Extrusion Bioprinting of Scaffolds for Tissue Engineering Applications

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To Qi Huang and Angel Chen
To Peter and Arlene Block

Preface

Over the past decade, considerable progress has been made in the development of various bioprinting technologies to fabricate scaffolds for tissue engineering applications, which has also led to several edited books to review and report these developments. Emphasizing the advances of various bioprinting technologies, these books are generally written for experienced researchers in this field. In addition, no books have been dedicated to extrusion bioprinting technologies although they are the most common way to fabricate scaffolds among the various bioprinting technologies available. Aimed to fill these gaps, this book focuses on extrusion bioprinting technologies and is particularly suited for both undergraduate and graduate students in universities or upper-division colleges as well as those who wish to become masters of this technology. It provides comprehensive/fundamental knowledge and practical applications of extrusion bioprinting technologies to fabricate scaffolds for tissue engineering applications.

After an overview of tissue engineering, Chap. 1 provides a brief introduction to the development of scaffolds for tissue engineering applications as well as various scaffold fabrication techniques. Chapter 2 presents the general requirements imposed on scaffolds and the scaffold design process. Chapter 3 discusses the properties of biomaterials important for extrusion bioprinting as well as the hydrogels commonly used. Chapter 4 focuses on the common methods/techniques to measure and characterize the mechanical properties of native tissues and scaffolds. Chapter 5 presents information on how to prepare biomaterial solutions with/without living cells for bioprinting scaffolds, while Chap. 6 is concerned with how to print scaffolds from the prepared biomaterial solutions. The last chapter (i.e., Chap. 7) introduces bioprinting-based and other approaches to create vascular networks within tissue scaffolds to facilitate their functions.

At last, but most importantly, I would like to acknowledge my current/former graduate students and researchers in the Biofabrication Laboratory at the University of Saskatchewan, Canada, whose assistance and perseverance made the completion

of this text possible. Specifically, Mr. Saman Naghieh co-authored Chap. 2, Dr. Fu You Chap. 3, Dr. Nahshon Bawolin and Dr. Nitin Sharma Chap. 4, Dr. Liqun Ning Chaps. 5 and 6, and Mr. MD Sarker Chap. 7. Finally, I also thank Mr. MD Sarker who drew the figures in Chaps. 1 and 3.

Saskatoon, Canada Daniel X. B. Chen

Contents

Chapter 1
Extrusion Bioprinting of Scaffolds: An Introduction

Chapter Learning Outcomes

- Understand the aim of and general principle used in tissue engineering
- Become familiar with the requirements imposed on scaffolds and their development
- Understand various scaffold-fabrication techniques
- Become familiar with extrusion bioprinting of scaffolds
- Know the advantages/disadvantages of extrusion bioprinting and recent achievements.

1.1 Introduction

Millions of people suffer from tissue/organs injuries or damage, such as peripheral nerve injuries and heart attacks. Tissue/organs transplantation is the gold standard to treat some of these types of injuries, but is severely restricted as an option due to the limited availability of donor tissue/organs. To address this issue, tissue engineering (TE) aims to produce tissue/organs substitutes to improve upon current treatment approaches, thus providing a permanent solution to damaged tissue/organs [1]. An analogy would be buying new parts at the mechanic to replace car parts that are broken or no longer functioning. Successes in tissue engineering would mean that someone who unfortunately suffers a tissue/organs injury could go to a hospital, have the engineered substitute implanted into his/her body, and then later completely recover the function of a healthy body with the help of the engineered substitutes.

The general principle behind TE is schematically shown in Fig. 1.1. Cells from a patient (or other resources) are harvested and then seeded onto or incorporated into an engineered substitute or scaffold (typically along with growth factors or other

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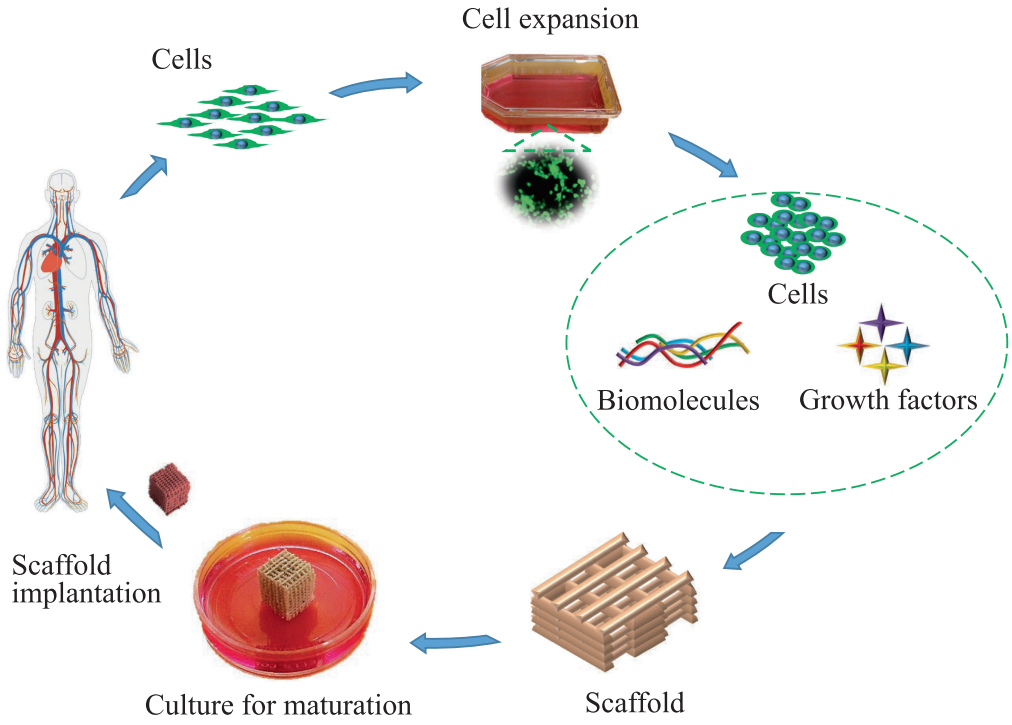

