Traction of 3D and 4D printing in the healthcare industry, from drug delivery and analysis to regenerative medicine

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ABSTRACT

Three-dimensional (3D) printing and 3D bioprinting are promising technologies for a broad range of healthcare applications, from frontier regenerative medicine and tissue engineering therapies through to pharmaceutical advancements, yet must overcome the challenges of biocompatibility and resolution. Through comparison of traditional biofabrication methods with 3D (bio)printing, this review highlights the promise of 3D printing for the production of on-demand, personalized and complex products that enhance the accessibility, effectiveness, and safety of drug therapies and delivery systems. In addition, this review describes the capacity of 3D bioprinting to fabricate patient-specific tissues and living cell systems (e.g. vascular networks, organs, muscles, and skeletal systems), as well as its applications in delivery of cells and genes, microfluidics and organ-on-chip constructs. This review summarises how tailoring selected parameters (i.e. accurately selecting appropriate printing method, materials and printing parameters based on the desired application and behavior) can better facilitate the development of optimised 3D-printed products, and how dynamic 4D-printed strategies (printing materials designed to change with time or stimulus) may be deployed to overcome many of the inherent limitations of conventional 3D-printed technologies. Comprehensive insights into a critical perspective of the future of 4D bioprinting, crucial requirements for 4D printing including programmability of a material, multi-

material printing methods, and precise designs for meticulous transformations or even clinical applications are also given.

KEYWORDS
Bioprinting; 3D-printed technology; 4D-printing technology; Microfluidics

1 Introduction

3D printing, or more correctly additive manufacturing, is a technique for the layer-by-layer fabrication of 3D objects from digital models, was established 30 years ago through the cooperation of various disciplines, including materials science, chemistry, robotics, and optics, and has since frequently been used in aerospace, automotive, and consumer goods production (1, 2). More recently, 3D printing technology has been adopted by the pharmaceutical industry, with the first 3D-printed drug product (Spritam® (levatirecam)) approved by the Food and Drug Administration in 2015 (3). 3D printing allows for the direct production of a finished dosage form, for example, a capsule or tablet (i.e. active pharmaceutical components and excipients) in a step by step manner, rather than requiring the combination of drug elements with excipients and processing such as needed in other drug manufacturing methods (4, 5). Research and manufacturing interests are now shifting towards these 3D-printed products, as more traditional pharmaceutical manufacturing methods, such as granulation or tablet compression, lack production flexibility, personalisation and process effectiveness (6). In contrast, 3D printing offers advantages in terms of producing on-demand, personalized and complex products that provide opportunities for enhancing the accessibility, spatiotemporal release and targeting, effectiveness, and safety of drugs (7). For instance, compared with conventional formulations of topical solutions or gels that are applied at high doses periodically, a drug-eluting personalized 3D printed oral

delivery device that releases the active compound over time would minimize washing away by saliva or overswallowing of drugs, thereby boosting treatment efficacy and reducing unwanted side effects. In addition, the possibility of controlling the locality of the drug-containing compartment in the mouthguard, as reported in Liang's et.al study, imparts spatial control over drug release, enabling preferential targeting of affected regions (8).

3D printing technologies have revolutionized the field of tissue engineering, which is largely focused on establishing new techniques to regenerate, repair and replace injured organs and tissues, as well as the creation of *in vitro* tissue models to evaluate disease development and drug screening (9-11). Conventional tissue engineering methods (e.g. porous scaffold development by gas forming, salt leaching, freeze-drying, and phase separation) have previously lacked precise control over the shape, composition and architecture of a scaffold, as well as control of pore size and distribution (12, 13). However, 3D bioprinting has gone some way in addressing these issues, with computer-aided design (CAD) software providing the capacity to fabricate patient-specific tissues from medical scans such as X-ray, magnetic resonance and computed tomography images. Furthermore, living cell systems can be printed by 3D bioprinters using specially designed and engineered 'bioinks', with or without additional support, which can mimic extracellular matrix components (9, 10, 14-17) allowing the additive manufactur of biomaterials and living organisms to create composite material-cellular constructs. 4D bioprinting is an emerging procedure that takes 3D bioprinting and incorporates a 'time' component (18, 19), where constructs undergo conformational change that can be triggered by one or more external stimuli including pH, temperature, or light (20, 21).

3D bioprinting is used to reproduce the compartmentalized structure of organs by the deposition of multiple biomaterials, cells, and biomolecules in predefined locations within 3D constructs.

Additionally, it allows different materials and cells to be deposited simultaneously, which is crucial given that the replication of organs and/or tissues requires biomimicry of the multi-component native cellular and extracellular components (22); for example, the creation of an extracellular matrix or extracellular matrix-like structure, or the inclusion of biochemical or physical cues similar to those in the native tissue. Here, the capacity of bioprinting to control the 3D distribution of multiple cell types in distinct spatial orientations is a major advantage compared to more traditional methods of fabricating tissue engineering scaffolds. However, maintaining cell viability (23) and functionality both during and after the 3D bioprinting process is one of the critical issues facing the technique (24). Of particular interest is cell arrangement and alignment, as this is a crucial factor in determining cellular function and behavior, which is relevant to various tissue engineering approaches, from neural to cardiac regeneration (25). Printing vascular networks is another challenge that has been studied extensively (14, 26). Mechanical strain is another important parameter that can influence cellular arrangement and affect functional tissue development (27-29) as discussed in sections 3 and 5 of the current work. The encapsulation of cells in 3D scaffolds or matrices, such as hydrogels, provides a biomimetic 3D microhabitat that facilitates cell-to-cell and cell-to-matrix interactions, and is therefore more representative of native tissue structures and *in vivo* conditions than traditional 2D cell culture (27-29).

The use of microfluidic devices for cell-loaded 3D hydrogels and sensor integrated bioreactors also offers significant potential to improve on conventional 2D and 3D cultures for applications in biomedical research. 3D printing is also being explored for the advancement of microfluidic bioreactors and organ-on-chip technologies, both of which have the ability to monitor cells' physicochemical qualities and provide an appropriate microenvironment for organoid culture. Such technologies can influence a broad range of clinical and biochemical studies, including point-

of-care diagnostics, efficient drug analysis, cancer biomarker screening, drug screening, and micro-physiological structure engineering (30-33). It should be noted that the manufacturing of microfluidic-based platforms is usually time-consuming, complex, and demands advanced cleanroom and costly facilities. As such, 3D printing offers an appealing alternative to conventional approaches (i.e. joining glass-polydimethylsiloxane and lithography) as it facilitates design duplication in the development phase as well as decreasing the associated costs of apparatus installation, physical work areas, and maintenance. The latest advances in 3D printing approaches allow for the production of highly complicated microfluidic devices via rapid and one-step procedures that lead to convenient accessibility of microfluidics to users (34-38). As aforementioned, examples of the practical application of 3D/4D printing techniques have considerably increased over the past decade (Figure 1).

The pace of development is shown through a considerable increase in publication numbers (Figure 1) of 3D/4D (bio)printed products, and in the growth of the small sub-fields of 3D/4D (bio)printing for "tissue engineering", "healthcare", "regenerative medicine", "pharmaceuticals" and "microfluidics." Throughout this review, a short overview on recent accomplishments, challenges and forthcoming perspective application of 3D/4D (bio)printing in these fields are summarized.

A variety of 3D printing technologies, each employing different operating principles and input materials, can be used for a broad range of applications. As mentioned previously, most of these techniques follow the same basic fabrication procedure to create final products from digital models (39-41). These aspects, common between both 3D printing and 3D bioprinting, are outlined in Figure 2.

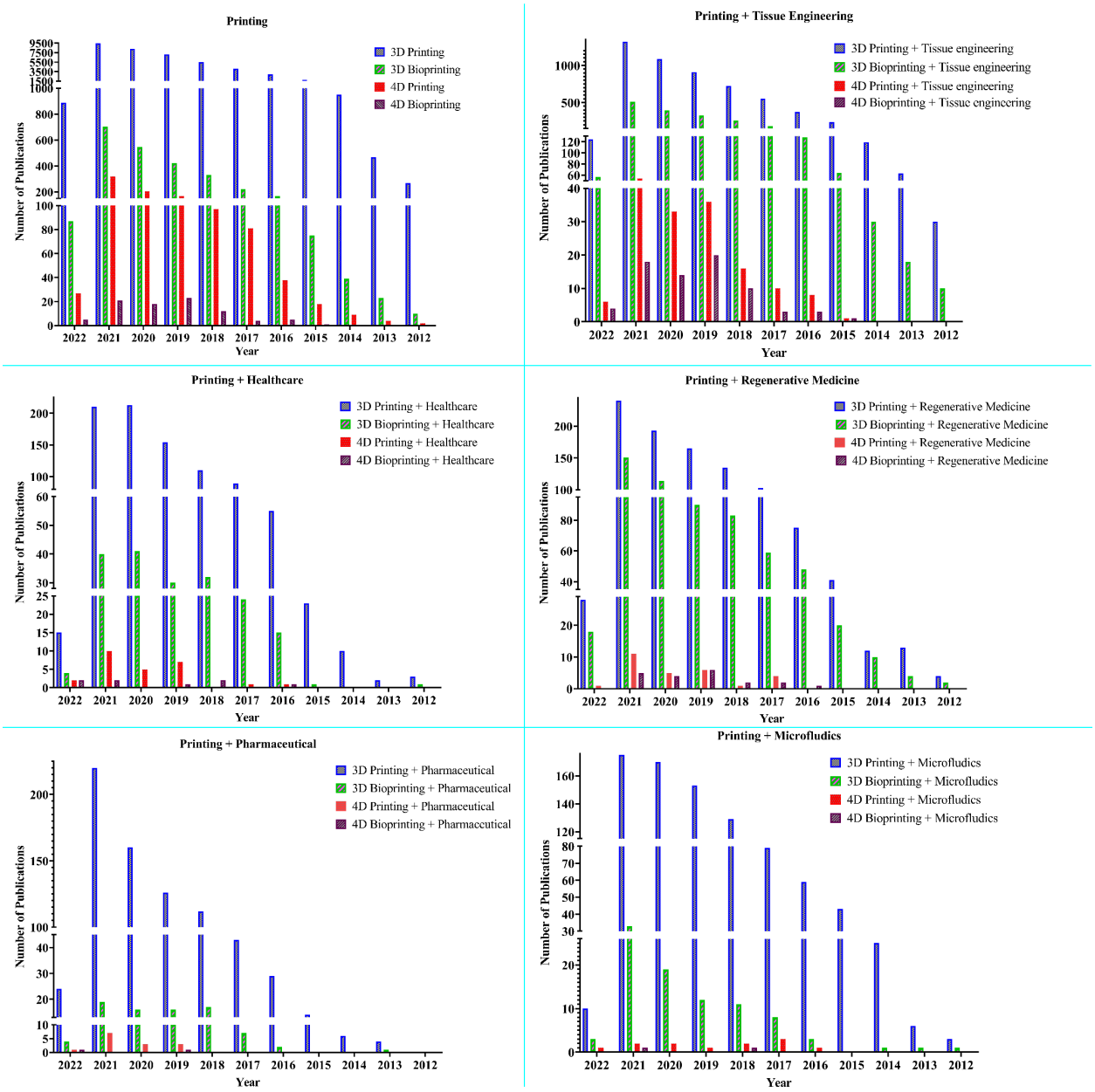

Figure 1: Publication trends derived from Web of Science over the last decade (2012-March 2022) with terms of "3D printing", "3D bioprinting", "4D printing", "4D bioprinting" in all fields of "tissue engineering", "healthcare", "regenerative medicine", "pharmaceuticals" and "microfluidics" in combination.

The various 3D printing techniques used for drug product manufacturing, cell and gene delivery, tissue engineering, and microfluidic applications are (I) inkjet or binder deposition printers (40,

42), (II) extrusion or fused deposition modeling (43), (III) material jetting (39, 40), (IV) powder bed fusion methods (44-46), (V) stereolithography or photopolymerization technique (47, 48), (VI) pen-based 3D printing (49, 50) and (VII) 3D printed molding or indirect 3D printing (35, 51-53).

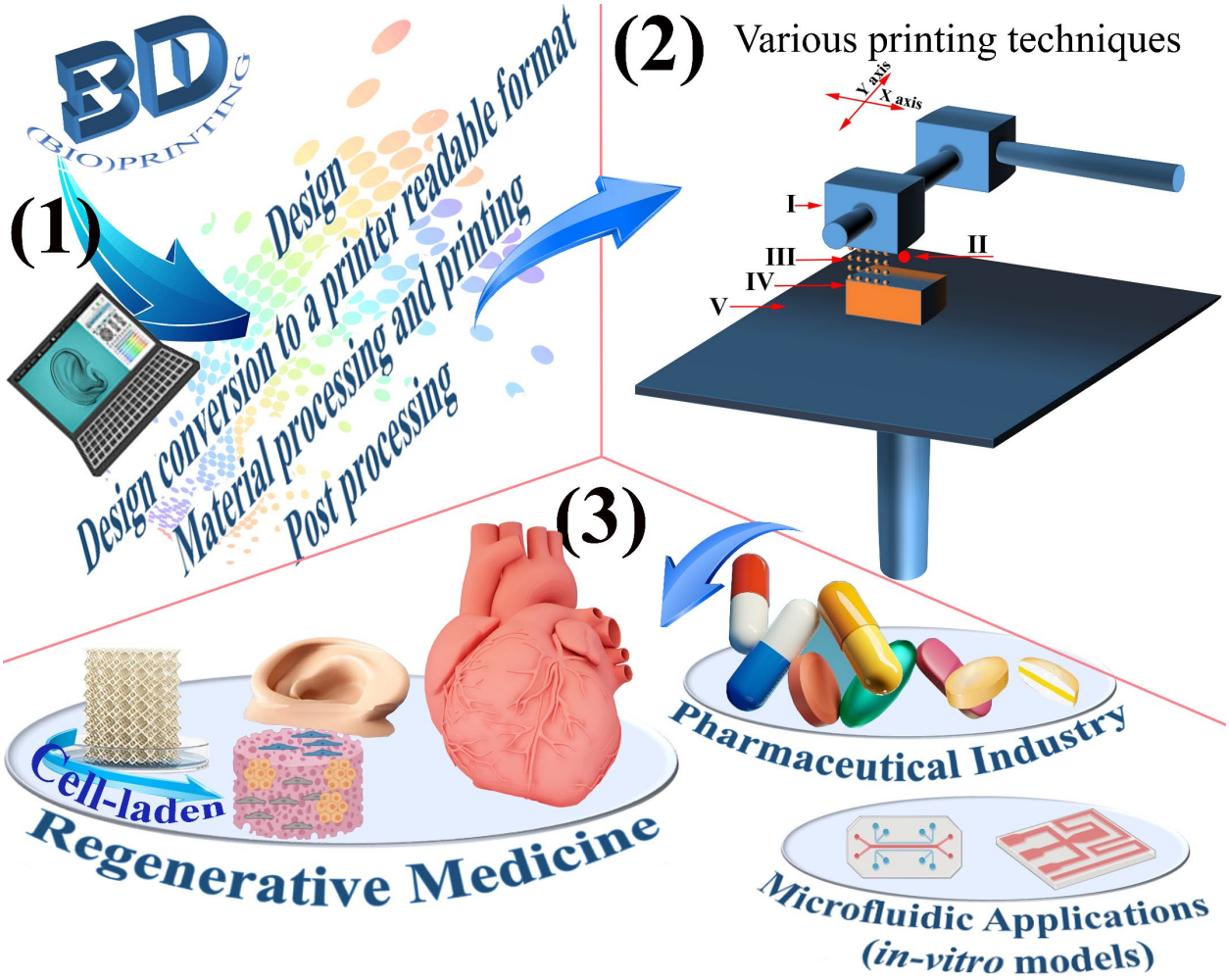

Figure 2: (1) Common aspects between various 3D (bio)printing methods: the expected product plan is digitally rendered with computer-aided design software, and rendered plans are converted to a 3D printer-friendly format, typically STL. Raw materials are then processed and automatically printed and solidified layer by layer to form the final product. (2) Schematic example of a 3D printing technology: A material jetting printer, consisting of I: Jetting head. II: UV curing light (optional). III. Droplets of build materials selectively jetted onto a build bed. IV: in-progress 3D printed sample. V: build tray. (3) 3D printing technologies have significant potential for various medical applications. Image credit Mr Karim Osouli-Bostanabad.

