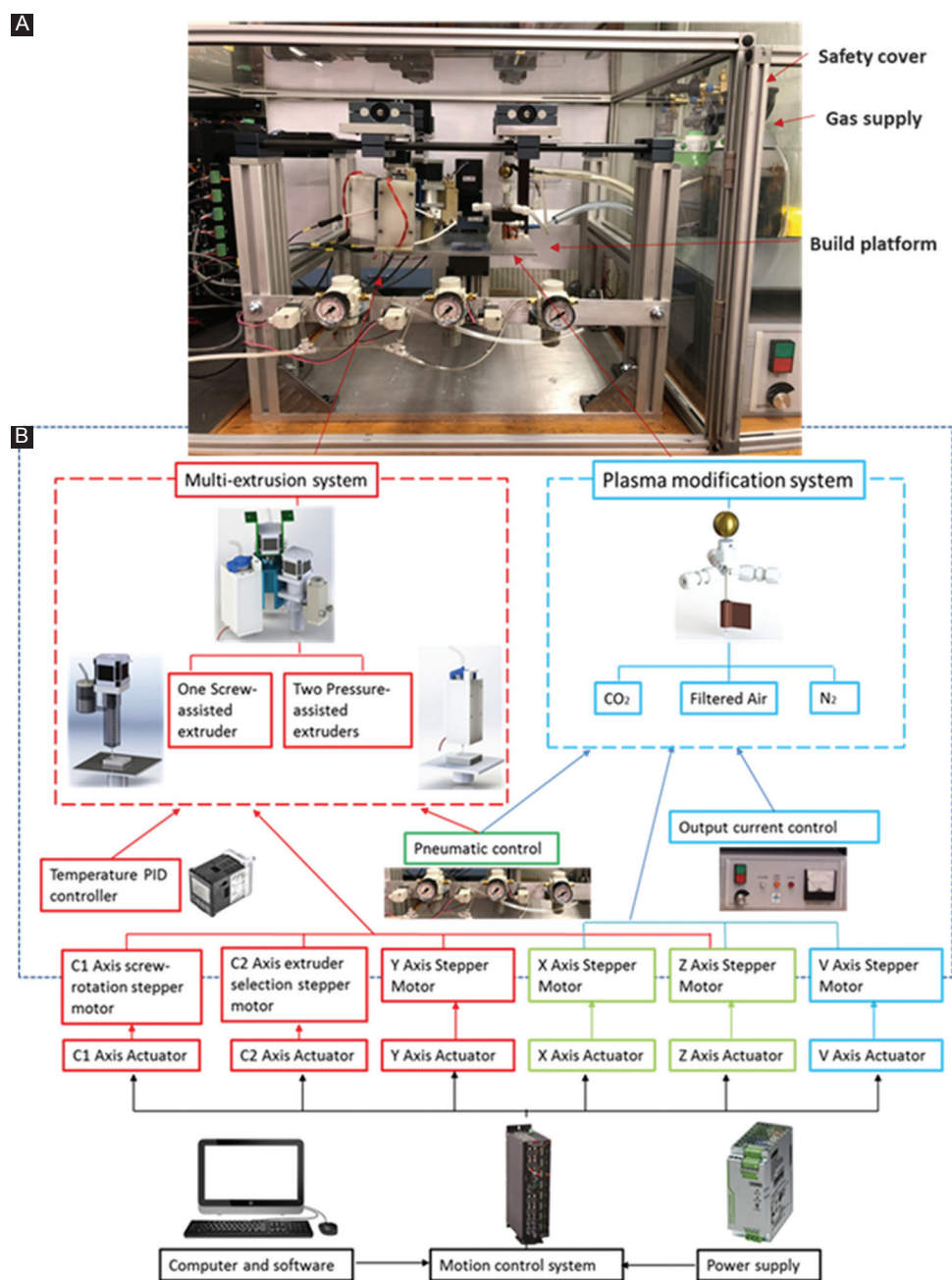


Figure 2. (A) Set-up of the plasma-assisted bio-extrusion system; (B) system diagram: Multi-extrusion system, plasma modification system, and control system.

distance from the bottom of the jet to the surface of the PCL filaments was 10 mm.

2.5. Morphology Characterization

Scanning electron microscopy (SEM) was used to assess the morphology and surface characteristic of printed scaffolds. The scaffolds were gold/palladium coated using a Q150T turbo-pumped sputter coater (Quorum technologies, UK) and imaged at 10 kV (Hitachi S3000N, Japan). These images were then analyzed using ImageJ software.

2.6. Wettability Measurement

Water contact angle (WCA) measurements on the flat surfaces of untreated and plasma-treated PCL scaffolds were carried out with a commercial KSV contact angle measuring 200 systems (KSV Instruments, Finland). The system is equipped with a CCD video camera and a micrometric liquid dispenser to drop 2 μ L of distilled water on the surface of the scaffold. The measurements of the contact angles are automatically calculated with the instrument software.

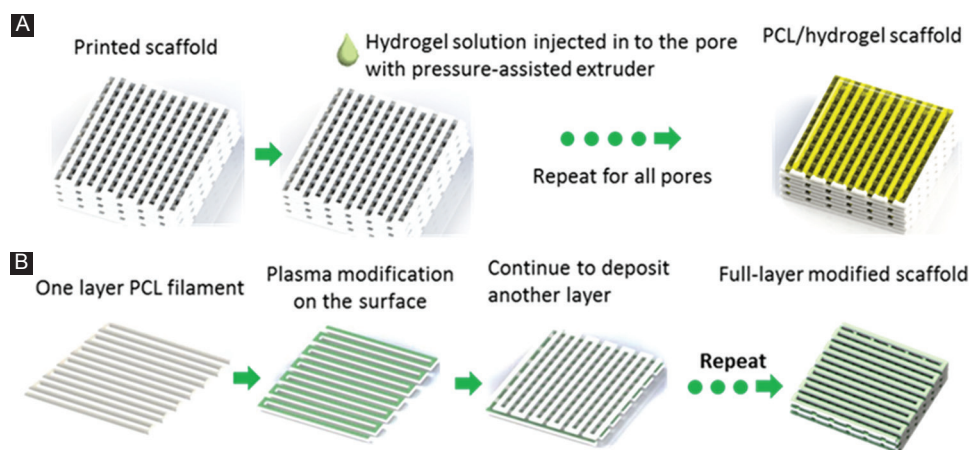


Figure 3. (A) Sequence of operations to fabricate hybrid polycaprolactone/hydrogel scaffolds; (B) sequence of operations to fabricate full-layer plasma-treated scaffolds.