Fig. 1.1 General principle behind tissue engineering

biomolecules to stimulate cell growth and functions); the cell-incorporated scaffold is then cultured to maturation, resulting in a functional construct that is then implanted into the patient to help repair or heal the damaged tissue/organs. Scaffold-based TE is an interdisciplinary field that involves applying the principles of life sciences and engineering to repair damaged tissues and organs with the help of scaffolds.

Made from biomaterials (such as polymers), a TE scaffold is used to support and facilitate cell/tissue growth and the transport of nutrients and wastes, while degrading gradually itself during the healing process. Several functional requirements have been identified as crucial for TE scaffolds in terms of architectural, mechanical, and biological properties.

Architectural properties of a scaffold refer to its external geometry and internal structure. Generally speaking, a scaffold's external geometry should mimic that of the tissue/organs to be repaired, while its internal structure should be highly porous to allow for cell growth and movement as well as facilitate the transport of nutrients into the scaffold and the removal of metabolic wastes out of the scaffold during the healing process.

Mechanical properties of a scaffold refer to its mechanical strength and degradation. During the healing process, the scaffold materials degrade as the cell/tissue grows and, as a result, the mechanical strength of the scaffold decreases with time. Concurrently, the cells grow and the tissue regenerates, which imparts mechanical strength to the combined construct of scaffold material and regenerated tissue. It is generally accepted that the mechanical strength of a scaffold at the initial stage of

implantation or of a combined construct of scaffold and regenerated tissue during the healing process should be similar to that of the tissue/organs being repaired.

Biological properties of a scaffold refer to its ability to support cell growth/functions (such as cell attachment, proliferation, and differentiation) and tissue regeneration, with limited or no negative effects (such as inflammation) on the host system (i.e., animal or human) in which the tissue/organs are to be repaired. The biological properties of a scaffold are typically evaluated using in vitro and in vivo tests. In vitro (literally "in glass") tests take place in a well-controlled laboratory environment, while in vivo tests are performed in the living body of an animal or human.

Depending on the TE applications or the tissue/organs to be repaired, more requirements may be imposed on the scaffolds. For example, scaffolds for peripheral nerve repair should possess a biodegradable and porous channel wall and incorporate viable Schwann cells [2], which greatly facilitate axon growth and thus functional recovery.

The development of TE scaffolds consists of three stages—design, fabrication, and characterization—as shown in Fig. 1.2. Based on the functional requirements, TE scaffolds should generally be designed with three-dimensional (3D) and porous structures of appropriate mechanical and biological properties, where the key is to design and/or determine the scaffold internal structure, scaffolds biomaterials, and living cells to be seeded on or incorporated within the scaffolds. Typically, scaffold design starts from an understanding and/or knowledge of the architecture of the tissue/organs to be repaired; medical imaging technology, such as computed tomography and magnetic resonance imaging, is a common tool for this purpose [3]. With such knowledge, scaffolds are designed with appropriate external geometries and internal structures as well as specifically chosen and spatially arranged biomaterials/cells so as to mimic the architectural, mechanical, and biological properties of the tissue/organs to be repaired.

In the second stage of scaffold development, scaffolds are created from biomaterials and living cells, as designed, by means of fabrication techniques. Scaffolds can be either fabricated from biomaterials and subsequently seeded with living cells, or fabricated from biomaterials incorporating living cells (known as biofabrication). Seeding cells onto scaffolds after they are fabricated impose limits on the ability to spatially place living cells into scaffolds as well as on seeding depth, i.e., the cells seeded into the scaffold remain near the scaffold surface. Advantages of incorporating cells in the fabrication process include the ability to produce a spatial distribution of cells, thus allowing the cell organization of the target tissue/organs to be mimicked. Sustaining the viability of living cells during the fabrication process is essential, and emphasizes the importance of sterile and gentle conditions for scaffold fabrication.

