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In situ three-dimensional printing for reparative and regenerative therapy

Nureddin Ashammakhi[1,2,3,4,5,6] · Samad Ahadian[1,2,4] · Ippokratis Pountos[7,8] · Shu-Kai Hu[1,2,3] · Nazzar Tellisi[7] · Praveen Bandaru[1,2,3] · Serge Ostrovidov[1,2,3] · Mehmet Remzi Dokmeci[1,2,3] · Ali Khademhosseini[1,2,3,4,9,10,11]

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Abstract
Three-dimensional (3D) bioprinting is an emerging biofabrication technology, driving many innovations and opening new avenues in regenerative therapeutics. The aim of 3D bioprinting is to fabricate grafts *in vitro,* which can then be implanted *in vivo*. However, the tissue culture ex vivo carries safety risks and thereby complicated manufacturing equipment and practice are required for tissues to be implanted in the humans. The implantation of printed tissues also adds complexities due to the difficulty in maintaining the structural integrity of fabricated constructs. To tackle this challenge, the concept of *in situ* 3D bioprinting has been suggested in which tissues are directly printed at the site of injury or defect. Such approach could be combined with cells freshly isolated from patients to produce custom-made grafts that resemble target tissue and fit precisely to target defects. Moreover, the natural cellular microenvironment in the body can be harnessed for tissue maturation resulting in the tissue regeneration and repair. Here, we discuss literature reports on *in situ* 3D printing and we describe future directions and challenges for *in situ* 3D bioprinting. We expect that this novel technology would find great attention in different biomedical fields in near future.

Keywords 3D bioprinting · Biofabrication · Bioinks · *In situ* 3D printing · Regeneration

1 Introduction

Tissue defects resulting from disease, trauma, or surgical resection require treatment to restore tissue structure and function. There are certain challenges related to reconstructing complex tissue defects, such as those affecting the skeleton or craniomaxillofacial area and those resulting after surgical debridement (Ashammakhi and Kaarela 2018; Datta et al. 2017). Such defects are difficult to treat using conventional and standard size implants. In particular, they need to be treated by using modified or trimmed implants or constructs. Three-dimensional (3D) printing offers the possibility to provide constructs that can be predesigned to precisely fit defect size and shape. These constructs are usually designed and engineered *ex vivo* and subsequently implanted into the body (Tellisi et al. 2018). However, this process has several

✉ Nureddin Ashammakhi
n.ashammakhi@ucla.edu

✉ Ali Khademhosseini
khademh@ucla.edu

[1] Center for Minimally Invasive Therapeutics (C-MIT), University of California - Los Angeles, California, Los Angeles, USA

[2] California NanoSystems Institute (CNSI), University of California - Los Angeles, 570 Westwood Plaza, Building 114, Room 4528, Los Angeles, CA 90095, USA

[3] Department of Radiological Sciences, University of California - Los Angeles, California, Los Angeles, USA

[4] Department of Bioengineering, University of California - Los Angeles, California, Los Angeles, USA

[5] Division of Plastic Surgery, Department of Surgery, Oulu University, Oulu, Finland

[6] School of Technology and Innovations, University of Vaasa, Vaasa, Finland

[7] Department of Trauma and Orthopaedics, University of Leeds, Leeds, UK

[8] Chapel Allerton Hospital, Leeds Teaching Hospitals, Leeds, UK

[9] Department of Chemical and Biomolecular Engineering, University of California - Los Angeles, California, Los Angeles, USA

[10] Center of Nanotechnology, Department of Physics, King Abdulaziz University, Jeddah, Saudi Arabia

[11] Department of Bioindustrial Technologies, College of Animal Bioscience and Technology, Konkuk University, Seoul, Republic of Korea

challenges related to logistics (3D printing in a location far from the operating room), sterility, and necessity to modify, trim, adjust, or combine different pieces to fit the shape and size of treated defect. In addition, the size and shape changes and thus ready-printed construct may not always fit following the debridement of defect. Alternatively, 3D printed implant may be installed on a subsequent second operation and this 2-stage procedure carries higher risks and problems.

