Review Article

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Additive manufacturing of PLA-based scaffolds intended for bone regeneration and strategies to improve their biological properties

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Abstract: Polylactic acid (PLA) is one of the most commonly used materials in the biomedical sector because of its processability, mechanical properties and biocompatibility. Among the different techniques that are feasible to process this biomaterial, additive manufacturing (AM) has gained attention recently, as it provides the possibility of tuning the design of the structures. This flexibility in the design stage allows the customization of the parts in order to optimize their use in the tissue engineering field. In the recent years, the application of PLA for the manufacture of bone scaffolds has been especially relevant, since numerous studies have proven the potential of this biomaterial for bone regeneration. This review contains a description of the specific requirements in the regeneration of bone and how the state of the art have tried to address them with different strategies to develop PLA-based scaffolds by AM techniques and with improved biofunctionality.

Keywords: polylactic acid, bone tissue engineering, composite materials

1 Introduction

1.1 Tissue engineering (TE): issues and strategies

TE aims to replace or restore damaged tissue by using artificial constructs that direct new tissue formation. This field integrates knowledge from biology, materials science, mechanical engineering and clinical sciences, offering new opportunities to treat patients that suffer from diseases and injuries affecting tissues like bone, cartilage, skin, nerves or even blood vessels (1–5). TE strategies commonly involve the combination and manipulation of cells, biodegradable constructs and bioactive molecules to induce the formation of new specific tissue. The final construct should resemble the structure and mechanical characteristics of the tissue to be regenerated in order to maintain the tissue functionality (6,7). One of the most promising approaches to reach the objectives of TE is the use of a scaffolding structure which would support the tissue during its growth (8,9). Scaffolds for TE applications must possess specific characteristics including biocompatibility, suitable mechanical properties, ease of sterilization, high porosity, high surface area and controllable interconnected porosity to enhance cell growth and support vascularization (6,10). Porosity and pore size play a major role on the functionality of 3D scaffolds, as the formation of new tissues depends on the characteristics of the interconnected network of the structure (11–14). Besides, it is imperative to consider the relationship between the mechanical and mass transport properties during its design stage (15). The scaffolding structure may be combined with living cells (16), growth factors (16), bioactive substances (17) or drugs (18) to increase the biological functionality of the implant. Efforts are even been made to design scaffolds that could mimic the functions of the extracellular matrix (ECM) by incorporating bioactive signals on the construct's surface with precise spatial distribution, thus opening the possibility of controlling cell response (19,20). In Figure 1, different research strategies for the use of PLA-based scaffolds for bone regeneration discussed in this work are summarized.

1.2 Biomaterials: interest of biodegradable materials

Scaffolds used in TE are usually made of titanium (21), bioceramic materials (22), natural or synthetic polymers (23)

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and composites (24). All of these are biomaterials, that is, materials that can be integrated in the surrounding tissue without eliciting any undesirable host reaction. While biocompatibility is a mandatory requirement for TE scaffolds, biodegradability of the engineered structure is a characteristic of utmost interest associated with the group of polymeric biomaterials. Complete integration of a degradable polymeric scaffold with new tissue can be achieved as the byproducts of the degradation process are excreted or reabsorbed into the patient's body, without the need of an explant surgical procedure or surgical revision (25,26). Biodegradable scaffolds are a really interesting option in TE applications, as they degrade at a rate that (ideally) matches the growth rate of the tissue to be regenerated. The degradation profile of these scaffolds should ensure sufficient mechanical support while the new tissue is being formed, as well as no immune or inflammatory response of the surrounding tissues due to the release of the scaffold's degradation byproducts (27).

Different materials have been explored to manufacture biodegradable scaffolds, including both synthetic and natural polymers. Among the latter, it is possible to highlight the work related to the use of collagen (28), chitosan (29) or alginate (30). Regarding synthetic polymers, the most promising ones are polycaprolactone (PCL) (31), polylactic acid (PLA) (32,33) and poly(lactide-*co*-glycolide) (PLGA) (34). Synthetic polymers have steady and standard properties than can be modified in a relatively easy way during their industrial production. In contrast, the properties of natural polymers are highly dependent on the source of the material (35). As natural polymers can be found in the ECM, polymers of natural origin have, in general, better biofunctionality than the synthetic ones, which lack in bioactivity, have lower hydrophilicity and, therefore, are not able to promote cell adhesion (36). However, synthetic polymers have higher processability, more controllable degradation rates and better mechanical properties, as they are stiffer than natural polymers. The combination of natural and synthetic polymers is a promising approach to overcome the above-mentioned limitations.

1.3 PLA as base material for scaffold manufacturing: advantages and disadvantages

PLA is a biodegradable thermoplastic aliphatic polyester that has been extensively used in TE applications as base material for scaffolds intended for bone, cartilage,

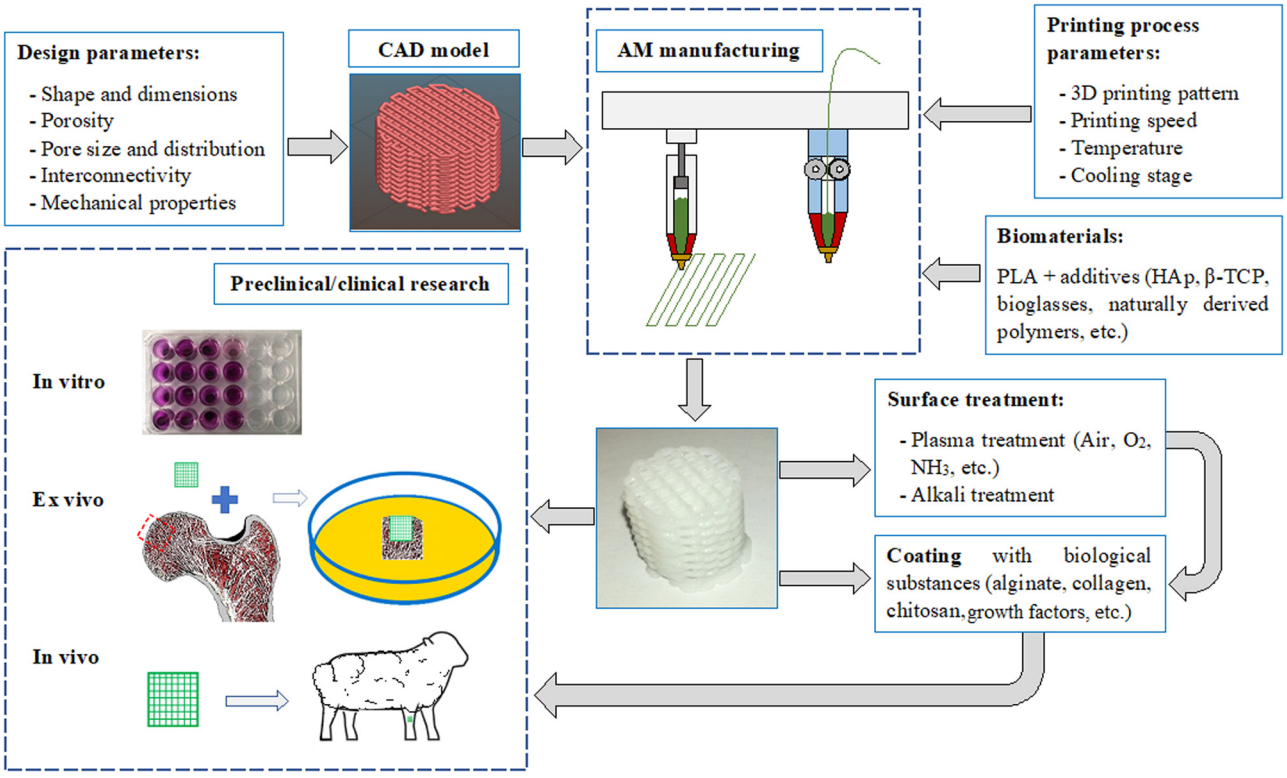

Figure 1: Research strategies for the application of PLA-based scaffolds in the bone tissue engineering field.

tendon, neural or vascular regeneration (26). This is a biomaterial that has been approved by the Food and Drug Administration for direct contact with biological fluids (37) and that can be obtained from renewable resources at relatively low costs. The most generally used methods to produce PLA are direct polycondensation of lactic acid and ring opening polymerization (ROP) of lactide, which is the cyclic dimer of lactic acid (38,39). Lactic acid (2-hydroxycarboxylic acid) is a chiral molecule with two stereoisomers: L-lactic acid and D-lactic acid (39). The direct polycondensation method is especially effective to obtain copolymers resulting from the combination of L-lactic acid with other monomers. Even though this method is a cheaper option than ROP, the process has a major limitation related to the simultaneous production of water and organic solvents, which can lead to the reduction of the molecular weight of the final product. Unlike the previous method, ROP allows to obtain PLA with high molecular weight, using lactides as precursors of the reaction. As lactides are chiral molecules, PLA can come in different stereochemical forms: poly(L-lactic acid) (PLLA), poly(D-lactic acid) (PDLA) and poly(D,L-lactic acid) (PDLLA) (40) (Figure 2). By modifying the initial composition, the properties of the polymer can be adjusted to the specific application (39). Properties of PLA also depend on its processing temperature and molecular weight.

An important property of PLA is the crystallinity ratio, which is influenced by the stereochemistry and thermal history of the polymer. Crystallinity influences many polymers' final characteristics, including mechanical properties, degradation rate, glass transition temperature and melting temperature (41,42). PLA with high crystallinity rate is obtained with a low content of D-enantiomer, while the polymer can be fully amorphous with D-content higher than a 20%. The glass transition temperature (T_g) of PLA is usually in the range from 50°C to 65°C, being the melting temperature (T_m) around 170–180°C. A decrease in the crystallinity rate of the polymer causes a decrease in both temperatures (43). On the other hand, the molecular weight of the polymer has an impact on the mechanical properties of the polymer and its degradation profile. The long time needed for the complete degradation *in vivo* of high molecular weight PLA samples has been pointed out as one of the causes in the appearance of inflammatory reactions in the surrounding tissues (44). For that reason, PLA with low molecular weight is preferred for TE applications because of its higher degradation rate, which in any case should match the rate of tissue growth and provide sufficient mechanical support.

Due to its biocompatibility, biodegradability and suitable mechanical properties, PLA has been extensively used in the biomedical field, including applications like suture, bone fixation material, drug delivery systems and TE (45). PLA has also a good processability by different techniques and, as we have mentioned before, its degradation rate, physical and mechanical properties can be adjusted over a wide range by modifying the molecular weight or the copolymer ratio (6). For example, PDLA is mainly used in drug delivery systems due to its faster degradation rate, while PLLA is the most chosen option for load-bearing applications because of its superior mechanical properties (46). PLLA has a T_g in the range of 60–65°C, a melting temperature of around 175°C and a mechanical strength of 4.8 GPa (26). PLLA also has a higher biological activity, showing more potential for its use in the TE field. On the other hand, because of its high mechanical stability and biocompatibility *in vivo*, PDLLA have recently gained attention as a base material for drug-delivery systems and scaffold manufacturing (46).