The last stage of scaffold development is scaffold characterization. By means of in vitro and in vivo tests, the performance or outcomes of scaffolds are examined and analyzed in terms of architectural, mechanical, and biological properties for various TE applications. In many cases, the scaffolds, once fabricated, need to be cultured in vitro prior to their implantation to facilitate their maturation for optimal in vivo performance or outcomes.

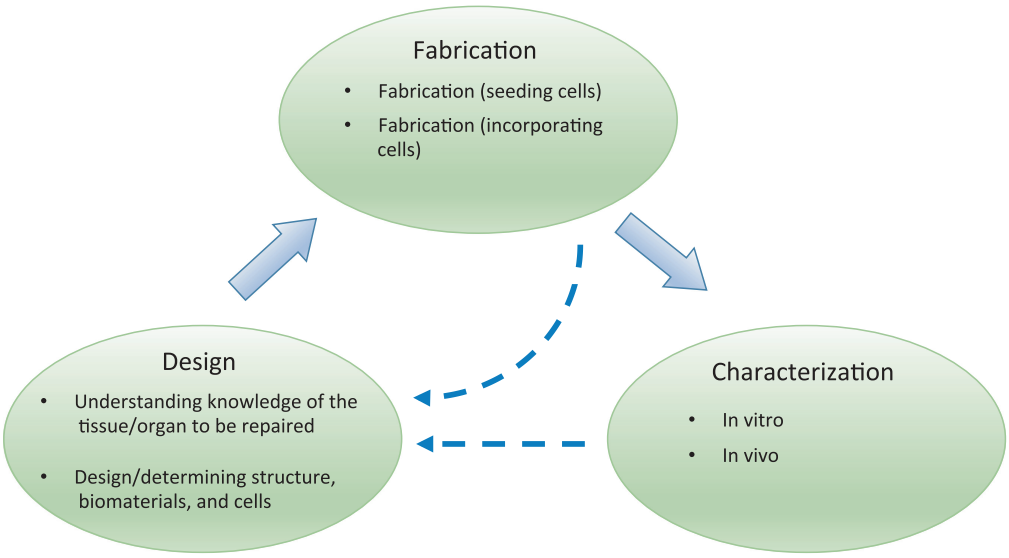

Fig. 1.2 Schematic of the development of TE scaffolds

Figure 1.2 shows the development of TE scaffolds as continuous and cyclic in nature. Scaffold development is not linear; that is, one does not necessarily achieve the best scaffold by simply proceeding from one stage to the next. The development of a scaffold for a given tissue engineering application is typically accomplished by iteration through the aforementioned three stages. For example, new discoveries in the relationship between the function and structure of tissue/organs help and improve our understanding of the architecture of tissue/organs to be repaired and therefore the scaffold design. Scaffolds should also be designed such that they can be fabricated by means of existing fabrication techniques. Advances in scaffold fabrication now allow for improvement over existing scaffold designs and more functional scaffolds. The performance and/or outcomes of scaffolds, as examined in vitro and in vivo, not only illustrate the effectiveness of the scaffold design and fabrication but also provide a means or feedback to refine the scaffold design as well as advance fabrication techniques to achieve better outcomes for a given TE application.

1.2 Scaffold Fabrication

A number of fabrication techniques have been applied to fabricate scaffolds from biomaterials and living cells. Generally, these techniques are divided into three categories, i.e., conventional, electrospinning, and 3D printing.

1.2.1 Traditional Techniques

Traditional techniques refer to those that are adopted from traditional fields to process biomaterials into scaffolds with a randomly generated pore structure. These techniques include porogen-leaching, gas foaming, phase separation, melt molding, and freeze drying.

Porogen-leaching. Porogen-leaching is one of the oldest polymer-processing techniques to make porous products and, in the early days of TE, was widely used to fabricate scaffolds. This technique involves dispersing a template (e.g., salt particles) within a polymer solution, gelling or fixing the template/polymer structure, and then removing or leaching the template from the structure so as to create a scaffold with a porous structure (Fig. 1.3a).

Gas foaming. During the gas foaming process (Fig. 1.3b), molded polymers are pressurized with gas-foaming agents, such as CO_2 and nitrogen; the release of pressure then results in nucleation and growth of gas bubbles and thus porous scaffold structures. This technique has the advantage of being an organic solvent-free process for scaffold fabrication; the major drawback is that the process may yield structures with largely unconnected pores and a non-porous external surface.