2 3D printing (additive manufacturing) techniques in pharmaceutical industry

Dosage forms typically comprise an active pharmaceutical ingredient and in most cases inactive excipients that ensure active pharmaceutical ingredients absorption, stability, patient palatability. In this field, additive manufacturing should not be confused with traditional additive processes such as film lamination, capsule filling or coating in pharmaceutical manufacturing (54, 55). 3D printing techniques allow for the direct production of a finished, highly personalised dosage form rather than requiring a step-by-step manufacture of active pharmaceutical ingredients and excipients (4, 5). 3D printing offers competitive advantages for made-on-demand, personalized and complex products that establish opportunities for enhancing the accessibility, effectiveness, and safety of drugs (7). The importance and relevance of each of these features is outlined below, along with recent trends in 3D printing that utilize these capabilities and may further advance drug therapies.

2.1 On-Demand Fabrication

3D printers have the ability to fabricate products with a range of defined spatial features on a short timescale (minutes to hours), depending on the size of construct, complexity, and type of printer utilised. This capability allows the printing of low-stability drugs for immediate intake, printing directly into/onto patients (for example, to print on-demand gels with wound healing properties, or tissue engineering scaffolds (56-58)), and printing in situations where time and resources are limited, such as ambulances, disaster zones, intensive healthcare units, surgery and emergency rooms, and military operations (59, 60).

To improve the development and delivery of drug products in a time-effective manner, pharmaceutical industries could feasibly embrace an approach from automotive fabrication, where 3D printing is employed to manufacture and evaluate numerous product iterations. One possible

strategy is the use of meshes that can be used topically and as an implant, using polymers with stimuli-responsive properties to actively release a drug when in contact with the target environment. This has been demonstrated in an elastic mesh for topical application as a drug delivery 'bandage' (61). The bandage consists of antibiotic-loaded nanofibrous meshes with embedded PEGylated-chitosan nanoparticles capable of responding to thermal stimuli (~37 °C). A flexible heater is also incorporated into the platform to enable control of drug release (Figure 3), while the final product is elastic, flexible, and conforms to easily attach to the skin in order to be used as either a local patch or as implantable support for wound healing.

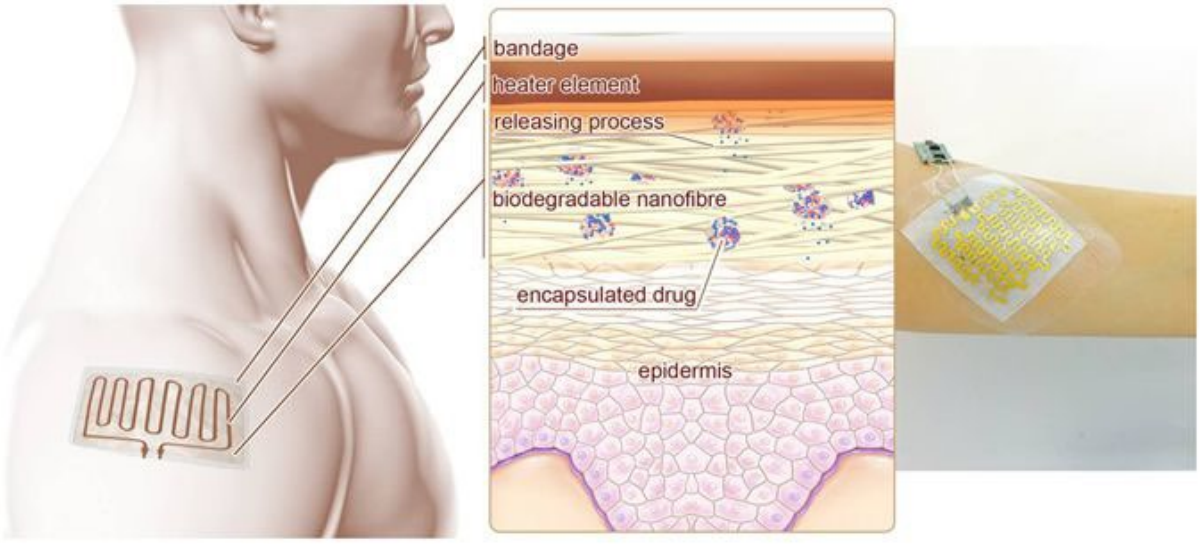

Figure 3: Components of an engineered flexible bandage drug delivery system with integrated electronics and heater (left and middle), where drug loaded thermo-responsive nanocarriers were placed within the nanofibers of the manufactured mesh, and the drug release was controlled by adjusting the temperature of the integrated heater. The miniaturized electronic control system is presented on the right. This image reproduced from [61] and used under the Creative Commons license permission (CC BY 4.0). Copyright 2017 Springer Nature

2.2 Personalisation

3D printing of drugs enables pharmacotherapy tailored to the individual characteristics of each patient. This technologyenables the rapid formulation of patient-centric medicines i.e. the right medicine/dosage form, for the right person, at the right time, in the right dose. For instance, paediatric patients are a particular population that needs special attention when designing dosage forms because of the necessity for medication adherence and clinical safety (62). In most cases, inadequately satisfying the therapy adherence results in sub-optimal health outcomes for these patients. Additionally, most therapeutic agents are not recomended for paediatric use, as children are excluded in the pre-development stage of clinical trials (63). The administration of tablets is the most suitable for paediatric patients (i.e. school age/ infants given Oral dosage forms < 2 mm), particularly those that can be effortlessly swallowed and dissolved in the oral cavity (64). Organoleptic characteristics, including taste, appearance, smell, or texture are also influential and foster adherence in paediatric patients. In addition, for paediatric product development, excipients selection should be considered in a way that is approved for these patients and therefore the formulation of dosage formats should be carefully designed (64). Ghanizadeh-Tabriz et al. used an extrusion-based 3D printer to fabricate fruit-chew designs containing bitter diphenhydramine hydrochloride using hydroxypropyl cellulose as a carrier and gelucire 48/16TM as a non-ionic surfactant combined with sucralose as a sweetener and strawberry flavor (65). A glass solution formation containing molecularly dispersed diphenhydramine hydrochloride inside the hydrophilic carriers was confirmed by physicochemical characterisations. The dissolution behavior of diphenhydramine hydrochloride from the 3D printed fruit-chew designs followed a burst release with >85% during the first 30 min. The sensory evaluation of the strawberry aroma and the sweetener intensity revealed a full taste masking of the bitter diphenhydramine

hydrochloride and showed a synergistic influence of the strawberry flavour and the sweetener with augmented fruity sweet strawberry, and aftertaste perception. It has been stated that these results can be benefited for paediatric dosage forms development with improved organoleptic characteristics, medication adherence and palatability (65).

One factor in the development of patient-centric medicines is palatability, which may have a significant effect on treatment outcomes, as well a patient's willingness to take the drug. Overall, the development of such pharmaceutical products with high quality, safety and clinical efficacy is extremely challenging and requires the implementation of novel manufacturing technologies which can address the aforementioned challenges but also to produce personalised dosage forms that fit the patient's clinical needs (e.g., dose, pharmacokinetics, palatability). However, patient willingness to use oral dosage forms, such as tablets in various forms, has yet to be entirely investigated. For example, patients prefer to take drugs in tablet forms (66-68), and preferences are often influenced by shape and size, but altering a tablet in any way to improve palatability or make it easier to swallow (e.g., dividing, crushing) could result in over/underdosing by changing the bioavailability. To address this, the effects of drug size and shape on palatability have recently been evaluated to determine how to best utilize the versatility of 3D printing to produce different drug shapes and formulations for improved patient outcomes. The majority of studies on 3D-printed drugs investigate the *in vitro* dissolution behavior of drugs based on their ingredients and design (69-72); however, one of the biggest superiorities of 3D printing procedures, the capability of producing numerous creative shapes or formulations to enhance patient acceptability, needs to be precisely evaluated. The palatability, size and shape design can improve the simplicity of swallowing as well as reduce the risk of esophageal injury. The desire and preference of target populations can also influence the shape and design of drug products. Goyanes et al. evaluated the

influence of size, shape and color of various 3D printed placebo tablets on palatability in 50 participants (73). Ultimately, patients showed a preference for the torus geometry, and printlets with a similar appearance to traditional formulations (discs and capsules) were also selected as easy to swallow. This finding highlighted that familiarity is an important quality for printlet acceptability. Printlets with smaller sizes were also found to be more desirable, although perception of size was found to be influenced by shape. Furthermore, it was found that printlet color can also influence patients' perception (73). However, patient willingness to use different oral dosage forms, such as tablets, capsules, lozenges, liquids or powders, is yet to be entirely investigated.

Chemotherapy and anticoagulation are amongst some of the most common therapies that require adaptable, personalized and on-demand frequent dose modifications to ensure optimal care. The accurate manufacturing capability and flexibility of 3D printed technologies permits frequent modificaitons of dose while enabling minimum effective dose administration of a the drug (74). Taking the example of warfarin, even though it compacts in small doses are available now, and doses can be adjusted by ingesting multiple tablets, or splitting or cutting tablets of a higher dose, this can lead to over/underdosing of the drug (75-77). To improve upon this current system, Vuddanda et al. used inkjet/binder deposition to demonstrate the capability of 3D printing for warfarin dosage individualization through engineering of easy-to-reproduce narrow/small-dosage warfarin (~ 50 μg) (78). This further supports the idea that ink formulations using appropriate solvents or nanosuspensions could lead to individualized and flexible dosage forms by 3D printing techniques (5, 57, 60, 68, 71, 75, 79, 80), although additional regulatory criteria must also be met for pharmaceutical applications. Additionally, in a study the flexibility of fused deposition modeling was utilized to produce personalized solid dosage forms tailored to an animal’s anatomy,

where warfarin therapeutics were fabricated in narrow/small doses and studied *in vitro* and *in vivo* (81). The study demonstrated that 3D printing methods have potential to engineer a highly responsive, accurate and dynamic anticoagulant delivery system capable of responding to even a constantly changing clotting profile of a patient. These studies indicate the vast potential of 3D printing methods to advance personalized medicine and the pharmaceutical industry more broadly.

Personalisation of dose ensures that pharmacokinetic and pharmacodynamic parameters are individualised for each patient, taking into account factors such as weight and age, to reduce the risk of side effects and overdosing (82, 83). To date, liquid oral dosages (for instance, suspensions and solutions) have been personalized using various straightforward dosing aids, such as droppers, scaled spoons or calibrated syringes. However, while these solutions are inexpensive when commercially available, they remain expensive if special manufacturing is necessary for the patient and human error remains an issue (84). As such, researchers have applied 3D printing techniques to formulate precise and reproducible liquid oral dosages. For example, fused deposition modeling has been used to print capsule shells, with the core of the shell loaded manually during the printing process (85). However, the manual loading in this example still risks a degree of human error. A fully automated 3D printing method was applied by Tochukwu et al. to print a liquid capsule (86), with shells of polymethacrylate polymer. The shell of the capsule was fabricated in a layer-by-layer manner and filled with a printed liquid dose during the printing process without compromising the integrity of the capsule shell or requiring any curing stage. The system was able to contain two active pharmaceutical ingredients (theophylline and dipyridamole) at the same time, indicating that it is a promising candidate for delivery of multiple solutions or suspensions. Furthermore, this method was capable of delivering both immediate and sustained drug doses, with release rate easily controlled by manipulating shell thickness and dispensed liquid dose volume

via the printing software. In clinical applications, this will enable healthcare workers to prepare a specific drug dose and release profile in personalized liquid capsules without the need to alter drug formulation.

We envisage that the utility of 3D printing will expand, enabling the manufacturing of complex drug products, for example, personalized implantable or wearable systems with controllable drug dissolution rates that exploit the benefits conferred by 3D printing materials. To maximise their potential, additive manufacturing can be associated with a 3D scanning system that extracts details of a patient's anatomy to make a customized tool that is specific to the patient such as drug-loaded patches that fit specific patient anatomies (87-90). However, current methods are inadequate in terms of standardization of approaches and materials, hampering the advancement of scalable and consistent 3D-printed drug delivery tools for use in healthcare industries (39, 59, 91, 92). This was demonstrated in 3D-printed individualized mouthguards that were manufactured based on personalized intraoral scans of dentition impressions with ability to deliver clobetasol propionate in the oral cavity and their *in vitro* drug release behavior was evaluated (Figure 4)(8). The drug dissolution behavior in humans was assessed from three kinds of mouthguards in varying material composition or design using vanillic acid (food-grade flavourant) instead of clobetasol propionate, where a sustained release profile was observed over the 6 h trial. Fused deposition modeling was used in this study as it does not use toxic photoactive polymers or organic solvents, which may facilitate the translation of these mouthguards to the clinic by restricting potential risks to human health (3, 93). Furthermore, polylactic acid and polyvinyl alcohol were selected as the mouthguard material, as these have pharmaceutical grades available for fused deposition modeling and clinical applications (8). This proof-of-concept study helps to confirm the potential of 3D printing as a platform for the translation and advancement of next-generation drug delivery tools for

individualized therapy. However, given that not all therapies or conditions are suited to oral release, there is still a need to further expand a more systematic process for other delivery routes, for example dermal, ocular, or internal release from implants.

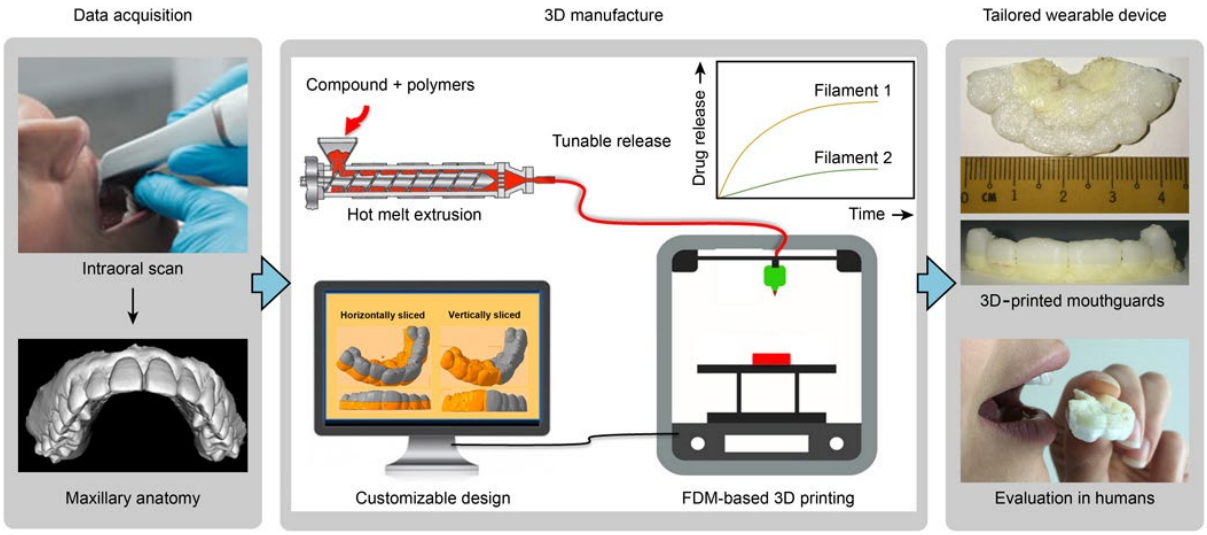

Figure 4: The 3D printing manufacturing process of wearable individualized oral drug delivery mouthguards manufacturing process using 3D printing. This process involves two phases, (I) the information acquisition step, in which the maxillary anatomy was acquired by an intraoral scan, serving as the pattern for 3D printing, and (II) the fabrication step, where a hot melt extrusion technique was used to manufacture the desired drug loaded printable filaments, which were then printed into customized devices using fused deposition modelling [8]. This image reproduced from [8] under the Creative Commons license permission (CC BY-NC-ND 3.0). Copyright 2018 Science

2.3 Complex Constructs

3D printing in the pharmaceutical industry could facilitate the development of new drug delivery systems with unprecedented accuracy and complexity (94), unlike traditional formulation methods

where fabricating structures with detailed spatial characteristics and controllable dissolution rates is difficult or impossible to achieve (95, 96). The flexibility and efficiency of 3D printing could propel the healthcare industry toward the goal of printing multi-medicine “polypills” to merge all of a patient's drugs into a single daily dose (70, 75, 86, 97). Given that the composition of a drug product can affect drug dissolution (98, 99), complex 3D printed structures establish novel possibilities for drug delivery systems, producing adaptable and complex geometries to provide controlled loading and dissolution behavior (70, 75, 97, 100). For example, dissolution is structure-dependent, and 3D-printed complex objects can be augmented to increase release rate compared to conventional immediate-release compounds, by printing shapes with higher surface to volume ratios (100, 101) or amorphous solid dispersions (87, 102-104). Although 3D printing can be applied for immediate release systems in this way, the majority of 3D printing studies are focused on controlled release systems (69, 79, 105-108). Aside from various 3D-printed drug products with complex structures, implants and scaffolds containing complex dissolution behavior can also be made using 3D printing technologies (109-112), which is an exciting approach to improve control of release patterns and drug targeting. Table 1 gives a summary of some dosage forms development using 3D printing techniques.

