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PH.D. THESIS

DESIGN OF 3D ADDITIVELY MANUFACTURED SCAFFOLDS AND COLLABORATIVE BIOMANUFACTURING SYSTEMS FOR TISSUE ENGINEERING

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“If you are not failing your time,
you are not doing R&D.
If everything works,
you are not asking tough enough questions”

Abstract

Additive Manufacturing (AM) technologies represent a useful and cost-effective tool for the timely fabrication of geometrically complex objects. The suitability of AM in achieving complex shapes, the accuracy, the reproducibility, and the high degree of automation of the processes, have contributed to affirm the great utility of these technologies in several contexts, including medical and healthcare. AM technologies enabled to rapidly fabricate medical devices meeting patient-specific requirements and, as a result, they have greatly enhanced routine clinical procedures.

This work provides an overview of the different AM classes as classified by the ISO reference standards, then focusing on the main applications of these technologies in the medical field. In the field of Tissue Engineering (TE), AM enables the designing and manufacturing of customized scaffolds with complex shapes, lightweight, and tailored properties, mimicking closely the heterogeneity and complexity of tissues and organs to substitute or promote tissue healing and regeneration.

Bone tissue regeneration has particularly benefited from these scaffold-based approaches. The usage of scaffolds as temporary three-dimensional frameworks to provide structural support for cell growth, proliferation and adhesion during the regenerative process, the ideal features of such constructs, are the main topics addressed by this work. A focus on the porosity effect on both biological and mechanical features of scaffolds is presented.

Magnetic nanocomposite scaffolds were designed and manufactured by means of AM to investigate the possible enhancing in bone tissue regeneration due to the magnetic characteristics of the constructs. The work reports the analysis of the role of magnetic features on biological performance. Even the mechanical characteristics of scaffolds were improved by using magnetic nanoparticles (MNPs) as reinforcement of the polymeric matrices.

Despite the encouraging outcomes of scaffold-based TE, the commercial translation of Additive Manufacturing technologies for scaffold fabrication is still a challenge. The production methodology

of 3D scaffolds for tissue regeneration is a complex and discontinuous process involving several stages going from the isolation of the stem cells to the in vitro dynamic cell culture. Even though in this scenario industries are increasingly implementing automated robotic systems, current technologies are not sufficient for the development of large industrial scale scaffold fabrication.

Accordingly, a relevant improvement could raise from the implementation of a modern collaborative workplace in an existing production line, combining strength endurance and accuracy of cobots, with intelligence, flexibility and adaptability of the human being. In a such system, the drawbacks related to the low level of process control, low productivity and risk of contaminations may be solved. Therefore, the current work also proposes a systematic approach to the design of collaborative biomanufacturing systems. The last chapter of this work provides a further insight into the potential to upscale the scaffolds manufacturing process, taking advantage of the possibilities given from the Human-Robot Collaboration (HRC), and gives evidence of critical features for workplace definition.

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Chapter 1. Additive Manufacturing for healthcare

1.1. Introduction

Over the last four decades, Additive Manufacturing (AM) emerged as a useful and cost-effective tool for timely fabrication of geometrically complex objects [1–3]. Additive Manufacturing, popularly known as 3D printing, was introduced in the 1986 by Charles Hull who filed the first stereolithography (SLA) patent. Comparing AM with conventional manufacturing processes, its main novelty consists in adding material instead of removing it. More in detail, Additive Manufacturing consists in the deposition of materials in a layer-by-layer manner to fabricate solid objects starting from three-dimensional (3D) model data [2]. Each layer is a cross-section, having a finite thickness, of the object derived from the model Computer Aided Design (CAD) data [4, 5]. A wide range of materials, e.g., polymers, metals, ceramics and bioinks, can be processed by means of AM realizing very complex geometry that could only be fabricated using AM. Moreover, complexity is very inexpensive in comparison to traditional manufacturing techniques. These technologies are currently used for several applications, including the engineering industry (e.g., aerospace and automotive industries) medicine, architecture, education, hobbies, toys, and entertainment.

Additive Manufacturing plays a crucial role in developing healthcare solutions, e.g., implants designing, surgical planning, and tissue engineering, due to its accuracy, repeatability, and reliability [2]. Additive manufacturing of organs, prosthetics, and pharmaceutical dosage, discovery and delivery devices are well-known application of AM in this context [6]. Hence, the growing use of AM in medical field is largely due to the biocompatibility of materials, cost effectiveness, accessibility, relatively short production time, collaboration, democratization, and easy customization [7–11]. Furthermore, AM enables the application of design approaches aiming to minimize weight, improving fatigue life, maximizing stiffness, and enhancing the longevity of medical implants. Another advantage related to the employing of AM technologies in the medical field is the possibility

to design tailor-made solutions, *patient-specific* implants or tools that perfectly fit the anatomy of the patient. The weak point is the slow speed making AM unsuitable for mass production [12–17].

To date, many AM technologies have been developed, however not all of these are equally employed for the manufacturing of medical devices. Metallic medical devices are usually realized through powder bed fusion processes: among these, selective laser melting, and electron beam melting are often used to fabricate medical devices starting from powdered metals. Biomaterials are also processed by powdered-based additive manufacturing, including selective laser sintering. Fusion deposition modelling is the extrusion-based process most widely used in this scenario. Biomaterials are mostly processed by means of Vat polymerization techniques, such as stereolithography.

The processes just mentioned above are classified according to the reference standards and then described in detail in Section 1.2. Then, an overview of most common applications of AM in the biomedical context is provided in Section 1.2.8. The biocompatible materials that are usually processed to realize medical devices are described in Section 1.4. Section 1.5 describes the approach to designing patient-specific medical devices and then, Section 1.6 presents a brief recapitulation and conclusions.

1.2. Classification of AM processes

Several techniques for AM have been developed since the 1980s. The ISO/ASTM 52900 [18] standard establishes general principles and a formal vocabulary for AM technologies providing a classification of process categories. The process categories are described below more specifically. Bioprinting is also introduced at the end of the paragraph even if it is not a specific additive manufacturing technique on its own, but a set of AM technologies mostly used for regenerative medicine and tissue engineering [19, 20].

1.2.1.Vat Photopolymerization

Vat Photopolymerization (VPP) is the first typology of AM process to be introduced and then commercialized by C.W. Hull in 1986. VPP involves the use of a bath of liquid photopolymer resin which is cured by means of thermal energy directly applied to the liquid material (**Figure 1.1**) [21].

A laser system is used to selectively cures, in a layer-by-layer manner, specific regions of resins according to the model geometry. In the following, the most used techniques using the main principles of VPP are described.

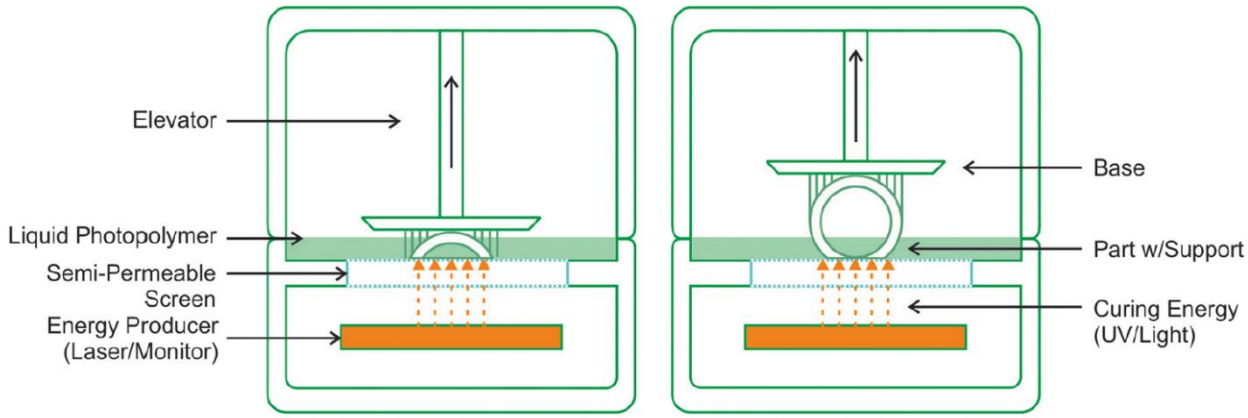

Figure 1.1. Vat Photopolymerization process schematic representation [21].

Stereolithography (SLA) is based on the selective photopolymerization of photosensitive resins through the irradiation of an ultraviolet (UV) light. SLA processes include two different irradiation methods: (i) mask irradiation; (ii) direct irradiation or photo-fabrication. The mask irradiation method consist in the solidification of each layer of the polymer by means the exposure to UV radiation. The UV radiation, generated by a lamp, is transmitted through a mask which is composed of transparent areas corresponding to the section of the model to be fabricated. In the direct irradiation method, a laser beam follows a precise pattern, causing polymerization of the resin and solidification of the material point-by-point and layer-by-layer until the entire structure is complete [33]. The SLA system for direct irradiation is composed of a container containing the photosensitive polymer, the movable construction table where the object is built, the laser system which irradiates the UV light and the dynamic optical system which focuses the laser beam. Hence, when a layer is complete, the platform descends into the container depositing a non-reticulated polymeric film which will be photopolymerized allowing the generation of the next layer.

Table 1.1 summarizes the above-mentioned process categories providing the typical commercial names for each one.

Table 1.1. Additive Manufacturing categories: classification according to ISO/ASTM 52900 and the related commercial names.

AM process categories	**Typical commercial names**
Binder Jetting	Powder Bed and inkjet Head, Plaster-based 3D Printing
Direct Energy Deposition	Laser Metal Deposition, Direct Metal Deposition, Direct Laser Deposition, Laser Engineered Net Shaping, Electron-Beam Freeform Fabrication, Weld-based Additive Manufacturing
Material Extrusion	Fused Deposition Modelling, Fused Filament Fabrication
Material Jetting	Multi-Jet Modelling
Powder Bed Fusion	Electron Beam Melting, Electron Beam Additive Manufacturing, Selective Laser Sintering, Selective Heat Sintering, Direct Metal Laser Sintering, Selective Laser Melting, Laser Beam Melting
Sheet Lamination	Laminated Object Manufacturing, Ultrasonic Consolidation
Vat Photopolymerization	Stereolithography, Digital Light Processing, Solid Group Curing, Projection Stereolithography

Table 1.2 reports a summarized comparison among the main AM technologies, focusing on some aspects related to the process: usable material, printing resolution, pros and cons.

Table 1.2. Comparison among AM technologies (adapted from [22]).