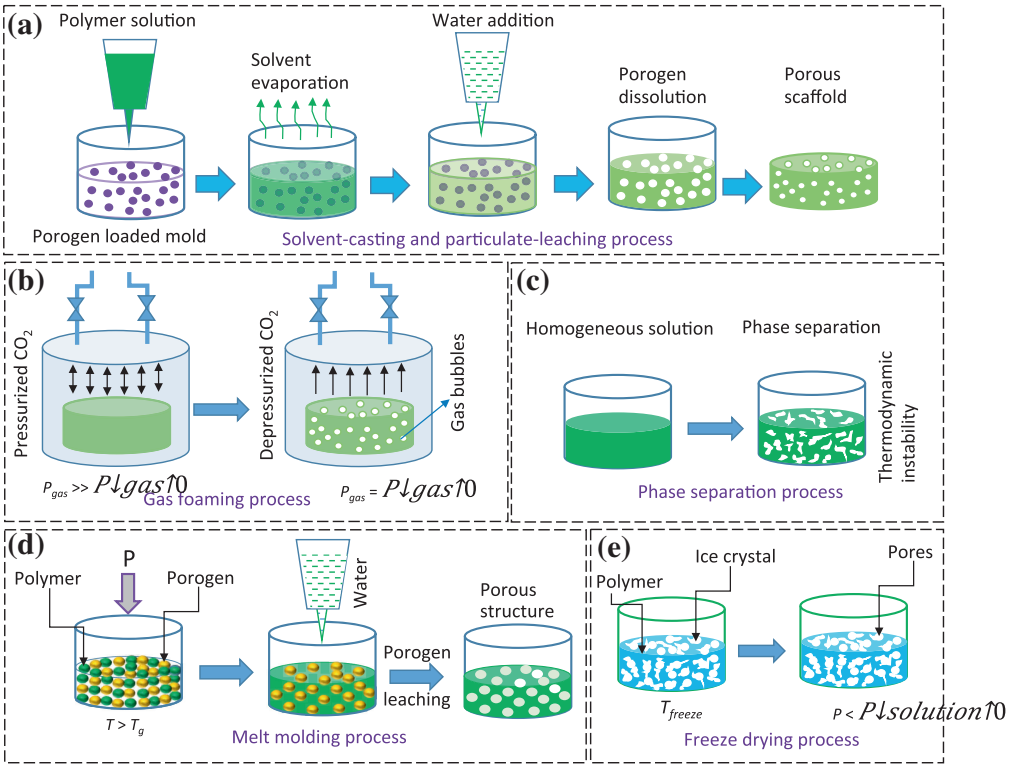

Fig. 1.3 Schematic of conventional scaffold-fabrication techniques: **a** solvent-casting and porogen-leaching process; **b** gas foaming process; **c** phase separation process; **d** melt molding process; and **e** freeze drying process

Phase separation. During the phase separation process (Fig. 1.3c), a polymer solution is quenched and undergoes a liquid–liquid phase separation to form two phases—a polymer-rich phase and a polymer-poor phase; the polymer-rich phase solidifies and the polymer-poor phase is removed, leaving a highly porous polymer network. The micro- and macro-structure of the resulting scaffold are controlled by varying process parameters such as polymer concentration, quenching temperature, and quenching rate. The process can be conducted at low temperatures, which is beneficial for the incorporation of bioactive molecules in the structure.

Melt molding. During the melt molding process (Fig. 1.3d), a mold is filled with polymer powder and a porogen component and then heated to above the glass-transition temperature of the polymer (T_g), causing the materials to bind together to form a scaffold in the shape of the mold. The porogen is then leached out, leaving a scaffold with a porous structure. Melt molding with porogen-leaching is a non-solvent fabrication process that allows independent control of morphology and shape. Drawbacks include the possibility of residual porogen and high-processing temperatures that preclude the ability to incorporate bioactive molecules.

Freeze drying. During the freeze drying process (Fig. 1.3e), a polymer solution is cooled to the temperature at which all materials become solid; the solvent is then sublimed from the solid phase to the gas phase by reducing the pressure to below the equilibrium vapor pressure of the frozen solvent. By doing so, the solvent is removed, leaving a scaffold with a porous structure. The scaffold structure depends on the concentration of the polymer solution, freezing rate, and applied pressure.

1.2.2 Electrospinning

Electrospinning is a fabrication technique to create fine fibers up to the nanometer scale from polymer solutions or melts. This technique was first developed in the 1930s, and since 1990 has found widespread applications in the fabrication of TE scaffolds. A typical electrospinning setup, as schematically shown in Fig. 1.4, includes three basic components: a spinneret (or a small orifice and flat-tipped needle), a voltage source, and a collector. During scaffold fabrication, a high voltage is applied to the polymer solution in the spinneret, while the collector is grounded; as a result, a large electric field is generated between the polymer solution and collector that causes the polymer solution to be continuously ejected from the spinneret. The jet travels spirally and then lands on the collectors, forming a 3D scaffold of fibrous architecture. Depending on the process parameters for spinning (e.g., the applied voltage and the distance between the spinneret and collector), the diameter of spun fibers typically varies between 200 nm and 5 μm.