Ideally, a customized implant should be produced and implanted during the same setting. However, this approach has been limited by various technical, procedural, and regulatory issues, thus far. This ideal solution would employ a commonly used 3D printing technique in the operating room and 3D print constructs *in situ*. This concept may involve the use of either handheld printers (Duchi et al. 2017; Di Bella et al. 2018; Hakimi et al. 2018) (Fig. 1) or robotic arms carrying printer nozzles, that are controlled by computers and scanners, which constantly measure the exact size of the defect (Wang et al. 2015a) (Fig. 2). The integration of developments made in robotics and computer-assisted interventions will also enable achieving more precision in printing procedure. These advances will enable developing more innovative solutions for *in situ* 3D printing to be employed in operating rooms. When fully deployed, the technology of *in situ* 3D printing will result in more accurate reconstruction of tissue defects and result in faster and more efficient healing of tissue defects.

In this work, we discuss technologies and materials used for *in situ* 3D (bio)printing. Applications of this novel technology to fabricate different tissues are described. We then highlight future directions and challenges toward wide applications of *in situ* 3D bioprinting in tissue regeneration.

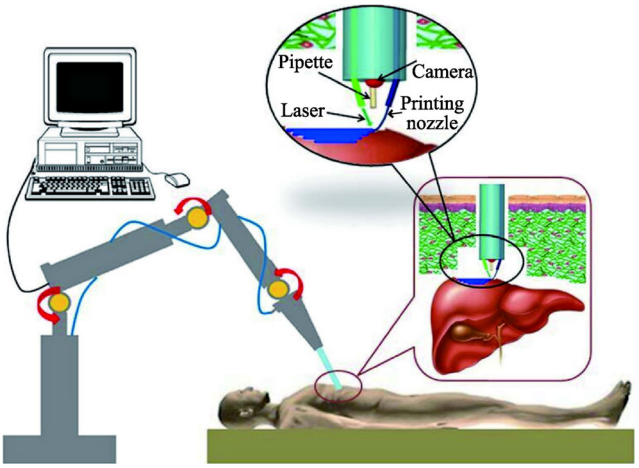

Fig. 2 Robot-assisted *in situ* 3D bioprinting. Schematic illustration showing the concept of robot-assisted *in vivo* 3D bioprinting in the frame of minimally invasive surgery. Reproduced with permission from Wang et al. (Wang et al. 2015b)

2 Technologies for *in situ* 3D (bio)printing

Although *in situ* 3D bioprinting is a rather novel approach, some facile and portable printing devices adapted for *in situ* applications have recently been reported (Di Bella et al. 2018; Keriquel et al. 2010; Sofokleous et al. 2013; Keriquel et al. 2017; Binder et al. 2010) (Di Bella et al. 2018; Keriquel et al. 2010; Sofokleous et al. 2013; Keriquel et al. 2017; Binder et al. 2010). For example, Sofokleous et al. (2013) developed a motorized and handheld spray gun equipped with electrohydrodynamic multi-needles capable of producing polymers as solid particles and fibers. The polymeric particles

