3D-BioPrinting: The future of Red Biotech

Every day Science moves little steps forward, contributing to the progress of our society. Sometimes, however, a single invention revolutionizes the world. Indeed, the invention of woodblock printing and development of industrial-scale printing-press in the 15th century have changed our society. 3D-printing is now boosting another revolution. The production of custom-made objects from a virtual model will trigger a rapid development of a more versatile, less expensive manufacturing sector for the on-demand market. The real revolution, however, is represented by 3D-printing in biomedicine. 3D-bioprinting represents the future of the Red-Biotech. This technology, indeed, will be able to build ex-novo organs using biocompatible materials and human cells; replace the allograft transplants, eliminating waiting lists that often make the difference between life and death; and provide more predictive, less expensive experimental models, replacing animal tests. The high innovation content of this technology, can make the difference between being obsolete and new

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Introduction

In the 15th century the 2D printing invention revolutionized society, facilitating information dissemination, alphabetization of people, and introducing the concept of communication which altered the very structure of society. Printing has been defined as the great contribution to civilization; today, the new frontier of the printing technology is 3D-printing. The inventor of 3D-printing was Chuck Hull, who first came up with the idea in 1983, when he was using UV light to harden tabletop coatings. Hull coined the term "stereolithography" in his U.S. Patent 4,575,330, – entitled "Apparatus for Production of Three-Dimensional Objects by Stereolithography" – issued in 1986. Production of 3D complex structures has been applied by industry to produce customized objects, such as pieces of bicycles and jewels.
Before the 3D-printer invention, industry could produce a specific object on industrial scale only, in fact production was a process made by many steps, each one carried out by a specific machine. It was not even possible to conceive the production of a single object for a single request. Now, thanks to this new technology any requested object can be imagined, designed and then produced by simply using the same machine, the 3D-printer. This technology has an endless potential, and the benefits of 3D-printing technology encompass manufacturing, scientific and biomedical fields. For example, scientists from the University of Central Lancashire in the UK have developed a 3D-printer that can "print" medicine tablets, that may be designed to custom-fit the needs of the patient who is going to take them; the advantage is that the 3D-printer can replicate any existing chemical in terms of weight and dosage fairly accurately [1].
In the last years many scientific discoveries and technological inventions in the field of biomaterial,

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microfluidics, engineering, nanotechnology, biochemistry and stem cell biology have made several 3D-printing applications possible in the biomedical field, yielding the real innovative value of this technology. In this field 3D-printers can be used to produce biocompatible, tri-dimensional porous structures that mimic human tissues, named scaffolds, or to print cells, biomaterials and biomolecules at the same time, in specific spatial positions and with a very high-resolution, moving from 3D-printing to 3D-Bioprinting. The ability to create a virtual design of a custom-made living-object and make it real, is a concept that, until a few years ago, also inspired science fiction. Bioprinting can be defined as the use of computer-aided transfer processes for patterning and assembling living and non-living materials with a prescribed 2D or 3D organization, in order to produce bio-engineered structures serving in regenerative medicine, pharmacokinetic and basic cell biology studies.

In spite of the crisis of BigPharma, Biotech represents an active and promising reality, but the major challenge is that pre-clinical studies to test the safety and efficacy of new drugs use laboratory animals and 2D cell culture, and neither of these methods are accurate reflections of how a drug will react in humans. In addition, alternatives to animal experimentation are sought. The possibility to test drugs and biotechnological products on 3D humanized constructs could represent the solution to this problem and, for its economical and innovative value, investing in this field could be the future of the so-called *Red Biotech* (the industries concerning with the discovery and development of innovative drugs and treatments in the field of biomedicine). Using 3D-bioprinting allows to set up tri-dimensional cell cultures, cultivate different cell types in the same structure, simulate cell to cell and cell to environment interactions *in vivo*, build human tissues for regenerative medicine applications, restore damaged organs, wound healings or correct maxillo-facial defects, and much more.