With current advances, spinnerets can be designed to deliver multiple polymer solutions. For example, a coaxial spinneret with an inner needle and an external needle can be used to apply two polymer solutions, respectively, forming fibers with a core/shell structure. Cells can also be added to the electrospinning solution to

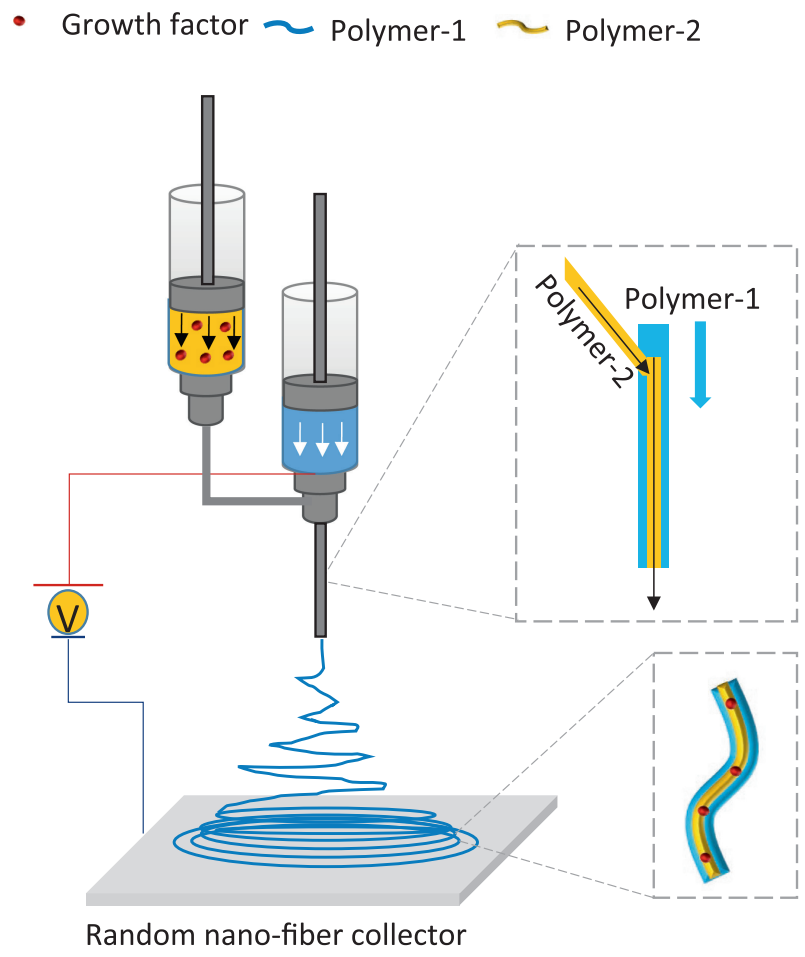

Fig. 1.4 Working principle of electrospinning

form cell-incorporated scaffolds. In cases in which solvent accumulation or toxicity is a concern, electrospinning polymers without solvents (via melting), called melt electrospinning, can be used to create scaffolds.

1.2.3 3D Printing

3D printing of scaffolds refers to the technique of depositing or patterning biomaterials in a layer-by-layer manner to create scaffolds with a 3D structure. Distinctive from the aforementioned traditional techniques, 3D printing offers reproducible control over the architectural properties of scaffolds due to the multilayer deposition of biomaterials. Other merits include the process (1) being easy and straightforward for the creation of scaffolds with porous structures, (2) being able to create complex structures to mimic those of natural tissue/organs, and (3) being

capable of incorporating living cells during scaffold fabrication. Based on the working principles, techniques used for 3D printing can be classified as either extrusion, ink-jet, or laser-assisted [3, 4].

Extrusion Printing. Extrusion printing is a technique to extrude or dispense continuous strands or fibers of biomaterials, layer-by-layer, to form 3D scaffold structures [5–7]. Extrusion printing is based on the principle of fluid extrusion or dispensing (Fig. 1.5a), by which the biomaterial solution stored in a syringe is driven by mechanical force (e.g., pressurized air) through a needle and then onto a printing stage.

Ink-Jet Printing. Adopted from the working principle of a commercial printer, ink-jet printing propels droplets of biomaterial solution (the ink in a printer) onto a printing stage (the paper in a printer) (Fig. 1.5b). As such, ink-jet printing is also known as drop-on-demand printing. The forces to propel the droplets of solution can be generated thermally or acoustically [4].

Laser-Assisted Printing. Laser-assisted printing is based on the principle of laser-induced forward transfer (Fig. 1.5c); when the laser pulses focus and hit biomaterials covered in an energy-absorbing substrate, high pressures are generated that propel the biomaterials onto a collector substrate. Laser-assisted printing is performed without the need for needles, thus avoiding the issue of clogging that can occur with other printing techniques.