2.7. Biological Tests

In vitro biological assessments were conducted with human adipose-derived stem cells (hADSCs) (STEMPRO, *in vitro* gen, Waltham, MA, USA). Before cell seeding, scaffolds were sterilized by soaking in 70% ethanol for 2 h. After sterilization, samples were rinsed twice in PBS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), transferred to 24-well plates and air-dried for 24 h at room temperature. 50,000 cells were seeded on each sample, including plasma-treated and untreated scaffolds.

Cell viability/proliferation behavior and the percentage of cells attached to the scaffolds (cell-seeding efficiency) were assessed through Alamar Blue assay (also termed the Resazurin assay, reagents from Sigma-Aldrich, UK). Cell viability/proliferation was measured at 1, 3, 7, and 14 days after cell seeding. For each measurement, cell-seeded scaffolds were transferred to a new 24-well plate, and 0.7 ml of Alamar Blue solution was added to each well, the plate was incubated for 4 h under standard condition (37°C, 5% CO₂, and 95% humidity). After incubation, 150 µL of each sample solution was transferred to a 96-well plate and the fluorescence intensity measured at 540 nm excitation wavelength and 590 nm emission wavelength with a spectrophotometer (Sunrise, Tecan, Männedorf, Zurich, Switzerland).

Cell attachment and distribution are assessed using laser confocal microscopy, with cell nuclei stained. At day 1 of cell culture and after 14 days, scaffolds were removed from 24-well cell culture plate, rinsed twice in PBS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), fixed with 10% neutral buffered formalin (Sigma-Aldrich, Dorset, UK) for 30 min at room temperature. After fixation, samples were rinsed twice with PBS for the removal of formalin, then permeabilized with 0.1% Triton-X100 (Sigma-Aldrich, Dorset, UK) in PBS at

room temperature for 10 min, rinse twice for the removal of Triton-X100. Cell nuclei were stained blue by soaking scaffolds in a PBS solution containing 4',6-Diamidine-2'-phenylindole dihydrochloride (Sigma-Aldrich, Dorset, UK) at the manufacturer recommended concentration. Samples were left in the staining solution for 10 min before removal, rinsed twice thoroughly with PBS. Confocal images were obtained using a 3D rendering mode on a Leica TCS SP5 (Leica, Milton Keynes, UK) confocal microscope, and cell colonization was quantified using a standard z-stack method. All images were taken at the center area of the scaffolds, and the experiments were performed three times in triplicate.

2.8. Data Analysis

All data are represented as mean $\pm$ standard deviation. Biological results were subjected to one-way analysis of variance (one-way) and *post hoc* Tukey's test using GraphPad Prism software. Significance levels were set at $P < 0.05$.

3. Results

3.1. Hybrid Scaffold Fabrication

The hybrid scaffold, consisting of PCL and hydrogel solution, was successfully fabricated using PABS. Figure 4A shows the image of the hybrid scaffold, where the porous structures hold the hydrogel solution without any deformation. Figure 4B and c present the top view and side view of the PCL/hydrogel scaffold, showing the position of the deposited hydrogel was precisely controlled and well adjacent with PCL scaffold.

3.2. Full-layer Treated PCL Scaffold

Figure 4B shows that the printed full-layer N₂ plasma treated scaffolds have a well-bonded interconnected

structure with a uniform pore distribution and pore size in the range of $\sim 500 \mu\text{m}$ (Figure 4C). The N_2 plasma treatment was conducted at a pressure of 0.689 bar and a flow rate of 5 L/mm. The deposition speed of the plasma jet was 3 mm/s, and each layer was subjected to the plasma treatment for 1 min. The distance from the bottom of the jet to the surface of the PCL filaments was 10 mm. Figure 4D presents the filament surface after N_2 plasma modification, where lines in the direction of plasma movement can be observed making the surface roughness increased.

3.3. Wettability Assessment of Full-layer Plasma Modified Scaffold

WCA measurements were performed on untreated and fully treated plasma PCL scaffold surfaces to determine the effect of plasma modification on the surface wettability. Table 1 highlights the WCA results at different time points after the droplet was dropped on the surface of the scaffolds. The results show that in the case of an untreated PCL scaffold, there are no significant changes in the WCA values with time with values varying between $83.2 \pm 2.0^\circ$ and $80.9 \pm 2.7^\circ$. For treated scaffolds, the WCA value, at 0 s, was lower ($63.0 \pm 3.1^\circ$), leading to a fully wetting value of $26.7 \pm 0.9^\circ$ at 0.5 s. At 3 s, the droplet was fully absorbed

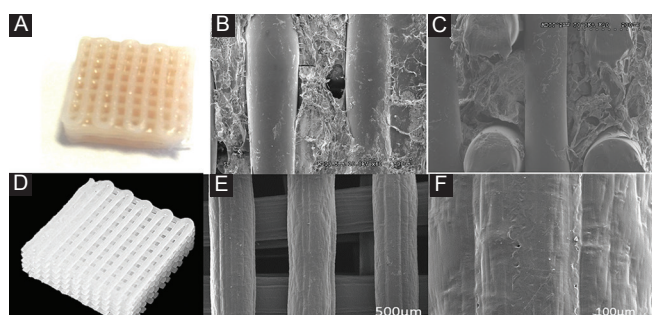


Figure 4. (A) Hybrid polycaprolactone (PCL)/hydrogel scaffold; (B) Scanning electron microscopy (SEM) image of top view of PCL/hydrogel scaffold; (C) SEM image of side view of PCL/hydrogel scaffold; (D) photo of a full-layer N_2 plasma-treated PCL scaffold; (E) SEM image of top view of full-layer N_2 plasma treated PCL scaffold; (F) SEM image of filament surface of full-layer N_2 plasma-treated PCL scaffold.

Table 1. Temporal variation of WCAs for treated and untreated scaffolds

Time	PCL scaffolds	N_2 plasma fully treated
0s	$83.2 \pm 2.0^\circ$	$63.0 \pm 3.1^\circ$
0.5s	$82.9 \pm 1.2^\circ$	$26.7 \pm 0.9^\circ$
3s	$80.9 \pm 2.7^\circ$	Fully absorbed

PCL: Polycaprolactone, WCAs: Water contact angles

3.4. Biological Assessment of Full-layer Plasma Modified Scaffold

The adhesion and proliferation of hADSCs cells on plasma full-layer modified PCL scaffolds were studied and compared with untreated ones. The biological characterization was assessed using Alamar Blue Assay. The fluorescence intensity of cell-seeded scaffolds measured at four different culture time points (Days 1, 3, 7, and 14) is shown in Figure 5A. Higher fluorescence intensity corresponds to more metabolically active cells. As observed, cell proliferation increases with time in all types of scaffolds, suggesting that they are suitable structures for cell attachment and proliferation. However, a fast proliferation rate is observed in the case of plasma-treated scaffolds. The different performance is statistically significant after day 3.