The crucial steps that led to bioprinting were the development of biocompatible, printable materials and cell-printing techniques. Early application of 3D-bioprinting was in cardiovascular diseases for stent and valve production for clinical usage.

This new promising technique has had a rapid and widespread development in the last few years, thus today there are many different bioprinters based on different technologies, from laser-assisted polymerization to microwave, that seed cells onto various solid substrate [2]. They use, for example, nozzle-based deposition of hydrogels and cells, drop-on-demand inkjet-printing of cell suspensions with subsequent cross-linking, layer-by-layer cross-linking of synthetic or biological polymers by selective irradiation with light and even laser-induced deposition of single cells. The choice of the technique, material, cell lineage to use depends on the final goal.

The 3D bioprinting is attracting our attention, as at Laboratory of Dosimetry & Metrology, FSN-TECFIS-DIM, we are actively involved in the field of tissue engineering research [3-5]. Indeed, we produced a scaffold (muscle acellular scaffold: MAS) (Figure 1 A), made up of native

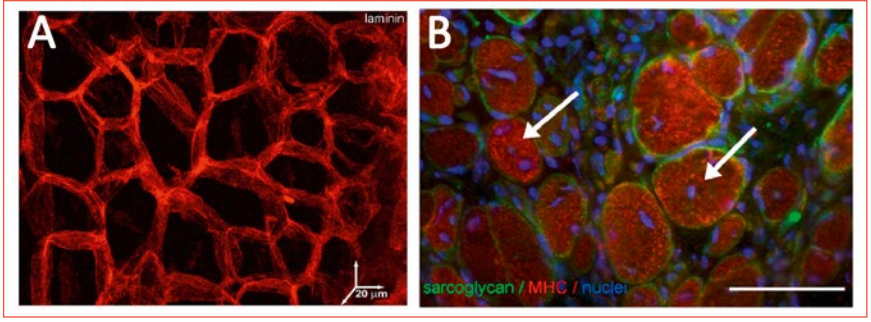

FIGURE 1 (A) Acellular skeletal muscle scaffolds (MAS), obtained by de-cellularization of a tibialis anterior muscle. 3D image stack reconstruction of confocal microscopy images of a 20 mm thick cryosection of MAS, highlighting the irregular, polyhedral tubular organization corresponding to that of the muscle fibers. (B) Staining of a cross-section obtained by MAS (muscle acellular scaffold) 21 days after implant in a mouse. Immunofluorescence analysis of muscle-specific molecular markers demonstrates that these cells (arrows) are generating skeletal muscle tissue inside the scaffold. Bar = 50 µm
Source: "The pro-myogenic environment provided by whole organ scale acellular scaffolds from skeletal muscle", Biomaterials, 32, 7870-7882, 2011

extracellular matrix (ECM) from skeletal muscle tissue [4]; this scaffold was able to recruit stem cells which differentiate, giving raise to new muscle fibers (Figure 1 B). ENEA holds a unique multidisciplinary expertise that would allow it to successfully conquer this field which up to now is being almost deserted in our country. Here we discuss on the state of the art of this technology, its application in biotechnological and biomedical field, and the challenges to overcome.

State of the art

Bioprinting begun from 2D ink-based printers modified to become cell-printers. The ink in the cartridge was replaced with a biological material and the paper was replaced with an electronically controlled elevator stage to provide control of the z axis (the third dimension in addition to the x and y axes). So bioprinting is the use of printing technology for deposition of biological material such as living cells, ECM components, biochemical factors, proteins or drugs on a solid or gel surface, by the help of *computer aided design* (CAD). To build entire tissue or organs that mimic natural organization in human, it is necessary to acquire a 3D image. Medical imaging technology is an indispensable tool used by tissue engineers to provide information on a 3D structure and function at the cellular, tissue, organ and organism levels. In tissue engineering, 3D-bioprinting can be essentially of two types: with or without incorporating living cells onto the solid surface. Different features of these technologies should be considered in the light of the most important factors in 3D bioprinting, namely surface resolution, cell viability, and the biological materials used for printing. Three concepts of 3D bioprinter are nowadays available: Inkjet bioprinters, Microextrusion bioprinters, Laser-assisted bioprinters (LAB) (Figure 2) [2].