Biodegradability is another important characteristic of PLA for its application as base material for scaffold manufacturing, as it can be completely degraded by random hydrolytic chain scission, generating monomers of lactic acid that are eliminated from the patient's body through the tricarboxylic acid cycle (42,47). Two mechanisms have been reported for PLA degradation: surface erosion, in which degradation is located at the polymer–water interface, and bulk erosion, in which a homogeneous degradation is observed over the polymer's surface (48). PLA degradation occurs by uptake of water followed by the hydrolysis of ester bonds (46). This hydrolytic degradation is initiated in the amorphous regions of the material, which ester links are more easily broken by its reaction with water. This first step is followed by the reorganization of the polymeric chains as a consequence of its increased mobility. Then, the degradation process advances towards crystalline regions of the material, initially less exposed to it due to their ordered structure (49). There seems to be also a contribution to the degradation of PLA caused by enzymes from the local environment, but it is yet clear if they act enhancing the degradation rate or simply favoring the removal of degradation byproducts (39).

The influence of the initial degree of crystallinity on the degradation rate of PLA is a controversial matter in the literature, as some authors have concluded that the hydrolysis of amorphous polymers is faster due to the lack of crystalline regions (46), while others defend the hypothesis that a higher crystallinity of PLA increases the degradation rate due to an increase in

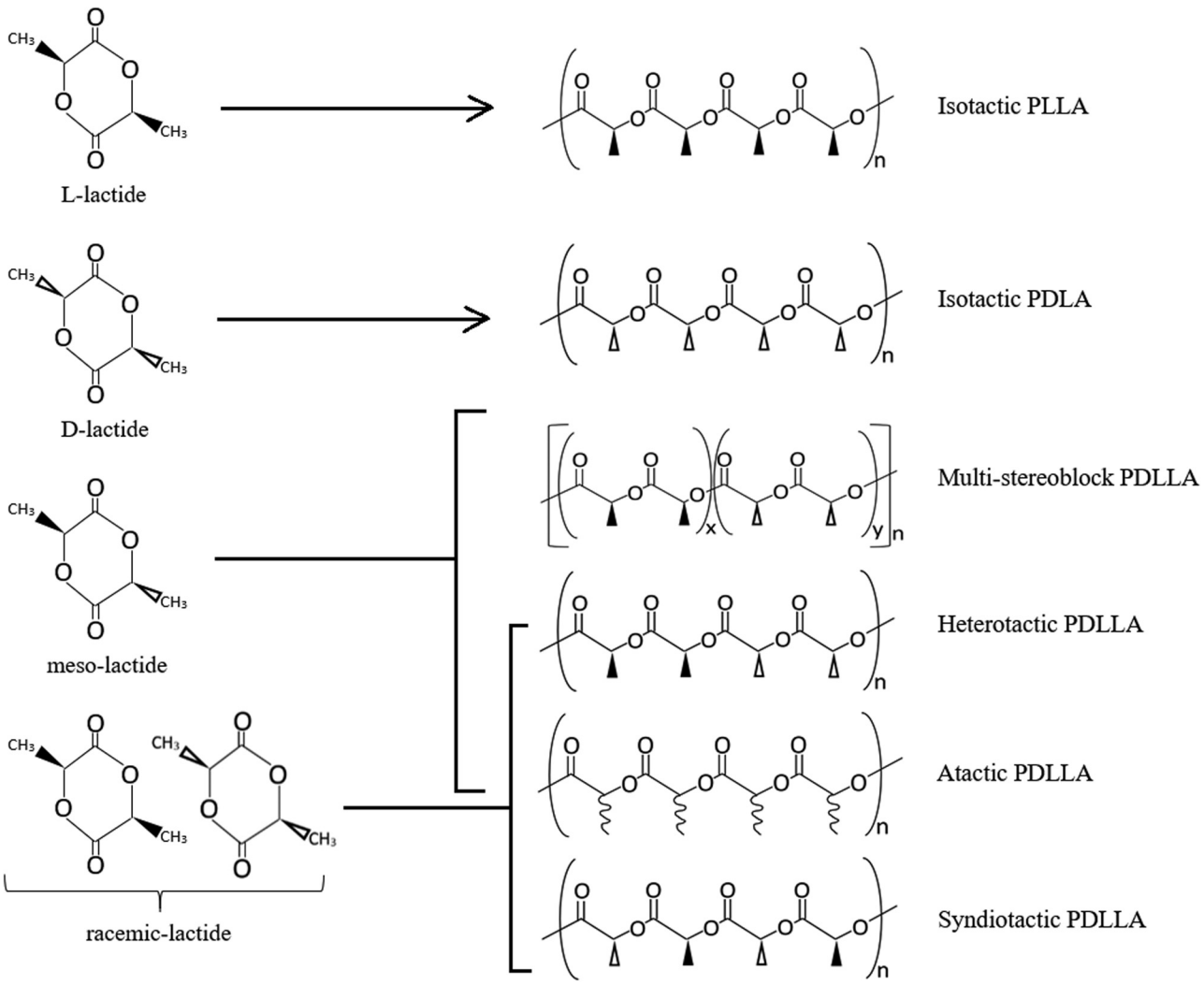

Figure 2: Lactide isomers and stereochemical forms of PLA.

hydrophilicity (50). In the end, the material properties should be adjusted so the PLA-based scaffold can be completely degraded while providing mechanical support and without causing any adverse tissue reaction until being eliminated from the human body through metabolic pathways. The generally slow degradation rate of PLA (sometimes up to several years) (44), the abrupt release of the acidic degradation byproducts or its accumulation because of an inefficient removal from the surroundings of the scaffold's location, could generate a strong inflammatory response, affecting cell growth and tissue regeneration (49). This is actually the major limitation for the application of PLA as base material for TE applications. Several strategies have been proposed in order to counteract the acidic byproducts and stabilize the pH of the surrounding tissues, including the use of low molecular weight PLA (51) and the combination of the polymeric matrix with basic compounds such as bioactive glasses (BG) and calcium phosphates (CaP) (52,53).

Some other important drawbacks of PLA are: (i) its poor toughness, as it is a brittle material with less than a 10% elongation at break (54); (ii) the lack of reactive side-chain groups, which make it difficult to induce surface or bulk modifications to improve its properties (37); and especially (iii) its low hydrophilicity (55), having a water contact angle of around 80°. The latter disadvantage generates poor wetting properties and a lack of cell attachment and interaction between the polymer and the surrounding tissues (56). Taking all these characteristics into account, PLA bioactivity has to be enhanced for its use in the target TE application. In this review, we discuss the published work describing the advances on the development of PLA-based scaffolds for bone tissue

engineering (BTE) obtained through additive manufacturing (AM) techniques. Strategies to improve the functionality of the scaffolds are also discussed.

2 PLA-based scaffolds for bone regeneration

2.1 Characteristics of bone tissue

The increasing ageing and life expectancy of global population is a challenge for the therapeutic alternatives currently available for the treatment of hard tissue affections, like those related to bone tissue loss due to degenerative (57), surgical or traumatic processes. During the last decades, important advances in surgical techniques for skeletal reconstruction have been presented, aiming to relieve patient's pain, improve their quality of life and also reduce social healthcare costs. BTE is the most promising technique to be used for bone regeneration, being an alternative to the conventional bone grafts. The latter strategy for bone healing includes auto-, allo- and xenografts, which application is hindered by some important drawbacks, e.g., donor-site morbidity and pain, difficult graft resorption, lack of osteoinductive properties, immune rejection and risk of pathogen transfer (6,58). BTE could eliminate the aforementioned issues by the implantation of porous scaffolds that mimic the natural bone ECM, being generally combined with osteogenic cells and morphogenic signals to promote cell growth, proliferation and differentiation (58). Hence, a broad knowledge of the characteristics of bone tissue is essential when designing the support structures for its regeneration.

Bone is a composite material with an organic phase representing around a 30% of the weight and an inorganic phase as the remaining 70%. The organic phase is mainly type-I collagen and water, but also contains small amounts of bone-resident cells (around a 2%) (59), glycoproteins, glycosaminoglycans and proteoglycans (60). The inorganic phase is composed of CaP, the majority of those being hydroxyapatite crystals (HAp, $Ca_{10}(PO_4)_6(OH)_2$). The Ca:P ratio in the mineral phase varies between 1.37 and 1.87, due to the heterogeneous presence of additional ions, such as bicarbonates, citrates, magnesium, potassium, strontium, zinc and sodium (2,60). The tensile strength and fracture toughness of the bone are related to the organic phase, while the compressive strength is provided by the inorganic one (60).

Regarding its structure, bone is a highly vascularized complex heterogeneous tissue with hierarchical organization levels. In a macro-scale level, bone has two main structural patterns: cancellous bone and cortical bone (58) (Figure 3). Cancellous or trabecular bone comprises the inner part of the tissue, which has a structure with high porosity (30–90%) (61) and host the majority of bone metabolic activities (59). Given its highly vascularized structure, cancellous bone represents only around a 20% of the bone mass. The remaining 80% corresponds to cortical bone, which in contrast roughly represents a 10% of its volume (2). Cortical bone comprises the dense boundary (less than 10% of porosity) (62) that surrounds and protects the inner more fragile trabecular part. The cortical bone possesses higher elastic modulus and stiffness, mainly due to its higher mineralization content, providing sufficient mechanical support for weight bearing. While the compressive strength of cortical bone is in the range of 130–220 MPa, with Young's modulus ranging from 17 to 20 GPa, these properties are in the range of 5–10 MPa and 50–100 MPa respectively, for cancellous bone (59,60). Cancellous bone is characterized by a higher level of toughness and, because of its porous structure, its great capacity for sudden stress damping (2), but also by a very low tensile strength (59). Micro- and nanostructure levels of bone hierarchical architecture comprise the ECM and its composite structure, in which collagen fibers reinforced by HAp crystals provide the compressive strength and high fracture toughness of bone (58).

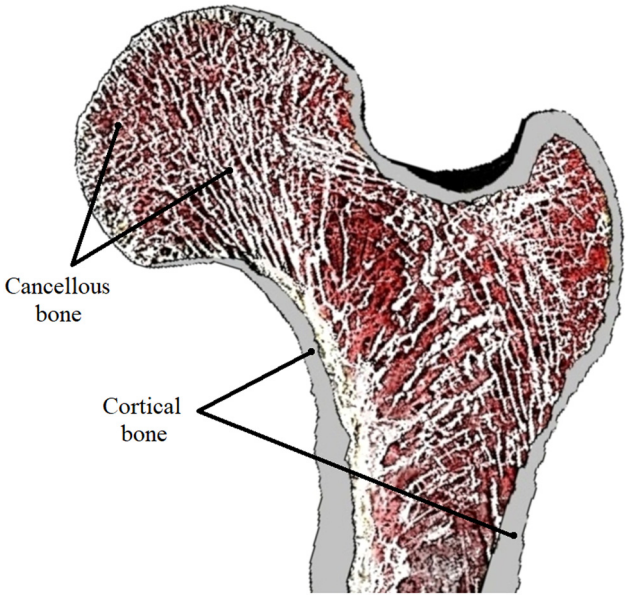

Figure 3: Cortical and trabecular bone structure.