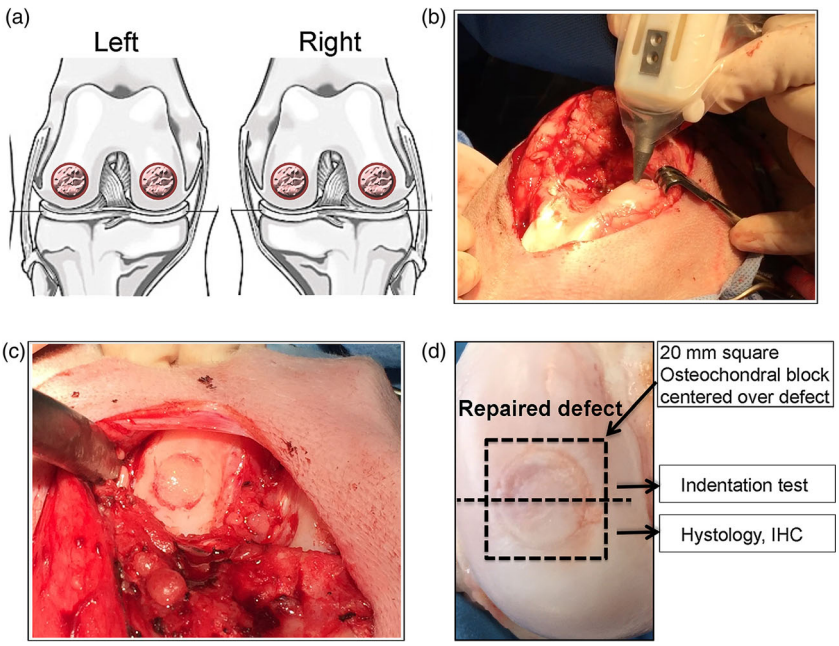

Fig. 1 Use of hand-held 3D bioprinting device for cartilage tissue regeneration. **a** A full-thickness cartilage defect was made in the medial and lateral femoral condyles of both stifle joints in sheep. **b** Images of intra-operative in situ 3D printing using a hand-held device. **c** Defect filled with HA-GelMA and MSCs and coated with fibrin glue spray. The defect was fully filled exhibiting curvature of the femoral condyle. **d** Macroscopic image of the retrieved treated cartilage defect. Reproduced with permission from Di Bella et al. (Di Bella et al. 2018)

and fibers were produced in sub-micrometer to micrometer range, which is physiologically relevant to mimic the extracellular matrix (ECM) structure. The use of multi-needles would enable the device to extrude multiple bioinks in a continuous manner for rapid fabrication of complex and heterogeneous tissue constructs (Liu et al. 2017; Miri et al. 2018). In another work, Cohen et al. (2010) used a robocasting-based additive manufacturing system to print alginate hydrogels onto cadaver calf femoral chondral and osteochondral defects. The ionic crosslinking of the gels is compatible with *in situ* repair. Cells can also be cultured in the hydrogels for potential applications in trauma surgery and facial reconstruction. However, other naturally-derived polymers (e.g., gelatin (Taira et al. 2018; Ahadian et al. 2015), gelatin methacryloyl (GelMA) (Colosi et al. 2016), and autologous platelet-rich plasma (Faramarzi et al. 2018)) should be added to the alginate to enhance its cell-responsiveness. Keriquel et al. proposed a laser-based fabrication technology adapted for *in situ* bone tissue regeneration with high precision and resolution using mesenchymal stromal cells (MSCs) (Fig. 3) (Keriquel et al. 2017). However, the technology was suitable to print on flat surfaces only and thereby new technological developments are required to obtain complex structures using such laser-based bioprinter.

3 *In situ* 3D (bio)printing in tissue regeneration

There are several reports on using *in situ* 3D (bio)printing to regenerate different tissues in the body (Table 1). An early report showed that nano-hydroxyapatite (n-HAp) could directly fill calvarial defects in mice in a minimally invasive manner (Keriquel et al. 2010). Later on, the same group

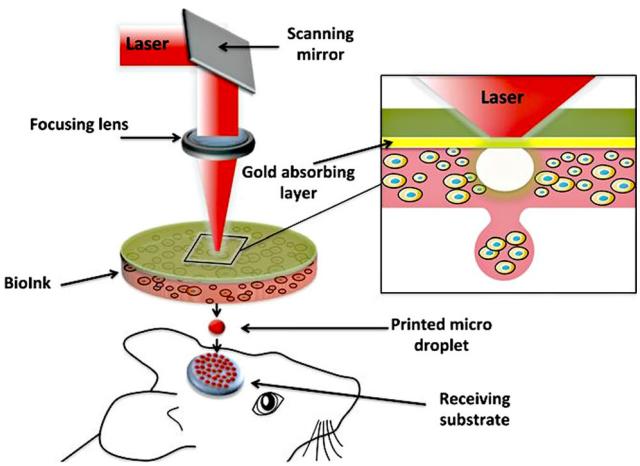

Fig. 3 *In situ* laser-assisted 3D bioprinting. Proof of concept for *in situ* printing using laser-assisted bioprinting for depositing of HAp into a critical-size mouse calvarial defect. Different geometric cell patterns were 3D bioprinted for guiding the regeneration of bone tissue. Reproduced with permission from Keriquel et al. (Keriquel et al. 2017)