Table 1: Different examples of 3D printing techniques for development of various pharmaceuticals

3D printing Method	Product (Dosage Form)	Excipiants/Materials	Active Pharmaceutical Ingredient	Pharmaceutical Usage	Ref.
Laser-based printing	Printlet	Candurin® NXT Ruby Red, Kollicoat® IR, Lactose monohydrate, Talc	Lopinavir	Solubility enhancement	(113)
	Oral tablet	Kollicoat IR, Eudragit L100-55, Candurin® gold sheen	Paracetamol (acetaminophen)	Immediate and modified release profiles	(114)
	Immediate-release (fast-dissolving) tablet	Mannitol, Silicon dioxide (colloidal SiO_2), Lactose, and Polyvinylpyrrolidone K30 (PVP K30)	Paracetamol (acetaminophen) and Alizarin yellow (dye)	Fast-disintegrating drug delivery devices	(115)
Inkjet printing	Controlled-release tablet	Beeswax	Fenofibrate	Controlled dissolution behavior for prolonged periods	(116)
	Controlled-release tablet	Polyethylene oxide (PEO) and Polycaprolactone (PCL)	Alizarin yellow and Methylene blue (dyes)	Controlled release drug delivery devices	(117)
	Controlled-release tablet	Polyethylene glycol (PEG)	Naproxen	Tablets with controllable dissolution kinetics	(118)
	Oral tablet	Cellulose powder, Eudragit® E-100, Eudragit RLPO, Lactose	Chlorpheniramine maleate	Controlled release drug delivery devices	(119)

Extrosion-based printing	Doughnut-shaped tablet	Hydroxypropyl methylcellulose E100, Ethyl cellulose, PVP K30, colloidal SiO_2	Acetaminophen	Tablets with linear release behavior	(120)
	Tablet	Poly(vinyl alcohol) (PVA)	Prednisolone	Extended-release tablets	(121)
	Tablet	Kollicoat® IR, Kollidon® VA64, Affinsiol™15 cP, Hydroxypropylmethylcellulose Acetate Succinate (HPMCAS) Benecel™	Haloperidol	Rapid-release tablets	(122)
	Tablet	Hydroxypropylmethylcellulose (HPMC) E5, Klucel™ HPC EF and LF, Aqualon™ EC N14, Soluplus®, Eudragit® L100,	Acetaminophen	Controlled-release tablets	(123)
	Caplet	PVA	Paracetamol/ Caffeine	Personalized oral dosage forms	(124)
	Tablet	PVA	5-aminosalicylic acid (5-ASA, mesalazine), 4-aminosalicylic acid (4-ASA)	Tablets with Modified-release profile	(125)
	Intravaginal ring	Polyurethane (Tecoflex™ EG-100A)	Clotrimazole	Sustained release dosage forms (delivery devices)	(126)
	Oral scaffolds	Acrylonitrile butadiene styrene (ABS)	Carbamazepine	Prolonged - release scaffold (zero-order release)	(127)
	Bilayer tablet	Hydroxypropyl cellulose (HPC), HPMCAS	Isoniazid, Rifampicin	Dual controlled release profile	(128)

	Tablet (hollow structured)	HPC	Domperidone	Sustained release tablets	(129)
	Tablets in torus-shaped	Poly(ethylene glycol) diacrylate (PEGDA), PEG, diphenyl(2,4,6-trimethylbenzoyl)phosphine oxide	4-ASA, Paracetamol (acetaminophen)	Modified-release (customizable) tablets	(130)
Stereolithography printing	Indwelling bladder devices	Elastic Resin, Magnesium chloride anhydrous, Urea, Gelucire® 48/16, Potassium dihydrogen phosphate	Lidocaine hydrochloride	Prolonged and localised delivery devices	(131)
	Polyprintlet	Hydrochlorothiazide, PEGDA, diphenyl(2, 4, 6-trimethyl-benzoyl) phosphine oxide (TPO), PEG	Irbesartan, Atenolol, hydrochlorothiazide, Amlodipine	Controlled release profile	(132)

3 3D printing techniques for biomimetic structures

Tissue engineering focuses on restoring the structure and function of damaged, dysfunctional or lost tissues through the engineering of functional tissue constructs (111, 133). Even with noteworthy progress in the field of tissue engineering and regenerative medicine over the last few decades, manufacturing of functional and complex tissue constructs capable of mimicking natural behavior remains a challenge (22, 134, 135). In particular, the development of 3D interconnected vascular networks within these tissue constructs remains a key issue requiring resolution, as it plays a vital role in enhancing the function of engineered tissues (135-137).

3.1 3D printing in tissue engineering

Adjusting bottom-up processes (i.e. the directed- or self-assembly of a scaffold from smaller elements or modules, feasibly with various modules aimed to perform distinct functions) for tissue engineering is a major challenge, and numerous attempts to print synthetic biodegradable/biocompatible scaffolds have been made since the first use of fused deposition modeling for tissue engineering applications (138, 139). Here, 3D bioprinting is a promising recent advancement, with the ability to simultaneously deposit single or blended supportive matrices and living cells (collectively called bioink), and the potential to assist in the fabrication of well-organized 3D vascular networks (25, 134, 137, 140). Various types of rapid prototyping methods have been established to enable the creation of macroscopic structures of deposited biomaterials, including gel deposition using syringe-based approaches, stereolithography, and solid freeform manufacturing. While both 3D printing and 3D bioprinting use a 3D prototype to fabricate constructs in a layer-by-layer manner, 3D bioprinting encompasses the use of both cell-loaded bioinks and other biological agents to build a living cell laden scaffold. Note here that the

polymeric 3D printed scaffolds that have a porous structure to allow cell seeding should not be confused with 3D bioprinting of cell-loaded bioinks (141, 142). In other words, 3D bioprinting is a method of manufacturing cell-loaded bioinks within functional tissue structures and organs from 3D digital prototypes.

3D bioprinting has numerous benefits in comparison with traditional tissue engineering approaches, which suffer from an inability to produce complex biomimetic constructs, with simple structures leading to unrealistic cell microenvironments (143). In contrast to this, 3D bioprinting has the ability to manufacture spatially sophisticated multi-modal, multi-component, multi-dimensional (nano to macro structures), compartmentalized biomimetic tissue constructs able to more acuretly mimic native tissue structure and function. In addition, 3D bioprinting inherently ensures good manufacturing practice processes via improved accuracy, automated and reproducible processing, and geometric freedom. These aspects of 3D bioprinting all combine to improve reproducibility and control of morphology, including key material properties such as porosity, pore size, compression modulus and general target tissue compliance. Additionally, spatial control over deposition of a wide range of biochemical elements, including growth factors, proteins, DNA, and drugs, in combination with cells, helps to further facilitate the fabrication of functional, precisely-controlled tissue repair constructs (17, 144). Several bioinks composed of various hydrogel substances, such as hyaluronic acid, collagen, gelatin, alginate, polyethylene glycol, chondroitin sulfate and others, have been used in bioprinting with high reliability, owing to their appropriate viscosity and biocompatibility (145-147). These bioinks are discussed in more detail in Table 2 and sections 3-6. One example of using several bioinks is the ‘BioPen’ (Figure 5, a droplet based bioprinting), developed by Han et al., which is capable of functional material deposition in a persistent, predetermined and scalable manner, offering micrometer spatial

resolution and nanoliter volumetric resolution (56, 148). As shown in Figure 5, this process is based on the ballpoint pen, where multichannel ink sources (i.e., solutions of the functional elements) and equipment with the capability of writing nucleic acids, proteins, living cells and other entities were substituted with the previously used channels. Point-of-care detectability using this method was demonstrated by using the BioPen to apply bioink onto paper, where the ink contained nucleic acid probes and gold nanoparticles modified on type-I human immunodeficiency virus (56). Furthermore, point-of-care usability of this technique was demonstrated by writing a persistent structure of functional living, interlinked cells with a determined extracellular medium in tissue engineering. Owing to the simplicity and accuracy along with the portability of this method, it can be economically applied for point-of-care biomarker detection, tissue engineering and deposition of patterned substances within surgeries. However, the manual operation of this handhold pen device means it is time consuming to use, lacks machine precision, and is not as scalable as other technologies, it is still an interesting technology. There may also be niche application in field medicine where portability would be a key priority, but these are outside the scope of the review. For our purposes, this simple technology highlights the core simplicity and attainability of 3D printing. Although often seen and discussed on a manufacturer scale, the underlying concept is noteably simple and flexible.

3D printing of muscle structures, particularly skeletal and cardiac muscles, is extensively researched (149, 150). Skeletal muscle damage occurring from surgery, tumour, trama, and degenerative disease exceeding 20% leads to denervation, scarring and loss of function (151). Independence and mobility restoration is a significant challenge in reconstructive approaches, where tissue engineering provides a solution towards the development of a muscle-tendon unit. Though numerous researchers have tried to design the muscle and tendon separately, few studies

have concentrated on advancing this type of composite construct (152, 153). To develop the muscle-tendon unit, Merceron et al. engineered a complex tissue structure using 3D bioprinting, fabricating a polymeric, mechanically heterogeneous scaffold structure that was relatively rigid on the tendon side and flexible on the muscle side (154). Further, it was capable of tissue-specialized cell distribution, with myoblasts on the muscle side and fibroblasts on the tendon side (154). These experiments confirmed the versatility of 3D unified organ printing systems to build integrated tissue structures with tissue-specialized mechanical and biological characteristics. Recent reports have demonstrated engineered 3D musculoskeletal tissues for fundamental research and drug screening goals (24, 149, 155-159).

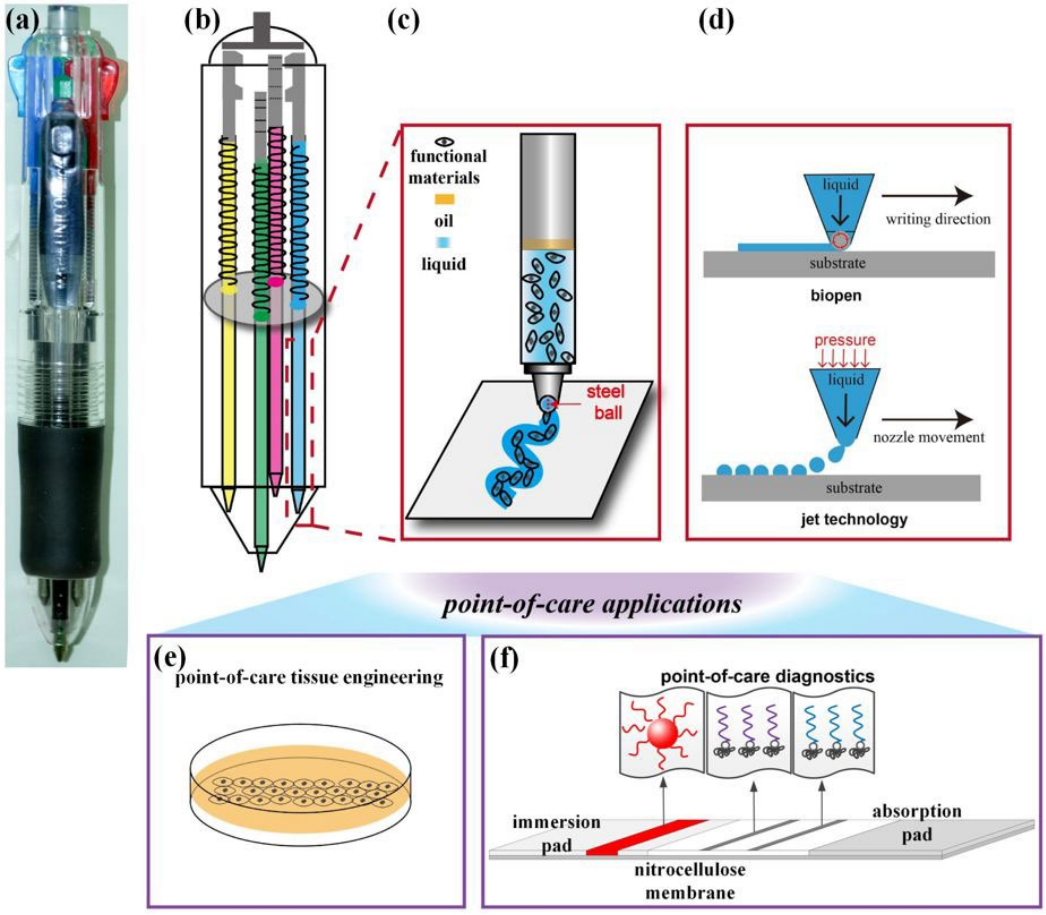

Figure 5: (a) The manufactured BioPen, with functional inks substituted into the ballpoint pen channels and optimized settings to write the bioink. (b) Multiple cartridges containing functional inks can be placed into one BioPen. (c) By adjusting the writing speed of BioPen, the functional ink suspension can easily be patterned onto various substrates. (d) Excellent connectivity can be achieved compared to the inkjet method, due to the continuous nature of the "writing" process, unlike the ink separated streams. (e, f) Accuracy and portability are both key advantages of this method for point-of-care diagnostics and tissue engineering [56]. This image reproduced from [56] under the Creative Commons license permission (CC BY-NC-ND 3.0). Copyright 2014 Springer Nature

Researchers are currently focussing considerable efforts on reproducing the highly complicated cytoarchitecture of complex functional patterns of the human central nervous system. Despite the fact that 2D cell culture approaches with human induced pluripotent stem cells (iPSC-derived neural cells) and animal models have presented promising insights into disease progression, developmental biology, and central nervous system networks functional dynamics, much of intrinsic complications in central nervous system of the human are not recapitulated (160). Lozano et al. used an extrusion based 3D printer to print brain-like constructs resemble as those in the cerebral cortex, with arginylglycylaspartic acid modified gellan gum hydrogel containing several layers of cortical neurons (161). A viability of about 80% has reported for the encapsulated neurons and they could differentiate into glia and neurons. A three-layered sandwich construct containing neurons in top and bottom layers and no cells in a middle layer was printed to evaluate the outgrowth of neurite between adjacent hydrogels from cortical neurons. Following 5 days of *in vitro* culture, the fabricated sandwich structure containing neurons extended axons, which penetrated around 100 μm within the middle acellular layer. Despite of the lack of a determined architecture in the printed construct, the capability of controlling the organization of extra cellular matrix and cells were sufficient to simulate multilayered brain-like neural circuits and to offer a way for recognizing neurodegenerative diseases and traumatic injuries of the brain (161). Hinton et al. fabricated an alginate-based brain model where complicated anatomy of a human brain, such as cerebellum and cortex, were printed with a resolution of 200 μm using CAD models based on optical, magnetic resonance imaging and computed tomography data (162). This study as a proof-of-concept showed the feasibility of fabricating the anatomical architecture of the brain tissue, although it should be noted the internal construct of the printed model was not built (162). Qian et al. reported an integrated layer-by-layer casting and 3D printing technique in fabrication of multi-

layered porous scaffolds composed of polycaprolactone and single- or multi-layered graphene (Figure 6) (163). The results showed that graphene incorporated electrically conductive 3D scaffolds could greatly enhance expression of neural cells both *in vitro* and *in vivo*. Where successfully axonal remyelination and regrowth was promoted after peripheral nerve damage.