AM Technique	**Material**	**Resolution**	**Pros**	**Cons**
Binder Jetting	Sand Metal powder	50–400 μm	Low cost, fast, color printing, no support structure needed, large objects	Low strength, requires post-processing, powders pose a respiratory hazard
Directed Energy Deposition	Metal Nylon	250–500 μm	Fast, composite materials, can patch defects on existing objects	Expensive, slow, low resolution, requires post-process machining
Material Extrusion	Hydrogels Thermoplastics	100–200 μm	Color, low cost, accessible,	Slow, anisotropy, lower resolution, nozzles

(FDM[1])	Ceramics Bioinks		composite materials, open source designs	impart high shear forces on cells
Material Jetting/Inkjet (MJ[2], DOD[3])	Photopolymer Bioinks	20–100 μm	Good resolution and cell viability	Slow, material waste
Powder Bed Fusion (SLS[4], DMLS[5]/SLM[6], EBM[7])	Thermoplastics Metal Powder Ceramics	100–200μm	Strong, fast, no solvents required	Most expensive, medium resolution, post-processing required
Sheet Lamination	Paper Ceramics Metal	~1 mm	Low cost, composite materials, no support structure needed	Slow, lots of material wasted, delamination
Stereolithography (SLA[8], DLP[9])	Photopolymer Bio-resin	1.2–200 μm	High resolution, fast, very good cell viability, nozzle free	Raw material toxicity, limited material selection, possible harm to DNA by UV
Spheroid assembly	Bioink Organoids	100–200 μm	Biologically active models, scaffold free, freeform fabrication	Fragile raw material, requires subsequent spheroid fusion

[1]Fused deposition modelling; [2]Material jetting; [3]Drop-on-demand; [4]Selective laser sintering; [5]Direct metal laser sintering; [6]Selective laser melting; [7]Electron beam melting; [8]Stereolithography; [9]Digital light processing.

1.2.2.Material Extrusion

Material Extrusion (MEX) is one of the most widespread AM techniques. Compared to Vat Photopolymerization, MEX allows extruding thermoplastics without the hazard related to the processing of some toxic resins [21]. MEX technologies use granules, filaments, or liquids as raw

materials. MEX processes involve the material extrusion through a nozzle or orifice. The printer head moves and selectively deposits material in a pattern that produces a part cross-section, layer-by-layer until the solid object is fabricated. **Figure 1.2** represents schematically the MEX systems.

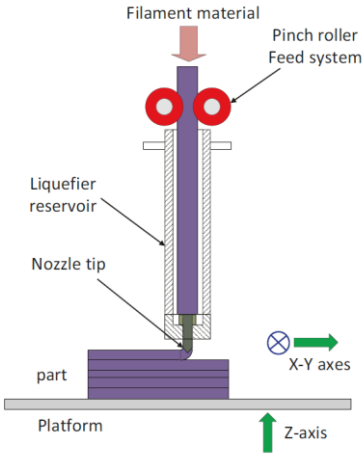

Figure 1.2. Schematic representation of Material Extrusion systems [1].

Fused Deposition Modelling (FDM) allows to fabricate 3D morphologically controlled structures. The technique consists in extruding a molten polymer filament through a nozzle. The moving nozzle deposits polymeric fibres onto a platform, building the physical model layer-by-layer.

The advantage of FDM is the possibility of processing polymeric material integrating a high quantity of particulate reinforcement phase [23]. However, the resolution of manufactured parts by means FDM techniques is its main limitation. The stratification thickness depends on the nozzle diameter. The smaller conventional nozzles have inner diameter of 0.254 mm. Reducing the volume of material which flows through the nozzle can improve the resolution of FDM techniques. Therefore, the manufacturing of submillimeter devices requires nozzles with diameters below 0.05 mm. Monzón et al. [24] present a paper introducing the possibility to realize micro-additive fused deposition (MAFD) by means the design of a nozzle with inner diameter 0.05 mm. They faced two main issues: (i) the reduced diameter affects the extrusion process which shows complications due to high shear stress, pressure drop and swelling; (ii) the cooling of thin filament is rapid and could obstruct the nozzle preventing the correct deposition.

Liquid Deposition Modelling (LDM) allows processing thermosets by using UV photopolymerization or hardening agents to induce curing. As shown by **Figure 1.3**, a viscous liquid is deposited by means a printer head filling the cross sections of the part layer-by-layer. UV light is used to initially cure the layer just deposited. Then, the thermal-curing process is completed into an oven to complete crosslinking.

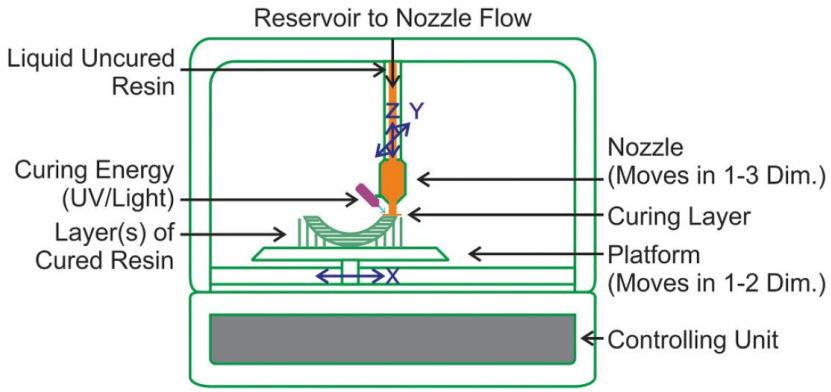

Figure 1.3. Schematic representation of Liquid Deposition Modelling [21].

1.2.3.Powder Bed Fusion

Powder Bed Fusion (PBF) is one of the first AM processes to be commercialized. It is very versatile as it is suitable for polymers and metals, and also for ceramics and composites [1]. PBF is a powder-based printing technique as well as BJT. The powder bed is the base material for the constructs. The powder is molten by applying thermal energy in order to selectively fuse powder particles, achieving a cohesive structure. The process is repeated layer-by-layer until the entire object is finished. Four different fusion mechanisms can be identified: (i) solid-state sintering; (ii) chemically induced sintering; (iii) liquid-phase sintering; (iv) full melting [25–27]. Moreover, PBF techniques can be distinguished by the different used thermal source (i.e., laser or electron beam) [25]. Consequently, considering the above, the following three PBF sub-categories emerged: (i) Selective Laser Sintering (SLS), (ii) Selective Laser Melting (SLM) and Electron Beam Melting (EBM). All PBF processes have a certain number of characteristics in common [28, 29]: fusion between powder particles is

induced through one or more thermal sources; the powder is selectively fused for each layer; the powder layers are added and smoothed by using special mechanisms.

Selective Laser Sintering (SLS) involves the use of an energy source, i.e., a laser, that is focused on a powder bed according to a precise path in order to melt the particles together as shown in **Figure 1.4**. Laser Sintering processes were originally used to fabricate plastic prototypes using a point-wise laser curing technique. This approach was subsequently extended to metal and ceramic powders; additional thermal sources are currently utilized, and variants for layer-wise fusion of powdered materials have been commercially introduced. As a result, a wide range of materials can be processed, including polymers, metals, ceramics, and composites, and used for direct manufacturing of end-use products, as the material properties are comparable to many engineering-grade polymers, metals, and ceramics made using conventional means [1, 30–32]. Regarding polymers, acrylic styrene and polyamide (nylon) are commonly used, also showing almost the same mechanical properties as the injected parts [30, 32, 33]. Polymer-based composites, e.g., fiberglass reinforced polyamide, may be also processed by using SLS, offering the possibility to use metals, e.g., copper, as reinforcement. Regarding the use of metals, a binder, e.g., a polymer binder, is used during the part building and then removed by heating. As reported in [32, 34], parts of alumina with high mechanical strength can be built using polyvinyl alcohol as binder.

SLS fuses thin layer of powder, typically in the range of 0.075-1 mm thick, which is spread across the build platform using a counter-rotating powder levelling roller. Nitrogen gas is used to fill an enclosed chamber where the part building process takes place, in order to minimize degradation and oxidation of the powder material. The temperature of the powder in the build platform is maintained at an elevated value, just below the melting point and glass transition temperature of the employed material. Moreover, the temperature around the part being formed is maintained elevated through infrared (IR) heaters placed above the build platform. IR heaters are used also to preheat the powder before to spread it over the platform aiming at maintaining an elevated and uniform temperature,

minimizing the laser power requirements for fusion and preventing warping of the part during the build.

According to [35], the laser power, laser scan speed and laser scan spacing affect the microstructure, physical and mechanical properties of the parts manufactured by SLS. Such parameters are directly related to the amount of energy on the powder surface of the manufactured part [25].

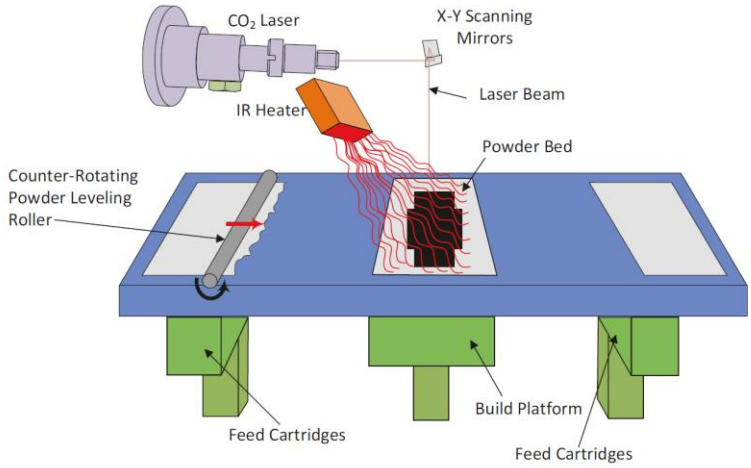

Figure 1.4. Schematic representation of the SLS process [1].

Selective Laser Melting (SLM) process is similar to SLS since it is based on the same concept, even if they differ in technical details. Parts that are realized by means of SLM, are obtained through powder melting instead of powder sintering. The melting of powder material is obtained through high power-density laser. SLM can be used to process several metallic materials, e.g., copper, tungsten, aluminium, and also ceramic and composite materials [36].

Electron Beam Melting (EBM) also referred to as Electron Beam Additive Manufacturing (EBAM) provides several advantages compared to other PBF techniques: the high energy efficiency, the moderate cost, the high speed for scanning operation. However, among the disadvantages it is worth mentioning the low process stability, the part defects and the high variable quality of the finished object [37, 38]. Compared to other PBF technologies, the thermal energy which is used to melt the powder particles come from the electron beam that is powered by a high voltage (30-60 kV). The

process takes place in a high vacuum chamber aiming at avoiding the oxidation problems (it is usually employed for manufacturing of metal parts).

1.2.4.Binder Jetting

Binder Jetting (BJT) technique involves the selective deposition of a liquid binder onto a bed of powder material (**Figure 1.5**). The binding droplets are sprayed over a thin layer of powder material to bind the powder particles. During the process, the printer head moves linearly, according to a precise path, over the powder bed and sprays the binding liquid in the form of droplets by means of multiple nozzles. The powder material (e.g., metal, polymer, ceramic, composite) is stored in a tank and it is deposited onto the building platform layer-by-layer by means of a layering-roller. The sprayed binding droplets bind the particles on a layer, the building platforms lowers by the layer thickness and the fresh layer is laid on the previous one. This process continues until the part is completed. The unbound powder can be re-used for new print.