printed MSCs either on the peripheral or central regions of discs made of n-HAp and collagen type I and applied to calvarial bone defects in mice (Keriquel et al. 2017). The deposition site of cell-laden hydrogel significantly affected bone tissue regeneration. The authors highlighted that the cells are a vital element of the 3D bioprinting process because poor bone regeneration was observed in the control groups that osteoprogenitor cells were not included in the bioink. In another work, Li et al. obtained the precise size of defect areas of bone and cartilage by the aid of high-resolution 3D scanning and then used *in situ* 3D bioprinting for defect regeneration *ex vivo* (Li et al. 2017). They used alginate and alginate-poly(-ethylene glycol) (PEG) hydrogels. The alginate-PEG hydrogels were polymerized using UV light, which may limit their clinical applications. In another study, Cohen et al. used a demineralized bone matrix and an alginate hydrogel for *in situ* repair of bone and cartilage defects, respectively (Cohen et al. 2010). Di Bella et al. treated critical-sized osteochondral defects in sheep (Di Bella et al. 2018). The bioink was composed of MSCs in a scaffold of GelMA and hyaluronic acid (HA) methacrylate hydrogels. Interestingly, *in situ* 3D bioprinting did not cause any intra- or postoperative complications. The above-mentioned proof-of-concept works on *in situ* bioprinting of bone and osteochondral tissues provide evidence that geographically complex pathologies could be treated with further advancement of this technology instead of salvage procedures such as joint replacement or joint fusion procedures.

Binder et al. used a model of full-thickness skin defect and bioprinted constructs containing keratinocytes and fibroblasts (Binder et al. 2010). They found that the produced skin was similar to the normal skin, and complete wound closure was noted. Next, Skardal et al. bioprinted amniotic fluid-derived stem cells and MSCs suspended in fibrin/collagen gel onto a full-thickness skin wound (2 × 2 cm). They reported a higher level of re-epithelialization and increased microvessel density and capillary diameters in wounds treated with the amniotic fluid-derived stem cells as compared to those treated with MSCs. The bioprinted cells did not permanently integrate with the tissue. Therefore, secreted trophic factors may be responsible for a favorable effect rather than direct cell-cell interactions. In a recent study, the same group bioprinted amniotic fluid-derived stem cells embedded in photocrosslinkable heparin conjugated HA onto full-thickness skin wounds in a murine model. They reported re-epithelialization, vascularization, ECM production, and wound closure in treated wounds (Skardal et al. 2017). In another study, Albanna et al. (2012) explored the healing potential of fibroblasts and keratinocytes suspended in fibrogen/collagen and compared the overall potential of using autologous versus allogeneic cells. They used 10 × 10 cm skin defects in a porcine model. In this preclinical study, the authors demonstrated that 3D bioprinters can provide precise and immediate cover to a large skin defect. In addition, supplementing the skin graft with cells involved in

Table 1 Summary of *in situ* 3D (bio)printing works for tissue regeneration applications

Animal model	Printing technology	(Bio)ink	Target tissue	Outcomes/comments	Ref.
Mouse ($n = 36$). Full-thickness excisional skin defect	Inkjet bioprinter	Human keratinocytes and fibroblasts	Skin	(1) Acceptable survival rate of cells after printing (2) Fast healing rate of the treated defects	(Binder et al. 2010)
Mouse ($n = 30$). Critical size calvarial defect	Laser-adapted printer	n-HAp	Bone	(1) *In vivo* bioprinting is possible (2) No effect on the animals' brain (3) Bone formation only occurred in some defects	(Keriquel et al. 2010)
Pig ($n = 6$). Skin defect	Inkjet printer	Autologous vs allogeneic fibroblasts and keratinocytes suspended in fibrinogen/collagen	Skin	(1) *In situ* skin bioprinting is a viable option for treatment of large skin defects (2) The utilization of autologous cells outperformed in healing potential compared to allogeneic cells	(Albanna et al. 2012)
Mouse ($n = 5$). Full-thickness skin wound	Inkjet bioprinter	Amniotic fluid cells and bone marrow MSCs suspended in fibrin/collagen gel	Skin	(1) The graft improved re-epithelialization with increased micro-vessel density and capillary diameters (2) Secreted trophic factors were responsible for the favorable effect, rather than direct cell-cell interactions	(Skardal et al. 2012)
Sheep (n = 6). Full-thickness osteochondral defect	Hand-held bioprinter (Biopen)	MSCs in GelMA and HA-GelMA	Cartilage	Bioprinting with the Biopen is feasible and resulted in early cartilage regeneration	(Di Bella et al. 2018)
Mouse ($n = 64$). Critical size calvaria defect	Laser-assisted bioprinter	MSCs on n-HAp and collagen	Bone	(1) The technology could print complex structures, which favored bone regeneration (2) Cell arrangement had a significant impact on bone regeneration	(Keriquel et al. 2017)
Rats. Circular skin defect	Droplet-based bioprinter	Rat primary dermal fibroblasts in collagen/fibrinogen	Skin	(1) Healing of defects was accelerated (2) Regeneration of functional skin with anatomically correct features was not achieved	(Gudapati et al. 2017)