Metin et al. printed graphene and gelatin-based nerve regeneration scaffolds/conduits, where mesenchymal stem cells (80%) revealed staining markers of Schwann cell and greatly enhanced the nerve growth factor secretion in existence of electrical stimulation (164). Additionally, Jakus et al. using an extrusion-based 3D printer fabricated a conductive composite scaffold of graphene-loaded polylactide-co-glycolide. Printed samples depicted high flexibility and mechanical strength, while preserving a conductivity more than 800 S/m. *In vitro* data revealed that 3D printed construct supported the human mesenchymal stem cell viability, adhesion, proliferation, and neurogenic differentiation, and considerably upregulated neuronal and glia genes (165). It been suggested that the electrical cues in the human nervous system could be used to inspire interactions between scaffold/neurons and lead those actions mimic that of a native tissue, such as the efferent limb: contraction of striated muscle, smooth muscle, and glandular secretion (166). Although significant development of novel bioinks has been reported, the viability and feasibility of these polymers/bioinks is still a challenge that must be answered, due to the need for printability, biofunctionality, biocompatibility, and in some cases, biodegradability. Ouyang et al. studied printability of bioinks employing a new semi-quantitative technique (167). This work revealed that viability of bioinks enhanced when printed in higher temperatures, high shear stresses, and lower concentrations of gelatin. Furthermore, biomaterials to be biofunctional must contain cell recognition sequences, consequently they will not immunologically be rejected *in vivo*.

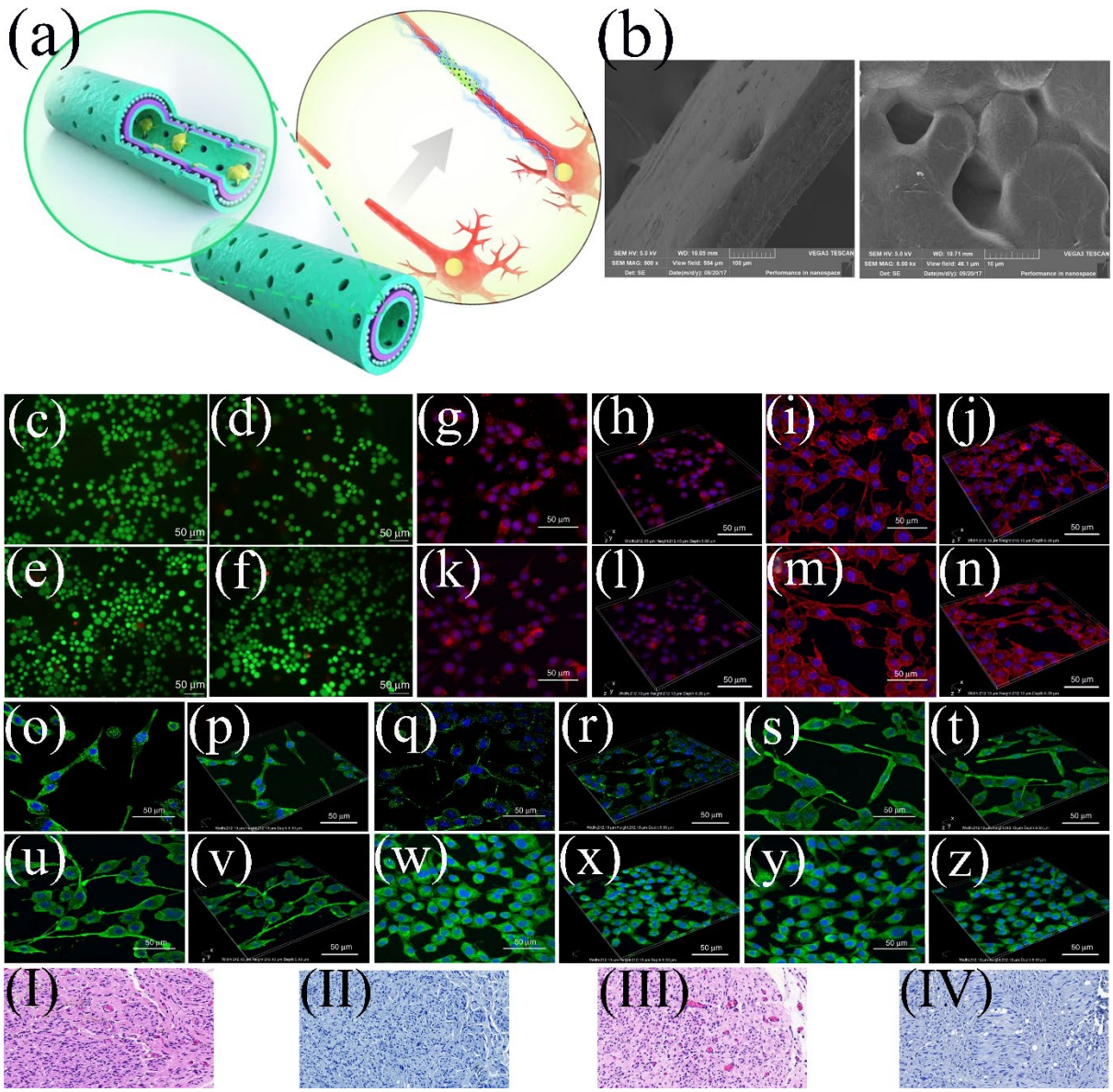

Figure 6: (a) Schematic presentation of layer-by-layer casting/printing of graphene nerve conduit; the interior and exterior green layers are mixed layers of polydopamine/ arginylglycylaspartic acid (PDA/RGD), the purple layer is a mixed layer of polycaprolactone (PCL) and single-layered (SG) or multi-layered (MG) graphene, and an illustration of the nerve conduit in a sciatic nerve injury model in the Sprague Dawley rats. (b) SEM images of the multi-layered and nanoporous 3D structure. (c-f) LIVE/DEAD cell staining for cell viability assessment on Schwann cells (SCs) by cell counting kit 8; (c) Live/dead/merge pictures for PDA/RGD-SG/PCL, (d) PDA/RGD-MG/PCL, (e) PDA/RGD-PCL, and (f) PCL (scale bar 50 µm). (g-n) Immunofluorescent staining for F-actin and nuclear protein Ki67 (Ki67); Ki67 expression of SC on (g, h) PDA/RGD-SG/PCL, (k, l) PDA/RGD-MG/PCL, Phalloidin staining on (i, j) PDA/RGD-SG/PCL, and (m, n) PDA/RGD-MG/PCL (scale bar 50 µm). (o-z) Immunofluorescent staining for glial fibrillary acidic protein (GFAP), class III β-tubulin (Tuj1), and S100; GFAP expression of SC on (o, p) PDA/RGD-SG/PCL, (q, r) PDA/RGD-MG/PCL, Tuj1 expression of SC on (s, t) PDA/RGD-SG/PCL, (u, v) PDA/RGD-MG/PCL, S100 expression of SC on (w, x) PDA/RGD-SG/PCL, and (y, z) PDA/RGD-MG/PCL (scale bar 50 µm). (I-IV) Hematoxylin & Eosin (I, III) and toluidine blue (II, IV) staining's for regenerated nerves after 18 weeks; SC-loaded (I, II) PDA/RGD-SG/PCL, and (III, IV) PDA/RGD-MG/PCL (scale bar 100 µm) (163). This image reproduced from [163] under the Creative Commons license permission (CC BY 4.0). Copyright 2018 Springer Nature

Lastly, the rheological characteristics of these substances (i.e. the balance between fluid and solid properties) must be matched to those of the target tissue (168). These limitations in combination present a challenging group of variables that restrict the range of biofunctional compounds available. Thus, even though many substances are intially considered as promising functional materials, they prove incompatible for *in vivo* applications. Polyaniline is a typical example of a previously common biomaterial in bio-related applications, due to its favorable persistence and conductivity at a broad range of pH. Polyaniline has the capability to easily create thin films, especially on nanostructured materials, which makes it an appealing candidate in biomaterial applications, but it lacks biodegradability and flexibility, while is possesses poor processibility and has been linked with chronic inflammation after implantation (169).

3.2 3D printing for gene/cell delivery

Gene/cell delivery is an integral part in regenerative medicine and tissue engineering research and this exemplified in the field of cartilage, bone and fibrous connective tissue (e.g. ligaments and tendons) research. (11, 111, 170, 171). There is a growing need for effective bone grafts globally, with over five hundred thousand patients suffering from bone defects and receiving restoration yearly in the US alone (172) and skeletal tissue manufacturing (e.g., cartilage and bone) via 3D bioprinting is a major advancement of regenerative medicine and tissue engineering efforts (173), with significant attention to integration of rhBMP-II (recombinant human bone morphogenetic protein-II, one of the most effective growth agents for bone development) within the scaffold as the most clinically relevant bone regeneration strategy. However, inconsistent results were obtained in clinical applications as rhBMP-II-loaded collagen sponges w showed a quick release rate that limited its osteoinductive activity (174). Additionally, rhBMP-II in high doses has resulted in postoperative problems such as inappropriate bone formation, osteolysis and

swelling of soft tissue (175, 176). Several studies have assessed the efficacy of 3D printed scaffolds in bone formation when integrated with different osteoinductive stimuli (177-180). Another approach to applying rhBMP-II is local gene treatment, which uses transduced cells to convey the BMP-II protein. This enables delivery of an osteoinductive growth agent alongside osteoprogenitor cells to a particular anatomic area where bone formation can be induced by transduced cells. Alluri et al. investigated the osteogenic potency of hyperelastic bone scaffolds prepared using 3D printing technology integrated with an extended osteoinductive growth signal using lentiviral gene treatment (181). *Ex vivo* local gene therapy allows for gene incorporation within host cells in a laboratory, which are then reimplanted into the host at a target site. Here, the osteoinductive BMP-II protein was released in a sustained manner (two weeks) from transduced cells, establishing the formation of a new bone.

Until now, numerous 3D printing techniques have been adapted to enable bioprinting with previously developed hydrogels and biopolymers. As the significance of individualized medicine is becoming more evident, the demand for new bioinks encompassing patient-specific (autologous) biological agents for tissue engineering is growing. Platelet-rich plasma is used as a patient-specialized resource of autologous growth agents that can be effortlessly integrated with hydrogels and printed within 3D structures. Platelet-rich plasma can improve angiogenesis, recruitment of stem cells and regeneration of tissue, as it contains a cocktail of growth agents. Faramarzi et.al. have engineered an alginate (1% w/v) bioink containing platelet-rich plasma (with concentration of 50 U of platelet-rich plasma per mL of the bioink) that, when printed and implanted, undergoes cross-linking through exposure to native calcium ions (182). The migration of stem cells and vascularization within these printed constructs can be augmented by controlling the release of

growth agents associated with platelet-rich plasma, which has been previously reviewed elsewhere (183-188).

Previous studies have indicated significant developments in tissue-engineered conduits integration to bridge defects of spinal cord injury using various strategies such as cell transplants, degradation products of glial scar or biological cues, and physical guides (189-202). The tissue of spinal cord is not structurally homogeneous, but consist of various types of neural cells organized in spatially complex orders (203, 204). The locally definite neuronal subtypes firmly affect axonal growth (205). Consequently, successfully recreating/fabricating patient-specific structures in appropriate clinical shape, size, and structural integrity have been progressed by integrating progenitor and neural stem cells with biocompatible 3D printed scaffolds to evaluate novel therapeutic cues for injuries in the spinal cord (198, 200, 203, 204, 206-212). 3D printing approaches are applied in two distinct fields of spinal cord scaffolds development i) seeding of cells on 3D printed scaffolds and ii) 3D printing of bioinks for constructs fabrication. Koffler et al. 3D printed a biomimetic hydrogel (PEGDA/GelMa)-based spinal cord scaffold to assist regeneration of a single type of neural progenitor cells following spinal cord injury using the abovementioned approach (cell seeding on a printed scaffold) (209), where cells were seeded on scaffolds with 2 mm in length and multi-channels in diameter of 200 μm. They showed that the printed constructs could support axon regeneration and create new neural relays throughout completely injured spinal cord sites *in vivo* in rodents. They found that after 4 weeks damaged host axons regenerated into 3D printed scaffolds and synapse onto neural progenitor cells implanted in the construct and that implanted neural progenitor cells in turn extended axons out of the printed biomimetic scaffold and below the injury, into the host spinal cord to reconstruct synaptic transmission and considerably enhanced functional outcomes (209). Studies concerning nerve

regeneration and scaffolds have proven that scaffolds with microchannel in diameters around 200 μm to 300 μm are effective in guiding axons linearly (213, 214), while channels with diameter more than about 450 μm led to reduction in nerve regeneration (215). As shown by Koffler et al. regenerated host axons indicated a linear pattern during growth guided by the architecture of microchannels of the printed scaffolds. On the other hand, acellular scaffolds (without any cell) represented only limited growth of host axons in the printed scaffolds, and grafting of neural progenitor cells (without scaffolds) extended axons in irregular/random directions (209). The biodegradability of implanted scaffolds made of synthetic poly(ethylene glycol) diacrylate enabled studying regeneration and remyelination of host axons in rats at 4 weeks. The degradation rate of the implanted hydrogel scaffolds was slow, where after 6 months the thickness of scaffolds was decreased by 49% and still structure of channels were unbroken completely filled with neural progenitor cells. Locomotor activity was determined to evaluate the functional recovery using the Bresnahan, Beattie, and Basso locomotor scale for more than 5 months. A compelling functional recovery was observed in rats implanted with scaffolds containing neural progenitor cells in comparison with scaffolds without any cell. The motor evoked potential responses recovery was seen in rats implanted with the cells-filled scaffolds at 5 months after injury, while rats with acellular scaffolds showed a baseline noise level, revealing new neural relays formation over areas of full spinal cord injury (209). For central nervous system, a few weeks would possibly permit move in capability to indigenous oligodendrocyte progenitor cells and the axons myelination (216). This biomimetic 3D printed construct could be tailored to be patient-specific in the size of spinal cord and geometry of the lesion with high anatomical accuracy by combining it with a magnetic resonance imaging.

It has recently been shown that the spatial placement of the transplanted neural cells and their homology to the host tissue are crucial to have successful specific tracts regeneration of the spinal cord (205). This hypothesises that the creation of particular cell kinds in precise orthotopic sites may be essential for better regeneration of spinal cord injury. In such a situation, seeding of cells in a preprinted scaffold is limited during particular neuron subtypes placing in desired sites, in particular onto a multichannel scaffold in micro diameter. Therefore, 3D bioprinting has been used to print patient-specific scaffolds with appropriate bioinks containing the required biomolecules/cells for positioning them precisely within the scaffold during printing. Using this approach particular neural subtypes can be placed in defined areas for best possible axonal innervation and orthotopic reconstruction connectivity of the damaged spinal cord (191, 193, 217, 218). Joung et al. used an extrusion based bioprinting method to fabricate a living model of spinal cord. To do so, a multichannel neurocompatible scaffold containing various kinds of stem cells derived neural progenitor cells (particularly, oligodendrocyte and spinal neural progenitor cells) with accurately positioned cells in specified places was printed (216). Both spinal neural progenitor and oligodendrocyte progenitor cells in suspensions of Matrigel were directly printed onto multichannel (150 μm in diameter) biocompatible silicone scaffolds. In this work, spinal neural progenitor cells were anticipated to differentiate into locally specified spinal neurons that thereafter create axons, and oligodendrocyte progenitor cells were foreseen to differentiate into oligodendrocytes that subsequently, across the channels of the scaffold, myelinate the axons, therefore forming a system of neural relays throughout the injury site. Both spinal and oligodendrocyte progenitor cells differentiated quickly and the spinal neural progenitor cells produced axons in channels of the bioprinted scaffold during 4 days. Additionally, this strategy allowed multiple kinds of neural cells to be printed simultaneously in a particular channel (clusters

of oligodendrocyte progenitor and spinal neural progenitor cells were positioned in a channel with a spatial distribution of around 200 μm). The printed spinal neural progenitor cells were indicated the ability to produce functionally active neuronal networks, which is a key for this therapy (216).