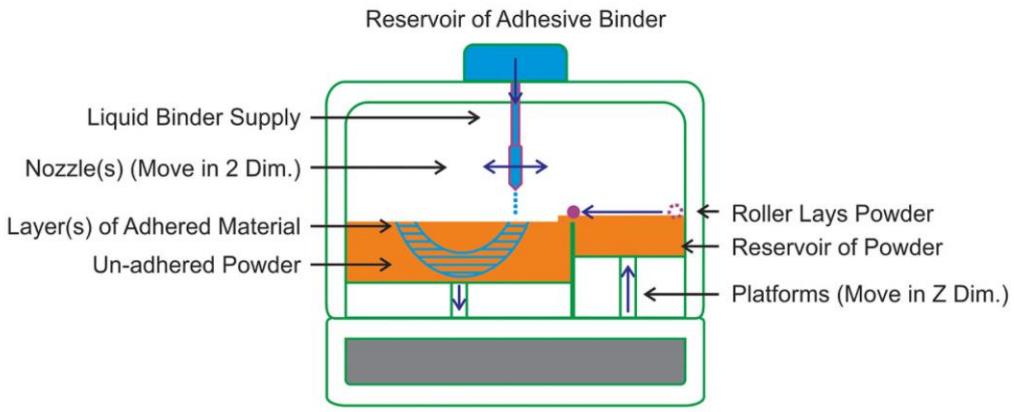

Figure 1.5. Schematic representation of Binder Jetting technology [21].

1.2.5.Directed Energy Deposition

Directed Energy Deposition (DED) techniques involves the simultaneous material deposition and melting. More in details, the power source, usually a laser beam, heats a narrow region and melts the substrate, and at the same time, further material is deposited onto the substrate and, consequently, melted [1, 25]. The raw material can be in wire or powder form. **Figure 1.6** schematically represents

a DED process that uses a laser as heat source and powder as raw material. DED is almost exclusively used to process metals for both research and commercial purposes. Due to DED unique characteristics, it is employed to repair and/or add features to existing parts. DED ability to create large, near-net-shaped freeform structures in a fast and cost-effective way compared to traditional casting and forgings has earned it the industrial interest [1].

Powder feed rate, beam power and traverse speed are the process parameters affecting the features of the manufactured object. The melt pool characteristics and the thermal history are responsible for warping, residual stresses, and surface roughness. Droplet kinematics play a crucial role, affecting the process quality.

The microstructure of parts realized through a DED process are similar to PBF processes, wherein each pass of the heat source (i.e., laser, electron beam) creates a track of rapidly solidified material.

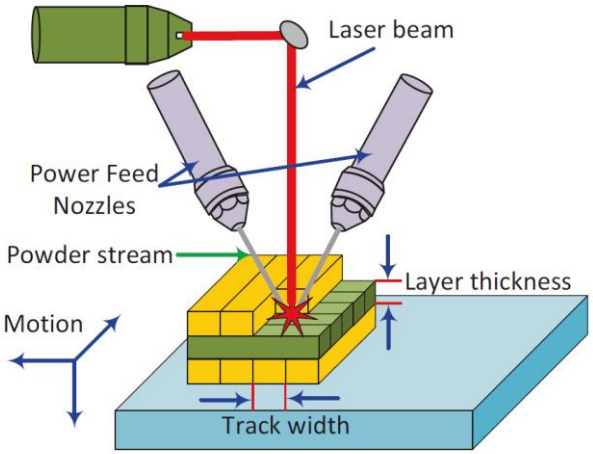

Figure 1.6. Schematic representation of DED process [1].

Laser Engineered Net Shaping (LENS) is one of the first developed and commercialized DED processes. Laser Engineered Net Shaping machines are made of an enclosed inert gas chamber where the materials are processed. The inert gas chamber typically contains argon which is several orders of magnitude cleaner than the inert gas system used in PBF processes. DED machines have historically used CO_2 lasers, which are economical and high-powered heat source. Since the absorption of most materials is much less at CO_2 laser wavelengths than for Nd-YAG or fiber lasers,

new DED machines currently use fiber, diode, or Nd-YAG lasers. However, the machines which use CO_2 lasers, apply a larger amount of laser energy to compensate for their lower absorptivity, resulting in a larger heat affected zone and overall heat input [1]. The residual stresses by uneven heating and cooling processes may represent a drawback playing a significant role in high precision processes like the repair of turbine blades [32, 34, 39].

1.2.6.Material Jetting

Material Jetting (MJT) process, which is schematically shown in **Figure 1.7**, involves the use of a photopolymeric resin which cross-links by means of UV-radiation. The resin is selectively deposited in the form of droplets through a printing head, onto a building platform. The sprayed resin is immediately cured by means of the UV light sources which usually moves together the printing head on the same carriage. Once a layer is completed, the building platform is lowered by the height of the layer, allowing to create the next layer. The process repeats until the part is completed. MJT allows realizing multi-material fabrication, multi-colour printing, and the deposition of dissolvable supports by using multiple printing head on the same carriage [21]. The supports are usually required because the deposited drops, before the cross-linking, cannot maintain their form stability on their own. MJT is a fast process compared to other AM technologies and allows to obtain very accurate 3D objects. However, there are some technical problems related to MJT. Droplet speed and size represent the most important factors since they play a crucial role in the deposition features [25]. Furthermore, the satellite droplets breaking off from the main droplets are characterized by not well defined boundaries [25, 40], along with the droplet splashing on impact, and should be limited in order to improve the quality of the parts fabricated by means of MJT [41]. For this reason, even the process temperature and fluid dynamics have to be properly considered in the optimization of the MJT process.

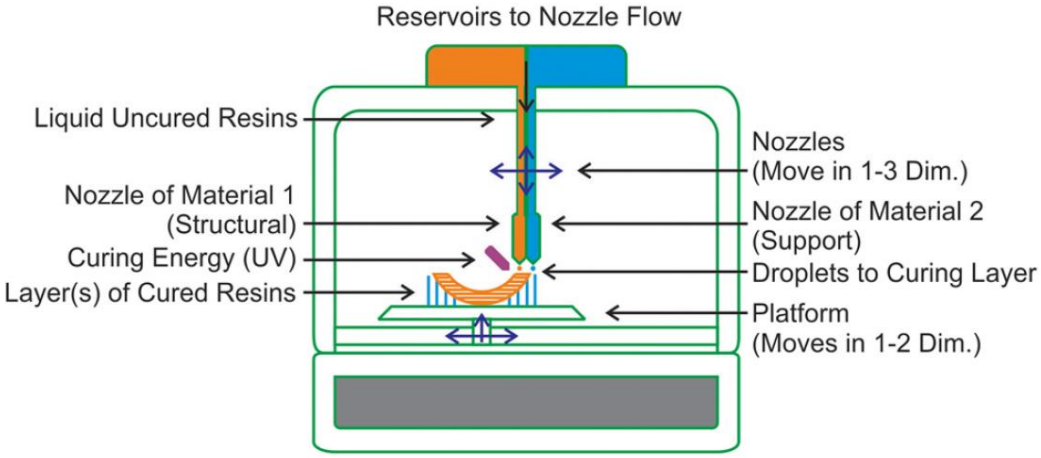

Figure 1.7. Schematic representation of Material Jetting process [21].

1.2.7.Sheet Lamination

Sheet Lamination (SHL) technology is schematically represented in **Figure 1.8**. SHL is also referred to as Laminated Object Manufacturing (LOM). Layers of materials in form of sheets are stacked and bonded together by using a bonding agent in order to realize the 3D object. The raw material is presented in the form of a continuous sheet wrapped around a spool. The sheet is spread over the building platform and then, it is made to adhere by using a heated roller. The bonding between successive layers occurs through several methods: (i) thermal bonding; (ii) gluing bonding; (iii) clamping; (iv) ultrasonic consolidation [25].

The contour of the cross-section in the specific layer is cut by means of a laser cutter or even a knife. The unused material is collected on a special roll for excess material. The building platform lowers layer-by-layer allowing the realization of the 3D object [42].

The sheet thickness and the accuracy of the cutting mechanism are the factors that most affect the precision of the realized part. A sheet layer can be from 0.04 mm and larger, whereas the new layer rolling speed is usually in the range of 13-40 mm/s with heat exposure of 5 – 20 s subject to the material.

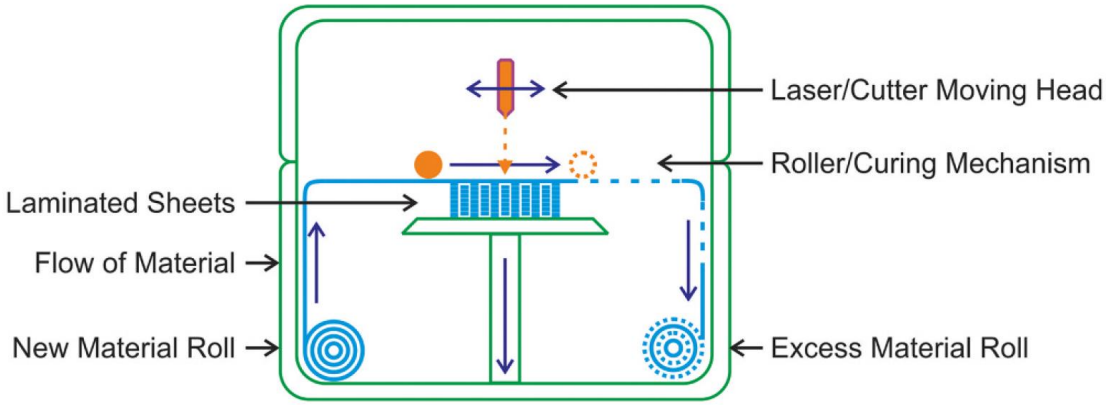

Figure 1.8. Schematic representation of Sheet Lamination process [21].

1.2.8.Bioprinting

Bioprinting is not part of the classification envisaged by the reference standard because it is not considered a specific additive manufacturing technique on its own. Multiple AM technologies used to 3D print living cells are included under the definition of bioprinting. Extrusion-based, inkjet-based, and laser-assisted bioprinting (**Figure 1.9**) allow depositing cells suspended in a bioink through nozzle-based or nozzle-free technique in order to fabricate 3D complex constructs in a top-down approach.

Extrusion-based bioprinting allows processing extremely viscous materials and cells of high density and depositing them to form 3D shapes. The extrusion of a continuous filament of bioink through a micro-nozzle is carried out by means of the applying of a continuous force due to a pneumatic pressure or piston or screw pressure.

Inkjet-based bioprinting uses hydrogel pre-polymer solution with suspended cells as bioink. The bioink is deposited in the form of droplets with high precision to coalesce into fibers onto the top of a substrate at a platform. The process continues in a layer-by-layer manner until the 3D structure is completed. The crosslinking process takes place between the deposition of one layer and the other. By controlling the drop size through a thermal or piezoelectric force it is possible realizing construct with a resolution of sub-100 μm [19, 22, 43].

Laser-assisted bioprinting takes place by transferring cell-suspended droplets in bioinks in the form through a receiving substrate, focusing a laser on the so-called ribbon, i.e., a membrane which is coated with cell-bioink on the side facing the printing surface, whereas on the other side is coated with an energy-absorbing layer (usually titanium or gold). Laser-assisted bioprinting is a nozzle-free techniques that does not create mechanical stress towards the cells during printing. The quality of this process depends on many factors, including the characteristics of the laser [44], the thickness and viscosity of the bioink layer, the air gap between the receiving substrate and the ribbon [45].