Confocal microscopy images (Figure 5B) present the cell attachment and distribution after cell seeding (day 1) and proliferation (day 14). It can be observed that plasma-treated scaffolds presented higher numbers of cells than untreated scaffolds. In addition, it is also possible to observe that the plasma surface-treated scaffolds presented best cell attachment and dispersion.

4. Discussion

The printed hybrid PCL scaffolds filled with hydrogel present the printability of PBAS, enabling the soft hard material integration. The photo and SEM images of the whole printed structure indicate that the plasma modification process does not affect the physical appearance of the structure and potentially no effect on the mechanical performances^[31]. Moreover, the SEM image of the filament surface confirms the increased surface roughness is due to the etching process^[32], which results in the stripping off the topmost layer of the polymer filament. From this study, the increase surface roughness is also resulted from the linear scratches attributed to the gas flow by the plasma jetting directly after the PCL deposition when the material is still in the molten status. As the printed material is not totally cooled down, when the plasma modification occurs, the gas flow effect is stronger, significantly influencing the surface topography. Moreover, the increase of surface roughness is beneficial for cell colonization in the scaffolds^[33].

The wettability results reveal that the hydrophilicity of the surface is dramatically improved due to the ionizable groups introduced on the surface by the N_2 plasma, which enhances the hydrogel bonding with water. Compared with the results published in^[28,34], the absorption speed is dramatically increased, due to chemical heterogeneity on the surface of each layer due to the plasma modification layer on layer. However, the effect of plasma modification can last within a certain period of time depending

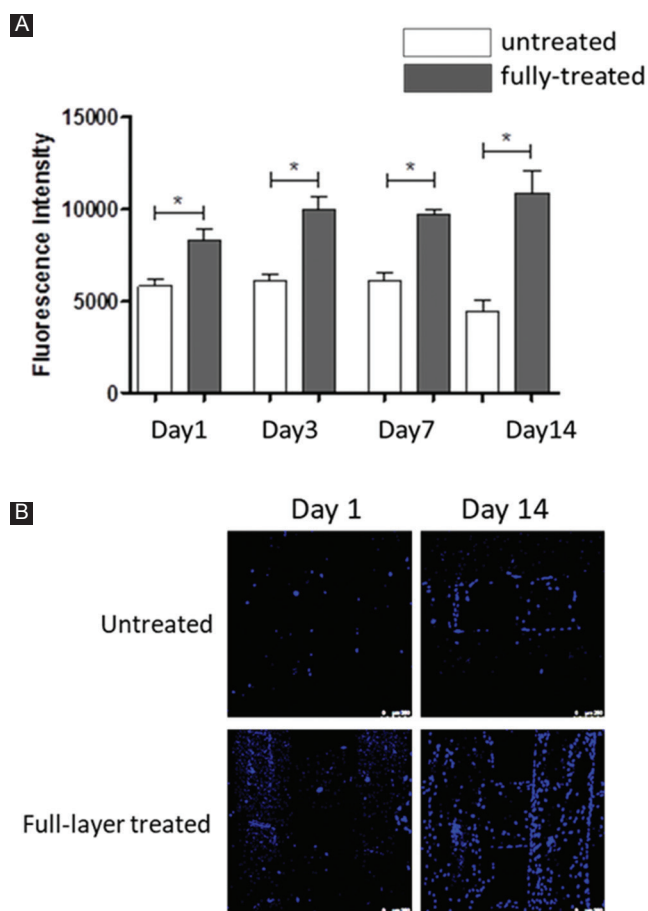


Figure 5. (A) Temporal variation of fluorescence intensity of cell-seeded PCL scaffolds with and without N_2 plasma treatment; (B) confocal microscope images of untreated and full-layer treated of cell-seeded scaffolds, 1 day and 14 days after cell culture. Scale bar 250 μm .

on the density of chemical reagents deposited on the surface, and the effect also decreases with time since the structural rearrangement of polymer chains resulting in the decreased surface energy^[35].

In addition, it is important to control hydrogels printability window (injection, deposition, and extrusion) during the printing process to mimic the native organ structures. To achieve precise printable properties, hydrogels have been designed to achieve adjustable viscoelastic behavior, and the SEM images confirm the liquid-like-gel structure before printing and quick gelation kinetics to produce a structure with good fidelity.

4.1. This Makes it Easy to Inject Hybrid Hydrogel System (Alg-Gel) MA

Consistent with the other studies^[36,37], a significant increase in the biological behavior is observed after plasma modification. Since the hydrophilicity has been improved with the plasma modification process, the environment is changed to be more suitable for cell

colonization and proliferation. Furthermore, with the full-layer plasma modification capability, the plasma active species can reach the walls of the pores on each layer of the scaffold, leading to the uniform cell distribution along the scaffold. This will enable a higher rate of tissue formation in the clinical research.

5. Conclusion

This paper presents a novel AM system comprising a multi-material printing unit and a plasma jet unit. To assess the system, hybrid PCL/hydrogel scaffolds and full-layer plasma treated PCL scaffolds were produced. The effect of plasma treatment on PCL samples was examined. WCA results confirmed that the hydrophilic character of the PCL samples increased due to the nitrogen groups introduced by the plasma jetting on the scaffold filaments. It was also possible to observe that the plasma treatment positively influences cell attachment and proliferation. Applications that may benefit from this technology include hybrid tissue, which has compositional variations depending on the region or organ-like structure that require the continuous vascular network to facilitate nutrient diffusion.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Discovering new 3D bioprinting applications: Analyzing the case of optical tissue phantoms

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Abstract: Optical tissue phantoms enable to mimic the optical properties of biological tissues for biomedical device calibration, new equipment validation, and clinical training for the detection, and treatment of diseases. Unfortunately, current methods for their development present some problems, such as a lack of repeatability in their optical properties. Where the use of three-dimensional (3D) printing or 3D bioprinting could address these issues. This paper aims to evaluate the use of this technology in the development of optical tissue phantoms. A competitive technology intelligence methodology was applied by analyzing Scopus, Web of Science, and patents from January 1, 2000, to July 31, 2018. The main trends regarding methods, materials, and uses, as well as predominant countries, institutions, and journals, were determined. The results revealed that, while 3D printing is already employed (in total, 108 scientific papers and 18 patent families were identified), 3D bioprinting is not yet applied for optical tissue phantoms. Nevertheless, it is expected to have significant growth. This research gives biomedical scientists a new window of opportunity for exploring the use of 3D bioprinting in a new area that may support testing of new equipment and development of techniques for the diagnosis and treatment of diseases.