To mimic structurally and mechanically the 3D environment of the native spinal cord, biodegradable composite of alginate-methylcellulose (6% (w/v) of low viscosity alginate and 18% (w/v) of medium viscosity methylcellulose) was used for multilayered scaffolds bioprinting containing dispersed cells in channels of the printed scaffold (216). As a polysaccharide-based hydrogel, alginate might lack the necessary protein components required for adhesion of cells, where cell attachments could be enhanced for long term survival using short peptide motifs, including arginylglycylaspartic acid, laminin-derived peptide (YIGSR), laminin alpha 1 chain Ile-Lys-Val-Ala-Val (IKVAV), RYVVLPR, and RNIAEIIKDI (219). It has been reported that mechanical cues resulting from extracellular matrices have significant influences on cellular characteristics, and therefore, are of importance in biomaterial's designing. A DNA cross-linked hydrogel with tunable stiffnesses (100 Pa to 30 kPa) was used to study cellular responses of neurons of spinal cord to substrate compliances. It was revealed that although primary dendrite lengths were not significantly affected by stiffness, more primary dendrites of spinal cord neurons extended; however, with rising stiffness, axons tended to shorten (220). When neurons were faced with stiffer substrates, there was a remarkeable decrease in focal adhesion kinase, suggesting its response to stiffness and involvement in neuronal neuritogenesis and mechanosensing (220). Khandaker et al. used poly(ethylene glycol) diacrylate with optimal rheological characteristics and revealed that the photoinitiator used for the polymerization process of this polymer is toxic for the printed samples containing DP147 dermal fibroblast cells (221). Cells viability tests for both poly(ethylene glycol) diacrylate gels having 0.2% and 0.6 wt% photoinitiator showed significant

differences after 7 days of incubation. Many unresolved issues remain in repair of central nervous system injuries regarding transplantation approaches and cell survival. Successful direct printing of various kinds of signaling molecules and neural progenitor cells onto channels of a particular scaffold will create a promising engineering approach for multicellular neural tissue developments, where the ability to control the differentiation, growth, and position of transplanted cells will be helpful in regenerating/repairing the damaged tissue. This progress not only reveals new opportunities in *in vitro* studying of interactions of multiple cell identities to model appropriate configuration of cell grafts, but even also enables the possibility of fabricating high quality organotypically organized, and spatially distributed cell transplants. Furthermore, considering inherent 3D structures of 3D printed scaffolds could even enable to print user-defined constructs using 3D bioprinting approaches and culture them as an organoid to grab more of the complicated interactions created within the development and cell identities.

A summary of commonly used bioinks and bioprinting methods in tissue engineering applications is outlined in Table 2. Printing accuracy or dimensional tolerance of a method less than ± 0.1 mm, less than ± 0.5 mm, and greater than ± 0.5 mm is considered as high, medium, and low, respectively. Scalability (i.e. capability to print constructs in various dimensions (build volume)) of different 3D-printed constructs depend on the selected printing material, printer model, and manufacturer. For instance, a printer scalability with a build volume greater than 10^3 cm^3, less than 1 cm^3, and 125 cm^3 could be considered as high, low, and medium. However, it should be considered that scalability and Printing accuracy depend on the selected printing material, printing device, and its manufacturer. Therefore, the range of available products/outputs was assessed and compared with each other. Cost was considered to include the printer cost and the entire process cost (from materials preparation to start printing and post-printing processings).

Table 2: Comparison of different 3D bioprinting methods in terms of printing accuracy, mechanical integrity, scalability, cell viability, and cost.

3D bioprinting methods	Example	Bioink	Printing accuracy*	Scalability	Viability of cells (%)	Cost	Ref.
Laser-based bioprinting	Bone	Collagen–/ polycaprolactone-nano hydroxyapatite	H	L	> 95	H	(21, 109, 173, 222, 223)
	Skin	Alginate hydrogel- human blood plasma and Ethylenediaminetetraacetic acid (EDTA)					
Inkjet bioprinting	Bone	Gelatin methacryloyl (GelMA)-PEG/ polylactic-co-glycolic acid (PLGA)/ PCL/ β-tricalcium phosphate (β-TCP)	M	H	> 80	L	(10, 149, 154, 155, 173, 224-232)
	Cartilage	Alginate / Nanocellulose/ PEGDA/ PEG-GelMA					
	Muscle	Polyurethane (PU)/PCL/ gelatin-methacryloyl					
	Lymphatic tissue	Modified collagen-alginate					
	Neural tissue	GelMA-poly(3,4-ethylenedioxythiophene) (PEDOT)-polystyrene sulfonate (PSS)-PEG					

	Liver tissue	Galactosylated alginate/ Collagen/ liver decellularized extracellular matrix					
	Skin	Poly (N-isopropylacrylamide-co-acrylic acid) (p(NIPAAm-AA)					
Extrusion based bioprinting	Bone	Alginate /Gelatin/Gellan gum-GelMA/ Nanosilicate/ magnesium phosphate-strontium/ PLGA/ PCL	L	H	40 - 95	L to M	(10, 87, 110, 173, 181, 223, 226, 233-241)
	Cartilage	Collagen Alginate/Nanocellulose/Agarose/ Polyethylene glycol monomethacrylate (PEGMA)					
	Muscle	Fibrinogen/Gelatin glycerol hydrogel/ hyaluronic acid					
	Neural tissue	Forkhead box D3 modified PU					
	Endocrinal tissue	Collagen/Gelatin / Alginate/PLA					
	Skin	Alginate/ Gelatin/ Collagen fibrinogen hydrogel/ polyvinylpyrrolidone (PVP)					
Stereolithography bioprinting	Bone	GelMA/ Graphene reinforced PU	H	M	25- 85	M	(47, 48,

	Neural tissue	Graphene nanoplatelets / GelMA	242-244)

H: high; M: medium ; L: low.

* Refers to how closely the fabricated constructs correspond dimensionally to the original CAD design

4 3D-Printed Microfluidics devicess/organ-on-chips

Microfluidic devices are a group of designed and manufactured microchannels that enable mixing of small amounts of reagents for various biological and chemical applications. These tools have been developed for point-of-care diagnostics, to analyze biological and chemical procedures, and for cell culture in a completely controllable microhabitat. Microfluidic devices significantly decrease the required volumes of biological samples and reagents (less invasive diagnostics), operation and manufacturing costs, as well as improving productivity, due to their ability to perform detection and separation with high sensitivity and resolution (245, 246). Alhtough to date, 3D bioprinting has been predominantly developed for therapeutic tissue engineering purposes (i.e. bone, skin, liver, blood vessels, cartilage and heart tissues) (154, 184, 247-253), engineered tissues in microfluidic devices can be used to simulate more complex physiological processes, functions and responses (34, 254). Furthermore, it is possible to imitate immune cell movement throughout the perfusable vascular system produced in the tissue prototypes (255), helping to clarify immune responses and behavior in the human body. 3D printing techniques for organ-on-a-chip technologies are an emerging area allowing the functional replication of tissues to improve biological analysis and drug screening (256). Organ-on-a-chip technologies are micro-designed biomimetic apparatuses that can replicate the crucial active units of human tissues by incorporating micro-manufacturing with microfluidics (257). Researchers have used soft lithography methods to develop organ-on-a-chip platforms that aim to reproduce various tissue functions, such as those performed by the gut, lung, brain, blood vessels, and heart (255, 258-260). These investigations have confirmed that organ-on-a-chip technologies can closely replicate native organ function, allowing for accurate predictions of drug and tissue behavior, and provide a valuable preclinical analysis tool for the progression of novel medicines. However, the complex and multistage chip

production procedures, including secondary cell seeding, glass-polydimethylsiloxane polymerization and chip bonding, make it laborious to produce chips for multiple cell types or to replicate different native extracellular matrix habitats with spatial heterogeneity similar to that of native tissues (261). Instead, 3D printing may provide an ideal method to solve these technical problems via an on-demand production process; for example, researchers have engineered vascular systems by creating perfusable channels within 3D tissue prototypes, by both direct and indirect printing with endothelial cells and fugitive bioink (262). Bioinks encapsulating parenchymal cells or, for instance, muscle or bone cells, have been used to fabricate vascularized, functional 3D tissue prototypes (254, 262, 263). A summary of advantages and disadvantages of commonly used bioprinting methods is outlined in Table 3, with comparisons between different features of the methods.

Table 3: Comparison of different 3D bioprinting methods in terms of printing resolution, speed, pros and cons.

Printing methods	Print resolution	Print speed	Pros	Cons	Ref.
Laser-based bioprinting	~ 20–100 µm	~ 10^6 $mm^3.h^{-1}$	Higher resolution Relatively higher fabrication speed Able to print metals Powders can act as a support base High porosity	Formation of a second phase Reletively poor mechanical strength because of high porosity Stair-step effect Due to high power laser (high temperature) High specific energy density	(21, 109, 173, 222, 223, 264, 265)
Inkjet bioprinting	~ 50–400 µm	~ 5×10^5 $m^3.h^{-1}$ - 25 $mm.h^{-1}$	High speed In a layer fabrication support constructs are included Relitevly high printing speed Printability of multiple materials Different kinds of raw materials can be used Capability to print reletively high porous constructs Low process cost	Post-processing is needed Relatively high waste of materials Trapped support removal from internal cavities is difficult Low viscous inks required (~ <0.25 Pa s) Reletively poor mechanical strength because of high porosity	(10, 149, 154, 155, 173, 224-232, 264, 265)

Extrusion based bioprinting	~ 100-300 µm	~ 10^5 $mm^3.h^{-1}$	Lower specific energy density Capability to fabricate ceramic molds Relatively low cost of process and the entry-level machines Different kinds of raw materials can be used Easy and versatile to customize Printability of multiple materials	Incapability to precisely control the size and direction (directionality) of droplets Grainy or rough surface finishing Relatively low level of resolution, precision and long fabrication time Incapability to print sharp external corners Printed parts have relatively anisotropic nature Supporting structures are required Fusion of interlayers can be affected in process by the filament circular cross-section Frequent clogging of the nozzle Relatively rough surface finishing	(10, 87, 110, 173, 181, 223, 226, 233-241, 264-266)

Stereolithography bioprinting	~ 50–200 μm	~ 10^6 $mm^3.h^{-1}$	Relatively higher resolution and fabrication speed Relatively smoother surface finishing Relatively low specific energy density	Post cleaning to remove the resin is required Low strain at break and opacity for advanced applications Fabrication of ink rheology Limitation on resin choice Possible cytotoxicity of residual photoinitiator and uncured resin	(47, 48, 242-244, 264-266)

5 4D printing for biomimetic biofabrication

Recently, *in vitro* tissue prototypes have received growing interest for application in drug trials and screening, providing a means of more precisely forecasting the behavior of and physiological responses to pharmaceutical compounds, and subsequently expediting the drug discovery procedure (111, 140, 256, 267, 268). Here, 3D bioprinting has emerged as a versatile method that can be successfully conducted to build biomimetic tissue structures with some degree of spatial accuracy (177, 183, 233, 269, 270). 4D printing is categorized as a procedure that links time with additive manufacturing. Actually, it has proposed that this method involves printing of a 3D construct capable of tolerating a controllable shape alteration (271, 272). In other words, 4D printing describes an evolution in 3D printed constructs, in terms of functionality, size, properties, and shape (272) that can be outlined as fabricating structures using additive manufacturing approaches with the ability to self-transform, in function or form, upon exposure to a predetermined stimulus such as heat, moisture, pressure, light, and electricity among others (273-275). The 4D bioprinting process, based on 3D bioprinting, but with embedded stimuli responsive capability (e.g. shape alteration), allows printed objects to more closely imitate the dynamics of native tissues. To achieve this, biomaterials with stimuli-responsive behavior can be incorporated into the 3D bioprinting strategy to create biologically functional structures that can adjust form in response to appropriate stimulation (276, 277). This shape transformation ability is considered to be the added '4^{th} dimension' to printed 3D objects. Crucial requirements for 4D printing include programmability of a material, multi-material printing methods, and precise designs for meticulous transformations (278). Remarkable further levels of complexity in a construct can be created using 4D printing approaches (e.g. changeability in functions and shapes) that cannot be achieved using any other current manufacturing techniques, including mechanical machining or focused ion beam

(279). While both 3D and 4D processes are almost similar and start with a 3D design and a printing stage, various key dissimilarities can be found between these two printing approaches that is illustrated in Figure 7, where 3D printing uses one or multiple materials to fabricate a static construct and 4D printing uses functional materials as well as some additional operating conditions to print structures with dynamic nature (able to respond to external stimulus). Firstly, 4D printing requires a facility with the capability of printing multi-materials since the variations in the material features (e.g. thermal coefficient or swelling ratio) are the base of creating stimuli responsive constructs. Secondly, to predict the alterations in the 4D printed structure and to consider it within the design step, mathematical modeling needs to be done to achieve a desired final product. Thirdly, an accurate stimulus is required to trigger functional or morphological changes in the 4D printed constructs. Common stimulus used in this regards are humidity, heat, light, electricity, or a combination of these stimuli. Finally, to achieve an effective 4D printed construct considering interaction mechanisms among used smart/functional materials as well as printing procedure is essential (e.g. to select appropriate printing device based on materials, to have knowledge about the stimulus duration and its intensity among others, and eventually if more than one stimulus is applied to know the order in the application of them) (19, 271-275, 279, 280). Additionally, printers need modifications (e.g. different binders or lasers based on the employed approach and material, modified nozzles) that enable multi-material printing and it is worth to mention that 4D printing approaches expands the range of additive manufacturing applications to those fields with a necessity of dynamical configurational changes (19, 271-275, 279, 280). Considering these, smart design and materials are vital in 4D printing, as 4D printed constructs need a comprehensive design including deformations prediction and analysis (i.e. digital data of the response) as well as smart materials with the ability to respond a particular stimulus by morphological or functional

changes. As is detailed in this section, these materials include hydrogels or polymers able to alter their size, structure, shape, or function in response to external stimuli (274, 275, 280-284).