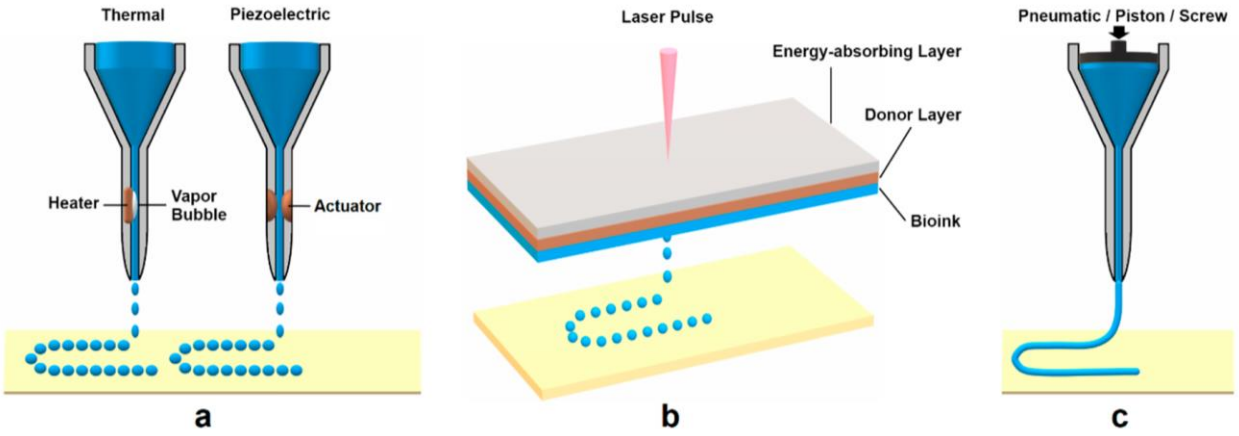

Figure 1.9. Bioprinting techniques: (a) inkjet-based bioprinting; (b) laser-assisted bioprinting; (c) extrusion-based bioprinting [19].

1.3. Biomedical applications of AM technologies

AM techniques allows fabricating geometrically complex object that is extremally difficult or even impossible to produce using conventional manufacturing techniques (e.g., formative, or subtractive techniques) in a fast and cost-effective way. To date, porous implants to repair human skull defects are commonly fabricated through a CAD/CAM approach involving the combination of reverse engineering and additive manufacturing techniques [46, 47].

Bioprinting is a unique and rapidly growing application of AM in the medical field. It allows to seed cells in 3D space according to a defined model, enabling the production of implantable tissue (e.g., skin [48], cartilage [49], bone [50]) and in vitro models for disease modeling or drug screening.

Therefore, it is clear that the role of AM in healthcare [2] became crucial in several applications, including pharmaceutical [31], therapeutic delivery [52–54], implant design [55], surgical planning [56] and tissue engineering [57–59]. AM application in the medical field, commonly employed materials, and the main steps of CAD/CAM approach for fabrication of 3D biomedical models and devices are described in the following.

1.3.1.Tissue Engineering

Tissue Engineering (TE) aims to replace and repair damaged or non-functional tissues by using engineered implants that combine biocompatible materials, live cells and growth factors to aid the normal tissue healing.

AM technologies gained great interest in the context of TE, since their suitability in realizing complex morphology with a high degree of automation, good accuracy and reproducibility [60]. The possibility of easily process polymeric materials, which are well suited to being used in biomedical field due to their customizable physico-chemical features, played a key role in the spreading of AM technologies in this context. AM enabled the realization of micro and nano-structured biodegradable construct suitable for several applications in the context of TE and regenerative medicine. 3D printed implants mimicking the microscopic network of connective tissue [61], porous implant aiming to promote bone regeneration [62], complex bioprinted 3D organoids [63] are some of the most common application of AM in TE scenario.

1.3.2.Bioprinting Tissues and Organs

Bioprinting is a special typology of AM technology that directly deposits biological material as bio-ink (e.g., living cells) through a computer-guided pipette, in a layer-by-layer fashion to fabricate artificial living tissues [3]. Bioprinting is currently used to realize organs and tissues which are suitable for transplantation. More specifically, this technology is employed for generation and transplantation of heart tissue, cartilaginous structures, bone, tracheal splints, vascular graft, multi-layered skin and so on [48, 64–66]. Moreover, it is possible to realize artificial tissue organoids mimicking real organs on a miniature scale in order to use them for medical research.

Bioprinting is mainly distinguished into the following three distinct types: laser-based bioprinting, jetting-based bioprinting, and extrusion-based bioprinting. **Table 1.3** compares the different AM technologies for bioprinting.

Table 1.3. Comparison of three category of bioprinting on distinct parameters [67].

	Laser-based bioprinting	**Jetting-based bioprinting**	**Extrusion-based bioprinting**
Biomaterial	Hydrogels and nano-hydroxyapatite	Alginate, fibrin and hydrogels	Alginate, collagen, fibrin and hyaluronic acid
Resolution	50 μm	50-300 μm	100 μm to 1mm
Cell viability	>85%	>85%	40-80%
Printing speed	200-1600 mm/s	1-10,000 droplet/s	10-50 μm/s
Material viscosity (mPa/s)	1-300	3-12 (low)	30-6 x 102 (high)
Manufacturing time	Long	Medium	Short
Applications	Skin	Skin, cartilage and vascular	Trachea and cardiac valve

1.3.3.Surgical Tools

Combining the recent advancements in the image acquisition with AM technologies it is possible to create CAD models of patient anatomy. This enables the designing and manufacturing of customized, patient-specific surgical tools [68]. Therefore, even though most surgical instruments are designed to work with the majority of patients, the ability to manufacture custom instruments allows for a more controlled and simplified operative experience, decreasing the risk of complications.

AM processes are increasingly used to develop various dental surgical instruments, orthopedic tools, and surgical guides. Benefitting from such a technological approach, clinicians can develop guides that strictly follow the patient's anatomy, while accurately positioning surgical instruments as well [3]. These additively manufactured devices can be used to operate in complex areas, ensuring full safety to patients [69].

1.3.4.Anatomical Models for Surgical Preparation

AM technologies are used to realize patient specific implant organ models aiming to help surgeons in planning and training. The first step concerns the acquisition of bi-dimensional images from magnetic resonance imaging (MRI) or computed tomography (CT) scans; then, converting them into 3D data, it is possible to fabricate models that precisely replicate the anatomical parts to be subject to surgery. This allows surgeons to practice before performing complicated surgeries. 3D printed anatomical models are widely employed in the neurological, maxillofacial, and orthopedic fields for planning treatments, assisting diagnosis, and fabricating customized prosthetic devices [5, 6].

1.3.5.Custom made implants and Prosthetics

AM technologies are also considered for the development of prosthetic limbs. The additive approach allows realizing customized prosthetic devices that perfectly fit the wearer in a short time, and with the same functionality as the devices produced by conventional techniques. AM solve many orthopedics problems and is usually employed to manufacture maxillofacial, cranial and mandible implants [3, 70].

1.3.6.Clinical applications in dentistry and orthopedics

AM technologies are widely used for dentistry application. Indeed, additive manufacturing is suitable both for maxillofacial and oral surgery, as well as for endodontics and orthodontics.

AM techniques provide high-quality restorations and comfort to dentists, since additively manufactured dental restorations are more robust in production compared to the restorations made by dental technicians through conventional techniques. Several AM techniques, e.g., FDM, SLS, LOM, are used in the context of dentistry, to fabricate dental pieces, bridges, crowns, etc. [71, 72]. **Table 1.4** reports the most common dental applications and the most used materials in this context.

3D printed models are also used in dentistry to plan and simulate oral surgery before starting the patient surgery [73]. Indeed, the adoption of a customized approach for the management of each patient is necessary due to the anatomical variability in dentistry. Moreover, in the last decades, the advancements in scanning and imaging technologies allowed to rebuild accurately in 3D

environments the patients' oral cavities. Therefore, combining the image acquisition techniques with AM technologies, it is possible to design, manufacture and employ custom oral prostheses and implants reducing waiting times and costs. For example, CAD models of patients oral cavities are commonly used to simulate the final teeth alignment, and to manufacture a corresponding mold for custom silicon prostheses [22]. A common approach in the maxillofacial surgery uses AM technologies to fabricate 3D custom biocompatible and osteoconductive implants to accurately repair, or substitute bone defects promoting the bone tissue regeneration. These implants are commonly called scaffolds and are described more in detail in Section 0.

The orthopedics is the first medical sector to use AM technologies to fabricate patient-specific models and implants for the management of injuries and the restoring of alignment, structural integrity and motion [3, 22, 74]. Similar to dentistry, also in the orthopedics field, CAD models of patient anatomy are generated through radiological imaging and then used to design and fabricate custom-fit implants by means of AM. Scaffolds aiming to integrate or even regenerate the own bone of the patient are used also in orthopedics. These additively manufactured orthopedic implants create tissue support and promote bone regeneration thanks their tunable features (e.g., implants porosity makes the implant penetrable to tissue and vessel ingrowth [75]).

Table 1.4. AM applications in dentistry and mostly used materials [73].

Dental applications	**Material**
Cost effective models of simple anatomical parts	ABS[1], PLA[2], PC[3], PEEK[4]
Orthodontic devices, surgical guides, bridges	Ceramic filled resins
Copings and bridges, metal crowns	Alumide powder, polyurethane rubber
Maxillofacial implants, drilling and cutting guides	photopolymers
Cell-laden scaffolds	Photopolymer resins, alginate

[1]Acrylonitrile butadiene styrene; [2]Polylactic acid; [3]Polycarbonates; [4]Polyether-ether-ketone.

1.4. Biocompatible materials for AM

In the context of biofabrication [76], referring to materials for AM, it is possible to distinguish between *bioinks*, i.e., cell-seeded material, and *biomaterial ink*, i.e., mostly used to print cell-free scaffold which can be directly implanted or subsequently seeded with cells [77].

Metals are also used to fabricate implants for a variety of applications, including orthopedic, dental and craniofacial.

1.4.1.Bioinks

Hydrogels are commonly used as bioinks as they have good biocompatibility, customizable properties and are very suitable for 3D cell cultures [78–82]. Hydrogels are suitable for extrusion-based bioprinting because of their non-Newtonian, shear thinning behavior. Despite this, they show some limitations when processed by AM. Low viscosities before crosslinking, for example, result in poor shape fidelity after extrusion and limited capacity to form large structures without collapsing [83, 84]. Alginate, agarose, collagen, cellulose, gelatin, gellan gum, fibrin and hyaluronic acid are biopolymer used for bioprinting. One of the most widely used hydrogels is alginate, a negatively charged polysaccharide used primarily for tissue engineering purposes. The crosslinking of alginate is obtained by adding divalent cations an thence it can be functionalized by adding arginine-glycine-aspartate (RGD) in order to promote the cells adhesion to the ECM [85]. Moreover, it is commonly blended with other biopolymers or reinforced with ceramics [86] in order to change its printing and biological features.

Another natural biopolymer frequently used in cell culture is gelatin. It is degradable, biocompatible and is also inexpensive compared to other bioink. Gelatine is usually modified to face its intrinsically limitations, including too slow gelation speed to guarantee shape fidelity. For example, methacrylate (GelMA) is used to promote UV crosslinking [87] and then used for several purposes [88–91].