the skin healing process could enhance the wound healing. These favorable results were demonstrated by 90% decrease in wound size, wound re-epithelization, and 20% reduction of the wound contractures compared to the controls. The authors reported that the use of autologous cells outperforms the use of allogeneic cells in terms of their healing potential. Despite these encouraging results from the aforementioned studies, the wounds were covered by scar tissue rather than normal skin. Nevertheless, they represent a promising option for developing treatment of large skin defects. Compared to other cell-based skin regenerative therapies, such as spraying keratinocytes (Sood et al. 2015) or keratinocyte sheet (Kirby et al. 2018), *in situ* printing shows the higher potential in addressing deeper wounds (i.e., deep into basal layer) by combining multi-material printing system. In addition, *in situ* printing can perform precise cell deposition for more efficient use of kerotinocytes since it is difficult to culture for large-scale production (Anderson et al. 2018).

4 Challenges and future directions

Three-dimensional bioprinting technologies have emerged as a method to create complex constructs for tissue regeneration and repair (Ahadian et al. 2018). It is now feasible to print large-scale and clinically relevant tissue constructs in a relatively short time (Gungor-Ozkerim et al. 2018). Other micro-scale technologies (e.g., microfluidic (Zhang et al. 2017) and fiber (Zhang et al. 2016) technologies) can also be combined with 3D bioprinting to achieve automated and controllable multimaterial bioprinting to recapitulate the heterogeneous structure and composition of native tissues. However, printed tissues still need to be matured using bioreactors *in vitro* and may require modifications in size and shape prior to their implantation *in vivo*. *In situ* 3D bioprinting has been developed to tackle these problems by directly printing tissues *in vivo*, where the natural environment in the body is used for tissue survival and functionality.

Current evidence on using *in situ* 3D (bio)printing for tissue regeneration is still limited. However, there is enough evidence to show that this procedure is possible, and has huge potential for use in tissue regeneration. It is expected that maturation, functionality, and integration of bioprinted constructs *in situ* are improved compared to engineered tissues *in vitro* because the microenvironment in the body is richer in signaling molecules than *in vitro* microenvironment. Furthermore, *in situ* 3D bioprinting procedures, within the framework of minimally invasive techniques, should lead to better patient outcomes, less pain, shorter time-to-recovery, reduced nosocomial infection, and reduced hospitalization (Ashammakhi et al. 2019). However, several issues still remain and should be tackled. For example, *in situ* 3D bioprinting requires fast gelation process, sufficient stability of printed construct *in vivo*, and friendly bioprinting environment (e.g., drying step by airflow). In particular, large size constructs need early integration with the host tissue in order to survive and function *in vivo (*Khademhosseini and Langer 2016). Hence, continuous development and optimization of bioinks and bioprinters, adapted for *in situ* printing are needed.

Development of portable bioprinters and mobile units may be a viable option for use in emergency situations and remote locations. Moreover, *in situ* bioprinting can be used primarily by surgeons for regenerative and reconstructive applications. Therefore, integrating *in situ* bioprinting with advanced robotic systems that utilize user-friendly interfaces is necessary for translation into common surgical procedures. In addition, advanced real time imaging with computational modeling will be useful for fast morphological assessment of defects and fabrication of tissues *in vivo*. In the future, various imaging modalities would be combined with advanced robotics to provide input for *in situ* printing interfaces. The use of stimuli responsive bioinks in *in situ* 3D bioprinting would also enable us to produce the so-called four-dimensional constructs (Li et al. 2016; Ashammakhi et al. 2018), which would be capable of recapitulating the natural morphological and structural changes in the tissues. All these developments will have impact on the future of *in situ* 3D bioprinting and its translation to clinic.

5 Conclusions

Thus far, research into the field of *in situ* 3D bioprinting has shown feasibility and usefulness. This novel research field provides great potential for tissue engineering and regenerative medicine applications where preoperative planning of construct size and shape is difficult or impossible to predict. Advances in robotic surgery, fused imaging, and computer-assisted medical interventions should also be integrated to develop future clinical *in situ* 3D bioprinting processes, which can be translated to products for a variety of surgical applications.

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Compliance with ethical standards

Conflict of interest The authors have no competing interests.

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