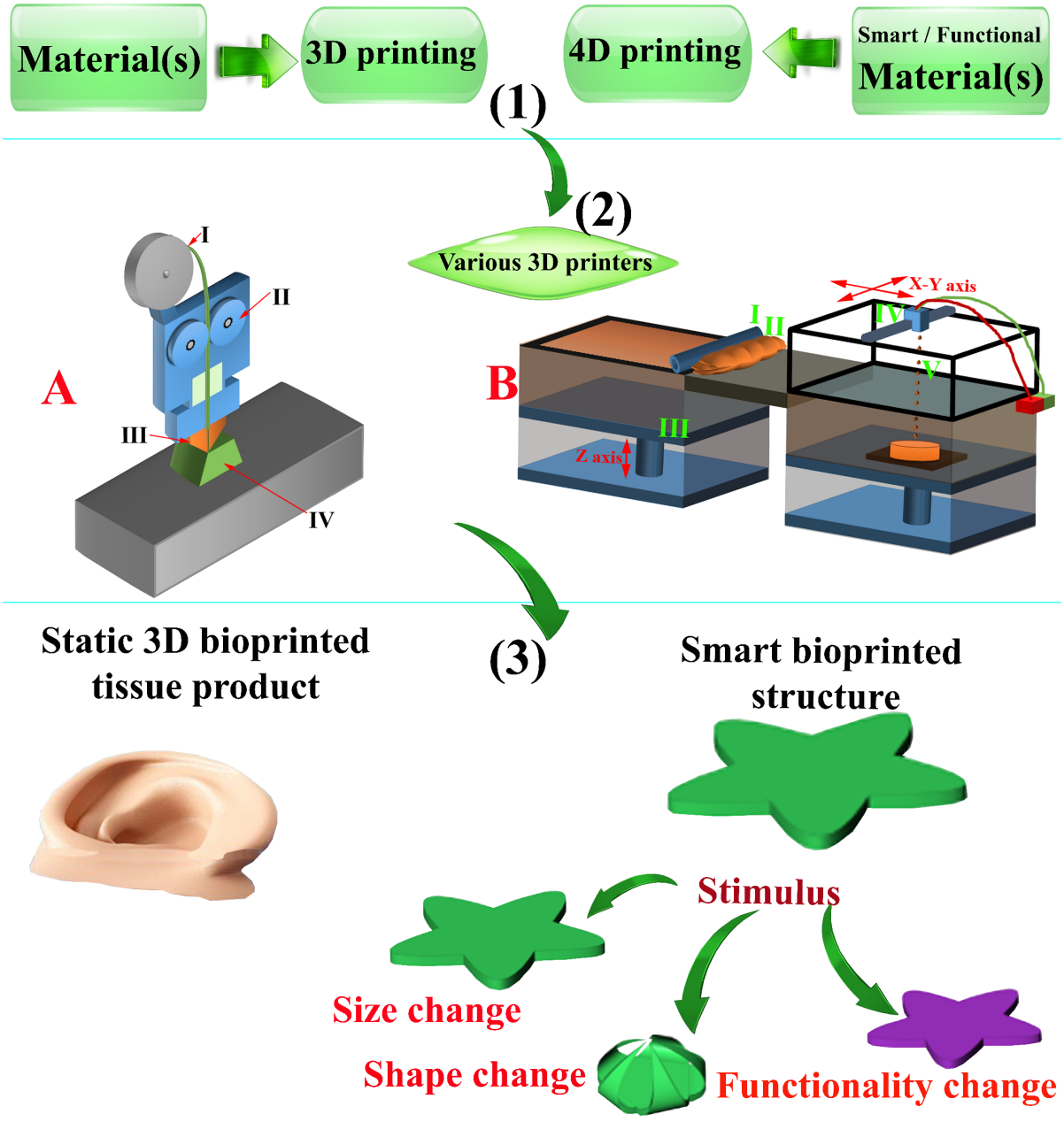

Figure 7: The general shematic of 3D and 4D printing including both (1) materials, (2) processes (A, an extrusion-based 3D printer using I: solid polymeric filament, which is processed through II. an automated gear system, forcing it through a nozzle at the base of the print head. III: This heated nozzle melts the filament and IV: molten extrudate is printed and fuses with the in-progress product upon cooling. B, Powder bed (binder deposition) printing using I: a powder roller that pushes thin and level layers of powder to the print surface under the inkjet print head (IV) II: powder composed of API. III: a height adjustable plate (moves in Z direction) to continuously provide new layers of powder at the appropriate height (moves in Z direction). IV: inkjet print head that selectively binds only powder in specific locations in the layer. V: unbound powder.) and (3) products (i.e. a 3D bioprinted static construct and a 4D stimuli responsive bioprinted construct with the capability to change its functionality, shape, or size. Image credit Mr Karim Osouli-Bostanabad

5.1 4D Materials

Autonomous structures that respond to their surroundings require mechanisms for actuation and sensing. There is equally a great need for new materials that are biodegradable and can be eliminated or cleared by the body without any adverse effects. Subsequent innovative studies in this field have developed biologically relevant tissue responsive materials, and led to the development of resorbable biomaterials, which experience resorption and chemical cleavage in body, allowing for the integration of native tissue as the structure breaks down (285).

Biomaterials must satisfy sophisticated conditions or requirements, which vary for different applications. As such, selection of an appropriate material with desired functions for a specific application is vital, and materials with tunable properties are therefore particularly desirable, as they can be adjusted as required. The fabrication of multifunctional biopolymers, which combine biodegradability and shape-memory effects, have received significant attention, particularly for *in vivo* applications (286, 287). Manufacturing and use of these biodegradable polymers (particularly those produced using renewable resources) have also increased due to the environmental challenges linked with polymeric materials usage (288). The most commonly used biodegradable polymers are composed mainly of polyesters, especially aliphatic polyesters, whose monomers can be derived from renewable resources, in addition to commonly used petrochemical sources (288).

5.1.1 Polymers with shape-memory characteristics

Polymers with shape-memory characteristics are finding applications in various sectors including biomedicine, healthcare, and engineering. These compounds have multi-shape (dual, triple, or more) memory based on their temporal or permanent shape transition number, when the material is subjected to an external stimulus. Light, pH, temperature, and redox conditions are the chief stimuli capable of initiating the material response, amongst others (289). However, the most

commonly investigated polymers with shape-memory characteristics react to temperature change, where the material changes from its temporary to permanent shape at the "transformation", "transition" or "switching" temperature (T_{trans}). The T_{trans} of a shape-memory polymer is dependent on the polymer's primary transitions, occurring either at the melting temperature (T_m) or the glass transition temperature (T_g), where polymers with shape-memory characteristics can be classified as T_m- or T_g-based polymers (290). Chemical and physical interactions (e.g., covalent bonds, physical bonds, or interpenetrating network formation) can fix the permanent shape of a polymeric material. The network that has formed by these interactions is able to develop a shape memory behavior, where the bonded areas are generally called net-points. Although both chemical and physical interactions could effectively create a polymer with shape-memory characteristics, polymers that are cross-linked chemically demonstrate a permanent shape with more stability. It is crucial to consider that any polymer with shape-memory characteristics shows two vital elements that could establish the triggering zones and permanent shape; these are the switching domains and net-points. Polymerization and crystallization of polymers with shape-memory characteristics can cause switching domain fixation, relying on T_g and T_m as the primary thermal transition temperatures (for detailed discussion readers are referred to (291, 292)). Various polymeric substances have been employed to produce polymers with shape-memory characteristics; however, biodegradable polymers have emerged as the most appealing compounds for biomedical applications, particularly for temporary devices (e.g., catheters, stents, and sutures) where biodegradability eliminates the need for detachment and removal from the treatment site. Another appealing aspect of these materials is that optimized synthesis of biodegradable polymers with shape-memory characteristics could allow for fabrication of polymers with a transition temperature similar to the temperature of the human body (293). One of the most commonly used

biodegradable polymers with shape-memory characteristics is polylactide/polylactic acid, and its derivatives. Poly(L-lactide) is a widely studied T_g-shape-memory polymer that has shown significant advantages including excellent biodegradability and biocompatibility, and facile processability where its crystalline part has been used as a permanent physical system (294). Additionally, shape-memory characteristics of physically cross-linked poly(L-lactide) and its copolymers, such as poly(lactide-co-glycolide), have been reported (236, 295, 296).

Another commonly used polymer with shape-memory characteristics is polycaprolactone, a biodegradable member of the polyester family with a relatively low T_m (60°C) and T_g (−60°C). Lorwanishpaisarn et al. fabricated a novel biodegradable blend of polycaprolactone/epoxy with dual self-healing and responsive shape-memory characteristics, indicating that the compound could be used as a coating or dual-triggered sensor for different applications, including medical devices (297). Both crosslinking and/or copolymerization methods have been reported to produce polycaprolactone with adjusted shape-memory and mechanical characteristics. Since the glass transition temperature is below zero, the shape-memory behavior is activated at the melting point. Therefore, adjusting the melting point of polycaprolactone using copolymerization, blending, or a covalent network addition alters the shape-memory characteristics of polycaprolactone (298-300). Other polymers used in this field are polyurethane, natural polysaccharide, starch and chitosan-based polymers, with numerous formulations used to advance or improve biodegradable substances (301-304).

5.1.2 Piezoelectric materials

Another example of these multifunctional materials is piezoelectric compounds, a class of materials that have electric responses (e.g., accumulation of electrical charge on the material surface) to mechanical stimuli. It has been revealed that this phenomenon is tightly linked with

crystalline structure of materials (305) and analysis of various materials from a crystallographic perspective has shown that a central symmetry absence in the unit cell results in the direct piezoelectric effect (306). Novel organic and inorganic piezoelectric substances with sophisticated characteristics and wider functionalities have recently been described (307, 308).

Biomaterials with piezoelectric characteristics are a particular group of smart/multifunctional materials that offer numerous benefits in comparison with common biomaterials, as they can readily transduce electricity to living systems in response to various procedures (e.g. body movements, cell migration, or external stimulation (e.g. vibration and ultrasound)). Additionally, materials can also provide reverse piezoelectricity, or the transformation of electrical stimuli to mechanical stresses (309). Numerous biomaterials with piezoelectric characteristics have been discovered for various biomedical applications, including collagen, polyvinylidene fluoride, poly(L-lactide), and poly (D-lactic acid) (310, 311). Examples of applications of these materials are given in section 5.2.

Different parameters influence piezo-transducer/material sensitivity, such as material selection, fabrication, activation, and post-treatment conditions. Furthermore, due to the processability, biocompatibility, and versatility of piezoelectric polymers, the application of these polymers is spreading in various fields such as tissue engineering and self-powering implantable instruments. The development of piezoelectric polymers that are compatible as cell culture surfaces or structures may further expand the relevance of these materials in tissue engineering and regenerative medicine. Piezoelectric biomaterials are also gaining considerable interest in healthcare industries for their associated mechano-electrical characteristics applicable in intriguing and novel approaches to cure, repair, and improve body functions. To reach these goals, the

fundamentals of the piezoelectricity of polymeric nanocomposites must be fully and methodically characterized, and their multifunctional design considered.

5.1.3 Thermoresponsive biomaterials

Polymers whose solubility changes in accordance with the ambient temperature are named thermoresponsive polymers. This solubility alteration is associated with conformational changes in the structure of the polymer. The solubility alteration temperature occurs at T_{trans}, which can also be called the critical solution point/temperature (T_{crit}). Thermoresponsive biomaterials are multifunctional compounds that can be used in various aspects of regenerative medicine and tissue engineering. Based on the substance, they can be employed as injectable *in situ* gelling compounds, as hydrogels for 3D printing, or as a biomaterial surface modifier for the engineering of cell sheets. Several thermoresponsive biomaterials have been used in biomedical applications and in thermoresponsive surface development including poly(N-isopropylacrylamide), its copolymers and derivatives, elastin-like polypeptides, pluronics, and poly(N-vinylcaprolactam) (312-315). Examples of applications of these materials are given in section 5.2.

5.1.4 Electrically conductive polymers

Electrically conductive polymers have optical and electrical characteristics similar to those of inorganic and metal semiconductors; however, they also show appealing characteristics comparable to those of regular polymers (e.g., easily synthesized and good processability), unlike metals (316). This is an important consideration for biomaterial applications, as it has been shown that endogenous electric fields have an important role in tissue regeneration (i.e. early embryonic advancements) and it is established that bioelectricity is an integral part of living systems (317); for example, developmental imperfections in the embryonic stage may appear because of slight

deviations from the embryo field potential (318). Endogenous electric fields may affect cellular operations including migration, chemotaxis, differentiation, and proliferation of cells. Furthermore, endogenous electric fields also influence intracellular communication, cell division, mechano-transduction, neuronal activities, epithelial/bone healing and ion transport (319, 320).

Considering the importance of bioelectricity, electrotherapy has been advanced for stimulation of deep brain, accelerated wound healing, improvement of musculoskeletal conditions, tissue regeneration, and bone fracture recovery (321). Electrically conductive polymers present supreme electrical characteristics and have been investigated in recent decades in different biomedical applications such as tailored release systems, neural prostheses, neural probes, and bio-sensors (322-325). Furthermore, researchers have demonstrated that cellular activities could be tuned via electrical stimulation (i.e. conductivities 10^{-4} -10^{-9} S/cm) of electrically conductive polymers. These activities, including cell migration, cell growth and controlled cell differentiation, resulted in significant attention on the use of these polymers and their derivatives in tissue engineering (326-330), which commonly deal with electrical stimulation responsive cells including bone, nerve, muscle and cardiac cells (323, 331, 332). Compatibility of electrically conductive polymers, such as polythiophene, polyaniline, polypyrrole and their derivatives, with biological molecules was revealed both *in vivo* and *in vitro* (323, 333). Electrical conductivity is the most significant property of these materials; accordingly, studying biological responses related to these electrical properties is important for biomedical applications. Usually, neurons using particular nerve endings or dendrites receive electrical signals and transmit them by means of nerve fibers (axons) to the body cells. Consequently, early works concentrated on the electrical stimulation of neurons using electrically conductive polymers as electrodes. The results revealed that these polymers could be employed as biological electrodes, and the growth of neurons could be increased using

an electrical field (334, 335). Recently, electrically conductive polymers have been used in nanocomposite design for tissue engineering (336, 337), with conducting nanocomposites shown to have the ability to adjust the rate of proliferation of various cell types, such as chromaffin cells (338), nerve cells (339), and endothelial cells (340). However, one of the main barriers to implementing these materials in tissue engineering is their inability to degrade, and as a result, maintaining electrically conductive polymers *in vivo* for a long period of time may provoke an inflammatory reaction and require surgical removal. Future work should focus on developing materials with both biodegradable and electroactive properties, as these would be extremely useful to and highly advantageous in the biomedical field.

5.2 4D printing for Biomedicine

4D printing has received significant attention both academically and industrially since 2013, although there is still a need for further research and development to commercialize this technology (341, 342). 4D bioprinting has great promise for applications in both therapeutic delivery and tissue engineering.

5.2.1 4D bioprinting application for therapeutics delivery

As previously discussed, a major point of interest in pharmaceutical research is the development of drugs that can be released in a clinically-relevant manner, in terms of both location and time. 4D bioprinting methods can be used to optimize control over temporal and spatial delivery of medicines, with, for example, the ability to print various apparatuses that can self-unfold or self-fold to release or encapsulate cells and/or therapeutic agents in a programmable way. For this purpose, multisomes (small oil drops encapsulating water-based droplets) were developed, which can be printed in water (343, 344), and then the encapsulated contents in the droplets can be

released by altering the temperature or pH of the surrounding solution. 4D printed materials can perform therapeutic functions via their responsive material properties, and could later be further enhanced with the inclusion of existing pharmaceutical compounds in the printed material. For instance, some mechanically robust, thermoresponsive hydrogels have shown up to 49% reversibility in their length at a heating/cooling cycle between 60 and 20 °C (i.e. when these hydrogels cooled from 60 to 20 °C, they swelled to their initial equilibrium conditions due to thermally induced actuation) that can be used to print cardiac valves with the ability to close the valve and reduce flow rate (~ 99%) by heat stimulation. Additionally, for treatment of heart disease or valve protection, the hydrogel can be loaded with desired drugs (345, 346). The same strategies can be applied to manufacture other micro- and nano-structures that are responsive to different micro-environmental alterations including osmolarity, light, humidity, or magnetic and electric stimuli (166). In one attempt, a composite of pentaerythritol triacrylate, poly(ethylene glycol) diacrylate, and magnetic nanoparticles (i.e. Fe_3O_4) was used to develop a biodegradable, biocompatible, and magnetically actuated hydrogel-based microstructure potentially applicable for targeted drug delivery (347). Another magnetically controlled and powered, hydrogel-based, enzymatically degradable double-helical structure responsive to the pathological markers was fabricated by Ceylan et al. for delivery of drugs or other therapeutics. It showed that matrix metalloproteinase-2 (MMP-2), at typical physiological concentrations, could fully degrade the designed structure in 118 h to nontoxic soluble products, where the sample quickly responds to MMP-2 by swelling and consequently releasing the embedded molecules. Furthermore, magnetic nanoparticles tagged with anti-$ErbB_2$ were released from the completely degraded double-helical structure for targeted labelling of human breast cancer cells *in vitro* (i.e. $SKBR_3$), with the aim of potentially using this construct further in medical imaging of cancerous tissues after drug delivery

(348). Using a similar approach, Bozuyuk et al. have proposed a double-helical, magnetically powered, polymer-based microswimmer that could release doxorubicin on-demand in response to stimulation of an external light source (349). In research to develop more versatile materials with a shape memory function, Liu et al. developed a chemically crosslinked polycaprolactone structure containing hydroxyapatite nanoparticles to deliver growth factor BMP-II in a controlled manner. *In vivo* evaluation of this construct in a rabbit mandibular injury revealed that BMP-II loaded scaffolds promoted regeneration of new bone and that the scaffold recovered its initial shape by body temperature stimulation after implantation (350).