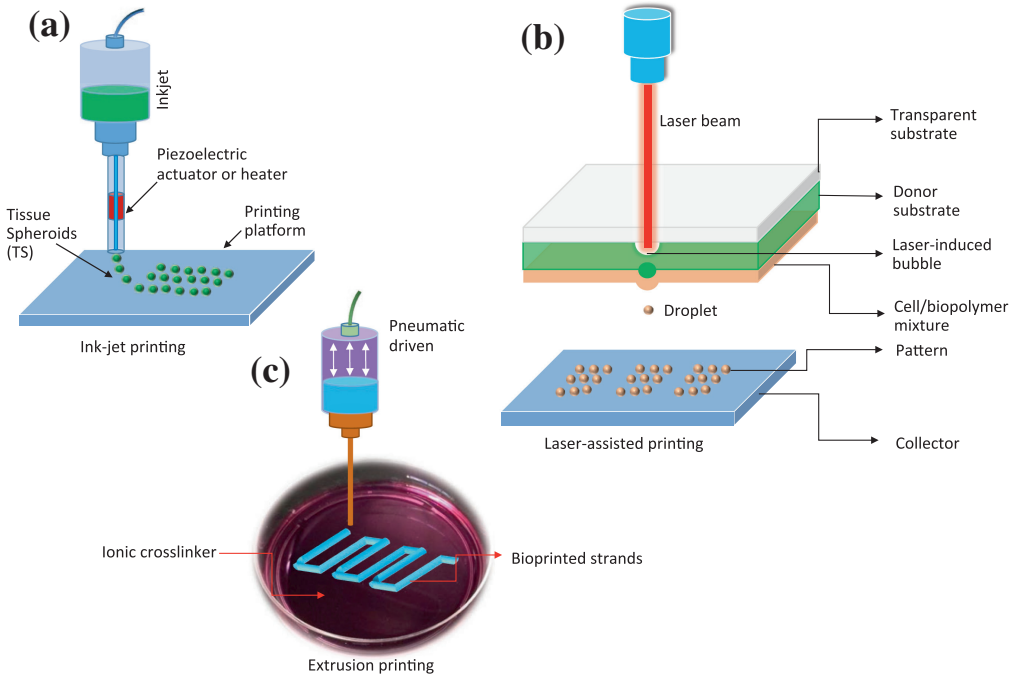

Fig. 1.5 3D printing techniques: **a** extrusion printing, **b** ink-jet printing, and **c** laser-assisted printing

1.3 Extrusion Bioprinting of Scaffolds

By 3D extrusion printing, scaffolds can be fabricated from biomaterials mixed with living cells. This fabrication process is referred to as extrusion bioprinting, and the biomaterial solution mixed with living cells is referred to as the bioink (similar to "ink" for a printer). An extrusion bioprinting system typically consists of a dispensing head, a positioning-control component, and a temperature-control component. A schematic of such a system is shown in Fig. 1.6. The system includes a dispenser mounted on the dispensing head, which can be controlled to move in three directions, a printing/supporting stage or platform to support the scaffold being fabricated, and three controllers interfaced to a host personal computer (PC) that controls dispensing, positioning, and temperature. During bioprinting, the bioink is loaded into the syringe and then driven by mechanical force (e.g., pressurized air) through a needle onto the printing stage, forming a layer-structure scaffold. Depending on the internal diameter of the needle used for printing, the resolution of strands that can be achieved is on the order of 100–150 μm. Typical scaffolds fabricated by such systems have 3D structures with repeatable layers of printed strands, as shown in Fig. 1.6b.

Bioprinting allows for the incorporation of living cells within scaffolds. Notably, living cells are dynamic structures with functions (e.g., growth and proliferation) that are affected by mechanical forces. During the biofabrication process, cells are subjected to sustained process-induced forces, such as pressure, shear stress, and extensional stress, which can cause the deformation and breach of cell membranes. Although cells have elastic abilities to resist a certain level of mechanical force, cell membranes may lose their integrity if the applied force exceeds a certain threshold; as a result, cells may be damaged and even lose their functions and viability [8].

To preserve cell viability, the solution used for bioprinting must be biocompatible while allowing rapid transport of nutrients/metabolites to/from incorporated cells. Hydrogels have been widely used for cell incorporation in bioprinting [3–8]. A hydrogel is a gelled or crosslinked (via either physical or chemical bonding) network of polymers, such as collagen, alginate, chitosan, or polylactic acid. The crosslinked network possesses high water content among polymer chains, which is used for cell incorporation provide a hydrated tissue-like environment, thus enhancing the cell viability in bioprinting. The crossed-linked network greatly facilitates the formation of a 3D structure when printing scaffolds. The gelation or crossing-linking of hydrogels takes time, and during this gelation period the hydrogel is in a solution or semi-solution form and is able to flow or spread on the printing stage. As a result, the printed structure of a scaffold may not be the same as the one designed. In some cases, the printed structures even collapse and fail to form a 3D structure; such hydrogels would be deemed unprintable. Examination of the difference between the scaffold design and the printed structures is a common practice to measure printability in bioprinting [9–11].

