Review Article

Overview and future perspectives on bone 3D bioprinting

ABSTRACT

3D bioprinting is a prominent topic in the field of tissue engineering. The progress made in the last decade is remarkable, but the technology still shows many drawbacks and limitations due to the relatively short introduction. In this paper we summarize the major 3D bioprinting protocols such as material jetting, material extrusion and vat polymerization, highlighting pros and cons of each technology, then we analyze different biomaterials and various components of the bio-inks compatible with the available 3D bioprinters. The goal of this review is to examine the existing tools to produce bone scaffolds and highlight future developments in the field of Guided Bone Regeneration (GBR) inside the oral cavity.

Keywords: bioprinting, 3D printing, dentistry, bone tissue engineering

INTRODUCTION

The skeleton supports and protects the internal organs of the human body. Bone is a living, vascularized and biologically very active tissue thought the process known as “bone turnover” it can reshapes itself via sequential phases of reabsorption of the old matrix and apposition of newly formed tissue [1].

This process is based on a delicate balance between the two phases that can be disrupted by conditions such as osteoporosis, Paget’s disease, cancer, major fractures or infections.[2], [3]

In those scenarios modern medicine can overcome even major defects with bone grafts of various nature and it is estimated that in a few years the global market share for bone grafts will be 5 billion US Dollars.[4]

Although bone grafting procedures are widely accepted, and their advantages are recognized by literature, there also are risks and limits [5] that must be considered such as infections, surgical complications and rejection of the graft.

The efforts of current research are aimed to combine bioengineering with medicine, allowing the creation of 3D printed scaffolds housing living cells and bioactive agents.

The creation of living human tissue via 3D bioprinting techniques opens very wide possibilities, not only in the surgical field, but also in the one of pharmacological testing, allowing to in vitro conditions much closer, if not identical, to the ones found in animal or human test subjects. [6]

These new technologies were achieved through years of research in the fields of nanotechnologies, cellular biology and medical and material engineering and can allow virtually limitless applications.[7]–[11]

Cells, extracellular matrix, growth factors and the 3D scaffold can be printed layer by layer all together to create a tridimensional graft.[12]

The scaffold itself should provide the perfect environment for all the other components; therefore, it must satisfy requirements of biocompatibility, biodegradability, porosity, stability and mechanical resistance. [13], [14]

Already currently some research verified that both internal and external structure of the bio-printed materials are extremely close to the natural counterpart and that the digitally assisted design provides the flexibility and the optimization requested to the graft to satisfy all clinical and anatomical needs.

Regenerative medicine, tissue engineering and 3D printing technologies developed in the last few years giving an important incentive to the advancements of 3D bioprinting. [15]

Bio-inks, made of scaffold, cells and growth factors, can be layered to create tissue-like structure following the criteria dictated by Bioengineering. [16]

To the clinician, these techniques allow the usage of different materials and a more efficient management of tridimensional architecture of the graft.[7]

The bone defect can be scanned using either radiological applications such as Magnetic Resonance Imaging (MRI) and Computed Tomography (CT) Scans or ultra-sounds based diagnostic tools (UI) [16]; afterwards, these images can be digitally recon-structed and a 3D model can be printed.[17] At this point, the best possible combination of cells, growth factors, extracellular matrix and scaffold can be chosen in order to mimic proprieties and function of the original tissue.

Once the scaffold is printed the cells can be positioned and the appropriate growth factors can be added to start cellular proliferation. [18]

2. 3D bio-printing technologies

Currently three 3D bio-printing technologies are available: material jetting, material extrusion and vat polymerization.[19]–[23]

2.1. Material Jetting

Material Jetting is divided in two categories: inkjet bio-printing and laser assisted bio-printing.

2.1.1. Inkjet Bioprinting

This bioprinting technique is fast, reliable and compatible with different cell types making it suitable to produce large grafts, in particular skin and cartilage. [24], [25] Its high-definition printing (up to 20µm) makes possible to print even the smallest blood vessel, contrary to other techniques that do not allow the reproduction of such precise details.

3D printing can be achieved modifying existing 2D inkjet printers, making this method more affordable and simplifying the programming of the printing algorithm.

The complexity of the cellular framework printable with inkjet is made possible by the droplet-by-droplet control of the bio-ink layer filled by cells.