In addition to printing existing materials to carry common therapeutics, another key aim of 4D printing is to improve existing therapeutic strategies by providing on-demand delivery of therapeutic agents, facilitating ongoing tissue repair or regeneration after treatment. One means of achieving this is printing carriers with shape-memory characteristics, which can be manipulated for easy ingestion or injection, and can then recover their initial shape in the body, serving as a drug depot. For example, Melocchi et al. have proposed a poly(vinyl alcohol)-based expandable drug delivery system with shape memory behavior and easy ingestion capability for gastric retention. Prototypes were printed in compacted form to be swallowed easily and it was reported that the prototypes recovered their original shape within a few minutes at body temperature in a 0.1 N hydrochloric acid solution, and that this approach prolonged the drug release (approximately 2 h) independent of manufacturing procedures and original shapes (351).

Although much research has been done to develop drug delivery systems and improve control over their shape deformation, drug localization, and biological functionality, few advancements have been made in translating these systems to the clinic. For 4D printed therapeutic delivery systems, there are rigorous criteria for clinical applications, such as high sensitivity and selectivity,

and accurate and rapid responses to stimuli. There is also a need to demonstrate robust efficacy and safety of 4D printed products, particularly for commercialization, approaches to scaling-up production. Consequently, while 4D printing techniques provide novel opportunities to effectively and efficiently manufacture drug, cell, or growth factor delivery systems, there is still much work to be done before these systems can be used in real world applications.

5.2.2 4D bioprinting application for tissue engineering

4D bioprinting technologies have the ability to create dynamic reprogrammable tissue structures that can encourage cellular growth and distribution uniformly. For instance, 4D polymeric-based cardiac constructs with adjustable curvature, on-demand light responsive shape changeability, and aligned microstructures were developed to imitate and repair myocardial tissue (352). Microgroove arrays with optimal widths were found via culturing mesenchymal, cardiomyocytes stem, and endothelial cells on the surface of the printed constructs, and evaluating their differentiation and proliferation profiles. The results revealed that 4D printed constructs have the ability to promote a remotely controllable and dynamic spatiotemporal transformation, distribute aligned cells uniformly, and promote myocardial maturation efficiently (352). Using the same strategy, Constante et al. reported the fabrication of shape-morphing scaffolds based on a combination of melt-electrowriting of polycaprolactone fibers and 3D printing of methacrylated alginate (353). The combination of these two methods permitted deposition of various compounds in a programmed way and manufacturing of high resolution constructs. It was also shown that the geometric shape, environment media, and concentration of calcium ions in the scaffold, as well as the patterns generated on its surface by polycaprolactone fibers, highly affect shape-morphing and cell alignments (353).

4D bioprinting is also demonstrating positive outcomes in the *in vitro* manufacturing of blood vessels, which has previously faced numerous challenges due to the physiological and anatomical characteristics of vasculature, particularly vessel reperfusion and structure. A combination of various cells, such as fibroblasts, mesenchymal stem cells, and endothelial cells, can be printed with hydrogels to form tubular constructs that imitate vasculature. Cell migration, proliferation and maturation occurs throughout the printed structures and consequently leads to vascularized constructs in which endothelial-specific definitive adhesion proteins and genes are expressed (25, 354). Heo et al. used gelatin methacryloyl hydrogels containing dorsal root ganglion to print conductive constructs for neurovascular applications. To achieve samples with a high electrical conductivity, an aqueous solution of polystyrene sulfonate:poly(3,4-ethylenedioxythiophene) was blended with polyethylene glycol diacrylate. These fabricated conductive structures provided sufficient structural support to transfer electrical stimulation toward encapsulated dorsal root ganglion cells and promote neuronal differentiation (231). Despite these advances, manufacturing of small-scale vascular constructs remains limited due to the restrictions on resolution using this approach. Other major challenges to the bioprinting of structures with capacity for vascularization include providing adequate nutrient exchange for the printed vasculature and the integration of these structures and vessels with host vasculature after implantation (355).

4D bioprinting can also be used for printing hard, morphologically rich yet macroscale structures like trabecular bone. For example, a combination of β-tricalcium phosphate, polycaprolactone, and poly(lactic-co-glycolic acid) was used to fabricate tailored synthetic scaffolds with a cell- laden mineralized extracellular matrix to mimic bone tissue and promote the biological activity of the printed structure (224). A flask bioreactor was used to culture the bone grafts with mesenchymal stromal cells derived from human nasal turbinates to stimulate a bone-like microenvironment. This

patterned bone-like structure indicated increased calcium deposition, cell differentiation, and upregulated expression of alkaline phosphatase (ALP), Runt-related transcription factor 2 (RUNX2), osteopontin and osteocalcin genes. Additionally, greater bone formation was observed *in vivo* in comparison to scaffolds that were not tailored (i.e. bare scaffolds). Prasopthum et al. have also demonstrated the versatility of 4D bioprinting for hard tissues, developing conductive and degradable polymer scaffolds to foster chondrogenic differentiation of chondroprogenitor cells. They demonstrated the feasibility of 4D-printed flexible, electroresponisve scaffolds in cartilage tissue regeneration (356). Despite promising research in this area, further work is still required to strengthen structures mechanically and improve their biomechanical characteristics.

4D bioprinting approaches have also been used to design and manufacture gland structures; a major unanswered oncological question is how to develop constructs to mimic efficiently the complex environments of a tumor *in vitro* for cancer studies. By using a self-folding approach, curved microstructures made of bilayers of photopatterned gelatin/co-polymerized poly(ethylene glycol) diacrylate were fabricated to accurately imitate acinus and duct geometries in the mammary glands, which, compared with existing flat dishes or 3D block-like models, showed that the 4D-printed structures are more reliable models for acini and ducts (357). The versatility and biocompatibility of this approach was highlighted by either culturing SUM159 human breast cancer cells after printing (i.e. postfabrication seeding) or encapsulating MDA-MB-231 human breast cancer cells in hydrogels, where cell viability was confirmed over 9 and 15 days, respectively (357). Amongst various 3D models including biopolymer scaffolds, *ex vivo* tissue slices and spheroid cultures, 4D bioprinting has a competitive superiority because of its ability to meticulously define and control the appropriate structure, print materials with shape

transformation ability to mimic the native tissue, and deposit multiple cell types in a high-throughput approach. For detailed information in this area, readers are directed to (358-361).

Taking into account the nanostructural characteristics of human tissues (362), 4D printing approaches have benefited from nanomaterials and smart/functional nano-bioinks for printing of tissue scaffolds (19, 273, 280). The incorporated nanomaterials may have interactions with functional materials that enhance 4D effects of the printed bio-tissues. For example, Cui et al. printed a brain model using smart nanocomposites (responsive to near-infrared light) to study 4D transformations, controllability of these transformations, and to explore the possibility of modifying behavior of neural stem cells using photothermal stimulation (280). In this work, nanoplatelets of a photothermal graphene were embedded in a shape memory thermo-responsive polymeric matrix, where the graphene acted as a thermal energy source by absorbing photons from a near-infrared light that eventually led to an observable transformation of the printed models. In comparison to direct thermally triggered shape shifting procedures, this smart nano-bioink is especially effective in fabrication of tissue scaffolds due to the fact that a long-wavelength, near-infrared light can penetrate efficiently into human tissues and is human benign (280). In future studies, it may be required to print the cell subtypes of the brain tissue, containing defined vascularization, and gradients of signaling factor for development of sophisticated brain models and their applications. Additionally, electroactive materials similar to other stimuli responsive materials can be integrated with a multiple responsive 4D printing approach, as a proof of-concept a graphene hybrid 4D structure was fabricated using stereolithography-based 4D printing as a smart nerve guidance conduit for nerve regeneration showing remarkable multifunctional properties, such as chemical cues, physical guidance, seamless integration, and dynamic self-entubulation (363). Additionally, the printed structure possesses shape memory characteristic. The

results of neurogenic differentiation of human mesenchymal stem cells on nanohydrid 4D printed conduits and their photo-cured counterpart revealed, while both had excellent neurogenic capability, the nanohydrid 4D printed constructs presented a remarkable aligned topography because of the microfeatures induced from printing. Moreover, it was found that the nanohydrid 4D printed conduit can considerably enhance neurogenic differentiation of human mesenchymal stem cells (363). By employing this printing approach, fabricating multiresponsive smart constructs, and demonstrating feasible application, 4D printing is thus an attractive promising candid in different high-value research fields, including spinal cord injury, neural tissue engineering, and peripheral neuropathy leading to muscular atrophy, but definitely not limited to, biomedical devices, and soft electronics.

Over recent years, other organs and micro-tissues have also been designed and fabricated by 4D bioprinting methods. For instance, cell-laden hydrogels for bio-artificial pancreases have been manufactured, where these cell-loaded structures must support and maintain cell viability while providing appropriate permeability and stability characteristics (364). Successful translation of this type of construct to the clinic may diminish or even eliminate the need for immunosuppressive drug therapies to avoid rejection of a transplant, as well as addressing donor tissue scarcities by using cells derived from xenogeneic or allogeneic sources (365, 366). Other works include the manufacturing of cardiac micro-tissues (25, 367), 3D human cancer structures or spheroids by cell-laden hydrogels, and tracheal-bronchial shape-memory stents (368, 369). These tracheal-bronchial miniature implants adjust their configuration and contours according to different micro-environmental variables, facilitating their physiological adaptation to the native tissue. In one study, three weeks after implantation in a child with tracheobronchomalacia, assisted ventilation of the site was avoided (370). Each of these examples highlights the enormous potential of 4D

printing strategies for clinical applications; however, further research and optimization of 4D bioprinting approaches is required before these technologies can be implemented.

5.3 3D and 4D bioprinting: Challenges and Opportunities

3D printing, which is a versatile technique, has been widely employed in numerous fields, including mechanical, biomedical, and electrical engineering. However, there are many associated challenges in applying this technology to biomedicine. For instance, bioink selection must be specifically tailored to a given application and printing technique, to ensure that the bioink retains adequate functionality in 3D, and that the integrated cells remain viable and are homogeneously distributed (371). Different bioprinting techniques have some issues on printing of cells, including enforced shear/mechanical stresses, laser or heat radiation induced changes, which subsequently result in cells viability decrement. Some studies reviewed the requirements of materials in cell printing, from rheological, structural fidelity, design parameters, and mechanical stability perspectives of printable inks (372-377). These reviews clearly discuss the issue from bioinks prespectives yet, there is a need to explore the challanges linked with cell printing methods. It has been mentioned that in droplet or extrusion based printing techniques, the droplet size should be at least larger than the cell wall diameter, even for a single cell printing, where the nozzle diameter for achieving a better resolution should be minimized, which it causes an increase in shear strains and consequently cell damages (378, 379). In conventional stereolithography methods, printing resolution relys mainly on laser exposure time and power, where focusing the laser beam for free radicals activation and better resolution achievement could lead to cells damage and reduce cellular viability. However, printing using two-photon polymerization approach is appropriate in comparison with stereolithography methods as infrared lights applied in this procedure are more safe to live cells (380, 381). The resolution enhancement also relies on shape fidelity preservation.

In extrusion based 3D printing methods, the shape fidelity could be disrupted by fusion or distortion of adjacent filaments from their overhang position. Consequently, cell distributions nonhomogeneously would be progressed in the printed platform, and because of lack of a nutrient flow and oxygen, proliferation and cell viability decrease (379).

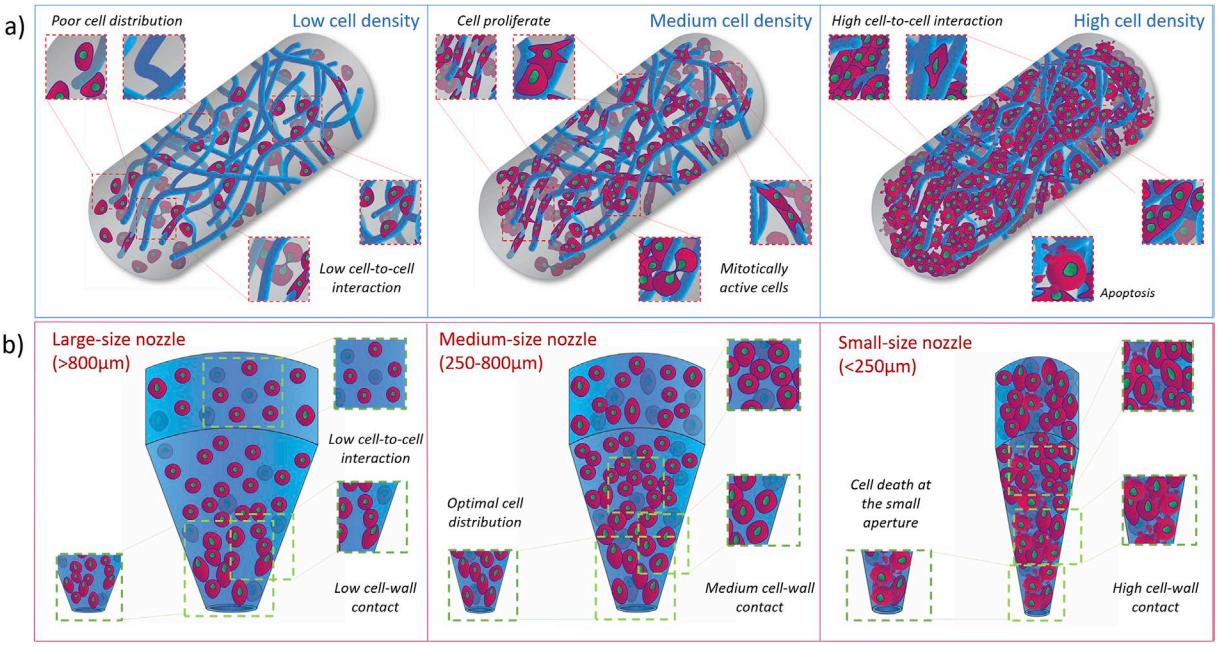

Figure 8: Effects of a) cell density and b) conical nozzle size on cell viability and printing resolution. This image reproduced from [382] under the Creative Commons license permission (CC BY-NC-ND 4.0, License Number: 5310690569636). Copyright 2019 Elsevier

Besides that, there is a direct correlation between cell distribution and its density with printability, as dense cell-laden hydrogels lead to impaired growth of cells and nonhomogenous distribution of cells in a hydrogel creates density variation in printed constructs with most resides population of cells at structures periphery as well as occurrence of a nonhomogenous oxygen distribution that affects the growth of cells (382). For instance, a cell density of about 5 -10 × 10^6 cells/mL serves as an ideal loading density in bone tissue engineering (382). Usually, hydrogels

with low modulus (<1 kPa) offers an ideal environment as bioink in 3D printing methods with an optimal proliferation ability and adhesion (382). It has been mentioned that strengthening of a polymer matrix could be influenced by cells in a high concentration (>5 × 10^6 cells/mL) while compromising interactions of cell-to-cell (Figure 8a) (382).