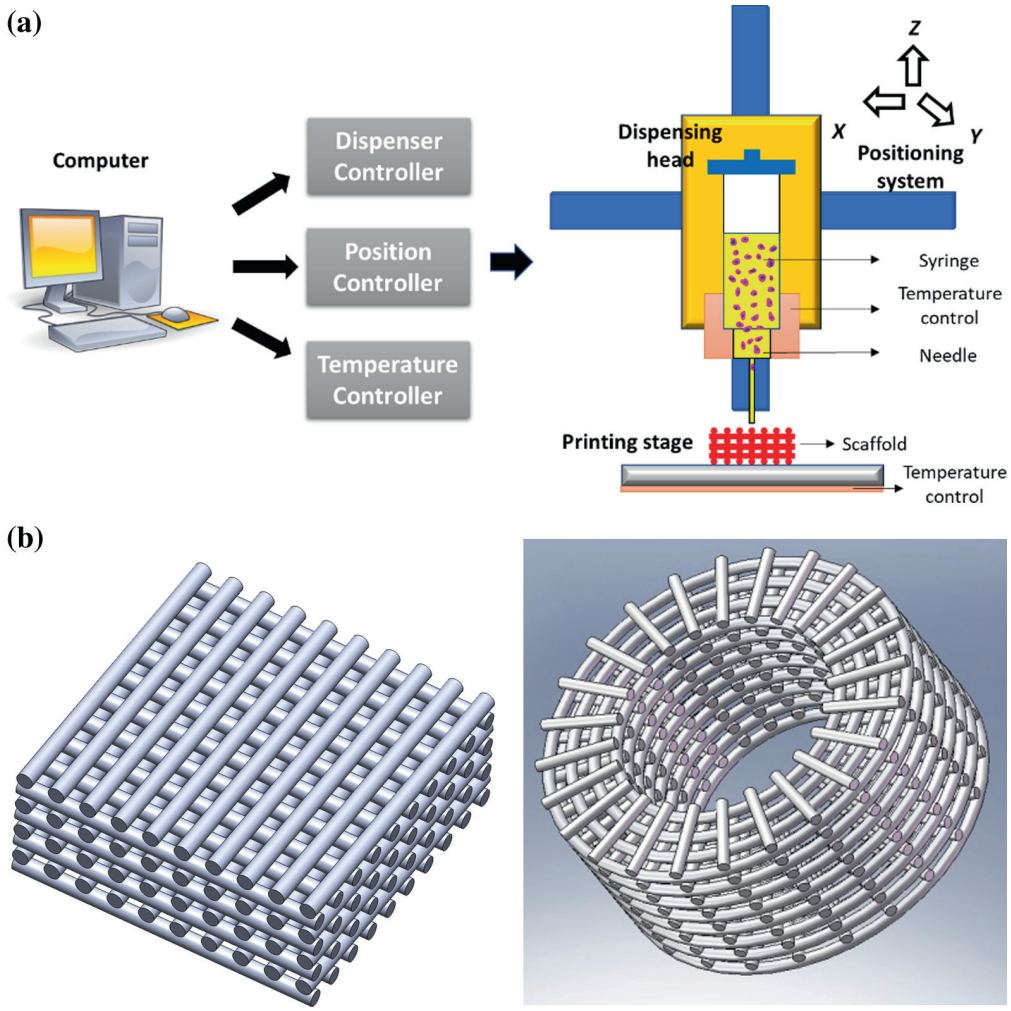

Fig. 1.6 **a** Schematic of extrusion-based bioprinting and **b** typical structures of printed scaffolds

A number of factors can be involved in bioprinting that affect performance, including the aforementioned cell viability and printability. These factors can be classified into two categories, i.e., biomaterial-solution properties and printing conditions. Biomaterial-solution properties include the physical properties (such as contact angle), flow behavior, and crosslinking mechanisms while printing conditions are the mechanical force (e.g., pressurized air) applied for printing, dispensing-head movement speed, and structural parameters (e.g., needle diameter and length). One can regulate both biomaterial-solution properties when preparing the biomaterial solution and printing conditions when designing the bioprinting process to achieve the best bioprinting performance, for example, in terms of cell viability and printability.

1.4 Advantages/Disadvantages of Extrusion Bioprinting and Recent Achievements

Over the last two decades, advances in both engineering techniques and life sciences mean extrusion bioprinting has evolved from a simple technique to one able to create diverse, yet complicated, tissue scaffolds. These scaffolds have been used in a wide range of tissue engineering applications, for example, to repair damaged skin, cartilage, bones, nerves, and spinal cords as well as to treat heart attack and stroke.

Extrusion bioprinting can produce tissue scaffolds using various cells types, including both primary cells and stem cells. Primary cell types, which are isolated from animals or humans and include cells such as osteocytes, chondrocytes, and keratinocytes, have been used in tissue scaffolds to faithfully represent tissues including bones, cartilage, and skin. In some cases, primary cells isolated from living tissues may be difficult or challenging to culture. In such cases, stem cells are often used as a substitute for primary cells in tissue scaffold bioprinting. Stem cells can self-renew and differentiate into specific cell types when certain cues are provided. The extrusion bioprinting technique has shown great potential for regulating and conducting stem cell growth and differentiation in many applications, such as those targeting brain tissue, gingival tissue, adipose tissue, and bone marrow tissue.