Keywords: competitive technology intelligence; additive manufacturing; biophotonics; scientometrics; patentometrics

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1. Introduction

Three-dimensional (3D) printing, also known as additive manufacturing^[1], was introduced >30 years ago, in 1984, when Charles Hull invented 3D lithography^[2]. 3D printing is defined by the American Society for Testing and Materials (ASTM) as a “process of joining materials to make parts from 3D model data, usually layer upon layer, as opposed to subtractive manufacturing and formative manufacturing methodologies”^[3]. The process starts with the creation of a 3D model on a computer, after which, it is sent to a 3D printer^[4].

The competitive dynamics of 3D printing may alter the *modus operandi* of multiple industries, and its effects will be observed in the nature of the design of

new products, placing special emphasis on how they will be conceived, manufactured, tested, and applied in many areas, such as the military, automotive industry, electrical device engineering, and medicine^[5]. One of the current technologies employing 3D printing for health care is biofabrication. This term refers to a relatively new process that focuses on the concept of personalized medicine. According to Jürgen *et al.*^[6], it can be defined as “the automated generation of biologically functional products with the structural organization from living cells, bioactive molecules, biomaterials, and cell aggregates such as microtissues or hybrid cell-material constructs, through bioprinting, and subsequent tissue maturation processes.” Under this approach, according to Mandrycky *et al.*^[7], 3D bioprinting is considered a

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“3D fabrication technology used to precisely dispense cell-laden biomaterials for the construction of complex 3D functional living tissues or artificial organs,” using an additive manufacturing strategy by depositing substrates such as living cells, nucleic acids, drug particles, proteins, and other biological components^[8].

Recently, 3D bioprinting has gained significant attention^[9], having wide utility in various areas of medicine, it enables the fabrication of living tissue with precise digital control^[10]. In addition, the interest of academia and industry in 3D bioprinting is growing^[11]. The applications of 3D bioprinting have spread from the development of tissue models for research, drug discovery, and toxicology^[12] to the possibility of developing functional tissues and organs for transplantation^[4]. Specifically, bioprinted models give a better understanding of physiological processes, including the mechanisms that can produce diseases, as well as those that are part of the prevention, detection, and treatment of those illnesses^[13-16]. Some future challenges for 3D bioprinting are related to technical factors, such as improving its resolution and printing speed, in addition to increasing the number of available materials^[12] to better mimic the biological, structural, mechanical, and even optical characteristics of organs and biological tissues^[17].

Biophotonics concerns the study of the interaction of visible light and biological systems^[18]. It is commonly used in medicine to study biological tissue to detect, diagnose, and treat diseases in a minimally invasive or non-invasive way^[19,20]. Biophotonics techniques have been shown to be less harmful than are other biomedical techniques that use ionizing radiation, such as X-rays^[21]. The light-biological tissue interaction is mediated through the optical characteristics of the biological medium. These characteristics are related to how the light travels and propagates through the biological medium (refractive index and scattering coefficient) and how it is absorbed by the medium (absorption coefficient), factors that determine the biological tissues' unique behavior in response to light. To mimic these properties, objects known as optical tissue phantoms have been used in biophotonics research and development (R&D)^[22]. A typical phantom consists of a base material, scattering, and absorber materials, and sometimes contrast enhancement agents, such as fluorophores^[23,24]. Optical tissue phantoms have been used as calibrators aiming to establish global standards for the measurement of biomedical techniques like imaging^[24]. Moreover, they have been utilized for the development of new techniques and prototypes for the diagnosis and treatment of diseases; furthermore, they are suitable for clinical trials, as well as for the improvement, and routine quality control of prototypes^[14-16,23,24,26]. Also, it is important to implement standardized protocols for the development of phantoms because they must present

minimum variation over time in their properties^[27], as any change in the phantom could be interpreted as an alteration in the performance^[25].

Most phantoms that have been used in laboratories are based on materials that do not entirely reflect the optical properties, heterogeneities, or complex, multilayered structures of biological tissues^[27,28]. In addition, the methodologies that have been proposed for their development generally consume a great amount of time, and the optical properties of the produced phantoms present significant variations^[28]. In general terms, phantoms have been developed in a single-layer way, with homogeneous optical properties that do not entirely reflect the complex behavior of biological tissue. Researchers' goal is to build multilayered phantoms with heterogeneities that better mimic the structure of biological tissues^[22]. The most commonly used methods of producing multilayer and heterogeneous phantoms are mold multilayer curing^[30], integration after mold casting, and spin coating^[31].

Not only the methodologies used to create optical tissue phantoms are important but also the materials play a key role. Phantom matrices represent >95% of the total volume of the phantom, and consequently, they have the most significant effect on its applications. Liquid, gelatinous, or solid substances can be used as phantom matrices^[22,32]. These materials are selected according to their properties, including their stability over time^[22]. Gelatinous materials have been shown to be the most viable option for the development of phantoms because they have thermal and mechanical properties that closely match those of biological tissue^[26,29] and their lifetime is longer than that of phantoms composed with liquid substances^[22]. They also allow the integration of a wide variety of substances to simulate both optical and biochemical properties^[22,26,33], making gelatinous materials ideal for biophysical studies and generating complex structures. Gelatins and agarose are commonly used materials that have been adopted in many laboratories since the mid-1990s^[22]. Thereafter, methods for the elaboration of phantoms have usually been carried out by hand.

Further technological advances have occurred, especially in the development of hydrogels, which began to be studied in the late 1990s for their use as phantom matrices^[26]. Hydrogels are biocompatible materials; they resemble tissue extracellular matrix^[34] and replicate some physicochemical properties of biological tissues^[35], and they can be used for drug delivery. This application is extremely attractive for enhancing phototherapies, such as photodynamic therapy^[36-38]. These phototherapies require the application of a substance known as a photosensitizer before irradiation with light for treating various diseases^[39]. Hydrogels can carry diverse types of photosensitizers to the target cells more effectively than the methods that are commonly used at present can^[36]. Among the hydrogels, polyvinyl alcohol (PVA) gel

shows the best performance since its optical scattering properties can be easily modified by adding microspheres or nanoparticles or through freeze-thaw cycles^[33,40,41], as well as for its ease in mimicking heterogeneities that can represent illness or a damaged tissue^[26,40].

Recently, 3D printing and 3D bioprinting have been proposed as methods for the generation of tissue phantoms^[42,43]. In comparison with conventional manufacturing processes, these technologies have multiple advantages, such as a short production cycle and freeform fabrication of objects with complex geometric characteristics and internal structures. Moreover, 3D bioprinting has been used for freeform fabrication of non-homogeneous, multilayered complex structures, such as skin tissue^[43]. It represents a very attractive solution to biomedicine since it allows the creation of layered structures that are similar to biological tissues^[14]. These features make it suitable for fabricating optical tissue phantoms for clinical applications. A window of opportunity can be opened in biophotonics where 3D bioprinting has not been adopted as in other fields^[44]. Here, 3D bioprinting would allow the development of more realistic phantoms.