Inkjet printers are the type of printer most commonly used for both non-biological and biological applications. Now, inkjet-based bioprinters are custom-designed to handle and print biological materials (bio-ink) at increasing resolution, precision and speed on a solid biocompatible surface (bio-paper). Inkjet printers make use of thermal or acoustic forces to eject drops of liquid onto a substrate, which can support, or form part of, the final construct. Advantages of acoustic inkjet printers include the capability to generate and control uniform droplet size and ejection directionality, as well as to avoid exposure of cells to heat and pressure stressors [2]. Different cell lineages can be printed at the same time using different nozzles and cartridges, and the acoustic waves allow the deposition of drops as large as a cell, giving a high resolution potential. Because of the availability of standard 2D inkjet printers, researchers can readily access and modify them. Moreover, commercially available inkjet 3D bioprinters are also relatively cost-effective owing to their simple components and readily available design and control software. The wide application of this technology by many groups has accelerated advances in inkjet bioprinting technology [2].

Microextrusion bioprinters are usually based on a temperature-controlled, material-handling and dispensing system that extrudes tubes or spheroids of materials, which are superimposed on one another and then cured through the addition of radiation or chemical reactions, or by time [2,6]. Nearly 30,000 microextrusion-based 3D-printers are sold worldwide every year, and academic institutions are increasingly

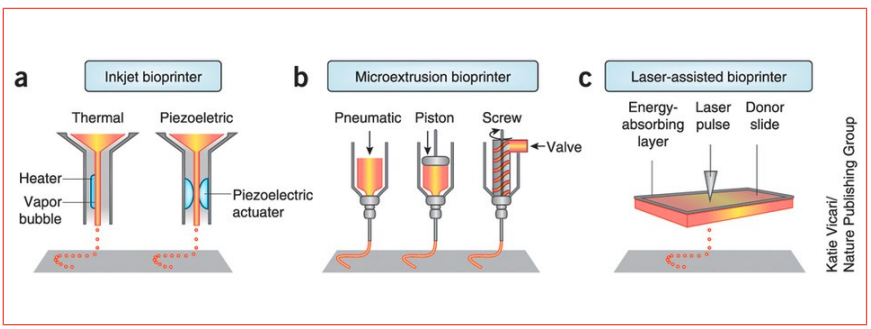

FIGURE 2 The three main bioprinting techniques: (a) Inkjet bioprinting; (b) Microextrusion bioprinting; (c) Laser-assisted bioprinting (LAB)
Source: "3D bioprinting of tissues and organs", Nat Biotech, 32, 773–785, 2014

purchasing and applying microextrusion technology in tissue and organ engineering research. Microextrusion-based printers are considerably more expensive but have better resolution, speed, spatial controllability and more flexibility in the material they can print, such as hydrogels, biocompatible copolymers and cell spheroids. A few systems use multiple print heads to facilitate the serial dispensing of several materials. The main advantage of the microextrusion bioprinting technology is the ability to deposit very high cell densities, covering one of the major goals for the bioprinting field: to achieve physiological cell densities in tissue-engineered organs. To create 3D tissue constructs with microextrusion printing, some groups used solutions comprised of cells only. Multicellular cell spheroids are deposited and allowed to self-assemble into the desired 3D structure. Tissue spheroids are thought to possess material properties that can replicate the mechanical and functional properties of the tissue ECM. Cell viability after microextrusion bioprinting is lower than that with inkjet-based bioprinting, due to the shear stresses inflicted on cells in viscous fluids. Although cell viability can be maintained using low pressures and large nozzle sizes, the drawback may be a major loss of resolution and print speed [2].