In addition to the ability of the extrusion bioprinting method to manipulate diverse cell types, various printed structures such as beads, filaments, fibers, channels, sheets, rolls, grids, and porous 3D constructs that mimic various tissue components have been successfully printed at the micro- or macro-scale. Among these structures, the formation of vasculature is a major challenge in tissue engineering. The function of vascularization is to supply oxygen, nutrients, and metabolites of cellular activities to ensure the long-term viability of cells and tissues. In extrusion-based bioprinting, vessel-like permeable channels have been produced and used to facilitate vascularization with the expectation of forming vascular networks. Supporting cells such as endothelial cells are often deposited in vessel-like channels during bioprinting to initiate the formation of vasculature and subsequently support their stabilization and function, which can further facilitate the angiogenesis of vessel networks.

Compared to ink-jet and laser-assisted printing, extrusion bioprinting has several advantages. It is able to dispense a wide array of biomaterials and cells, including both native and synthetic hydrogel polymers, cell aggregates, and decellularized extracellular matrix, while other printing techniques are limited to bioprinting hydrogel polymers with suspended cells [12]. Depositing biomaterials with physiological cell density, which is a major challenge for other bioprinting techniques, is feasible with the extrusion-based bioprinting method. Due to its fast deposition speed, extrusion bioprinting has also often been used to produce large-scale scaffolds.

Extrusion bioprinting also has several disadvantages. It has a limited strand resolution (typically greater than 100 μm), mainly due to considerations related to the mechanical force required to drive a scaffold solution through the nozzle and the nozzle mechanical strength to bear the pressure induced inside the needle.

Organizing deposition at the microscale is also challenging compared to other bioprinting techniques. For example, laser-assisted bioprinting can reach the highest resolution of 1 μm [13], and ink-jet-based bioprinting produces droplets less than 50 μm in diameter [14]. In addition, the printability of hydrogels is heavily dependent on their crosslinking capability and/or the printing conditions; biomaterials with a slow crosslinking speed may not be appropriate for use in bioprinting due to difficulties related to forming 3D structures. In addition, needle clogging with biomaterial solution is another problem in extrusion bioprinting that may cause complete interruption of biomaterial deposition and therefore affects the integrity of the resulting scaffold structure.

Summary

Tissue engineering aims to produce tissue/organs substitutes that improve upon current treatment approaches, thus providing a permanent solution to damaged tissue/organs. In scaffold-based TE, the scaffold is used to support and facilitate cell/tissue growth and the transport of nutrients and wastes, while degrading gradually itself during the healing process.

Several requirements have been identified as crucial for TE scaffolds in terms of architectural, mechanical, and biological properties. The architectural properties of a scaffold are characterized by its external geometry and internal structure, mechanical properties by its mechanical strength and degradation, and biological properties by its ability to support cell growth/functions and tissue regeneration with limited or no negative effects on the host system. The biological properties of a scaffold are typically evaluated using in vitro and in vivo tests.

TE scaffolds can be fabricated by conventional, electrospinning, and 3D printing techniques. Traditional techniques are those adopted from traditional fields to process biomaterials into scaffolds with a randomly generated pore structure. These techniques include porogen-leaching, gas foaming, phase separation, melt molding, and freeze drying. Electrospinning is a fabrication technique to create fine fibers up to the nanometer scale from polymer solutions or melts. 3D printing refers to extrusion, ink-jet, and laser-assisted printing techniques that are able to deposit or pattern biomaterials in a layer-by-layer manner to create scaffolds or constructs with a 3D structure. Distinct from traditional techniques, 3D printing offers reproducible control over the architectural properties of scaffolds.

Extrusion bioprinting can fabricate tissue scaffolds with various structures by incorporating primary cells and/or stem cells. These fabricated scaffolds have been widely used in applications including the repair of damaged skin, cartilage, bones, nerves, and spinal cords as well as the treatment of heart attack and stroke. Extrusion bioprinting has numerous merits and demerits compared to other printing techniques.

Problems

1. Explain the general principle used in scaffold-based tissue engineering to heal damaged tissue/organs and the role that the scaffold plays in the healing process.
2. Name the three requirements imposed on TE scaffolds; perform a literature review on one requirement and illustrate your understanding of this requirement.

3. Name the techniques that can be used to fabricate tissue scaffolds; perform a literature review on one technique (except extrusion bioprinting) and explain its working principle and its merits/demerits for use in scaffold fabrication.
4. Briefly explain the process of extrusion bioprinting of scaffolds and one aspect of bioprinting performance you see as the most important.
5. Name and explain one achievement accomplished by means of extrusion bioprinting that has been reported in the literature.
6. Name and explain one advantage and one disadvantage of extrusion bioprinting compared to ink-jet and laser-assisted printing.

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