Bone undergoes a continuous process of remodeling controlled by the action of osteocyte, osteoblast and osteoclast cells, which interact with growth factors, hormones and signaling molecules to maintain the bone health (60). Osteoclasts are responsible for bone resorption, while osteoblasts simultaneously carry out the formation of the new tissue (62). A balance between osteoclastic bone resorption and osteoblastic bone formation should exist to get a good remodeling process. The formation of new tissue, also called osteogenesis, can occur by two different pathways: endochondral ossification or intramembranous ossification. Intramembranous bone formation involves the direct differentiation of mesenchymal cells into osteoblasts, while in the endochondral process the mesenchymal cells first differentiate into chondrocytes, which then deposits a cartilaginous layer that is finally mineralized and replaced by new bone tissue (60). Upon micro- or nanofractures, the bone is self-repaired by bone remodeling, following the intramembranous and endochondral processes, without the formation of scar tissue (58,63).

2.2 Bone scaffolds design strategies and criteria

Scaffolds intended to be used for bone regeneration should possess a similar composition, mechanical properties and hierarchical structure of that of natural bone, and more importantly, they must mimic the physiological functions of the ECM. Ideal scaffolds for bone regeneration have the following characteristics (6,59,60):

- Biocompatibility and biofunctionality
- Non-immunogenic and nontoxic
- Biodegradability, with a degradation rate that matches the tissue regeneration growth rate
- Osteoconductivity, having favorable surface characteristics for cells to adhere and proliferate
- Osteoinductivity, which implies the recruitment and stimulation of immature cells to differentiate them into preosteoblast cells to later induce new bone ingrowth
- Osteointegrity, ensuring a strong adhesion between the scaffolds, the new bone tissue and its surroundings
- Be easily manufactured at relatively low cost
- Biomaterials composition similar to that of native bone tissue
- Highly interconnected pore structure, with engineered porosity and pore size to enhance cells proliferation and allow mass transfer (nutrients and metabolic waste)
- Potential to encapsulate biomolecules (growth factors, stem cells, anti-inflammatory agents, etc.)
- Adequate mechanical integrity until complete degradation of the structure.

One of the most important parameters for scaffold design is the pore size of the final structure. For bone regeneration, pore sizes reported in the literature range from 20 to 1,500 μm, with no general consensus about the optimal value to maximize the osteogenic process while ensuring the necessary mechanical support (47,64). Generally, pore sizes in the range of 75–100 μm promote the formation of unmineralized bone tissue, while smaller pores are prone to occlusion and can only be penetrated by fibrous tissue (6,59). Mineralized bone tissue can be formed by using scaffolds with pore sizes larger than 200 μm, but values higher than 300 μm are required in order to ensure vascularization (47,62). Pore sizes in the range of 300–500 μm should be suitable to enhance bone formation and avoid osteochondral ossification (6). Pore interconnectivity is another important geometrical parameter to take into account the design of bone scaffolds, as it directly influences the diffusion of nutrients and the removal of metabolic waste, being at the same time a critical parameter to ensure continuous bone tissue ingrowth (65–67).

Porosity levels higher than 50% are generally needed to allow vascularization, with values even exceeding 90% for some bone scaffolds found in the literature (68). Porosity also affects cell attachment and biodegradation rate, as they depend on the available surface area for the interaction with cells. Therefore, higher values of porosity would positively affect the biofunctionality of the scaffold, but it would affect at the same time its mechanical properties. The mechanical strength of the structure decreases by increasing the porosity, which hinders the use of highly porous scaffolds for its application in the regeneration of load-bearing applications. On the other hand, cell–material interactions for bone tissue ingrowth are not only influenced by the mechanical properties of the scaffold (69) but also by its surface properties, including topography, surface chemistry, hydrophilicity and surface energy and charge (59,70–72). The selection or design of biomaterials for bone scaffold manufacturing should take into account all these properties to ensure a good performance from the mechanical and biofunctional point of view.

2.3 Scaffolds with functional properties intended for bone tissue regeneration

A wide range of biomaterials has been used in the replacement of bone tissue. The most traditional group of materials are metals, especially those based on titanium and its alloys (73). Titanium scaffolds and prostheses provide excellent biocompatibility and mechanical performance, being widely used for bone defects treatment (13,74). However, its non-biodegradability limits the prospects of use of this biomaterial in the field of BTE. Besides, titanium-based implants have a mismatch on the mechanical properties with the surrounding tissue due to their high elastic modulus. This difference causes the appearance of the stress shielding effect (25), which aims to be reduced by using porous structures manufactured by AM techniques such as electron beam melting (75) or selective laser melting (76). In addition, titanium is a bioinert material, being unable to interact with the bone. Different strategies have been proposed to overcome this drawback, as the one presented by Song et al. (74), who applied a surface activation and HAp coating method to improve the bone-material integration of 3D printing titanium scaffolds.

In this sense, bioceramic is the type of material with a higher potential to interact effectively with the host tissue, since HAp represents around 65% of bone mass (77). Although HAp is a widely used biomaterial for bone regeneration because of its great osteoinductive capacity (13), its poor biodegradability is a major limitation that restricts its clinical use. Apart from HAp, calcium carbonate (78), bioglasses (79) and CaP (80) are also promising substances in the TE field. In the latter group, we can find one of the biomaterials that have recently attracted more interest for bone regeneration, which is the β-tricalcium phosphate (β-TCP, $Ca_3(PO_4)_2$). Unlike HAp, β-TCP presents complete bioresorbability, apart from a good processability by 3D printing techniques (81). Regarding bioglasses, they possess excellent bioactivity and bone binding ability, promoting the formation of a HAp-like layer after the scaffold is implanted (82). However, ceramic-based scaffolds exhibit poor mechanical properties (high brittleness), which hinder its application in the field of BTE as requirements for load-bearing bone regeneration cannot be fulfilled.

In contrast to metals and bioceramics, polymers have great design flexibility, being possible to adjust their composition, degradation rate and structure to the specific requirements (13). Besides, in order to mimic the natural bone structure and composition, there is a trend to combine ceramic materials and polymeric materials, using natural or synthetic polymers as base materials for scaffold manufacturing. Some examples of natural polymers used as base materials of bone composite scaffolds include collagen (83), alginate (84), chitosan (85), hyaluronic acid (86) and silk (87). Among the synthetic polymers, PLA (68), PCL (88) and PLGA (89) are the most commonly used as base materials, mainly due to their biodegradability, biocompatibility and good processability. The work of Domingos et al. (90) represents a good example of this type of composite materials. These authors achieved an enhancement of the mechanical properties and *in vitro* biological performance of PCL-based scaffolds by the incorporation of micro- and nano-hydroxyapatite particles. Definitely, synthetic biopolymer-based composites are of special interest as they possess the required strength to match the properties of bone (2). In the next sections, we will focus on the use of PLA as base material of scaffolds intended to regenerate bone tissue.

2.4 Different techniques for manufacturing of PLA-based porous scaffolds

PLA has been extensively investigated in BTE applications due to its biocompatibility, good processability, adequate mechanical properties and tunable degradation rate, among other favorable properties already commented in Section 1.3. A great number of fabrication processes have been successfully used to manufacture 3D scaffolds using PLA as base material. In Table 1, a summary of PLA-based scaffolds reported in the literature for bone tissue regeneration and their manufacturing process is shown. Particle/salt leaching (91–93), solvent casting (94,95), phase separation (96,97), gas foaming (98), freeze-drying (99) and electrospinning (100,101) are some of the most extensively used methods for bone scaffold manufacturing. However, these conventional methods have some limitations that hinder their application for BTE (102,103):

* Poor reproducibility
* It is difficult to control pore shape, size and geometry
* Uncontrolled and sometimes limited interconnectivity
* Cannot allow full control of scaffolds shape and dimensions
* Use of toxic solvents.

AM techniques have gained great attention in the last decade for bone scaffold manufacturing as a strategy

to overcome these limitations. The possibility of customizing porous constructs with a precise architecture to adapt them to the patients' needs made these processes a powerful tool for TE applications. In Table 1, no AM techniques are included, as the application of these manufacturing processes to obtain PLA scaffolds for BTE will be discussed in depth in the next section.

3 Additive manufacturing of PLA-based scaffolds for bone tissue regeneration

AM techniques are based on building geometrically complex structures by a sequential layer-by-layer deposition of material controlled from computer-designed models (124). 3D models can be created by using image data acquired from biomedical imaging techniques or by a computer-aided design (CAD) software, allowing the design of fully customized3D structures (125). The file obtained is then converted to STL (that stands for "STereoLithography" or "Standard Tessellation Language") or another format that can be suitable for the AM machine. Once transferred to the equipment, the mesh data is digitized and divided into 2D layers to obtain the sequence of material deposition. The setup of the manufacturing process could include parameters like temperature, layer thickness, nozzle diameter or power source or material flow. After the layer-by-layer deposition, some AM methods need a postprocessing step for supporting material removal (126).

According to ISO/ASTM 52900:2015 Standard, AM technologies can be classified into the following categories: (1) vat photopolymerization, (2) powder bed fusion, (3) material extrusion, (4) material jetting, (5) binder jetting, (6) sheet lamination and (7) directed energy deposition. With this set of techniques, it is possible to obtain TE constructs with patient-specific

Table 1: Examples of different techniques for PLA-based bone scaffolds manufacturing

Manufacturing process	Composition	Ref.
Salt leaching	PLA/β-TCP nanoparticles	91
Particle leaching	PLLA/β-TCP	92
	PLLA (chitosan-coated)	93
Porogen leaching	PLLA (HAp/collagen coated)	104
Solvent casting/salt leaching	PDLLA/nHAp	94
	PLA/CaP glass	95
	PDLLA (CaP-coated)	105
Solvent casting/particulate leaching	PLA/pennisetum purpureum	106 and 107
Solvent casting/particulate leaching/sol-gel	PLA/HAp/lignocellulose/BG	108
Thermally induced phase separation (TIPS)	PLLA/HAp	96
	PLLA/HAp	97
	PLLA/nHAp	109
	PLLA/β-TCP nanoparticles	110
	PLLA/chitosan/P24 peptide	111
	PDLLA (plasma-treated)	112
TIPS/salt leaching	PLLA/β-TCP nanoparticles	113
TIPS/gelatin leaching/supercritical CO_2 drying	PDLLA	114
Rapid volume expansion phase separation	PLA/β-TCP	115
Gas foaming	PLA/phosphate glass	116
	PLLA/HA and PLLA/β-TCP	98
Freeze-drying	PLA/collagen/nano-HAp	99
	PLA/chitosan/gelatin/nHAp	117
In situ precipitation and freeze-drying	PLA/chitosan/HAp	118
Freeze-drying/porogen leaching	PLLA/collagen/dexamethasone	119
Freeze extraction/porogen leaching	PLLA/PCL/nHAp	120
	PLLA(HAp-coated)	121
Electrospinning	PLA/mesoporous bioglass	100
	PLLA (mineralized)/strontium	101
	PLLA/siloxane-doped vaterite	122
	PLLA (plasma-treated)	123

design that could also be adapted to the surgeons needs for its implantation (103). The great control that they offer over the pore size, pore shape and porosity of the structure allows to tailor the structural, physical and biological properties of the scaffold to mimic native tissue function (127). As mentioned in Section 2.2, porosity plays a key role in cell–cell communication and cell–ECM interaction, nutrients and metabolic waste diffusion and in the mechanical properties of the structures. The possibility of controlling these parameters offers a great advantage compared to other manufacturing techniques commonly used to obtain bone scaffolds, such as the ones listed previously in Table 1.