Laser-assisted bioprinting (LAB), albeit less common than other methods, is being increasingly used for tissue- and organ-engineering applications. LAB is based on a pulsed laser beam that acts on a laser-energy-absorbing layer (gold or titanium) and a layer of biological material (cells and/or hydrogel) prepared in a liquid solution. LAB is compatible with a range of viscosities and can print cells with negligible effect on cell viability and function. LAB can deposit cells at a high density with microscale resolution of a single cell per drop. The application of LAB to fabricate a cellularized skin construct demonstrated the potential to print clinically relevant cell densities in a layered tissue construct, but it is unclear whether this system can be scaled up for larger tissue sizes [2].

As described above, different techniques use different materials, and have a different application potential. In the field of tissue engineering, materials used for bioprinting must have certain characteristics: printability (easy handling and deposition by the bioprinter); degradation kinetics (degradation rates should be matched to the ability of the cells to produce their own ECM); biocompatibility (avoiding undesirable local or systemic responses from the host, actively contributing to the biological and functional components of the construct, giving rise to nontoxic degradation byproducts); structural and mechanical properties (based on the required mechanical properties of the construct, depending on the tissue to construct, such as muscle, bone or skin); cell interaction properties (facilitating cell adhesion and interaction, cell proliferation and tissue formation); and permeability (allowing nutrient exchange and diffusion of wastes) [2,6].

Owing to the wide spectrum of mechanical and biochemical properties of the native tissue, a variety of materials are being developed to mimic specific cell and tissue niches. Hydrogels are the materials most commonly explored for fabricating the complex 3D cellular microenvironments, as they can be tuned for ideal degradability and mechanics, and functionalized by incorporating biomolecules of interest. They are generally considered to have high biocompatibility and non-immunogenicity. The optical clarity of hydrogels permits the use of a vast assortment of photochemical methods to fabricate material structures or pattern biomolecules within the hydrogel matrix. Similarly, their high water content creates an environment conducive to the encapsulation of cells. Alternatively, many synthetic polymers capable of forming hydrogels have also been developed to act as blank-slate materials, the biochemistry and mechanics of which can be custom-tailored via simple chemical modifications. Although poly(ethylene glycol) (PEG) is the synthetic polymer most commonly explored, owing to its innate protein repulsiveness and the fact that it is already FDA approved for certain applications, several other hydrogel-forming polymers have been investigated [7].

Organ and tissue printing

3D-bioprinting of tissues and organs is based on three central approaches: biomimicry, autonomous self-assembly and mini-tissue building blocks [2].

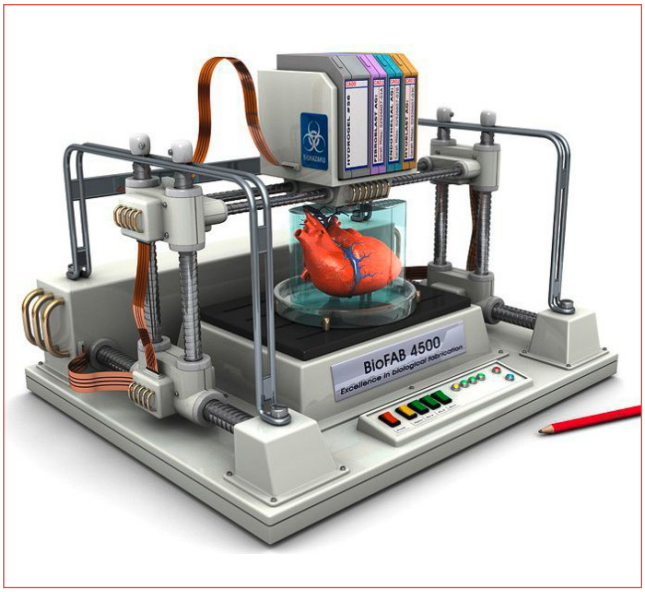

FIGURE 3 Bioprinting Challenges. The major challenge for 3D-bioprinting is to build up functional human entire organs, such as heart, in order to replace allograft transplantation avoiding waiting list
Source: "Bioprinting toward organ fabrication: challenges and future trends", IEEE Trans Biomed Eng, 60, 691-699, 2013