This paper presents a competitive technology intelligence study of the presence of 3D printing and 3D bioprinting to develop optical tissue phantoms, using a methodology created by Rodríguez-Salvador *et al.*^[11], for identifying the scientific and technological trends regarding methods, materials, and uses, as well as determining the most prolific countries, organizations, and journals in this domain.

2. Methodology

Systematic research on the state-of-the-art and determination of trends and time horizons of 3D printing and 3D bioprinting applied to the manufacturing of optical tissue phantoms were carried out, based on the approach of Rodríguez-Salvador *et al.*^[11]. It consists of iterative processing of information that includes a planning stage, source determination, data gathering, and analysis; every step was supported by expert feedback, as explained below.

2.1 Planning Stage

It consists of an organization process to define the objectives, participants, timing, and specific activities of the study. In this case, the objective was to identify the presence of 3D printing or 3D bioprinting to produce optical tissue phantoms for biophotonics; determining methods, materials, and uses, as well as the most prolific countries, organizations, and journals in this field.

2.2 Data Source Determination

It starts with the identification of primary and secondary sources. The first one is based on the opinion of experts in

the area; in this case, it involves the development of optical tissue phantoms and 3D printing or bioprinting while secondary sources include scientific and technological documents. This research involved scientific papers, conference proceedings, and patents. Web of Science (WoS) and Scopus were analyzed using scientometric tools. Patents were examined using a patentometric platform, PatSeer. This software was designed for research, analysis, and project management and allows access to nearly 120 million records from the main patent offices worldwide^[45].

2.3 Data Gathering

This phase begins with the determination of a search strategy, first by identifying the appropriate terms and keywords, which will ensure that the most relevant information is collected. In this study, the keywords were identified from a state-of-the-art review and with the support of experts in the field. It is important to note that the main query terminology was obtained from the research study of Rodríguez-Salvador *et al.*^[11], where the principal technologies and applications to the health sector of 3D bioprinting were researched; for completing the query, experts were also consulted. Data collection in this study focused on the terms that are applied to the use of 3D printing for developing optical tissue phantoms. After the literature review and expert validation, three main categories were identified, as follows: (i) Terms referring to 3D printing and 3D bioprinting; (ii) those exclusively concerning optical tissue phantoms; and (iii) those involving the applications of biophotonics, which, according to the experts consulted, should be subdivided into diagnosis and phototherapy. The obtained terms were utilized for the development of different search queries, adapted according to the consulted databases. Boolean operators and exclusion terms were used. In addition, the study considered a time interval from 2000 (since 3D printing applications in health care emerged by that time^[46,47]) to 2018 (specifically, until July 31, 2018, as the collection activity ended on that date).

2.4 Analysis

In this research, this step involves the use of scientometric and patentometric tools. First, a manual examination to rule out duplicated and non-relevant documents were carried out. Then, grouping information and a statistical analysis were performed. Expert feedback during this process was essential. Scientometric and patentometric analyses were developed using PatSeer platform and special software programmed in R language, to mine data from the papers that were found.

Using this iterative process and adapting the search query from Rodríguez-Salvador *et al.*^[11], changing and adding suitable keywords, and using Boolean operators,

We design the following query: (((3d) OR (3d)) OR ((three dimensional) OR (threedimensional))) AND ((*print* OR manufactur* OR fabricat*) OR (rapid prototyp*) OR ((layer by layer) OR (layerbylayer))) AND ((optic*) AND (mimic* OR bio* OR simulat* OR tissue) AND (phantom*)) AND (((tissue OR bio* OR diagnos*) AND (diffus* OR reflectance OR fluorescence OR imaging) AND spectroscopy) OR ((phototherapy) OR (photodynamic AND (therapy OR treatment)))) AND NOT (surgic* OR nuclear OR ultrasound OR radiotherapy OR (xray)). The global query was adapted according to each of the databases consulted.

3 Results and Discussion

3.1 Scientometric Analysis

After a detailed examination to rule out non-relevant documents, 81 papers were found in the Scopus database and 58 papers in the WoS database from January 1, 2000, to July 31, 2018. The previously mentioned designed query was adapted for each database. Subsequently, a de-duplication process was performed to find any possible repeated documents between the two databases. In the end, a total of 108 documents were identified.

A research relating to a creation of a 3D optical tissue phantom to analyze epithelial cancer was also found. While

it does not entail the formal definition of 3D bioprinting^[4,6,7], it represents an interesting effort to build biologically functional 3D structures, from the early 2000 s. This paper was authored by Sokolov *et al.*^[48], and its process involves cervical cells embedded in a collagen matrix where blood cells are added later. Furthermore, a layer of epithelial cells is placed on top of the phantom to completely simulate the cervical tissue, even in different stages of cancer.

The results obtained showed the early incursion of 3D printing into developing optical tissue phantoms; however, the presence of 3D bioprinting to create these phantoms was not detected. Tables 1-3 present the most representative articles that were found, ordered by techniques or methods, materials, and applications.

The global results of the scientometric analysis are presented in Figure 1A-1D.

Curve fitting allowed us to find the behavior and trends of the number of publications per year on 3D printed optical tissue phantoms. An exponential regression was performed excluding the data from the year 2018. The obtained equation that describes the data growth is shown in Equation 1, with a value of the coefficient of determination, $R^2 = 0.9527$:

$$y = (2.097 \times 10^{-268}) e^{0.3071x} \quad (3.1)$$

If the growth rate continues with the same behavior, then the total number of papers will be 29 for 2018, and

Table 1. Methods used for 3D printed optical tissue phantoms

Paper	Institution/country	Description
Wang <i>et al.</i> ^[13] “3D printing method for freeform fabrication of optical phantoms simulating heterogeneous biological tissue”	Center for Biomedical Engineering, University of science and technology of China/China	A 3D printing method was developed for the fabrication of tissue-simulating phantoms with a multilayer structure that consists in selectively depositing the phantom materials layer by layer using spin coating. The goal was to develop a skin tissue phantom as a standard for testing biomedical optical devices
Lurie <i>et al.</i> ^[49] “Three-dimensional, distendable bladder phantom for optical coherence tomography and white light cystoscopy”	Department of Electrical Engineering, Stanford University/United States	A new spin coating protocol was developed to mitigate the nonuniformity of 3D model topology. The 3D printed phantom mimics the size, structure, microscale surface topology, and optical properties of a cancerous bladder for performing optical coherence tomography tests