In this section, PLA-based scaffolds for bone regeneration obtained by AM techniques are highlighted. In particular, we will focus on vat photopolymerization, powder bed fusion and material extrusion methods, as these are the AM technologies that have been extensively employed for polymeric-based scaffold manufacturing (127–129).

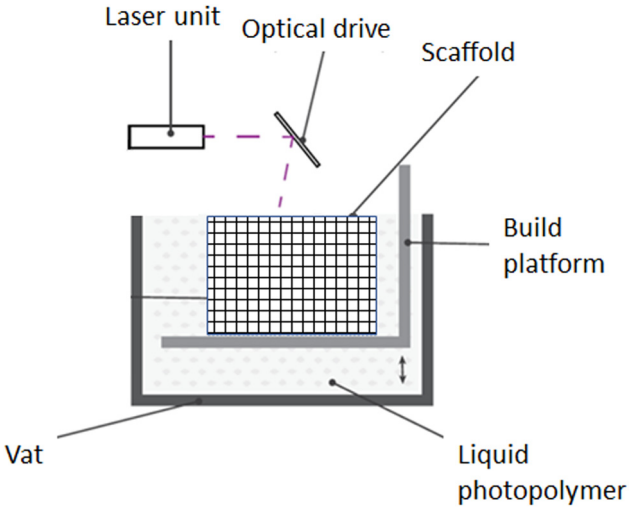

Figure 4: Vat photopolymerization by laser light source (SLA technique).

3.1 Vat photopolymerization

Vat photopolymerization is an AM process where the build of each layer of material is produced by the photocrosslinking of the monomers in the resin, which react to create a solid structure. The radiation needed to cure each layer of material can be applied through two different methods: by a laser-based approach (Figure 4), commonly known as stereolithography (SLA) (130), or by a digital light projection approach, commonly referred to as DLP (Figure 5). The resolution of this technique at commercial level is around 50 µm (130,131), which is below the one obtained, for example, by extrusion-based processes (132), allowing the manufacture of more complex 3D designs. Because of its high resolution and the precision of the geometries that can be obtained, SLA has been one of the earliest 3D printing methods used in BTE (13). However, the main limitation for a wide implementation of this technique for TE applications is the need of biocompatible photocurable materials, which may exhibit inadequate biodegradation rates and biocompatible behavior.

PLA oligomers can be functionalized with methacrylate end groups in order to obtain a photocurable resin (133–135). The methacrylate groups are introduced in the structure normally by previously creating hydroxyl-terminating oligomers, which are able to react with methacrylic anhydride (134,136,137). As the reactive species must be in the liquid state, PLA resins need to be diluted in solvents. When the viscosity of the medium is high enough to allow a suitable solidification of the material, the solvents used are usually reactive, such as methyl methacrylate, butane-dimethacrylate and *N*-vinyl-2-pyrrolidone (134). These substances are not easily biodegradable, so their introduction in the formulation limits the biomedical application of the final parts. For this reason, nonreactive diluents are desirable, especially those with a suitable polarity to be washed with biocompatible liquids to remove any trace during the postprocessing stage. Nevertheless, when nonreactive solvents are used, problems related to shrinkage may appear. Melchels et al. (134) used ethyl lactate as solvent and they analyzed the importance of the chemical structure's design of the oligomers on the properties of the final additive-manufactured structures, demonstrating the relationship between the degree of swelling of the parts and the arm length of the star-shaped oligomers. Another issue regarding the creation of innovative chemical structures in order to obtain a photocurable polymer that can be processed through SLA, is the ability of the body to remove their degradation products, as kidneys are not able to remove water-soluble polymer above 200 kg/mol (136). Melchels et al. (138) have analyzed this matter through spectroscopy and they concluded that the products have a suitable molecular weight to ensure renal clearance.

An alternative approach in the design of PLA-based scaffolds through SLA is the use of copolymers of this material. For example, Seck et al. (137) used a copolymer

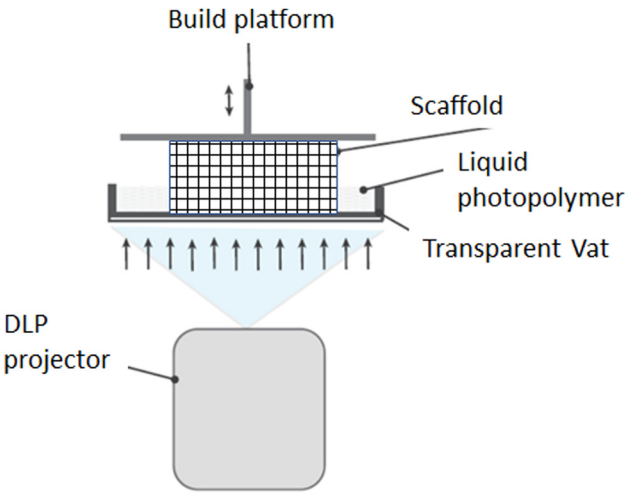

Figure 5: Vat photopolymerization by controlled area light source (DLP technique).

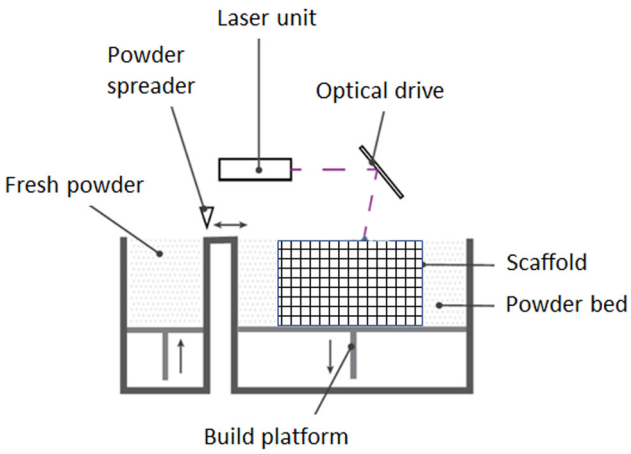

Figure 6: Laser powder bed fusion (SLS technique).

of poly(D,L-lactide) and poly(ethylene glycol) (PEG) to obtain structures manufactured through SLA techniques with relatively good results. Regarding the possibility of using composite materials, Ronca et al. (133) reported the manufacturing of structures containing up to 20% of nanosized hydroxyapatite. As expected, the energy of the curing light must be increased with the concentration of the additive (139). Although it is possible to find in the literature complex scaffolds for BTE obtained by SLA, some limitations of the technique have hindered its use for this application, like restrictions on the layer thickness and laser irradiation to avoid overcuring or potentially cytotoxic effects when working with encapsulated cells (13). This method is also generally more expensive and time-consuming than other AM techniques, requiring complementary instrumentation in order to produce biomedical devices.

3.2 Powder bed fusion

Powder bed fusion, also known as selective laser sintering (SLS), is an AM technique based on the melting of powder particles by the action of a focused laser beam, which sinters the material to create 3D structures according to a computer-designed layer-by-layer pattern (15,140). After printing a layer, new powder is added to the vat of the equipment and then sintered (Figure 6). Thin layers with heights in the range of 20–150 μm can be deposited. Once the process has finished, the manufactured part is removed and cleaned to eliminate any trace of powder. SLS parts may need further postprocessing (such as polishing or drying) depending on the specific application (127). This approach is ruled by the energy parameters of the laser beam and the material characteristics, such as the particles size and the viscosity of the molten material. Some of the advantages of SLS include its high resolution, the lack of need of a support material or structure that must be removed later and the possibility of avoiding the use of organic solvents. This technique is also relatively fast and cost-effective. These characteristics enable the manufacturing of complex 3D constructs using a variety of materials, including polymeric, metal and ceramic powders. Metallic powders are generally used to obtain 3D structures that could be applied in BTE for the regeneration of load-bearing bones, while the polymeric materials are preferred for non-load-bearing applications (13).

In the biomedical field, several disadvantages of SLS are related to the high temperature needed in this process, as it limits the selection of biomaterials for scaffold manufacturing, prevents the combination of these materials with cells and could lead to the degradation of the material by chain scission, cross-linking or oxidation processes (13,15). Different authors have also reported the presence of two levels of porosity in SLS parts: the macroporosity obtained through the CAD design (which will determine the mechanical properties of the parts and their vascularization) and the microporosity that is a consequence of the incomplete melting of the material during the sintering process due to high melt viscosity (141,142). This hierarchical architecture and the high microporosity of the final structure will affect the biological performance of the scaffold and its mechanical properties. On the other hand, the final pore size depends on the spreading characteristics of the powder, which in turn depends on the size of the particles used (13). The need of a small

and homogeneous particle size of the raw material could be considered the main limitation of this technique, as the optimal range for SLS processing is between 10 and 100 μm (143). Furthermore, the particles must be rounded to ensure material fluidity and prevent their agglomeration (144).

The use of PLA for the manufacturing of 3D scaffolds by SLS remains limited compared to other biodegradable polymers, such as PCL (143). Commercial PLA is typically delivered in the form of millimeter-sized pellets, so a method for preparing particles with smaller size before the SLS process is essential to ensure a high resolution of the 3D constructs. PLA particles in the micro- and nanoranges can be obtained through different techniques, such as emulsion/extraction, salting out, spray-drying, microfluidic techniques or mechanical milling (145). Solvent-related methods allow a good control of the size and shape of the PLA particles, but the use of toxic organic solvents for dissolving the polymeric chains (dichloromethane, chloroform, etc.) hinders medical approval and industrial-scale application. In contrast, organic solvents are not used in mechanical milling methods, which can also be upscaled more easily. However, particle size reduction of PLA by milling can only be achieved till a certain extent and the shape of the particles is generally highly irregular (144).

Despite the aforementioned limitations, some examples of PLA particle size reduction can be found in the state of the art. In this regard, Zhou et al. (141) used an emulsion/solvent evaporation technique to create PLA/carbonated HAp nanospheres. They used poly(vinyl alcohol) as the emulsifier and dichloromethane as the organic solvent, obtaining with this procedure PLA particles with sizes between 5 and 30 μm. To avoid the use of toxic solvents, Gayer et al. (144) have proposed to substitute this procedure by a mechanical one, developing PLA/calcium carbonate powders with suitable properties for SLS. The composites were prepared by dry impact milling and later sieving. Approximately, 25% of additive content was used in combination with four different inherent viscosity grade PLA (PLLA or PDLLA). The powder that includes the PLA with the lowest value of this property (1.0 dl/g) was the one that showed the best results in terms of processability, having also the smallest average particle size diameter (50 μm). Samples manufactured by SLS using this composite powder promoted cell viability of osteoblast-like cells, confirming the potential application of this scaffold for BTE applications.