Collagen, gelatin and fibrin are natural components of ECM and therefore are commonly used to rebuild artificially ECM for cell culture scaffolds, even if the majority of chemical and biological signals from natural ECM are missing. Moreover, ECM is decellularized (dECM) to produce a

functional bioink even if the decellularization reduces the mechanical characteristics and therefore blending with synthetic or natural materials is necessary [92, 93].

1.4.2.Biomaterial inks

Biomaterial inks are thermoplastic polymers, e.g., biodegradable such as polycaprolactone (PCL) or non-degradable such as polypropylene (PP), inorganic materials such as cements, ceramics and metals mostly in powder form. The main requirements for being classified as biomaterial inks are the ability to be processed by additive manufacturing technology and not contain cells. Cells may be subsequently seeded. Resins commonly processed through SLA for scaffold fabrication are also classifiable as biomaterial inks.

Synthetic hydrogels have non-Newtonian properties and are printable by extrusion-based AM processes like biopolymeric hydrogels, but on the other hand they are generally not suitable for direct cell seeding. A commonly used synthetic hydrogels is Pluronic which is used as support material for structures with protrusions and as sacrificial core to produce hollow structures [94–99]. Elastomers are interesting materials due to their mechanical properties mimicking the viscoelasticity of native tissues.

Thermoplastics are the most used material in the context of AM, with a huge range of applications in several fields. Thermoplastics are suitable to be processed by several AM techniques. PCL, PLA, polyvinyl alcohol (PVA) are used to additively manufacture medical devices for direct implantation in vivo or for supporting of cell-seeded hydrogels. They allow producing engineered devices with high resolution, good shape fidelity and controlled porosity.

Ceramics materials consist of a mixture of inorganic salts, including calcium and phosphate, mainly adopted for dentistry and orthopedic applications, or for bone tissue regeneration due to their osteoconductive characteristics. The main feature of ceramics is brittleness which makes handling and implantation difficult. Indeed, they are often mixed with a polymeric binder for extrusion or powder-based additive manufacturing. Tricalcium phosphate (TCP), hydroxyapatite (HAp), biphasic calcium phosphate (BCP), poly (methyl methacrylate) (PMMA) and bio glass are commonly

processed ceramics. In [100], 3D printed scaffolds are manufactured by using functionalized PCL: the powdered ceramic and polymer are mixed, melted and rendered in filament form to be extruded. The natural ceramics used to functionalize PCL is derived from decellularized bone matrix and Bio-Oss, i.e., a bone mineral derive from bovine, outperforming synthetic ceramics osteoinductive capability.

Metal implants for medical purposes are usually manufactured through traditional methods such as casting, forging and machining. AM enables the production of metallic patient-specific implants from reconstructed 3D imaging data. SLM allows producing high quality devices, with intricate lattice structures from metallic powders, aiming at overcoming the problems of surrounding stress shielding which arise in hip implants.

1.5. The designing of patient-specific devices

In the medical context, the ability to personalize and rapidly vary the design of a product without a substantial cost increase is an important opportunity.

Figure 1.10 summarizes the approach to the design of patient-specific medical devices showing the main involved steps.

The first step consists in the acquisition of data regarding the geometry of certain human body parts. The acquisition of data concerning the external geometrical characteristics of the human body, rather than the features of internal organs, is needed when designing ergonomic products or prosthetic and orthotic devices [2]. For these purposes, image acquisition techniques such as 3D scanning are usually adopted. The three-dimensional shape of objects can also be obtained by adopting the photogrammetry technique consisting in the acquisition of a series of photos which are then processed through dedicated software [101].

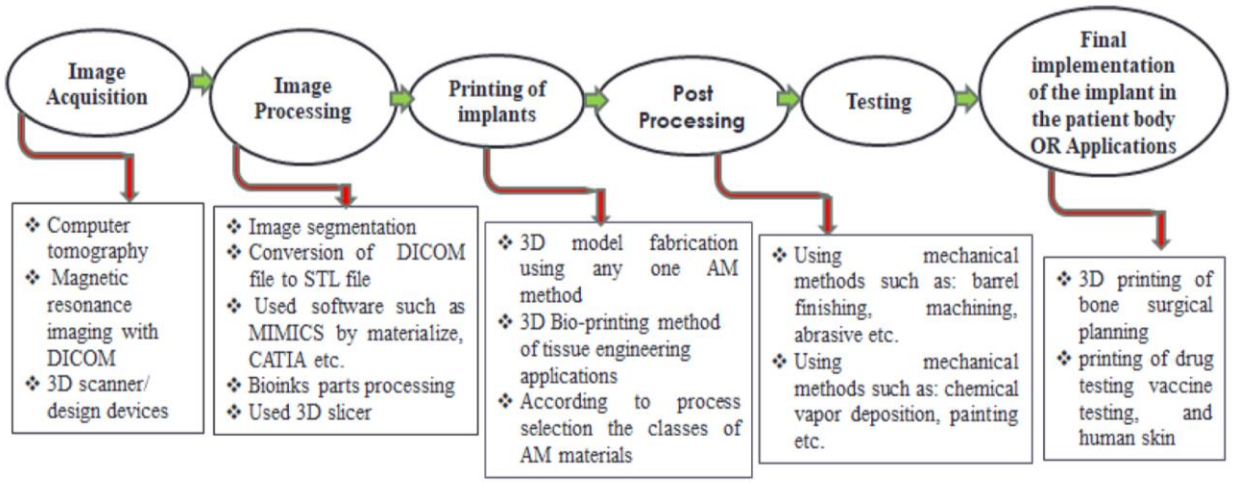

Figure 1.10. The main steps involved in the fabrication of additively manufactured medical devices [3].

The acquisition and reconstruction of the shape of internal body organs are required in order to design patient-specific medical devices such us implants, surgical guides or instruments. For these reasons, medical imaging modalities, i.e., magnetic resonance imaging (MRI) and computed tomography (CT), are usually employed in this context. Both MRI and CT produce a stack of 2D images, usually in DICOM (Digital Imaging and COmmunications in Medicine) format, that include all parts of the body within the field of view. By using dedicated medical image processing software an following a number of image processing steps, it is possible to create a 3D computer model of a specific body part from the acquired 2D stack of images. Materialise Mimics, Simpleware and Amira are examples of commercial software, whereas ImageJ and DeVide are non-commercial software developed for these and similar purposes.

Image segmentation is the first step of the process aiming to separate the body part of interest from other tissues or organs. Then, the 3D model is obtained by connecting and interpolating the detected contours of the desired body part. Hence, the resulting 3D model is usually represented into STereoLithography (STL) format and exported to CAD (Computer-aided design) software to be modified, adjusted, and perfected.

An exception to the above-described process occurs when a patient has lost a large part of his tissue or organs in a traumatic event and therefore the native geometry is unavailable. The same problem

occurs when a tumour has disfigured the original anatomical shape of the organ. In these cases, usually, the contralateral side is used to estimate the anatomy of the traumatized or disfigured organ. On the other hand, *statistical shape models* (SSM) are used when the trauma is too large and no information concerning the native anatomy is available [102, 103].

Once information related to internal or external body part are acquired, designers can customize the design through a parametric [104–106] or partially non-parametric approach. After the analysis and optimization, parts are prepared to be additively manufactured: part orientation, slicing scheme and process variable optimization are the main three steps to follow at this point. A post-processing step may be required before the component can be implanted.

1.6. Conclusions

Additive Manufacturing is a rapidly growing technology in the medical sector. AM technologies offer unprecedented levels of freedom in the designing of medical devices and biomaterials.

It plays a crucial role in the biomedical field and provides a great number of advantages, including cost savings, customization, optimization of product design, shorter supply chain, ability to develop devices with complex geometry, for a wide range of applications. Additively manufactured implants, surgical instruments and custom-made devices can have a major effect in terms of time required for surgery and patient recovery time. Moreover, AM represents a great opportunity to aid pharmaceutical and medical companies generating more specific drugs and changing the way surgeons and technicians plan patient-specific procedures.

Bioprinting tissues and organoids, surgical instruments, custom-made prosthetics, anatomical models for surgical preparation, scaffolds for tissue regeneration are the major core applications of AM in the medical field. To date, craniofacial implants, knee, hip and spinal implants, prosthetic dentistry and several medical instruments are manufactured by additively manufacturing the most suitable materials according to the application. AM techniques allowed to apply biologically inspired principles in the design of medical devices and biomaterials.

Although AM offer many advantages compared to traditional manufacturing, it still presents some limitations. Some applications such as organ printing, for example, still further research efforts. Even from an economic viewpoint, the convenience of using AM is not taken for granted. Indeed, only when the clinical benefits for patients and clinicians is clear the use of AM techniques could be justified. This is partly due to the costs related to the manufacturing that are relatively low in relations to the total cost. Most of the cost for patient-specific AM medical devices is associated with the designing process and not with the manufacturing.

Other issues are related to image acquisition. High-resolution images need long scanning times resulting in increased costs and more ionizing radiations for patients.

Therefore, the main limitations to be addressed in the context of AM for medical purposes are the following:

- The choice of materials is limited: the materials employed for biomanufacturing must be always biocompatible and sometimes also biodegradable. Currently, there are a limited number of materials that could meet these requirements, particularly given the stringent biocompatibility requirements of internally implantable medical devices.
- Additively manufactured devices usually have low mechanical properties: the medical devices to be implanted require reasonable mechanical feature, e.g., compress and tensile strength.
- The dimensional accuracy is low: the shrinkage of the components during the cooling usually results in a low dimensional accuracy which prevents the component fitting.
- AM is not suitable for mass production: AM becomes expensive and not enough fast in the context of mass production.

Some approaches that address these limitations involve combining AM with traditional subtractive and formative process. The combination of AM with conventional manufacturing techniques aims to obtain better control of the tolerances and surface finish in a shorter production time. Additively manufactured negatives could be used as models for formative processes in a huge number of biomedical applications. The combination of technologies could allow leveraging the strength points

of AM, such as free-form manufacturing, and conventional techniques, such as the availability of a wide range of materials.

In conclusion, the employing of AM technologies in the medical context has opened up an enormous number of possibilities in the most disparate applications. However, many issues and challenges are still open and require to be addressed.

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Chapter 2. The designing and manufacturing of scaffolds for hard tissue regeneration

2.1. Introduction

TE uses *scaffolds* as temporary three-dimensional frameworks to provide structural support for cell growth, proliferation and adhesion during the regenerative process [1]. Scaffolds provide sites for the anchorage of stem cells, that in the right microenvironment, can reorganize and form the extracellular matrix (ECM), providing structural support for the newly formed tissue. An ideal scaffold should be able to preserve cells and growth factors, offering adequate mechanical support on a side and allowing tissue vascularization and diffusion of cell nutrients and oxygen on the other. Some basic requirements that a scaffold should possess, can be identified: (i) the interconnected porous structure is crucial to allow the mass transfer of metabolites and to provide the required space for remodelling of the new tissue; (ii) the biodegradability should have a controllable kinetics so that the scaffold acts as a support until the neotissue (i.e, cells plus organized ECM without vascularization) is formed [2]; (iii) the biocompatibility of the material is fundamental since it has to enhance the initial cell attachment as well as avoid the host immune response; (iv) the mechanical properties should be similar to those of the tissue to be repaired, (v) the geometry of the scaffold should allow to substitute the anatomic defect or damage.