Biomimicry involves the manufacturing of identical reproductions of the cellular and extracellular components of a tissue or organ. The replication of biological tissues on the microscale is necessary for this approach to succeed. Thus, understanding of the microenvironment, as well as the nature of the biological forces in the microenvironment, is needed [2].

Autonomous self-assembly is based on the use of embryonic organ development as a guide. The early cellular components produce their own ECM, appropriate cell signaling and autonomous organization and patterning to achieve the desired biological micro-architecture and function. This approach requires a deep knowledge of the developmental mechanisms of embryonic tissue genesis and organogenesis [2].

The concept of **mini-tissues** is relevant to all of the above strategies for 3D-bioprinting, because organs and tissues comprise smaller, functional building blocks, or "mini-tissues", that can be defined as the smallest structural and functional component of a tissue [2].

Current Challenges

Today the major problem to be overcome for high-dimension organ printing is vascularization. Although the past work has generated methods to create artificial skin, cartilage, tracheas and bladders, these represent relatively simple structures compared with the complex architectures of heterogeneous or vascularized organs and tissues [7]. Most of the current fabrication strategies have developed scaffolds that are capable of culturing cells only for short periods of time in relatively small constructs. In order to allow the development of thicker tissues and prevent the formation of a necrotic core within a scaffold, it is very important that perfusable vascular networks allow the exchange of gases, nutrients, and metabolic products. Therefore, both current and next generations of biofabrication techniques need to address the challenge of fabricating a network of microvessels within a scaffold to allow the formation of larger and thicker tissue constructs. The anticipated potential of providing tubes with tailored branching geometries made of biocompatible or biological materials pushes future visions of patient-specific, vascularized tissue substitutions, tissue-engineered blood vessels, and bio-based vascular grafts [7]. The most straightforward approach to perfusable tissue might be the generation of a network of interconnected channels within the tissue matrix. Such channels may be used as supply system for cells within the surrounding matrix, and may additionally be seeded with endothelial cells. Early works used moulds for preparing sacrificial structures in order to fabricate microfluidic networks, which then allowed the transport of macromolecules into surrounding hydrogels under low driving pressure differences [8].

Anyway, overcoming this challenge would allow to develop organs *in vitro* on demand, thereby lowering or completely eliminating the need for organ donation from individuals [7].

Other applications of 3D-bioprinting include developing high-throughput, 3D-bioprinted tissue models for research, drug discovery, and toxicology.

This great interest in the development of 3D-biorpinting and the economic and innovative potential of this field are giving a boost to every other sector directly or indirectly linked, such as biomaterials, microfluidics, bioengineering, stem cell research and industrial