In addition to the limitations related to the particle size, some authors reported the low mechanical properties of PLA sintered scaffolds, proposing the introduction of different additives to overcome this limitation. This is the approach followed by Shuai et al. (146), who added phospholipid-coated nanodiamond particles to improve the mechanical properties of PLLA-based SLS scaffolds. The compressive strength, compressive modulus and Vickers hardness of the sintered composite structures greatly increased compared with unmodified PLLA scaffolds (by 162.8%, 163.2% and 88.2%, respectively), due to the higher dispersion of the nanodiamond particles promoted by the phospholipid coating. A better dispersion is achieved since the hydrophobic tails of the phospholipids repel each other, after being the hydrophilic heads bonded to the nanodiamond particles surface. A decrease in the water contact angle of the scaffold is also reported when the percentage of additive particles is increased, favoring as a result of cell adhesion, proliferation and differentiation. The use of composite powders to improve the mechanical strength of the scaffolds has also been evaluated by Gayer et al. (147), who analyzed the properties of SLS-manufactured PDLLA/β-TCP 50/50 scaffolds. The effect of particle size, filler particle size and polymer molecular weight on the processability was also assessed in this study using the same composition of biomaterials. Again, the best results in terms of processability were obtained for the composite powder with lower particle size (around 35 μm) and melt viscosity, leading to scaffolds with lower porosity and, therefore, higher mechanical strength.

3.3 Material extrusion processes

Extrusion-based processes consist of the layer-by-layer deposition of materials through a nozzle tip, following a designed pattern, to obtain complex 3D structures. This technology is commonly known as fused deposition modeling (FDM) when low-melting-point thermoplastic materials are used. The material is fed to the AM equipment in the form of a continuous solid filament (generally with a diameter of 1.50 or 1.75 mm), being melted in a heated printhead and extruded over a build plate using a pinch roller system (148) (Figure 7). Each printed layer adheres to the previous one, hardens as it is cooled by a fan and then binds with the layer that is added on the top to form a solid construct. The printhead moves in the x–y plane to deposit the polymer in a semimolten state and then advances to an upper layer by its movement in the z-axis. Typically, stepper motors are

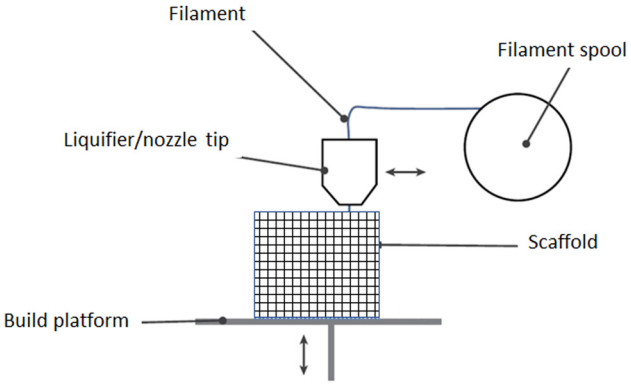

Figure 7: Material extrusion of thermoplastic material (FDM technique).

used to move the extrusion head and adjust the flow rate (149).

FDM is one of the most widely used AM techniques for scaffold fabrication, as it allows the manufacturing of 3D custom-made constructs. It is at the same time a flexible, low-cost and easy to implement technology, with immediate availability of printing materials. These advantages, coupled with the possibility of obtaining scaffolds with controlled porosity and pore size, are some of the reasons why FDM is nowadays a technique with great potential in the biomedical field. Regarding its drawbacks, the use of relatively high processing temperatures prevents the combination of cells or temperature-sensitive biological compounds with the polymer matrix during the scaffold's production process (bottom-up approach) (13). Therefore, a top-down approach is required, consisting in the seeding of cells onto the surface of the scaffold after its manufacturing by the FDM method. Another important limitation is its low resolution when compared to other AM techniques (127), despite FDM-manufactured scaffolds have been obtained with high resolution (150). Furthermore, support materials are needed when the structures to be printed have sharp or long overhangs. A two-nozzle printer is required in these cases (148).

For biomedical applications, PLA, PCL, PLGA and their blends with other biomaterials, are among the preferred options to obtain FDM-produced scaffolds (13). PLA is indeed one of the most spread materials for the fabrication of parts by this technique not only for the biomedical sector, but also for general applications (151). PLA scaffolds intended to be used in the biomedical field can be even obtained with low-cost FDM-based printers (152). Apart from the aforementioned advantages of PLA as a base material for scaffold manufacturing, this biomaterial has suitable thermal characteristic for FDM processability, being generally extruded at temperatures between 200°C and 230°C (148). The process conditions should be adjusted so the material do not suffer from excessively high shear stress during extrusion, as this could promote the degradation of the polymer or affect its biocompatibility (13). PLA-based composite materials can also be processed by FDM with the objective of improving the matrix characteristics (153,154). The final properties of the 3D printed structure will depend on the biomaterials used and the manufacturing process parameters.

The type of printing pattern used to manufacture the FDM scaffold for BTE has great influence on its vascularization, mechanical properties and cell ingrowth. Most of the proposed scaffold structures do not attempt to mimic the damaged tissue. Instead, they follow a uniform rectangular pattern with regular porosity and pore size. One example of scaffolds manufacturing using this configuration is found in the study of Grémare et al. (42), who developed and characterized rectangular-pattern PLA scaffolds obtained by FDM. Regardless of the pore size of the structure (150, 200 and 250 μm), both reasonable mechanical properties and human bone marrow stromal cells (hBMSCs) growth were obtained.

More complex printing patterns have been used in order to better resemble the architecture of natural bone, such the honeycomb-like structure with controlled porosity and pore size designed by Hutmacher et al. (155). The authors concluded that the honeycomb design conferred suitable mechanical properties to the structure for its use in BTE applications. The material used in this study was PCL, being the first work where the FDM technology was applied for the manufacturing of TE scaffolds. A more recent example of the use of this designed pattern is the work of Zhao et al. (156), in which the influence of the honeycomb structure characteristics on the mechanical properties of PLA scaffolds was discussed. Scaffolds with 90% porosity and compression modulus of 70.4 ± 11.4 MPa were obtained. An alternative complex designed configuration is the gyroid printing pattern, which gives as a result a mesh with curved-shape branches and nodes with four junction points. Germain et al. (157) used this configuration to produce PLA scaffolds by FDM and compare them to commonly used strut-based structures. The porosity of the scaffolds manufactured was in the range of 70%–75% with pore projections of around 800 μm, thus ensuring a good tissue vascularization. The spring shape of these structures showed great potential for bone regeneration, due to its isotropic behavior

regarding compression, robustness and mechanical energy absorption capacity. Unlike rectangular-patterned scaffolds, whose mechanical properties are strongly influenced by the printing orientation of the struts (158), the gyroid scaffolds could bear compressive efforts coming from any direction with the same effectiveness. According to the analysis of the stress–strain curves obtained from the compression test, the scaffolds with gyroid pattern had an apparent compression modulus of 50 MPa and a more deformable behavior compared to strut-based scaffolds.

Apart from the modification of the printing pattern, there are other strategies to modify the internal and external configuration of the scaffolds when trying to mimic the hierarchical architecture of bone. One example is the combination of FDM and gas foaming techniques proposed by Song et al. (159), which allow them to obtain a final structure with two different pore size levels with interconnected porosity. Briefly, PLA was combined with poly(vinyl alcohol) (PVA) to obtain continuous filaments suitable for FDM. After manufacturing the composite scaffolds, they were subjected to a gas foaming process to generate micropores in the structure. Finally, the temporary PVA phase was water-etched to extract it and create open pores. The final PLA scaffolds had macropores ranging from 100 to 800 μm and micropores in the range of 2–10 μm. Despite this promising configuration for BTE, the mechanical properties of the scaffolds manufactured using this procedure are too poor to ensure the support of the growth of the new bone tissue.

Numerous studies can be found in the literature regarding the influence of other printing parameters on the mechanical strength of the PLA scaffolds obtained by FDM. For example, Ouhsti et al. (149) concluded that there is a strong dependence between the deposition angle, the extruder temperature and the printing speed with the final mechanical properties of the scaffolds. Specifically, they analyzed the tensile strength and Young's modulus of PLA 3D printed scaffolds. On the other hand, Dave et al. (160) assessed the influence of layer height, infill density and printing speed on the mechanical properties under compression load. Their results suggest a high dependence of the compressive strength on the infill density, while no significant effects were obtained by modifying the layer height or the printing speed. On the other hand, Murugan et al. (161) pointed out that the extrusion temperature used to manufacture the constructs also affects their tensile strength and Young's modulus. Too high processing temperatures could also lead to an important reduction of the polymer's molecular weight (42).

Aside from the need of ensuring sufficient mechanical support, PLA-based scaffolds obtained by FDM should possess the appropriate biological properties to promote cell ingrowth. In this regard, the biocompatibility of PLA can be maintained after the FDM process, showing the constructs no cytotoxicity toward osteoblast-like cells. For example, Grémare et al. (42) cultured hBMSCs onto 3D printed PLA scaffolds, obtaining good results in terms of metabolic activity and cell distribution over the porous structure. Regarding cell differentiation, the micro- and nanotopography of the scaffold surface is one of the most important factors affecting osteogenic processes, supporting the differentiation of mesenchymal stems cells (MSCs) toward specific lineages (162). In contrast, macropatterns generated by the FDM equipment have not seem to induce this effect, as concluded by Alksne et al. (163). In order to stimulate cell differentiation, one possible approach is the combination of the PLA matrix with bioactive coatings. In a recent work, Teixeira et al. (164) manufactured PLA scaffolds by FDM and then coated them by immersion in polydopamine (PDA) and type I collagen (COL) solutions. The PDA/COL-coated PLA scaffolds showed improved cell adhesion and enhanced metabolic activity of MSCs during the first week of culture. Also, ECM components, specifically collagen and calcium, were deposited in a higher extend (after 14 days) when the coating was applied to the structures. At day 21, despite obtaining no significant difference in terms of cell proliferation and ECM compound deposition, coated scaffolds showed an alkaline phosphatase (ALP) activity 500 times higher compared to the unmodified samples. These results are a good indicator of the ability of the proposed scaffolds to stimulate osteogenic differentiation.

An alternative modification of PLA scaffolds manufactured by FDM in order to improve its biological properties is the combination of the base material with natural or ceramic additives. In a study of Zhang et al. (165), a comparison between PLA/HAp scaffolds, β-TCP ceramics 3D structures and partially demineralized bone matrices (DBM) was conducted regarding cell proliferation and differentiation *in vitro*, as well as bone repair capacity *in vivo*. The PLA/HAp printed scaffolds were designed with a pore size of 500 μm and porosity around 60%. These constructs possessed good biocompatibility and cell viability according to the results, promoting cell adhesion and proliferation of the bone marrow stromal cells (BMSCs) seeded onto the structures. Furthermore, cell differentiation was also enhanced by the PLA/HAp according to the ALP test and the gene expression analysis of osteopontin and type I collagen osteogenic markers. On the other hand, for the *in vivo* evaluation of

the scaffolds a critical-size rat calvarial defect model was used. PLA/HAp scaffolds showed again a favorable biocompatibility, higher degradation rate and improved osteoinductivity.