However, each tissue requires a specific scaffold design with defined characteristics. The design of scaffolds for bone tissue repairing, for example, starts with the analysis of bone biology.

2.1.1.Scaffolds for bone tissue engineering: basic concepts

Bone tissue is a complex and hierarchical structure which consists of an inner cancellous bone and an outer cortical bone. Its mechanical properties are different depending on its location and function. Bone is a non-uniform porous structure composed of several trace elements (e.g., carbonate, manganese, potassium) [3–5]. This heterogeneous tissues can be considered as a composite consisting

of inorganic nanoparticles of hydroxyapatite (HA), collagen – which constitutes over 90% of the organic phase - and water [6]. Non-collagenous proteins join together constituting the nanostructured extracellular matrix (ECM) which is responsible for several cell (i.e., osteoblast, bone lining cells, osteocytes, and osteoclast) adhesion, proliferation and differentiation [7–9].

Bone tissue shows an innate capacity for regeneration after injuries [10, 11]. Despite this, larger and more complicated defects can impede complete healing. Hence, specific treatments are needed before the tissue can regenerate repairing the defeat. In this scenario, polymeric 3D scaffolds are largely used to promote bone regeneration overcoming the drawbacks related to the conventional bone graft [12]. These devices act as mechanical supports, providing the structural stability which is required for bone healing process.

Porous scaffolds are designed to mimic the structure of the bone replicating similar Young's modulus, compression strength and biocompatibility. Bone ingrowth, indeed, strongly depends on size and shape of pores and on the randomness of the distribution of pores. Pore size determines the available space for new tissue to proliferate and whereas the pore shape can affect the permeability rate and consequently the bone ingrowth [13, 14]. The porosity also affects the mechanical properties and the scaffold capability to respond to stresses. The ideal situation involves a scaffold that has mechanical properties very close to those of real tissue in order to reduce the "stress shielding" that weakens the bone around the implantation region. Young's modulus and compression strength for cortical bone are in the ranges of 7-20 GPa and 100-250 MPa respectively [15–17], whereas for trabecular bone, the values are respectively in the ranges of 2-5 GPa and 11-24 GPa [18–21]. Scientific literature reports that a negative correlation occurs between elastic modulus and compression strength [22]. However, by controlling the porosity of the scaffold, it is possible to manage the relationships between these two parameters [23]. Therefore, scaffold features have to be modulated to address several needs as per the application scenario.

Manufacturing technologies play a crucial role in the definition of scaffold features [24]. Conventional techniques such as solvent casting combined with particulate leaching, freeze drying,

gas foaming, melt moulding, fibre bonding, phase separation techniques, electrospinning are largely used. Although these methods allow manufacturing small pore and are suitable for large-scale production, they do not allow controlling macro shape, micropore, internal architecture and curved channels.

AM enables the control of pore shape, the pore size, the porosity rate, and the whole geometry by computer design. The porosity can be controlled punctually; complex geometries with gradient pore distribution (similar to bone tissue) can be manufactured in an efficient and economic manner [25]; the mechanical and chemical properties are customizable choosing among a wide range of biocompatible materials (solid-, liquid-, powder-based material) which can be easily processed by means of AM technologies

2.1.2.Additively manufactured scaffolds: composition and most used AM techniques

The most of 3D scaffolds that are used for bone tissue regeneration are polymers, bioactive ceramics, and composites. These can be injectable or rigid depending on their composition or intended use [26]. Metallic scaffolds are also used. However, these may cause bone resorption and fracture due to the great mismatch among the mechanical characteristics of the implant and the bone.

Natural polymers, e.g., fibrin, chitosan, collagen, hyaluronic acid, are biocompatible and osteoconductive but have a degradation rate that is difficult to control. Collagen 1 is widely used in bone tissue engineering. However, collagen has low mechanical properties and thus it is usually reinforced with hydroxyapatite (HA) particles to obtain higher robust material [27].

Synthetic polymers exhibit a controllable degradation rate, mechanical integrity, and customizable features. Compared to natural polymers, synthetic ones have worst bioactive properties. Poly lactic-co-glycolic acid (PLGA) and polycaprolactone (PCL) triggered interest in clinical practices due to the low toxicity of degradation product. Poly(ε-caprolactone) is an aliphatic polyester and represents one of the most used synthetic polymers, due to its biodegradation rate, processability, high chemical and thermal stability [28–31]. Polypropylene fumarate (PPF), polyanhydride, polylactic acid (PLA), poly(glycolic acid) (PGA) and polyether ether ketone (PEEK) are common synthetic polymers.

Hydrogels are an important class of polymers for bone tissue engineering. They consist of hydrophilic polymer networks that allow cells to adhere, grow and differentiate. Both synthetic and natural hydrogels can mimic bone ECM topography. However, the application of this kind of material, in the context of hard tissue regeneration, is problematic due to the low stiffness and low capacity in bearing relevant loads.

Bioactive ceramics (e.g., calcium silicate, coralline, hydroxyapatite (HA)) exhibit high compressive strength, low ductility, and high resistance to deformation; at the same time, ceramics are characterized by brittleness [32]. Ceramic materials are usually used as reinforcement in the production of polymer matrix composite scaffolds for bone tissue engineering. This allows to obtain biomimetic constructs (bone is also a composite made of organic collagen fibers and inorganic crystals of hydroxyapatite) and improve both biological and mechanical properties of the scaffold [33]. Indeed, neither natural nor synthetic polymers display high load-bearing strength compared to composite scaffolds.

Hence, composites scaffolds are mainly obtained by combining polymers with highly durable materials to improve the load-bearing strength of the constructs. Inorganic inclusions such as ceramic particles, carbon nanotubes, alloy particles, magnesium metallic are largely used in the literature [26, 28]. Depending on the size of the inorganic reinforcing fillers, nanocomposites can be distinguished from conventional composites. As far as regards bone tissue engineering, it is proved that the employing of nanofillers better replicate the natural bone structure, enhance the mechanical properties and, moreover, induce a more efficient cell response [34, 35].

It follows from the above that the polymers are the most used and promising materials in the context of bone tissue engineering. Therefore, **Table 2.1** is presented to summarize the benefits of polymeric 3D scaffolds for bone tissue engineering. It lists four main categories of additively manufactured scaffolds: (i) natural polymer-based scaffolds, (ii) synthetic polymer-based scaffolds, (iii) natural polymer-based composite scaffolds and (iv) synthetic polymer-based composite scaffolds.

Table 2.1. Polymeric 3D printed scaffolds for bone tissue engineering (adapted from [5]).

Polymeric scaffold category	Biopolymer	Benefits	References
Natural polymer-based scaffolds	Collagen	80% cell viability, with highly porous microarchitectural properties.	[36]
	Collagen-fibrochondrocytes	High mechanical resilience and fiber-structural constructs.	[37]
	PEI coated alginate	Increasing cellular proliferation.	[38]
Synthetic polymer-based scaffolds	PCL	Significant bone repair and regeneration.	[39]
	PLGA	Good biocompatibility and osteoconductive properties in a rabbit model; Osteoconductive properties with comparable human osteoblast proliferation.	[40]
	PLA	Cellular targeting properties for bone repair	[41]
Synthetic polymer-based composite scaffolds	HA nanoparticles into PPF	High compressive strength with microporous constructs.	[42]
	PCL-PPF-HA	Substantial bone regeneration and mechanical durability	[43]
	PCL and β-tricalcium phosphate (β-TCP)	Potential for customization coupled with load-bearing implant.	[44]
	PCL-calcium phosphate	Cytocompatibility with suitable mechanical features for bone tissue repair (in vitro).	[45]
	HA, TCP, Bio-Oss (BO) and/or	Bioactivity and cellular adhesion of PCL scaffold	[46]

	decellularized bone matrix (DCB)		
	PPF with calcium phosphate coating	Bone substitute material for segmental bone defects	[47]
Natural polymer-based composite scaffolds	Gelatin/α-TCP/SF (silk-fibroin)	Significant mechanical properties, cellular proliferation and differentiation.	[48]
	Chitosan-based hydrogel	Greater osteoblast cell proliferation, improved biodegradation and mechanical integrity.	[49]
	α-TCP/collagen	Good cellular proliferation and excellent delivery system for bone tissue regeneration	[50]
	Collagen with ABS impregnated	Improved mechanical properties	[51]

In this context, scaffold manufacturing is usually performed by means of a Computer Aided Design and Computer Aided Manufacturing (CAD/CAM) approach; this consists of four main steps: (i) the patient's medical images are acquired by means of non-invasive techniques, i.e., Magnetic Resonance Imaging (MRI) and Computerized Tomography (CT), and the geometry of the defect is transformed into digital data; (ii) the 3D custom scaffold is designed by means of CAD tools on the basis of the previously acquired and processed medical images; (iii) digital data are converted into a format (typically a G-CODE) that is readable by an AM machine; (iv) datasets are transformed into solid objects by means of a machine using a specific AM technique.

Most used AM techniques for scaffolding are SLA, FDM and SLS. Stereolithography allows to realize scaffolds through the photopolymerization of a photo-sensitive resin layer-by layer. The manufacturing process ends washing off the uncured resin and post-curing the scaffold under UV light. In [52] SLA is used to realize an osteoconductive nanocrystalline HA material. The work shows

good results in terms of adhesion, proliferation and osteochondral differentiation of cell bone marrow-derived mesenchymal stem cells (MSCs). Micro-stereolithography (micro-SLA), Two-Photon Polymerization (TPP) and Digital Light Processing (DLP) are evolvement of SLA technology, which enable more efficient and higher speed building generation. Micro-SLA uses a single photon beam, smaller diameter of laser spot and a submicron resolution of movements. In [83], PFF scaffolds fabricated by means of micro-SLA exhibit mechanical properties close to those of human trabecular bone. TPP ignites the solidification of the photo-curable resins thanks to the absorption of two radiations photons enabling nanometric resolution. It involves the use of a femtosecond laser which emits IR. In [53] TPP is used to fabricate a biodegradable PLA scaffold. This star-shaped scaffold supported *in vitro* osteogenic differentiation and *in vivo* bone tissue formation. DLP processes a whole layer at time is cured by using dynamic masks. A digital light projector is used instead of lasers. The process allows filling a large amount of ceramic particles into photo-curable resins. The major drawback of this technique is related to the cytotoxicity of the resins, and therefore materials with better in vivo biocompatibility (i.e., vinyl esters resins) are explored in other researches [54].

FDM allows controlling porosity of 3D scaffolds by modifying the material deposition amount, the fiber diameter, the spacing among the fiber, and the layer height. No toxic solvents are required, and the results is scaffold with high porosity and good mechanical properties. This technique shows flexibility in material processing. PCL-HA o PCL-TCP composites scaffolds are realized by means of FDM for bone tissue regeneration.

SLS is used to realize scaffolds starting from powder material. A laser sinters powder, binding materials to obtain a solid architecture. SLS allows realizing metallic and ceramic scaffolds. Also powder of polymeric materials are studied and tested to ascertain their laser sinterability. Thus, 3D scaffolds made of PCL and a combination of PEEK and HA are realized for bone tissue engineering. These are characterized by a porous interior structure and an anatomically shaped external architecture and therefore are suitable when mechanical strength and high fracture toughness are

required. The necessity of removing trapped powder in the post processing phase on a side, and the required high operating temperature, are the main disadvantages related to SLS technologies.