3D: Three-dimensional

Table 2. Materials used for 3D printed optical tissue phantoms

Paper	Institution/country	Description
Zhao <i>et al.</i> ^[16] “3D printing of tissue-simulating phantoms for calibration of biomedical optical devices”	Department of Precision Machinery and Precision Instrumentation, University of Science and Technology of China/China	An optical tissue phantom with mechanical and optical heterogeneities was created using 3D printing. The process uses gel wax polydimethylsiloxane and colorless light-curable ink as matrix materials, titanium dioxide (TiO ₂) powder as the scatterer, and graphite powder and black carbon as absorbers
Kim <i>et al.</i> ^[50] “3D printing-assisted fabrication of double-layered optical tissue phantoms for laser tattoo treatments”	Electrical and Mechanical Engineering, Pukyong National University/Korea	A double-layered phantom made of gelatin and agar as matrix materials and a mixture of TiO ₂ powder as the scatterer and coffee as the absorber was developed. Then, 3D printing for precise control of the thickness of each layer was used
Sangha <i>et al.</i> ^[51] “Adjustable photoacoustic tomography probe improves light delivery and image quality”	Weldon School of Biomedical Engineering, Purdue University/United States	A depth-profiling 3D-printed phantom was created using PVA and polyethylene tubes. The PVA was treated with a freeze-thaw cycle to modify its optical scattering properties. This phantom can be used for acoustic and optical analysis

PVA: Polyvinyl alcohol, 3D: Three-dimensional

Table 3. Uses of 3D printed optical tissue phantoms

Paper	Institution/country	Description
Ghassemi <i>et al.</i> ^[52] “Rapid prototyping of biomimetic vascular phantoms for hyperspectral reflectance imaging”	FDA/United States	The 3D printing of a human retinal vasculature phantom filled with hemoglobin solution was developed. The purpose was applying the phantom in tests of a near-infrared hyperspectral reflectance imaging system for the analysis of the human retina
Bentz <i>et al.</i> ^[53] “3D printed optical phantoms and deep tissue imaging for <i>in vivo</i> applications including oral surgery”	School of Electrical and Computer Engineering, Purdue University/United States	In the study, 3D printed mouse phantoms were created for use in deep fluorescence imaging tests. These phantoms represent an alternative to animal experimentation in these types of tests
Lv <i>et al.</i> ^[54] “Design of a portable phantom device to simulate tissue oxygenation and blood perfusion”	School of Engineering Science, University of Science and Technology of China/China	An optical tissue phantom was fabricated using 3D printing to mimic blood vessels. The phantom was used to calibrate and validate medical optical devices

FDA: Food and drug administration, 3D: Three-dimensional

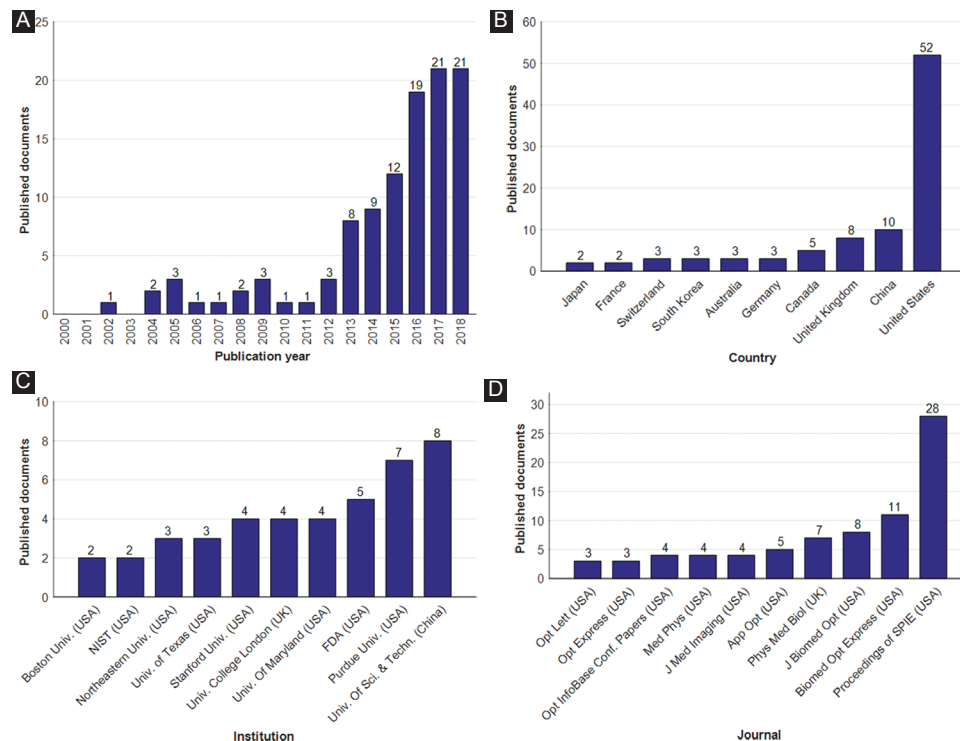


Figure 1. Summary of published papers on 3D printed optical tissue phantoms, which are indexed in both Scopus and Web of Science databases, grouped by (A) publication year, (B) affiliation country, (C) institution of principal author, and (D) journal in which the paper is published. In B-D, only the 10 most frequent papers are shown. Note: National Institute of Standards and Technology (NIST), Food and Drug Administration (FDA).

54 will be published in 2020. As shown in Figure 1A, the number of articles referring to the fabrication of optical tissue phantoms using 3D printing technology has increased since 2013.

Insights exhibit that the United States (52 documents) is the top country for publications on 3D printed optical tissue phantoms, representing 48.15% of all publications, followed by China (10 documents) and the United Kingdom (8 documents). As shown in Figure 1C, while eight of the leading institutions in terms of publications in this field come from the United States, the institution with the highest number of papers in the area is the University of Science and Technology of China (8 documents), followed by Purdue University of

the United States (7 documents). It is interesting to note that, of the top 10 publishing institutions, two belong to the United States government, namely the Food and Drug Administration (FDA), which is responsible for the control and supervision of products related to food and health^[55], and the National Institute of Standards and Technology, which “promotes innovation and competitiveness by advancing measurement science, standards, and technology”^[56]. Since optical tissue phantoms are intended to become standards for the measurement, calibration, and approval of biomedical devices, both institutions are interested in carrying out research in the area. Finally, the results reveal that most publications, 25.93% of all documents, were issued in

the Proceedings of the SPIE, a conference record of the Society of Photo-Optical Instrumentation Engineers. It is also important to note that, of the 108 documents found, 35 are conference proceedings and 73 are journal papers.

An association map among authors, keywords, and journals contained in the documents analyzed was also developed. For this task, a software programmed in R language was created. The map is shown in Figure 2.