Another extrusion-based AM process is the one known as 3D bioprinting, which consists in the continuous extrusion of biomaterials by an air-pressure, piston-assisted or screw-assisted system to build 3D constructs according to a CAD-designed model (166). The most common method involves the application of pressure from a compressed gas to an ink-containing syringe in order to extrude the material through a micronozzle (167). The base material used in 3D bioprinting is generally a soft hydrogel, such as gelatin, collagen, laminin, fibronectin, alginate, chitosan, silk fibroin or gelatin methacryloyl (GelMA) (166,168). A good integrity of the 3D printed structure is ensured by using cross-linking methods after or even during the manufacturing process. Chemical- and photo-crosslinking (using UV light) after deposition are among the preferred options. This technology is implemented in most commercial units of bioprinters and bioplotters (Figure 8), in which viscous natural hydrogels can be printed in combination with synthetic polymers (hybrid scaffolds), ceramic materials (composite scaffolds) or even cells (cell-loaded scaffolds).

The possibility of incorporating cells during the manufacture of the scaffold is the major advantage of 3D bioprinting, avoiding their seeding onto the structure afterward (157). Cell-laden hydrogels are commonly referred to as "bioinks." The incorporation of cells and other bioactive compounds is feasible since this technique do not involve a heating process. Biomaterials with high cell densities can be deposited without negatively affecting the material processability or cell viability. In this regard, a recent study by Diamantides et al. (169) showed that the density of the cells incorporated affects the rheological properties of collagen bioinks, obtaining an improved printability as this parameter is increased. The proposed constructs for cartilage regeneration were seeded with chondrocytes up to a concentration of 100×10^6 cells/mL, maintaining high cell viability through a 14 days test. Some other aspects of 3D bioprinting include the ability to print high viscosity materials and struts with increased thickness by tuning the process parameters (flow rate, pressure, etc.).

Regarding its limitations, 3D bioprinting has a poorer resolution compared to FDM-related techniques. In order to overcome this drawback, narrower nozzles and higher driving pressures could be used, but a potential decrease in cell viability is generated as a consequence of these modifications due to shear stress effects on cells (170). Therefore, the optimization of the resolution-printing speed balance is required when the objective is to obtain highly porous structures which can provide a suitable environment for cell growth. Biomaterials with shear-thinning characteristics are an interesting option to fulfil this objective, as they possess high flowability under high shear rates, but become a viscous gel when they exit the nozzle and the shear stress is removed (166). On the other hand, although some studies have demonstrated that the appropriate selection of the process conditions can lead to the manufacturing of highly vascularized structures (171), this is still a matter of concern for 3D bioprinting application. One approach to address this issue is the use of sacrificial material, which are removed after the printing process to generate the vascularization channels (168). Sacrificial materials, such as gelatin or carbohydrate glass (168), are incorporated simultaneously during the printing material deposition, providing mechanical support for the upper layers of the structure. This methodology increases the complexity of the process since two different printheads are needed. Also, the number of methods for sacrificial material removal is limited because of the requirement that they should not elicit any cytotoxicity effect on the final structure.

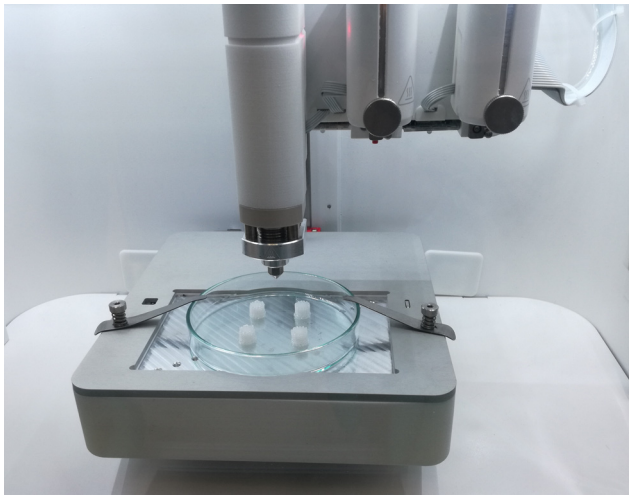

Figure 8: PLA scaffolds obtained by 3D bioprinting using a BioX 3D bioprinter (Cellink, Sweden).

3D bioprinting has been applied for the manufacture of scaffolds intended to regenerate vessels (171), neuronal tissues (172), cartilage (168) and bone (173). For the latter tissue and in order to meet the mechanical requirements, which generally cannot be satisfied using a sole material, the use of multicomponent bioinks and hybrids scaffolds has been widely proposed (168).

Despite the literature regarding the application of PLA for scaffolds manufacturing using 3D bioprinting is quite reduced, some examples of the use of PLA for bone regeneration as base material can be found. In that sense, Serra et al. (150) developed high-resolution 3D printed PLA-based scaffolds with added PEG and CaP glass particles. PEG was used as a plasticizer to improve the material processing. The CaP glass particles were mixed in a 1:1 relation with a 95/5 w/w% blend of PLA and PEG particles. The scaffolds obtained by 3D bioprinting at low temperature (40°C ± 5°C) showed high interconnectivity and uniform distribution of the additive glass particles, which increased the roughness and hydrophilicity of the surface. Both improvements promoted MSCs adhesion. Scaffolds containing both additives showed a very well-spread morphology of the cells. In a later work of Serra et al. (174), the authors extended the study to combinations of this biomaterial comprising a 5%, 10% and 20% of PEG in its blend with the PLA matrix. No CaP glass particles were added this time, as the objective was to analyze the influence of PEG on the scaffold final properties. It was concluded that the mechanical properties of the structure decreased with the increasing amount of PEG particles, while the degradation of the scaffolds is enhanced. Hence, the properties of the constructs can be tailored by modifying the percentage of plasticizer incorporated to the formulation of the blend. Taking into account the results of both studies, the best combination proposed by the authors is the combination of PLA, PEG and CaP glass particles, both from the biological and mechanical points of view.

In a totally different approach, the use of PLA as cell-laden microcarrier (MC) in 3D bioprinting constructs has been explored by Levato et al. (175) MCs are particles designed to promote attachment and proliferation of cells thanks to its high specific surface area. In this work, MSCs-laden PLA MCs were encapsulated in gelatin methacrylamide-gellan gum (GelMA-GG) bioinks. The results obtained from the characterization of the 3D printing structures showed that the PLA-MCs improved the compressive modulus and at the same time stimulated cell adhesion, bone matrix deposition and osteogenic differentiation. Mechanical reinforcement and enhanced cell viability were achieved without lowering the processability of the base bioink. A proof of application of the methodology proposed was presented in the form of a biphasic osteochondral scaffold, which consisted of a bone part with MC-laden bioink and a cartilage part made by using the GelMA-GG bioink without the MCs.

4 Strategies to improve PLA biological properties for bone tissue regeneration

As explained along the previous sections, PLA has favorable properties for its application in the biomedical field, including its biocompatibility, biodegradability, good processability and mechanical properties. However, its use in regenerative medicine is limited due to its hydrophobicity, which hinders cell adhesion and proliferation, and the release of acidic byproducts during the degradation process. In order to counteract these drawbacks and increase the bioactivity and osteoconductivity of PLA bone scaffolds, a variety of methods have been presented in the literature. In this section, the incorporation of additives, the application of surface treatments and the use of surface coatings with bioactive compounds are reviewed. We will focus on the use of these methods to improve PLA-based scaffolds properties obtained by AM techniques and intended to be used for bone regeneration.

4.1 Use of additives

The design of composite materials allows to tailor and optimize the biological and mechanical properties of PLA-based scaffolds, also offering the possibility of adjusting the biodegradation profile and rate of the manufactured structure (6). The biomaterials that have shown more potential for this purpose are bioceramics, specifically HAp, β-TCP, ceramic bioglasses and other CaP compounds. The incorporation of ceramic additives to the PLA matrix has been demonstrated to improve the hydrophilicity, osteoconductivity, mineralization upon implantation and mechanical properties of the 3D structures. Furthermore, given the basic nature of bioceramics compounds, they act as buffer agents during the degradation process, counteracting the pH decrease in the surroundings of the scaffolds and reducing the risk of formation of localized areas with an acidic environment (148) that could lead to an inflammatory response. Among the biomaterials mentioned, HAp is the one that has attracted more attention as an additive of PLA-based scaffolds. In the study of Niaza et al. (176) HAp was incorporated in the form of micro- and nanoparticles to the PLA matrix. Firstly, a mixture of both biomaterials was extruded to produce a continuous filament containing a 15% w/w of HAp. Then, this