The concept of creating 3D scaffold through an additive approach, even incorporating living cells during the manufacturing process raised significant attention in recent years. 3D Printing (3DP) and Bioprinting (also named 3D plotting or Direct-Writing) are facing remarkable developments.

The 3DP belongs to Binder Jetting AM technologies. It consists in the ink-jet print of a liquid binder onto a powder bed at room temperature. 3DP is used to directly fabricate the actual scaffolds, or to print molds. Pharmaceutical and biological agents, e.g., peptides, proteins, polysaccharides, DNA plasmids and cells, can be incorporated into powder materials and processed due to the manufacturing process takes place at room temperature. This can improve the process of bone tissue formation.

Bioprinting gained interest since it allows accurately controlling cell distribution by dispensing together small units of cells and biomaterials with micrometer precision. Tissue-like structures are additively manufactured in a scalable and cost-effective way. Hydrogels are currently the most processed materials by means of bioprinting technologies. Jetting-, extrusion-, laser-based printing are the most used bioprinting techniques.

Jetting-based bioprinting allows realizing 2D or 3D structures by spraying picolitre bio-ink droplets onto a substrate. Piezoelectric ink-jetting, electro-hydrodynamic and acoustic wave jetting are some examples of material jetting techniques. Low costs, high printing speed (the printer heads support parallel work mode) and relatively high cell viability (80/90%) are the main advantages related to these techniques. However, there are also disadvantages to take into account: the print head nozzle frequently clogs due to variations in viscosity as the temperature changes; heating causes uneven particle arrangements.

Extrusion-based bioprinting systems is used to fabricate 2D or 3D scaffolds by dispensing hydrogels mixed with cellular material (i.e., bio-ink) in the form of continuous filaments. The bioink is extruded through a micronozzle by employing pneumatic pressure and following a defined path. Then the material solidifies by means of chemical or physical processes. The three-dimensional structures are

realized by assembling 3D patterns layer-by-layer. Extrusion-based bioprinting allows high viscosity bio-inks to be processed through the micronozzle. Furthermore, the devices that are realized by means this technique exhibits high cell viability (greater than 90%). The main drawbacks are the shear stress on cellular materials and the restricted resolution obtainable.

Laser-based bioprinting employs the energy of pulsed laser to transfer cell-suspended materials from a "ribbon" (glass typically covered with a layer of gold or titanium) to a receiving substrate. A pulsed laser beam is focused on the "ribbon" causing the evaporation of the liquid biological material composed of hydrogels and cells. Consequently, the evaporated material can reach the receiving substrate in droplet form. This procedure does not require nozzle, and therefore the problems of nozzle clogging with cells or materials is solved. Moreover, biomaterial with a high range of viscosities (1-300 mPa/s) can be processed by means of this technique [4, 55].

2.1.3.Additively manufactured magnetic scaffolds

A recent approach in TE involves the use of magnetic nanoparticles (MNPs) for the manufacturing of magnetic responsive scaffolds. In the medical context, MNPs exhibit interesting physical properties and provide appealing possibilities because of their size, ranging from a few nanometres up to tens of nanometres. These dimensions are comparable to several biological entities, e.g., protein, gene, cell, or virus. MNPs below 30 nm in size, present superparamagnetic behaviour, exhibiting the ability to be magnetized by applying a magnetic field without remanence once the field is turned off [56–58]. The use of magnetic nanoparticles as reinforcement of polymeric matrix scaffolds, combined with an external magnetic field appears to be a viable strategy specially to promote bone tissue regeneration. Magnetic scaffolds could address the problem of mimicking endogenous growth factors production by achieving controlled delivery and release, rather than growth factors seeding prior to implantation. These bone graft substitutes could be magnetically switched on/off enabling its usage for delivering biomolecules *in vivo* and for the stimulation of adhesion, proliferation, and differentiation of cells [59].

Zhao et al. [60] distributed magnetic nanoparticles in macro-porous ferrogel scaffolds embedded in iron oxide nanoparticles aiming to optimize porous structure of the scaffolds for cell delivery. In [61] the authors realized magnetic poly (ε-caprolactone)/iron-doped hydroxyapatite nanocomposites for both repairing damaged tissues and hyperthermia treatment. The authors' key idea was to utilize a poly(ε-caprolactone) (PCL) matrix reinforced with iron-doped Hydroxyapatite (FeHA) nanoparticles to realize a fully biodegradable structure with high mechanical properties. In this way it is possible to realize a porous structure, manufactured using rapid prototyping technique, which do not require to be removed surgically but is able to biodegrade once the treatment is done. Panseri et al. [62] proposed hydroxyapatite/collagen magnetic scaffolds for orthopedic tissue engineering with the aim of attracting growth factors and cells attached to other MNPs. Magnetic scaffolds can be seen as static stations that can be reloaded again and again after implantation, every time the healing process requires this action, providing the unique possibility of customizing the scaffold activity to the needs of the healing tissue. Such a strategy aims to mimic the real endogenous growth factors production of the human body in order to enhance the tissue regeneration and angiogenesis processes.

In this context, superparamagnetic material allows to realize magnetic scaffolds capable of reaching magnetization values up to 15 emu g^{-1} at 10 kOe for the adhesion of ferrofluids or MNPs when an external magnetic field is applied [57], and then capable of being magnetically turned off by simply by removing the applied magnetic field.

Benefiting from AM technologies and from MNPs features it is possible to design and manufacture magnetic polymer-based nanocomposite scaffolds. De Santis R. et al. [63] shown the possibility to embed superparamagnetic PVP-coated Fe_3O_4 nanoparticles in a PCL matrix in order to realize a magnetic scaffold and provide a programmed bio-factor release by means of an external magnetic field. However, the long-term effects of the iron-oxide-based phases such as maghemite or magnetite in the human body are still unclear [64, 65]. Therefore, the research efforts focused on the necessity of have non-toxic MNPs for this kind of applications. A bioresorbable and biocompatible superparamagnetic-like phase is realized by Tampieri et al. [56] through doping hydroxyapatite with

Fe^{3+}/Fe^{2+} ions (FeHA), minimizing the formation of magnetite as secondary phase. FeHA nanoparticles in a PCL matrix is already used by Gloria et al. [61] to realize nanocomposite substrates which were evaluated in magnetic and biological performances.

Relating to the use of magnetic devices in the medical context, it is also reported that the presence of a moderate external static magnetic field can have positive effects on osteoblast cells, wound healing and pain release even without the presence of magnetic nanoparticles [66–68].

2.2. Scaffold Design for AM

AM technology enable the precise fabrication of geometry-based constructs at the level of cellular design, e.g., body-centered-cubic/octahedron (BCC/OC), Diamond/face-centered-cubic (FCC), rhombic dodecahedron (RD) and so on. Parametric design, such as Voronoi and Triply Periodic Minimal Surface (TPMS) can also be achieved by means of AM technology. Scaffolds that are based on these structures presents high randomization, functional internal connectivity, and excellent mechanical characteristics. Gradient porosity is also realizable in order to mimic the whole structure of natural bone and its excellent mechanical property distribution.

In the following, the approaches to realize porous scaffolds are classified into cellular design, including non-parametric and parametric design, and whole design, including uniform, gradient and topological optimization-based design.

2.2.1.Cellular Design

The basis of the porous construct is defined unit cell. Non-parametric design involves geometric and structural design. Parametric design involves the generation of cellular structures according to specific algorithms. The various designs have different performance.

Non-parametric design is based both on 3D structures and plane structures. The 3D structure-based design uses BCC, Diamond/FCC and other polyhedron structures as unit cells. The most common plane structural based design is honeycomb.

The *body-centered-cubic/octahedron* is frequently used due to the relatively ease of design and manufacturing due to the proper inclination of all struts which minimizes the warping effect during

the AM process, including the SLM process. The original geometry of BCC/OC is represented in **Figure 2.1a**, and it is obtained by connecting the center of a hexahedron with eight vertices. The Young's modulus of the orthopedic scaffolds which are designed using BCC/OC geometry, could be reduced by 75-80% [69]. Furthermore, these scaffolds have highly predictable size effects and, therefore, the mechanical features corresponding to porosity can be accurately deduced. Concerning the mass transport properties, when any fluid passes through the strut cross, its speed slows down, and generally, the low flow rate is beneficial for cell proliferation. Hence, as showed by [70] the BCC/OC scaffolds have advantages in bone ingrowth. Despite this, these BCC/OC based scaffolds have not satisfactory compressive properties. Some variants of the classic BCC/OC geometry are proposed in literature, including the pillar BCC (see **Figure 2.1d**) with the eight vertices connected to obtain a reinforced structure [69, 71], the BCCz unit (see **Figure 2.1b**) with four reinforcements in the z-axis [72, 73], the BCC unit modified by adding vertical stiffeners through the center of the BCC/OC unit (see **Figure 2.1c**) [74]. All these variants exhibit better compressive properties and Young's modulus than the original BCC/OC unit. However, the addition of stiffeners has an impact on the anisotropy and on the fatigue life of the scaffolds. The combination of FCC and BCC, the face and body-centered cube cell with vertical struts (FBCC/FBCCz), is another variant which allows to reach higher stiffness than the original BCC/OC [74]. Therefore, on one hand, BCC and its reinforced design have excellent mechanical features and easy to manufacture characteristics, on the other, they have insufficient internal surface area and relatively low anisotropy.

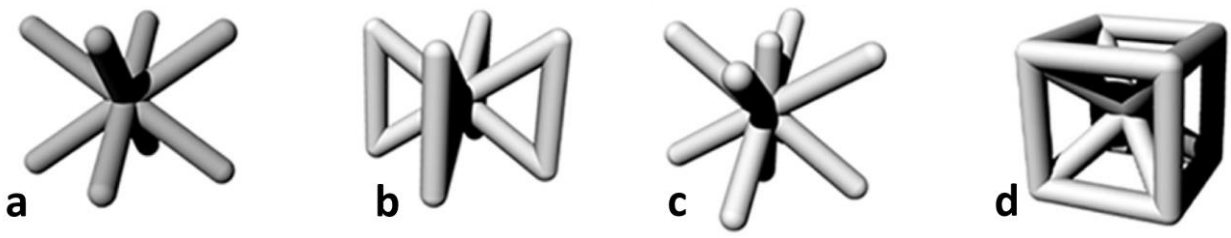

Figure 2.1. The BCC unit and its variants: (a) the original BCC unit; (b) the BCCz unit; (c) the reinforced BCC unit by adding vertical stiffeners through the center of the unit; (d) the pillar BCC unit (adapted from [3]) .