Each link between authors, keywords, and journals is represented by a single line. When the number of interactions increases, the lines become thicker. The frequency of each author, keyword, and journal title is also shown, represented by the height of the rectangle placed next to each word. It is observed that the top author is Erbao Dong from the University of Science and Technology of China, with 6 papers published, followed by Shuwei Shen from the same university, Jianting Wang from the FDA (USA) and Brian Z. Bentz from School of Electrical and Computer Engineering of Purdue University (USA) who published 5 papers each. Keywords comprised four categories. The first one refers to phantoms proper, where the following terminology was detected: *Phantoms*, *biomimetic tissue phantoms*, *tissue phantoms*, *optical phantoms*, *tissue-simulating phantoms*, *tissue-mimicking phantoms*, and a special type of phantom, *the biomimetic vascular phantom*. The second category covers methods applied to produce optical tissue phantoms; in this case, the results show that the spin coating is the most frequent process. The third classification focuses on determining optical properties, illustrating that the coefficients related to absorption and scattering and turbid media (a term that describes a material with strong optical scattering and absorption characteristics

like the biological tissues^[57]) predominate. The fourth group covers keywords related to applications of 3D printed optical tissue phantoms. In this regard, it can be observed that phantoms are mainly used in techniques for detecting illnesses, such as biophotonic imaging, hyperspectral reflectance imaging, near-infrared spectroscopy, and optical coherence tomography. In terms of journals, the map shows that the highest number of publications on this topic comes from the proceedings of the SPIE.

Specifically, the use of 3D bioprinting for optical tissue phantoms development was not detected in the documents obtained. Conversely, the use of 3D bioprinting to produce phantoms to be applied in other areas (not for optical applications) is present, especially for ultrasound imaging^[58-63]. Since 2010, these phantoms have been referred to as “biophantoms,” and they have begun to be commonly used for ultrasound research^[58].

Conventionally, phantoms have been made by hand using materials that can mimic optical properties, such as intralipid, TiO₂, inks, and resins, among other materials^[22], instead of using living cells. Unique advantages of using 3D printing for the development of optical tissue phantoms were detected in this research, including easy production, complex multilayer fabrication, ability to add substances to mimic heterogeneities of biological tissues, low cost, and lifecycle environmental friendliness, including reusability of phantoms' parts and materials, and avoiding toxic resources.

3.2 Patentometric Analysis

A total of 34 patients were obtained in the search period established from January 1, 2000, to July 31, 2018. The query presented in previous sections was applied and adapted to

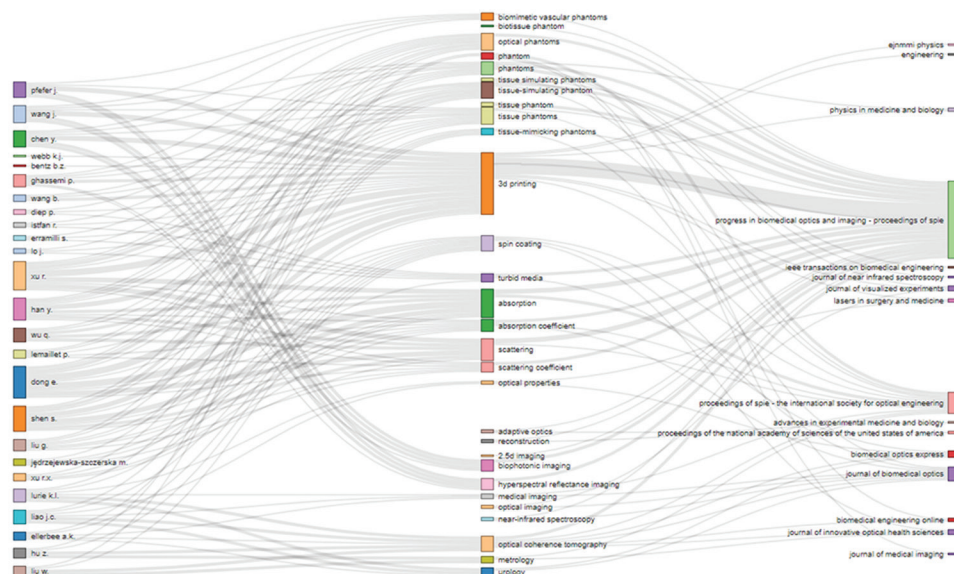


Figure 2. Association map showing the top authors, keywords, and journals or conference proceedings. Keywords are grouped relating to phantoms, methods, optical properties, and applications.

be used on PatSeer software. After reviewing each patent in detail and carrying out a de-duplication process, the total number of documents was reduced to 23. Finally, the patents were grouped by family, resulting in only 18 patent families. As in scientometric analysis, there are no documents related to using 3D bioprinting to produce optical tissue phantoms, but there are for 3D printing. Figure 3 shows the number of patent families published during the time interval analyzed and their priority countries.

From the documents analyzed, it was found that the early patent involving the use of 3D printing to create optical tissue phantoms was published in 2009. Following this, there is a time gap until the publication of a patent in 2015. Four patents were published in 2016, and six patents each were published in 2017, and the first half of 2018. Although there is a growing trend in the number of patents, there are not enough data for estimating a mathematical function that describes the growth behavior. The main reason for the small number of patents could be that 3D printing technology is not yet fully developed for optical tissue phantoms and it takes longer to publish a patent than it does to put out a scientific paper.

Similarly to the scientometric analysis, the most prolific country in the area is the United States, having eight published patent families, followed by South Korea with five, Germany with three, and finally, China with two. In terms of assignees, the Korea Photonics Technological Institute and Pukyong National University (both in South Korea) have the highest number of patent families; they coauthored three patents, followed by Siemens (Germany) with two patents. Table 4 shows the patent families' assignees ordered by country position.

Finally, only three institutions were detected that had published scientific papers and patents on 3D printed optical tissue phantoms, which are the following: Purdue University (seven scientific papers and one family patent published) and Georgia Institute of Technology and Pukyong National University (one scientific paper and one family patent each).

3.3 Global Trends in 3D Printed Optical Tissue Phantoms

The main focus on the development of optical tissue phantoms through 3D printing was determined after a detailed analysis of the scientific papers and patent families. The results are categorized in Table 5 according to methods, materials, and uses.