development of more advanced technologies to help researchers to overcome these numerous challenges in the near future (see Fig. 3 as an example).
This future is already present in some countries. For example, to an Indian cancer patient, who has had a substantial section of his palate removed after undergoing surgery to remove a tumor, a new set of teeth has been given with the help of 3D printing [9]. Scientists at University College London are using 3D printing to create ears to be implanted onto children with severe disfigurements, and the next stage is to trial the operation in Mumbai, India, where there are already a dozen children ready to undergo the surgery [10]. At the University of Toronto some engineering students have developed a 3D-bioprinter that can rapidly create artificial skin grafts from a patient's cells to help treat burnt victims [11]. Indeed, among others, also the US Army is hoping to soon begin clinical trials with 3D-printed skin, in order to help soldiers better recover from war injuries.
This technology is revolutionary mainly in the field of regeneration of those human tissues unable to self-regenerate, such as bone, cartilage, nervous system. Today it is possible to build artificial bone from calcium phosphate, which is a component of both human bones and teeth; the printer's product should be able to integrate directly into a patient's body, where it will fuse with the existing bone.
Due to clinical, commercial, industrial interest of this area of research several industries and start-ups based on bioprinting are rising worldwide, enjoying great success and vast profit, such as Organovo (which has doubled its turnover in about one year), founded in California, in 2007, thanks to *Ink-jet Printing of Viable Cells* patented by Dr. Thomas Boland at Clemson University in 2003.
Bioprinting "fever" is gradually affecting the globe and certainly we will witness a strong scientific and industrial development in the next few years, in a great race where yesterday's future is tomorrow's present, leap-frogging to breakthrough innovation.

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references

[1] J. Stanton. (2014), "3D Printing Technology Produces Printable Medicine Tablets", *Home TestingBlog* hometestingblog.testcountry.com (updated at 11/11/2014), Lancashire, UK

[2] S. V. Murphy & A. Atala (2014), "3D bioprinting of tissues and organs", *Nat Biotech*, 32, 773–785, Nature Biotechnology, Winston-Salem, North Carolina

[3] B. Perniconi, D. Coletti, P. Aulino, A. Costa, P. Aprile, L. Santacroce, E. Chiaravalloti, L. Coquelin, N. Chevallier, L. Teodori, S. Adamo, M. Marrelli and M. Tatullo (2014), "Muscle acellular scaffold as a biomaterial: effects on C2C12 cell differentiation and interaction with the murine host environment", *FrontPhysiol*, 5, 354, Frontiers, Rome, Italy

[4] B. Perniconi, A. Costa, P. Aulino, L. Teodori, Sergio Adamo, Dario Coletti (2011), "The pro-myogenic environment provided by whole organ scale acellular scaffolds from skeletal muscle", *Biomaterials*, 32, 7870-7882, Elsevier, Rome, Italy

[5] L. Teodori, A. Costa, R. Marzio, B. Perniconi, D. Coletti, S. Adamo, B. Gupta, A. Tarnok (2014) "Native extracellular matrix: a new scaffolding platform for repair of damaged muscle", *FrontPhysiol*, 5, 1-9, (Epub ahead of print), Rome, Italy

[6] C. M. O'Brien, B. Holmes, S. Faucett, and L. G. Zhang (2014), "Three-Dimensional Printing of Nanomaterial Scaffolds for Complex Tissue Regeneration", *Tissue Engineering* Part B, 00, 1-12, Mary Ann Liebert Inc, Washington

[7] P. Bajaj, R. M. Schweller, A. Khademhosseini, J. L. West, and R. Bashir (2014), "3D Biofabrication Strategies for Tissue Engineering and Regenerative Medicine", *Annu Rev Biomed Eng*, 16, 247-276, Annual Reviews, Urbana, Illinois

[8] E. Hocha, G. E.M., T. and K. Borchers (2014) "Bioprinting of artificial blood vessels: current approaches towards a demanding goal", *Eur J Cardiothoracic Sur*, 46, 767-778 European Journal of CardioThoracic Surgery, Stuttgart, Germany

[9] S. Linning (2014), "Indian cancer patient forced to have his upper jaw removed gets entirely new set of teeth built by a 3D printer", *MailOnline* www.headline1.com, Bengalore, India (updated at 11/11/2014)

[10] R. Singh (2014), "Making 3d printed ears for disfigured children", *BBC news* www.bbc.com (updated at 11/11/2014), London, UK

[11] C. Claude (2014), "Treatening severly burned people with 3D printing is now possible with PrintAlive Bioprinter, a Canadian prototype that prints bandages that resemble human skin", *Makery* www.makery.info (updated at 11/11/2014), Toronto, Canada