Cells in a very low concentration (<1 × 10^6 cells/mL) lead to the polymer matrix relaxation and decreased *in vivo* integration (382). However, the excessively low viscosities of some bioinks may cause heterogeneous cell densities within bioprinted constructs (383). In contrast, bioinks with high viscosity may result in augmented shear stresses within the printing procedure, influencing cell functions and viability (384). In order to solve these challenges, a microfluidic process has recently been suggested to deposit bioinks with low viscosity, by which the sheath flow of a carrier was used to aid bioink extrusion through the print head core (385). Additionally, a more recent strategy named embedded bioprinting allows for direct 3D prototype writing in an anti-gravity manner on a supporting platform, from which the printed objects can be selectively removed or retrieved (386-388).

Figure 8b shows the impact of nozzle diameter and its effects on the viability and distribution of cells. Nozzles in large diameters (> 800 μm) ascertain minimal cell-to-nozzle wall and cell-to-cell interactions that subsequently reduces the shear stress at the nozzle orifice and enhances cell survival rate chances. However, larger droplets are created using a larger nozzle, which hinder the printing resolution and nozzles in narrower diameters (< 250 μm) provide better printing resolution, while causing more shear stresses that result in the cell viability reduction. Nozzles with medium sizes (250–800 μm) offer a balance between the cell viability and print resolution (382). Cells selection for incorporation in bioinks relies on several parameters, including cells capability to withstand and tolerate printing modalities as well as postprinting crosslinking

mechanisms; proper proliferation ability of cells and their differentiation controllability in the 3D bioprinted platforms. The highest care should be given to cellular functions maintenance after printing and their biological signal paths identification within interactions with multiple cells at the host tissue during choosing the appropriate cell type for using in bioprinting techniques. Besides that, before starting the bioprinting, cells should reach to an appropriate confluency during the *in vitro* culture. An ideal cells selection would leads to obtain cellular homeostasis and enhance the probability of printed constructs biocompatibility with the organ or host tissue (389).

Furthermore, extrusion pressure has a correlation with printing speed that ultimately influences cell viability. A higher extrusion pressure is often correlated with higher printing speed that results in lower cell viability. In this regard, Fakhruddin et al. have optimized a printing speed of 4 mm s−1 for their ink formulation on the extrusion-based bioprinting technique (390). In extrusion-based bioprinting, shear stress generation inside the nozzle wall is expressed by a power law function for non-Newtonian fluids that correlates apparent shear rate with shear stress. However, the shear rate on nozzle wall is directly proportional to the deposition velocity and inversely proportional to the nozzle radius (391). The detrimental effect of shear stress on cell viability has been already mentioned.

As previous studies dealing with stem cells have mostly been done in 2D, there is a need to investigate and address the unknowns about culturing stem cells in 3D environments (e.g., cell viability, homogeneous distribution of cells). These techniques can then be used for high-efficiency organoid printing, for predictive disease modelling and personalized drug screening (392-398). In bioprinting applications, stem cells are promising candidates due to their stress-induced differentiation capability. It has been shown that high shear forces or mechanical pressures of extrusion-based or inkjet bioprintings, promote mesenchymal stem cell differentiation. Laser-

assisted bioprinting techniques can maintain stem cell multipotency and ultimately use other mechanisms for stem cell differentiation. Furthermore, stem cells' differentiation ability can also be regulated by scaffolds elastic modulus, where scaffolds with modulus in the range of 0.1–5 kPa are useful for adipose tissues and neuronal cell differentiation. Comparably, scaffolds with modulus between 8-30 kPa are favorable for bone tissue, cartilage, and muscle (399). Three types of stem cells (i.e. embryonic, induced pluripotent, and mesenchymal stem cells) are used in bioprinting techniques each with particular supremacies and limitations. Among them, mesenchymal stem cells are broadly employed because of their procurement simplicity. However, in comparison with embryonic stem cells, they lack in multipotency. Ethical challenges and issues linked with immunogenicity have limited the application of embryonic stem cells on a broad range of applications. Compared to mesenchymal stem cells, induced pluripotent stem cells have enhanced multipotency. However, some works showed there is a possibility of tumorigenesis promoting using these stem cells (399). Mesenchymal stem cells offer the differentiating advantage into other cell types, including adipocytes, osteoblasts, smooth muscle cells, cardiac cells, chondrocytes, endothelial cells, neural, and hepatocytes cells (389). Literature reviews showed that although high density of stem cells initially results in tissue formation enhancment, in the long run it decreases proliferation and viability of cells after printing. In contrast, a low density of stem cells is also not practically beneficial as it results in poor functionality. High cell density provides superior cell-to-cell interactions that help the differentiation toward sought cell types. However, seeding of stem cells in a high density needs a large amount of expansion *in vitro* that alleviates the alteration risk in cells phenotype. Additionally, high loading raises the bioink viscosity and negatively affect the capacity of waste removal and nutrient exchange of encapsulated cells. Consequently, the cell density selection also relies on the employed bioink to

achieve a desired functionality and directly correlates with the bioink viscosity of and considerably influences the printability (382, 400).

Generic cell types, including stromal cells, neurons, and endothelial cells are omnipresent in multiple organs. The coexistence of multiple cell types is also visible in the native organs. The need to imitate the process of incorporating multiple cell types with bioprinting is endurable. It has the self-organization capabilities, especially in the organoids. However, the coexistence depends on a defined line of inclination within the guest cells, along with an optimum host and guest cell ratio (401). Maiullari et al. constructed multicellular heterogeneous cardiac 3D constructs through 3D bioprinting. It is well established that myocardial functions are also governed by nonmuscular cells like fibroblasts and vascular cells. Hence, bioprinting of heterotypic human umbilical vein endothelial cells and induced pluripotent stem cells derived cardiomyocytes provide enriched vascular networks. Human umbilical vein endothelial cells impose a high orientation index through different geometries and helps in the integration of the host's vasculature (402). Kuss et al. mentioned that commonly cocultured endothelial cells with mesenchymal stem cells possesses limited regeneration capabilities for craniofacial constructs (403). Bourget et al. experimentally proven that in laser-based bioprinting of endothelial cells and mesenchymal stem cells, mesenchymal stem cells provide the flexibility of maintaining the printed pattern over time. This study is useful for the selective migration of cells along with the study of the trophic factors (404). Datta et al. highlighted that complex dynamic cancer microenvironments can be imitated by utilizing bioprinting with multiple cell types. Bioprinting provides the advantage of controlling and observing multiple cell types behavior in 3D architecture. This multiple cell type for tumor model includes fibroblasts, adipocytes, patient-derived cancer cells, endothelial cells (405).

The inability of many 3D bioprinting systems to produce biological constructs with integrative biomimetic complexity is another major barrier. Therefore, in recent years many attempts have been made to facilitate multi-material bioprinting (406-410). While these two problems demand more technological advancement in instrumentation and materials, the third challenge represented here has led to the emergence of 4D printing. That is, the established bioprinted 3D systems do not have the capability to show appropriate biological reactions, since the constructs are largely static, unlike the extremely dynamic morphologies of native tissues that respond to endogenous stimuli (411, 412). Thus, it has been necessary to create novel strategies for bioprinting of objects that can cope with the transformations required, by effectively integrating current 3D bioprinting systems with various biomaterials that are known to respond to stimuli, leading to the emergence of 4D bioprinting; however, more work is required to optimize this technology.

This capacity for material shape transformation, along with degree of application-defined programmability of a material, multi-material printing methods, and precise designs for meticulous transformations, are crucial requirements for 4D printing (278). The opportunity to manufacture artificial bio-structures with function and configuration that better imitate the physiological characteristics of natural tissues may revolutionize tissue regeneration and therapeutic production approaches in the future (Figure 9). However, despite significant progress, there are still several challenges for 4D printing technology compared to 3D alternatives in terms of affordability, scalability, manufacturing, and ease of application; 4D printing techniques are more expensive than their 3D alternatives, and the use of tissues, smart materials, and viable cells is still limited. In the coming years, the development of more novel stimuli-responsive bioinks and smart biomaterials for 4D bioprinting and *in vivo* demands will be required to broaden the therapeutic

opportunities of 4D printing. Overall, the 4D bioprinting field is still emerging and we envision its rapid expansion in the coming years.

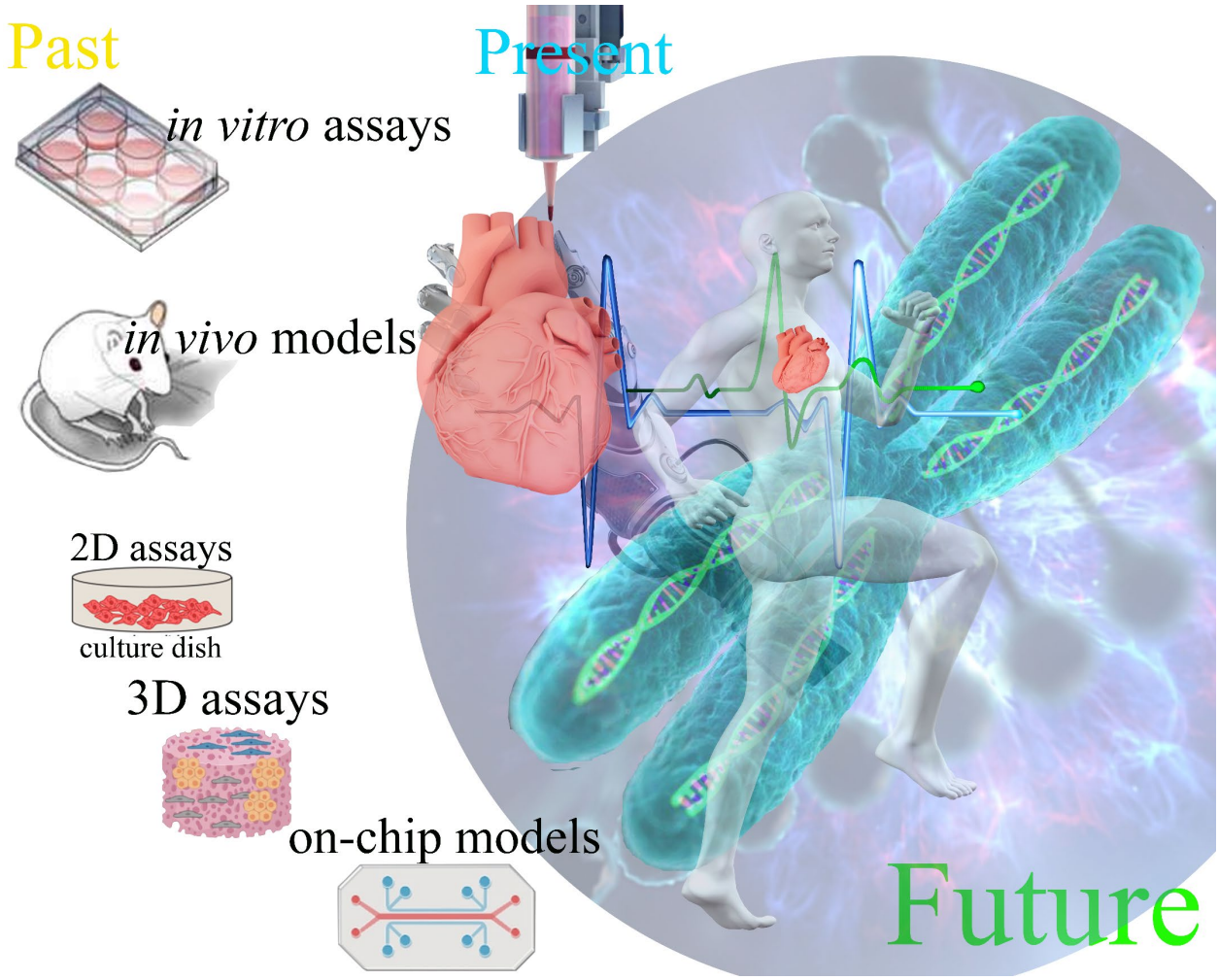

Figure 9: Schematic illustration that is highlighting the potential impact and future research directions that will likely occur in application of 3D/4D bioprinting techniques. *In vitro/in vivo* and 2D assays nowadays can be approached using various 3D assays as well as on chip models thanks to 3D/4D printed constructs. The opportunity to manufacture artificial bio-structures with various embedded cells, therapeutic agents and growth factors having functions and configurations that better imitate the physiological characteristics of natural tissues may revolutionize tissue regeneration and therapeutic production approaches in the future and gain numerous clinical applications. Image credit Mr Karim Osouli-Bostanabad. Chromosome adapted from

https://www.verywellhealth.com/chromosome-16-disorders-2860706.

6 Conclusion and Perspectives

3D printing and 3D bioprinting are emerging strategies that have facilitated the development of new drug delivery systems, implants and scaffolds with high accuracy and complexity, for applications in the biomedical and pharmaceutical industries. Moreover, recent advances in 3D printer aided gene/cell delivery, tissue engineering and regenerative medicine have provided the ability to create various human organ constructs (e.g. skeletal, vascular, and muscular systems). In addition, the combination of 3D printing technologies in microfluidics applications and the emergence of 4D bioprinting to mimic the dynamics of a native tissue have created novel opportunities to effectively and efficiently manufacture dynamic reprogrammable tissue structures and organ-on-a-chip systems, as well as allowing for the delivery of drugs, cells, or growth factors. Recently, there has been substantial advancement in the 3D printing arena, but despite numerous publications highlighting successful 3D printing/bioprinting of drug products, organ-on-a-chip systems, tissue types and microfluidic apparatuses, taking the procedure from the bench to the bedside demands concentrated attempts on numerous fronts. There remain several limitations that must be overcome before these strategies can be successfully translated to clinical applications, including:

I. Computational analysis for tissue fusion or its growth assay
II. Improved scalability of these methods to produce tissue at human-scale quantities
III. Advancement of hybrid systems by integrating or combining various 3D printing/bioprinting modalities
IV. Novel bioink formulation with adjustable rheological and mechanical qualities
V. Evaluation of cell and bioink interaction by mechano-biological methods

VI. Use of stimuli-responsive (smart) hydrogels in 4D bioprinting for bioprinting of personalized medicines

VII. Further study regarding the social, regulatory, and ethical aspects of 3D/4D bioprinting techniques. As 3D/4D printing becomes more clinically relelvent, existing social and ethical issues around the use of stem cells may require further attention

Once these challenges are addressed, we believe that the improved functionality and utility of 3D and 4D bioprinted structures have the potential to be used in various applications, including regenerative medicine, tissue engineering, bioelectronics, actuators, robotics, medical devices, and even personalized medicine.

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ABBREVIATIONS

3D: Three-dimensional
ALP: Alkaline phosphatase
ABS: Acrylonitrile butadiene styrene
CAD: Computer-aided design
EDTA: Ethylenediaminetetraacetic acid
GelMA: Gelatin methacryloyl
HPMCAS: Hydroxypropylmethylcellulose Acetate Succinate
HPC: Hydroxypropyl cellulose
MMP-2: matrix metalloproteinase-2
PEGMA: Polyethylene glycol monomethacrylate

PEGDA: Poly(ethylene glycol) diacrylate
PU: Polyurethane
PLGA: polylactic-co-glycolic acid
PCL: Polycaprolactone
PSS: Polystyrene sulfonate
PVP: Polyvinylpyrrolidone
PVP K30: Polyvinylpyrrolidone K30
PEG: Polyethylene glycol
PEDOT: Poly(3,4-ethylenedioxythiophene)
PEO: Polyethylene oxide
p(NIPAAm-AA): Poly (N-isopropylacrylamide-co-acrylic acid)
RUNX2: Runt-related transcription factor 2
rhBMP-II: Recombinant human bone morphogenetic protein-II
SiO_2: Silicon dioxide
TPO: diphenyl(2, 4, 6-trimethyl-benzoyl) phosphine oxide
T_{trans}: Transformation", "transition" or "switching" temperature
T_m: Melting temperature
T_g: Glass transition temperature
T_{crit}: Critical solution point/temperature
β-TCP: β-tricalcium phosphate

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Table of Contents