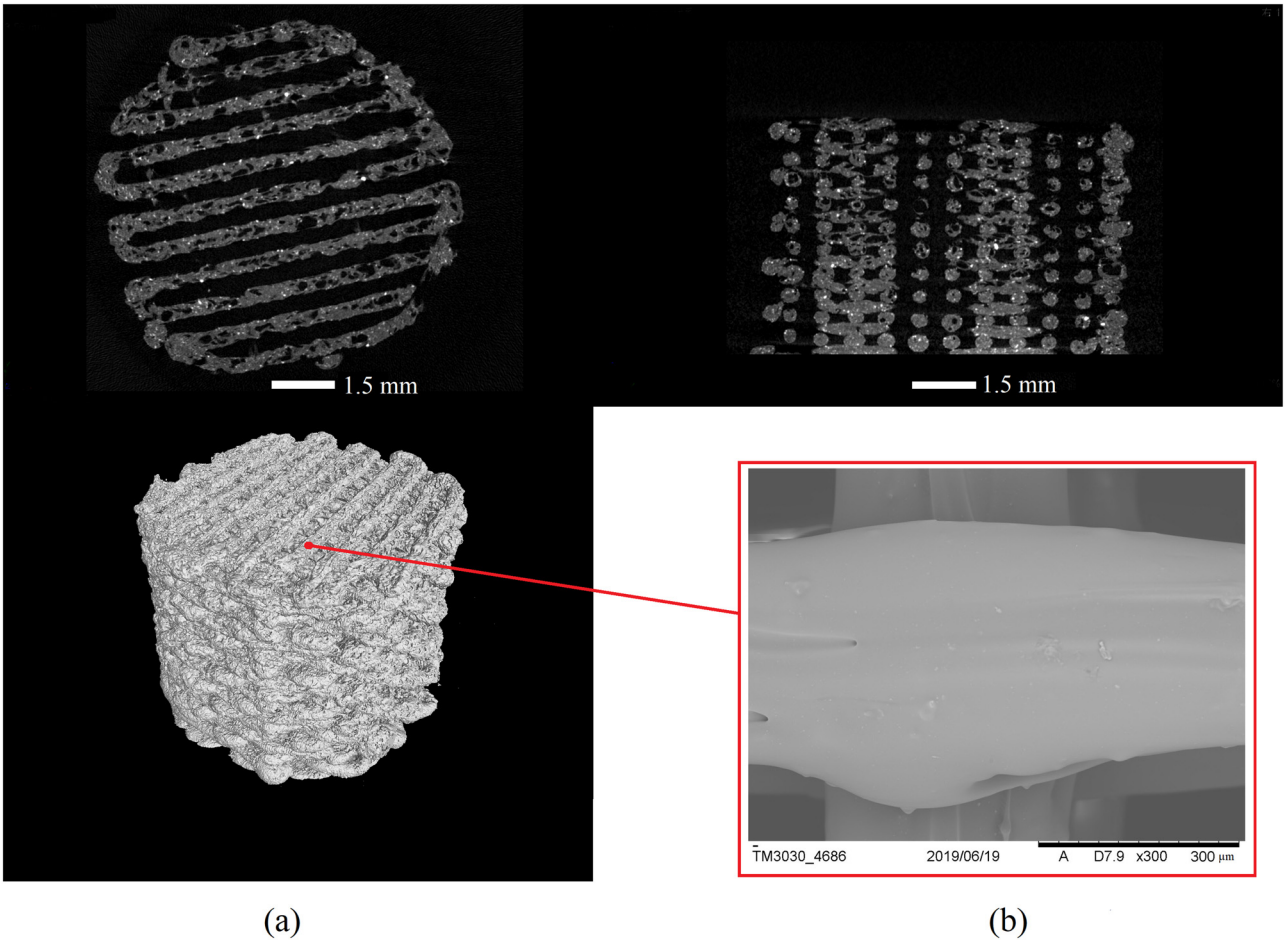

Figure 9: Images of 3D printed PLA-based composite scaffold, using β-TCP particles as additive, obtained by (a) scanning electron microscopy (SEM; Hitachi TM 3030 at an acceleration voltage of 15 kV) and (b) micro-computed tomography (micro-CT; Y. Cheetah, YXLON Ltd).

filament was used to manufacture rectangular-shape porous scaffolds by FDM. The final porosity of the structure was barely of a 30%. Despite having a porosity lower than the range recommended in the literature for cancellous bone regeneration (61,68), these scaffolds showed promising results in terms of mechanical properties, which were improved by the HAp nanorods introduced in the formulation. As an example of the incorporation of ceramic additives to the PLA matrix, Figure 9 shows images related to the morphological analysis of PLA-based composite scaffolds, containing β-TCP as additive, that have been recently developed by our group.

With the objective of improving not only the mechanical characteristics of the PLA matrix, but also its bioactivity, Esposito Corcione et al. (177) studied the feasibility of producing high HAp-loaded filaments to be 3D-printed using low-cost FDM equipment. Continuous composite filaments were successfully obtained using a twin-screw extruder after feeding it with particle mixtures comprising 5%, 15%, 30% and 50% of HAp. By using this solvent-free method, a good dispersion of the ceramic particles was achieved even at the highest concentration level studied. Samples manufactured by FDM technique showed no significant modification of the properties measured for the filaments (glass transition temperature, melting point, degradation temperature and crystallinity rate). The same authors recently presented another work (153) where they delved into the manufacturing of scaffolds by FDM using filaments containing a 50% of HAp. In this case, the additive was incorporated in the form of microspheres synthesized by spray drying and then mixed with the PLA by an extrusion process to obtain the filaments. Thermogravimetric results confirmed the presence of the additive in a 50% concentration, while the glass transition temperature and the degree of crystallinity

of the PLA matrix were not affected by the incorporation of the additive. From the morphological point of view, a homogeneous dispersion of HAp microspheres was confirmed, as well as an increase in the surface roughness. Although the theoretical porosity of the structures according to the CAD model was equal to 50%, scaffolds containing only PLA showed a porosity value of approximately 39%, while the result for the composite scaffolds was about 55%. The higher porosity of the PLA/HAp constructs led to a decrease in the mechanical properties, assessed by measuring the Young's modulus of the structures under compression testing (238.98 ± 19.05 MPa and 124.04 ± 25.21 MPa for PLA and PLA/HA scaffolds, respectively). Taking all the results into account, the authors concluded that the incorporation of a high load of HAp induced the formation of porous and rough strands, increasing the available surface area for cell adhesion, while lowering the mechanical properties of the structure to a value still in the range reported for cancellous bone (178). Therefore, composite scaffolds with improved osteoconductivity for BTE applications can be obtained with this method.

A deeper mechanical characterization of PLA-based composite scaffolds was carried out by Senatov et al. (179), who studied both compression and shape memory properties of 3D printed PLA/HAp 15 wt% scaffolds. These scaffolds had an average pore size of 700 μm and a porosity of 30%. Samples were subjected to compression–heating–compression cycles repeated up to the fracture of the sample, alternating compression forces at a strain rate of 15% and temperatures up to 80°C. The use of HAp particles as additive of the PLA matrix led to a significant increase of yield strength, strength at 15% strain and Young's modulus. In addition, no significant cracking of the structure or delamination of the layers was observed after the first compression test. Then, the PLA/HAp scaffolds were able to withstand three compression–heating–compression cycles, showing shape recovery rates in the range of 96–98%. In contrast, PLA samples were destroyed after two cycles. Despite the good results in terms of shape recovery, the mechanical properties were decreased in a proportion of a 20% after each cycle. Despite the promising capacity of these structures to "heal" the cracks that could appear during their *in vivo* application, the temperature needed to activate this recovery process (53°C according to the results of this work) is far above the internal temperature of the body (37°C). Other strategies should be applied to reduce the shape memory onset temperature without reducing the favorable mechanical properties of the proposed scaffolds.

Apart from ceramics additives, an alternative approach is to combine the PLA matrix with natural polymers or their derivatives. A good example is the work of Wei et al. (180), who investigated the microstructure and mechanical properties of scaffolds comprising PLA and different amounts of *o*-carboxymethyl chitosan (CMC). The scaffolds were manufactured by FDM after extruding continuous filaments of 100/0, 90/10, 80/20, 70/30, 60/40 and 50/50 w/w% PLA/CMC blends. The experimental work was complemented with the findings obtained by applying a molecular dynamics simulation method, revealing the molecular interaction mechanism between different components in PLA/CMC composites. The addition of CMC improved the hydrophilicity of the scaffold's surface, as can be concluded from the water contact angle measurements. The value of this parameter was reduced from approximately 75° to about 30° when the composition was PLA/CMC 50/50 w/w%. The introduction of the additive also reduced the fractional free volume and chain motion capability, resulting in a better processability of the material. Regarding the mechanical characterization, the tensile modulus increased with the concentration of CMC used, confirming the capacity of the additive to act as a reinforcement agent of the PLA matrix. On the other hand, the maximum tensile strength was obtained for samples containing a 20% of CMC, which were attributed to have the strongest intermolecular interaction between PLA and CMC components, being the experimental results in agreement with the calculated ones. Higher concentrations of the additive led to a decrease of this property due to the phase separation of the materials and the subsequent aggregation behavior of CMC. These results confirm the possibility of tuning the morphological, mechanical and biological properties of PLA-based scaffolds for BTE applications by incorporating additives in a controlled proportion.

4.2 Surface treatment

In spite of the suitable properties of PLA in terms of biocompatibility, the hydrophobicity of this material limits it interaction with extracellular proteins and cells. In order to improve the biological properties of PLA constructs for bone tissue regeneration, one effective approach is to apply a surface treatment to the 3D structure, aiming to modify its topography or surface chemistry. These surface changes can induce a positive effect on the attachment of cells and biological

compounds to the structure (181,182). Surface treatments can be used as the final modification of the 3D construct or as a previous step before coating the structures with bioactive compounds, as they allow an effective immobilization of these substances with the polymeric matrix (183,184).

As the coating strategies for PLA-based scaffolds in BTE will be discussed in the next section, we will now focus on the surface treatments generally used, being alkali treatments one of the most common options. This method basically consists on the immersion of the structures into a sodium hydroxide (NaOH) solution with an optimized concentration and during a certain time to obtain the desired surface modifications. One example of its application can be found in the work of Martin et al. (185), who treated PLA scaffolds manufactured by FDM using a 1:1 NaOH 0.25 M and ethanol 96% (v/v) solution. The samples were immersed during 4 h at room temperature with continuous stirring, then washed with citric acid 0.5% (w/v) and deionized water. Different collagen mixtures containing antibiotics and citrate-HAp nanoparticles were used as bioactive coatings of the treated structures. Unlike in the traditional alkali treatment, where hydroxyl groups are chemically incorporated to the PLA surface by nucleophilic attack to the ester bonds, the use of citric acid after hydrolysis also induced the formation of carboxyl groups bonding. In this way, a significant increment of the hydrophilicity and surface roughness has been reported (186). There are also references in the literature about the use of alkali treatments without further modifications to the structure, as proposed in the work of Nam et al. (187) These authors assessed cell adhesion on PDLLA and PDLLA/PLGA films treated by immersion in a 1 N NaOH solution. A strong influence of treatment time on surface wettability and, consequently, on cell affinity was confirmed.

Despite its proved efficiency, alkali surface treatments could introduce undesirable morphology changes and, more importantly, affect the bulk mechanical properties (188). Plasma treatment, in contrast, is one of the most explored techniques used to modify the surface chemistry of PLA-based constructs without changing the bulk properties of the material (189). This treatment is able to create functional groups with a higher water affinity, such as carboxyl (–COOH) and hydroxyl (–OH) groups (190,191). Therefore, the hydrophilicity of the surface is increased, as experimentally confirmed by the reduction of the water contact angle of the treated material (Figure 10). In the study of Nakagawa et al. (190), the authors obtained a decrease of this parameter from 77.4° to 39.8° after applying an air plasma treatment over PLA samples manufactured by injection molding. This modification induces an improvement in the biological performance of the scaffolds by enhancing cell adhesion capacity. Similar results regarding the hydrophilicity increase of the surface were obtained by Jordá-Vilaplana et al. (192), who also studied injected molded PLA samples treated with plasma. In addition, the authors observed topography changes in the surface due to some material removal, enhancing the roughness of the structure in a nanometric scale. Apart from air, other gases can be used for the plasma treatment of the samples to functionalize the surface. In the work of Yang et al. (182), an anhydrous ammonia (NH_3) plasma treatment was applied to porous PLA-based scaffolds to improve their hydrophilicity and cell affinity. This objective was fulfilled after the incorporation of N-containing groups to the treated surface. Not only cell adhesion and proliferation can be improved by these methods, but also cell morphology, as concluded by Yamaguchi et al. (193), who observed a close-contact extensive spreading of epithelial cells on plasma-treated PLLA constructs compared to unmodified samples. For the latter, cells showed small and round morphology and proliferated separately from one another.