As can be observed in Table 5, the most used method for developing optical tissue phantoms by 3D printing is spin coating, mainly because this process enables the creation of multilayered structures with micron-thick layers, such as biological tissues^[49]. In contrast,

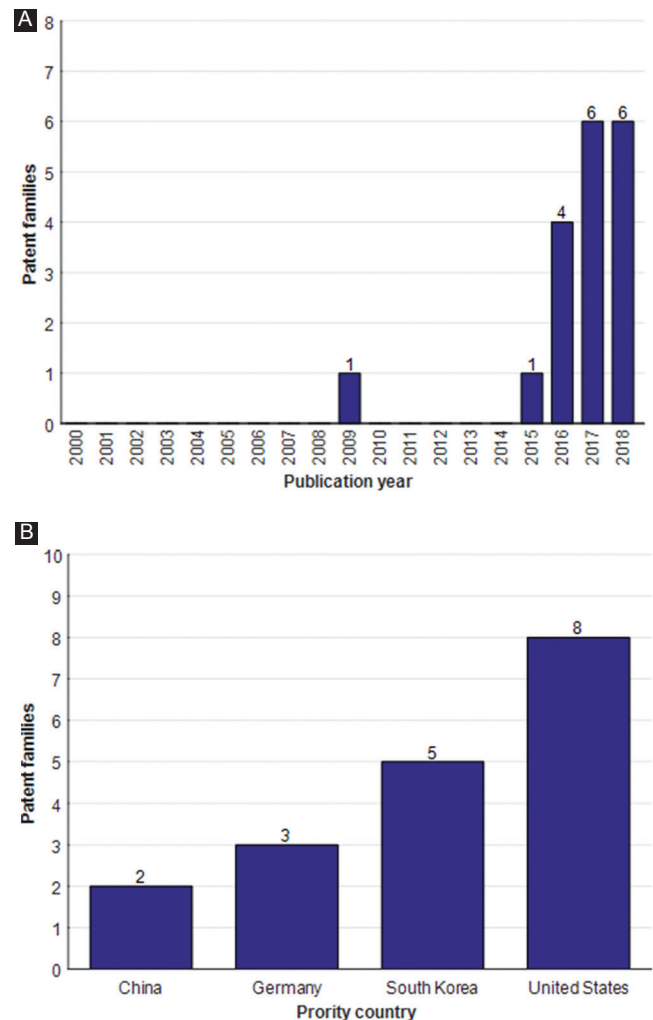


Figure 3. Summary of published patents on 3D printed optical tissue phantoms from January 1, 2000, to July 31, 2018, grouped by (A) the number of patent families published and (B) priority countries.

Table 4. Patent assignees by country. The number of patent families for each assignee is in parentheses

Country	Assignee
United states	Board of Trustees of Leland Stanford (1)
	Erica Burgett, Rebecca Howell (1)
	Georgia Tech Research Institution (1)
	Mayo Foundation (1)
	Purdue University Research Foundation (1)
	Siemens Corp. U.S. (1)
	University of Massachusetts (1)
	University of Indiana Res. Tech. Corp. (1)
South Korea	Korea Photonic Technological Institute (3)
	Pukyong National University (3)
	Samsung Life Public Welfare Foundation (1)
	University of Ulsan (1)
Germany	Max Planck Gesellschaft (1)
	Siemens AG (2)
China	Shenzhen Institute of Advanced Tech. (1)
	Taishan Medical University (1)

Table 5. Global trends for optical tissue phantoms development using 3D printing

Category	Trend
Methods	Spin coating process predominates because this technique allows thin layers to be generated (30–60 μm in thickness), which better mimic the layer structure of biological tissues
Materials	Polydopamine, polydimethylsiloxane, and the hydrogel, PVA, are present to elaborate matrix tissue phantoms. To mimic scattering properties, TiO_2 powders, intralipids, and PVA (involving a freeze-thaw treatment) are used. Finally, those applied to mimic absorption properties include India ink, dyes, graphite powder, hemoglobin solutions, and coffee
Uses	Applications are divided into the following: Diagnosis techniques, specifically: (1) Medical imaging procedures: confocal microscopy, optical coherence tomography, optical diffusion tomography, hyperspectral reflectance imaging, fluorescence imaging and photoacoustic; and (2) spectroscopy (diffuse reflectance and near-infrared spectroscopy); and Phototherapy techniques, especially in tests for tattoo removal using laser

PVA: Polyvinyl alcohol, 3D: Three-dimensional

the predominant materials for the matrix of 3D printed optical tissue phantoms include both polymers and hydrogels since they facilitate the addition of other substances to improve the optical properties of the phantom^[31] and can be used easily for 3D printing^[16,51]. Materials of non-biological origin (TiO_2 , India ink, dyes, graphite powder, and PVA) are preferred over biological materials (such as intralipid and hemoglobin solutions). Finally, two categories of phantom applications were identified, namely diagnosis and phototherapy, with the first predominating; the dearth of research on optical tissue phantoms for phototherapy may be because phantoms do not yet possess thermal properties and dynamics like blood flow in tissue, which is crucial for phototherapies^[64]. However, efforts are being made to develop phantoms that include vascular tissue filled with blood or hemoglobin solutions to mimic the tissue oxygenation and blood perfusion^[31,54,65].

4 Conclusions

In this study, scientometric and patentometric analyses were carried out to identify trends in the use of 3D printing or 3D bioprinting to develop optical tissue phantoms, focusing on the following key elements: Methods, materials, and uses, as well as predominant countries, institutions, and journals. The results revealed that 3D printing is already used for the development of optical tissue phantoms, where the spin coating is the most frequently employed method. Materials such as polymers and hydrogels prevail as the phantom matrix; meanwhile, to mimic optical properties, the use of synthetic materials such as TiO_2 , India ink, or dyes outweighs that of biological materials (intralipids, hemoglobin, etc.). Finally, it was identified that 3D printed optical tissue phantoms are mainly focused on diagnostic purposes rather than phototherapy.

The insights obtained in this study illustrate that the more active countries in R&D on optical tissue phantoms using 3D printing are the United States and China. The main institutions that publish scientific papers in this area are located in the United States; meanwhile, in terms of patents, the leading institutions come from South Korea. Only three institutions were detected that published

both scientific papers and patents in the field - Purdue University and Georgia Institute of Technology, both in the United States and Pukyong National University in South Korea. Specifically, a quarter of the total scientific papers identified was published in the proceedings of the SPIE.

Regarding limitations, this research was restricted by a lack of information, due to the novelty of applying 3D printing in the development of optical tissue phantoms. This issue led to obtaining few scientific papers and especially patents, and it was not possible to identify a clear trend of the behavior of patents published in this field. In terms of 3D bioprinting, no documents using this technique to produce optical tissue phantoms were detected, which limited the study to analyzing publications on 3D-printed phantoms.

Since the applications of both 3D printing and 3D bioprinting are growing, future analysis will be based on a higher number of publications, especially for the latter. Indeed, 3D bioprinting is already utilized to produce phantoms, but in other areas, such as ultrasound imaging, where they are known as “biophantoms”^[63]. Ultimately, 3D printing technology has a disruptive potential for the development of optical tissue phantoms; this technology has distinct advantages over traditional methods, such as the development of complex multilayered structures, easy production, and low cost. Unique biophotonic prototypes can be built using 3D bioprinting, thereby facilitating diagnosis tests, equipment validation, and so on^[66]. The 3D bioprinting domain is still in development, and its uses and applications in many areas have not been completely studied, so this research reveals a new window of opportunity to explore.

Conflicts of Interest

No conflicts of interest are reported by the authors.

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