The diamond cell is a typical unit cells for AM of orthopedic scaffolds. This cell, as showed in **Figure 2.2**, possesses FCC basic configuration with tetrahedral angles of 109° between each element; it has sixteen equal edges and fourteen vertices. In [75, 76], the authors evaluate the mechanical properties of a Diamond/FCC scaffold made of Ti6Al4V and show that its Young's modulus is similar to natural bone. The Diamond/FCC scaffolds with a low porosity have Young's modulus and compressive strength similar to cortical bone, whereas the scaffold with high porosity presents the same characteristics similar to cancellous bone [77]. Therefore, the mechanical properties of the Diamond/FCC scaffold make it suitable for bone implants. The Diamond/FCC scaffolds made in degradable materials also show excellent mechanical properties. Magnesium (Mg) and iron (Fe) are usually used as reinforcement of biodegradable materials. Li et al. [78] studied the mechanical features of degradable porous magnesium scaffolds, showing that, even after four weeks of biodegradation in vitro, the Diamond/FCC designed structures have the Young's modulus in the range of the cancellous bone (700-800 MPa). Concerning the biological properties, [79] designed Ti6Al4V Diamond/FCC scaffolds with different pore sized in order to evaluate their biocompatibility *in vitro* and osseointegration *in vivo*. [79] shows that the pore size most suitable for bone integration is in the range of 300-400 µm, whereas [80] shows that, for titanium and tantalum (Ti/Ta) scaffolds, the pore diameter of 500 µm allows excellent osteogenesis.

Figure 2.2. The Diamond unit [3].

Taniguchi et al. [81] identified the pore diameter of 600 µm as the most suitable size both for bone ingrowth and mechanical properties of Diamond/FCC titanium scaffolds. These differences in results

are due to the different materials used for manufacturing, but, at the same time it also indicated that the Diamond/FCC structures with reasonable pore diameter are suitable for the adsorption and proliferation of osteoblast. Hence, the Diamond/FCC is a cellular design with excellent mechanical characteristics and good bone growth. Moreover, the Diamond/FCC scaffolds show an almost isotropic behaviour as the mechanical performance are almost the same in different directions, and therefore, they could be applied to the situation of under multidirectional stress in orthopedic applications.

Rhombic Dodecahedron (RD), truncated cube (TC), Octet, and Rhombic Cube Octahedron are other polyhedron structures commonly used in orthopedics. Among these, it is worth describing the RD scaffolds properties: RD is a central symmetric structure (see **Figure 2.3**) which shows the same mechanical properties in the three main directions [82]; the Young's modulus and the compressive strength of the RD are close to the cancellous bone in high porosity [83]; fatigue life is sufficient to be used to realize orthopedic scaffolds. Moreover, RD scaffolds exhibit good biological properties since the RD geometry allows providing the required nutrition and oxygen supply for cells, resulting in an excellent osteogenic microenvironment for the integration of osteoblasts [83].

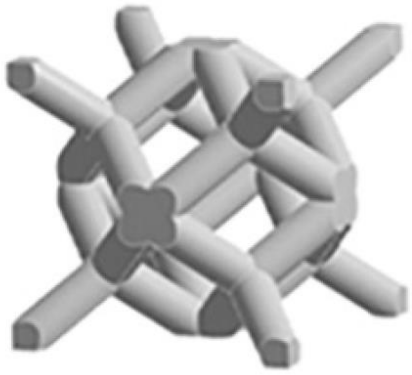

Figure 2.3. The rhombic dodecahedron cell unit [3].

Honeycomb structure has low weight, high porosity, high stiffness, and it is used as for structural and biomedical applications. By modifying the honeycomb porosity, Young's modulus can be adjusted between cortical and cancellous bone [84].

Parametric design consists in generating a porous structure according to algorithms. Voronoi-Tessellation and TPMS are the two main methods to design porous constructs according to algorithms.

The Voronoi structure reproduces closely the microstructure of bone in morphology, generating a mesh structure based on random discrete points which are connected to be a network structure [85]. The Voronoi unit cells are shown in **Figure 2.4**. In literature, Voronoi based scaffolds are built in several manners [86], including reverse creating from tomography (CT) images [87], or using B-spline curves to represent the boundaries of the irregular shaped pores [85]. The Voronoi tessellation method used in [88] allowed to design porous scaffolds by means of computer design software by matching the histomorphometric indices of trabecular bone, i.e., trabecular thickness and separation, trabecular number, bone surface to total surface ratio, bone volume to total volume ratio. Although this method allows obtaining an isotropic porous interconnected model, it shows inevitable problems such as poor repeatability, long cycle time and energy consuming. Sharma et al. [89] manufactured Voronoi based aluminium scaffolds which exhibited a high load to weight ratio which is one of the main parameters for these devices. Gradient porosity, ranging from 60% to 95%, allows reaching excellent mechanical properties for Voronoi-based scaffolds. Moreover, these kinds of scaffolds show remarkable fluid properties, allowing to achieve excellent cell adhesion, migration and bone ingrowth. Concluding, the main advantage of Voronoi design is the similarity with cancellous bone in terms of bionics, mechanical features and bone ingrowth.

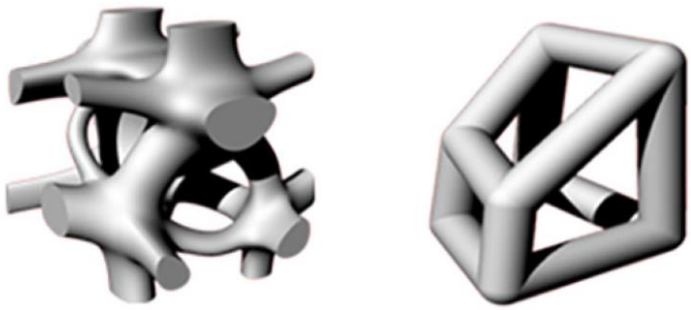

Figure 2.4. The Voronoi unit cells [3].

Structures with Triply Periodic Minimal Surface (TPMS), i.e., skeletal TPMS and sheet TPMS, are made by repeating elements with the minimum possible area. They are infinite smooth surfaces, in which space is divided into two disjoint sub volumes without self-intersection; furthermore, they are periodic in three independent surfaces [3]. Theoretically TPMS is an excellent structure for cell proliferation [90]. Several variants of TPMS structures are proposed in literature, including Diamond, Primitive, Gyroid skeletal TPMS which have the most flexible design space [91, 92]. The TPMS is in general a very good choice for orthopedic scaffolds. The variants of TPMS exhibit properties close to natural bone in terms of Young's modulus, compressive strength, porosity rate and permeability. It is possible to distinguish between stretching TPMS structures that have excellent mechanical properties and bending structures that have better performance in permeability.

2.2.2.The whole Design

The whole design section aims to focus on the whole design of the porous structure instead of the unit cells, discussing the effect of porosity on biology and mechanical properties from a macroscopic point of view. The proposed whole design definition includes four categories: uniform design, layered gradient design, continuous gradient design and design based on topological optimization.

Uniform design allows obtaining the same porosity for the entire scaffold. Therefore, there are two main situations: high porosity scaffold mimicking cancellous bone, and low porosity scaffold replicating cortical bone. The management of the porosity rate is crucial to obtain the desired mechanical and biological scaffold properties. In [93], Wielding et al. manufactured a porous Ti6Al4V scaffold with porosity in the range of 60-80%, which shows a Young's modulus of 6-8 GPa. Both elastic modulus and porosity are remarkably similar to cancellous bone. Torres-Sanchez et al. [23] replicate cortical bone through titanium scaffolds with porosity value of 27-37%. Regarding the bone ingrowth, over 60% porosity and pore size larger than 300 μm promote bone formation [79, 94, 95] probably due to the high permeability. Nevertheless, living tissue are not homogenous structures and, therefore, uniform design of scaffolds may lead to sub-optimal results.

Layered gradient design comes from the idea of mimicking the layered structure of bone [96]. This approach to scaffold designing consists in realizing a scaffold with low to high porosity in different layers. Various methods can be used to design the layered gradient structures. Shi et al. [97] design and fabricate a three layer gradient scaffold by changing struct diameter and achieving a porosity rate from 68.5% to 88.2% and an elastic modulus of 12-18 GPa. In general, the layered structure is the best structure to simulate bone. Both mechanical and biological characteristics of layered gradient scaffolds are better than the scaffold with a uniform porous structure. However, the stress transition and the connection between the layers represent a problem to solve.

Continuous gradient design represents an approach that aims to solve the above-mentioned issues related to layer gradient design. Continuous gradient structures can be generated by progressively modifying the strut diameter of a BCC unit cell [98]. Small pores in the core of the structure and large pore in the outer surface allows on the one hand to increase mechanical strength and on the other hand to increase cell penetration and proliferation. The continuous gradient structure provides a way for the efficient transfer of nutrients, exhibiting excellent osteogenic performance. Therefore, in general, continuous gradient scaffolds show mechanical properties changing continuously with the gradient, an elastic modulus within the range of bone Young's modulus, a compressive strength higher than the bone. Bone ingrowth also appears to benefit from continuous gradient design.

Design based on Topological Optimization (TO) aims to optimize the material distribution within the design space according to a given set of loads, boundary conditions and constraints in order to maximize the performance of the scaffolds. In the medical field, the most common approach for Topological Optimization is the continuum approach, which is a micromechanics theory-based method that considers the design space as an artificial composite material with a huge number of periodically distributed small cavities. The finally optimized model presents small hole regions which are filled and areas with large apertures which are considered empty [99, 100]. Hollister et al. [101] presented an image-based approach to design and fabricate patient-specific craniofacial scaffolds directly from MRI or CR data. In their work, Hollister at al. use voxel density distribution to describe

scaffold topology. By combining Topological Optimization with Finite Element Analysis (FEA) it is possible to identify high-stress regions and properly distribute material to maximize the stiffness for a given material volume fraction [102]. TPMS can also be used as a Topological Optimization main unit [103]. Thus, concluding, the TO approaches in orthopedics are able to make the scaffold porosity and the stress distribution more acceptable to prevent the stress shielding effect.

2.3. Conclusion

In the context of bone tissue engineering, scaffolds are used to substitute or repair damaged tissue with the aim of improving cell viability, adhesion, proliferation, osteogenic differentiation, vascularization, and load bearing. AM technologies enabled the possibility to manufacture complex TE scaffolds with several compositions and internal architecture, allowing to tune both biological and mechanical properties.

AM offers the possibility of achieving good reproducibility and control over the microstructure and shape of the scaffolds, and therefore, it enables the development of customized advanced tissue engineered constructs that meet specific requirements in terms of geometry, pore size, pore interconnectivity, anatomical size and shape. Furthermore, AM techniques allow for high automation of the manufacturing process compared to the traditional scaffold fabrication techniques.

Due to the versatility of AM technologies a wide range of materials can be processed. To date, scientific community is still working on ideal materials for developing 3D constructs that mimic bone (trabecular, cortical, cancellous bone) characteristic. Synthetic-based composite scaffolds are very similar to natural bone. The use of certain particles reinforcement in polymeric matrices allows obtaining unique features in the scaffolds. Fully biodegradable scaffolds made of polymeric matrices and MNPs reinforcement are presented in literature. The use of magnetic forces as a means of achieving controlled assembly of tissue regeneration appears as a promising approach. The literature shows that magnetic scaffolds have a great potential to meet several clinical requirements for bone tissue regeneration.

The design of the porous structure is crucial as it affects both mechanical and biological features. The main approaches, starting from the description of the single cell units to the whole design, are presented above.

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Chapter 3. Design of 3D additively manufactured scaffolds with integrated functionalities for bone tissue engineering

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