For BTE applications, Wang et al. (194) have investigated the use of cold atmospheric plasma (CAP) technique to treat the surface of PLA scaffolds obtained by 3D printing. The objective was to modify the roughness and chemical composition of the constructs in the nanolevel, aiming to mimic the ECM properties of bone tissue. Different exposure times (0, 1, 3, and 5 min) were studied and the scaffolds were treated both on their top and bottom sides. Results showed that the different CAP treatments applied to the structures increased the hydrophilicity, roughness and oxygen to carbon ratio of the surface. The modifications introduced on the surface chemistry and nanoscale morphology effectively promoted the attachment and proliferation of osteoblast and BMSCs. Interestingly, the most promising results were obtained for the PLA scaffolds treated with plasma for 1 min. These findings showed the great potential of surface treatments to enhance the biofunctionality of PLA-based to be applied in BTE applications.

On the other hand, the main disadvantage of this method is its nonpermanent effect, as there is a progressive loss of treatment's effectiveness with time due to surface chemical rearrangement (195,196). In addition to this, plasma treatment can affect the degradation rate of the PLA matrix, as concluded by Wan et al. (197), who observed that an increase in the treatment time or power supply led to an enhanced the degradation of PLA scaffolds. Another limitation of these

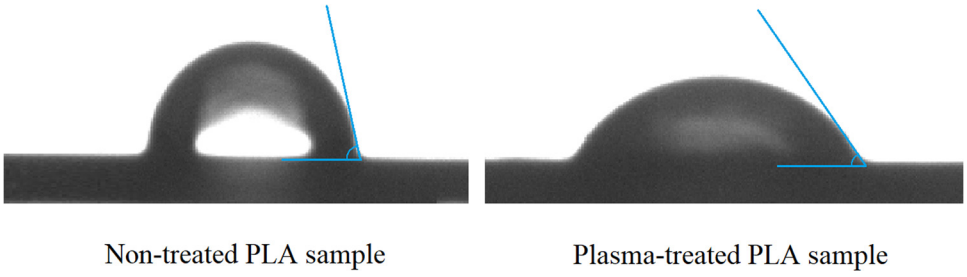

Figure 10: Water contact angle images of sessile drops over the surface of PLA and oxygen plasma-treated PLA samples obtained using a Krüss DSA100 contact angle measuring device (Krüss GmbH, Hamburg, Germany).

methods is the difficulty to generate a homogeneous modification on the samples to be treated. In this regard, for the specific case of 3D complex scaffolds, one of the challenges is to ensure that the surface modification takes place throughout the entire structure, since it is not always possible to reach the inner part of a scaffold with complex internal architecture or with small pore size (121,196,198).

4.3 Coating with bioactive substances

When the objective is to improve the biofunctionality of PLA-based scaffolds, the application of a bioactive coating to their surface is a promising approach. Several examples can be found in the literature regarding coated PLA scaffolds intended for bone regeneration. Some of the bioactive compounds investigated with this purpose include chitosan (199), alginate (200), collagen (201) or calcium phosphates (105), among others. In this section, different coating strategies to improve the biological properties of PLA-based bone scaffolds will be reviewed. The focus will be put on scaffolds manufactured by AM techniques.

In a recent work, Kao et al. (202) concluded that an improvement on stem cell adhesion, proliferation and differentiation could be obtained by coating the 3D printed scaffold surface with polydopamine (PDA). Specifically, human adipose-derived stem cells (hADSCs) were used to assess the biofunctionality of the PLA scaffolds modified by this mussel-inspired surface coating. The scaffolds were printed by using an FDM-based technique. The obtained structures were immersed into a dopamine (DA) solution with continuous stirring at room temperature. Two different concentrations were studied for this solution: 1 and 2 mg/mL of dopamine in 10 mM pH 8.5 Tris buffer. Finally, the scaffolds were soaked in the dopamine solution for 12 h. The results obtained were promising for the application of this method to enhance the properties of PLA-based bone scaffolds. A better performance of the PLA scaffolds coated with the DA solution of higher concentration (2 mg/mL) was confirmed. Some important findings in this work include the enhanced adhesion, proliferation, type I collagen secretion and cell cycle of hADSCs cultured on PLA/PDA scaffolds compared to the unmodified constructs. In addition, ALP activity and osteocalcin concentration were significantly improved after the application of the proposed coatings, being osteocalcin an osteoblast-specific protein hormone (203). According to the results, the expression of ang-1 and vWF angiogenic proteins was also significantly enhanced. The application of a PDA coating to 3D printed PLA scaffolds is also assessed in the work of Teixeira et al. (164) already mentioned in Section 3.3. These authors evaluated not only the effect of the PDA coating on the biological properties of the constructs, but also its ability to immobilize type I collagen (COL) onto the scaffold surface. With this purpose, FDM-manufactured structures were immersed into PDA and/or COL solutions after an alkali treatment. According to the results, COL immobilization increased by 92% when the PDA coating was applied. The combination of both coating steps led to an improved osteoinductivity of the 3D printed PLA scaffolds, as confirmed by the viability, adhesion and metabolic activity tests carried out using BMSCs. In contrast to the methodology used by Kao et al. (202), who directly applied the PDA coating, a previous surface treatment is used in this work. The promising results of these studies show the feasibility of both strategies to improve the scaffold properties (Figure 11).

In the already mentioned work of Martin et al. (186), 3D printed PLA scaffolds with enhanced biological properties were developed by coating the structures with bioactive compounds after applying an alkali surface treatment. Different collagen-based coatings were assessed in this work. In all cases, the treated PLA scaffolds were immersed in the coating solution for 24 h at room

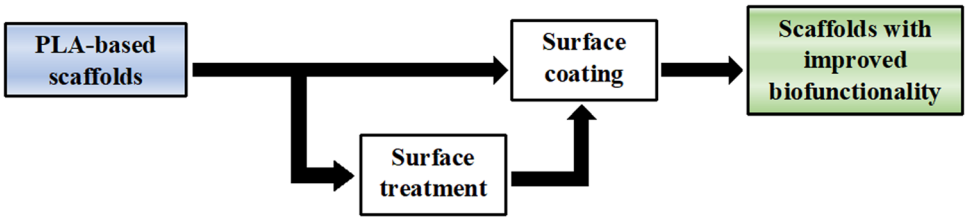

Figure 11: Application of a surface coating to scaffolds with or without previous surface treatment.

temperature and under stirring. The best results were obtained for a combination comprising collagen (COL), minocycline antibiotic (MH) and bioinspired citrate-hydroxyapatite nanoparticles (cHAp). Functionalized PLA scaffolds with this coating reduced the bacterial biofilm formation while favoring cell adhesion, proliferation and osteogenesis-related gene expression of hBMSCs. The proposed constructs could be suitable for bone regeneration, as they also showed adequate wettability and mechanical properties that match the ones reported for trabecular bone. Disparate results regarding the mechanical performance of the proposed scaffolds were obtained in the work of Fernández-Cervantes et al. (200), who presented a mathematical model to design 3D scaffolds for BTE applications. The numerical solution of this method takes into account the spatio-temporal changes that occur during the bone remodeling process (bone mass, osteoblast and osteoclast populations, etc.). The scaffolds developed were composed of PLA, sodium alginate and HAp, showing a microstructure that resembles the architecture of trabecular bone. Firstly, a mixture of HAp and the sodium alginate solution was stirred to induce the gelation process of the latter, due to its interaction with the divalent cations of the ceramic compound. Then, PLA constructs manufactured by 3D printing were immersed into the coating in a batch stirring reactor to produce the composite scaffolds. Despite their suitable morphological properties, the mechanical properties of the PLA/alginate/HAp scaffolds tested in this work do not match with the reported ranges for trabecular bone in terms of compressive strength and elastic modulus. However, the authors stated that these properties can be easily tuned by the application of a simulated body fluid (SBF) treatment. Samples immersed in SBF and incubated at 37°C for two weeks showed an improvement in compression resistance due to the induced mineralization of HAp crystals on the surface of the composite scaffolds.

The coating of PLA-based scaffolds with bioactive compounds has also been proposed for osteochondral regeneration. With this objective, Holmes et al. (204) proposed the use of biphasic 3D printed PLA-based scaffolds for osteochondral regeneration. With the aim of mimicking the architecture of natural bone and cartilage tissues, they designed 3D structures composed by two distinct parts, which varied in pore size, pore density and printing pattern. In order to increase the mechanical strength of the final construct and prevent its failure at the engineered interface, tubular-shaped structures with the length of the scaffold were incorporated in the CAD design. The authors stated that this innovative methodology allow them to improve the integration of the bone and cartilage parts, resulting in a more effective method than the conventional procedures for assembling the osteochondral unit, which are based on the separate manufacturing of the layers and their subsequent union by using glue, suture or thermal methods (205). Compression and shear test results confirmed the enhanced mechanical characteristics of the designed scaffolds. On the other hand, with the objective of improving the biocompatibility of the PLA matrix, the 3D printed scaffolds were coated with acetylated collagen. The method used for chemical functionalization of the surface involved successive immersions of the structures into different solutions, resulting in the linkage between PLA and ethylenediamine and then between the latter and glutaraldehyde. The surface coating process was completed after type I collagen binds to glutaraldehyde. The best results after 5 days in a proliferation test using hBMSCs were obtained with the collagen-coated PLA scaffolds. However, regarding chondrogenic differentiation, structures with and without coating displayed similar synthesis capacity of glycosaminoglycan, type II collagen and total protein content.

5 Conclusions and future trends perspectives in this field

PLA has been extensively applied in TE because of its good biocompatibility, biodegradability and mechanical

properties. Furthermore, PLA is very suitable to be processed by AM, which provides many advantages for bone scaffold manufacturing (customization, hierarchical and porous structures, repeatability, functional graded manufacturing, etc.). Different AM technologies have been reported for PLA-based scaffolds processing, such as material extrusion, powder bed fusion and vat photopolymerization. Nevertheless, the use of PLA in TE requires addressing some issues related to the release of acidic byproducts and their accumulation due to an inefficient removal from the surroundings of the scaffold's location. This accumulation can generate inflammatory conditions, negatively affecting tissue regeneration. This review reports some approaches to stabilize the pH, including the use of low molecular weight PLA or composites formed by PLA and bioactive glasses or calcium phosphates.

Another relevant topic shown in this review is the discussion of the different strategies to improve PLA properties. This paper highlights the use of additives to increase the mechanical properties and enhance the osteoconductivity of the matrix (HAp, β-TCP, CMC, etc.), the application of surface treatments to increase the surface hydrophilicity (alkali treatments, plasma treatments) and the use of surface coatings with bioactive substances to promote cell bioactivity (chitosan, alginate, calcium phosphate, PDA, collagen, etc.).

The future of PLA as biomaterial for bone scaffolds manufacturing is linked to the further development of some specific features for the improvement of the efficiency. Some relevant research lines include:

- Development of innovate composite materials to be used as feeding in bioprinting systems
- Improvement of the bioprinting process to enable the production of multifunctional graded scaffolds combining PLA with other biomaterials or bioinks
- Combination of AM techniques with other technologies, such as electrospinning, taking advantage of the benefits of each of them for bone scaffold manufacturing
- Loading of drugs or antibiotics for associated infections.
- Possibility of integrating sensing materials into the scaffold, aiming to monitor the properties change through time (pH level of the surroundings, mechanical stress of the structure, etc.)
- Implementation of theoretical degradation models of PLA to predict medium/long-term *in vitro* and *in vivo* behavior.

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