Based on the different activation method of the droplet, inkjet bioprinting is classifiable in thermal and piezoelectric.

The first method relies on electrical current to locally heat the bio-ink and print each droplet, the latter uses the deformation of the piezoelectric crystal to create droplets at regular intervals and electric impulses of various intensities to modulate the ejection pressure and the stress levels at which the cellular substrate is printed.

Applying heat and pressure to living cells obviously has an impact on viability and proliferation.

2.1.2. Laser-assisted Bioprinting - LAB

Developed 10 years after the first inkjet bioprinting technique, Laser-assisted Bio-printing (LAB), is mostly used to print DNA, peptides, and cells. [26], [27] This method is not widely adopted as inkjet, but its usage is on the rise.

The system is typically composed of a pulsed laser beam, a scanning mirror, a set of focusing lens, a “ribbon” (a substrate of glass coated with laser absorbing materials such as gold, titanium or non-metallic alternatives on which a layer of bio-ink is applied) and a receiving substrate.

The laser beam hits the “ribbon” creating a bubble inside the bio-ink. The cavitation effect generated by the bubble propels a droplet of bio-ink onto the receiving substrate.[28]

As the metallic coating can lead to vaporization of the metal with contamination of the bio-ink, alternative materials have been introduced.

2.2. Material extrusion

Material extrusion printing techniques rely on gas, pistons or screws to continuously eject a set amount of a mixture of cells and hydrogel, forming filaments then cured to achieve a 3D structure.

Compared to gas driven extrusion techniques, the mechanical ones are more complex and expensive, but also more direct and precise due to the absence of the delay caused by gas compression. Between the mechanical extrusions, screw and piston are both suitable for high viscosity bio-inks, but the first allows a finer spatial control.

2.3. Vat polymerization

2.3.1. Stereolithographic (SLA) 3D bioprinting

The Stereolithographic (SLA) 3D bioprinting process starts with the preparation of the bio-ink by mixing living cells with a solution of photosensitive polymer. A 3D scan, such as magnetic resonance imaging (MRI) or computer tomography (CT), of the desired model is converted in 2D slices. The bio-ink is laid down in a Petri’s dish at the desired thickness of the layer and a source of light projects the image of the 2D slice onto it guiding the polymerization. With this technique the light source is the most important aspect concerning precision and resolution. The printing process repeats these two steps until the complete model is obtained.[29] SLA bioprinting is used to quickly create precise bone and cartilage grafts based on the CT scans of the patient showing high fidelity in re-producing tissue structure and architecture. [30]–[33] It also has a variety of implementations in the construction of models to study cell growth and tumor behavior. Different types of SLA 3D bioprinting are classified based on the illumination source and the projection pattern.[29] Visible light and UV wavelengths can be used in the SLA technique and the Digital Light Processing (DLP) system. Infrared wavelengths can be used in two-photon polymerization (TPP or 2PP) printing process, even if it is not directly classifiable as an SLA bioprinting. [29], [30], [34], [35]

2.3.2. Digital Light Processing 3D bioprinting - DLP

The DLP 3D bioprinting system consists in an array of microscopic mirrors the size of 5 to 10 um that can rotate and change inclination in order to reflect the light beam coming from the lamp. This system is called digital micromirror device (DMD). The lamp produces wavelengths in the UV and visible range, a Graphics Processing Unit (GPU) analyzes the pattern and a controller changes the position of the array manipulating the reflection to a specific pattern and intensity. DLP based SLA printing is fast, has high resolution, that can be enhanced even further with focusing lenses, and can even print complex structures thanks to the DMD arrangement. [36]–[38]

2.3.3. Direct Laser Writing Bioprinting

Direct laser writing bioprinting cannot be considered a SLA process because it does not rely on a layer-by-layer printing, despite it use a polymerization process. The pulsated laser beam, focused by a set of lenses, operates inside the volume of hydrogel allowing for an extremely localized polymerization. Compared to Digital Light Processing, Direct Laser has higher costs and lower printing speed, since it is based on a point-by-point printing process; on the other hand, the high efficiency and precision of the beam makes the polymerization process very fast, allowing to partially compensate for the initial speed deficit.[29]

After analyzing the various printing methods available today, we will now address the printing materials currently being studied in the field of Tissue Engineering mostly focusing on bone scaffolds.

3. Biomaterials

Bone tissue is characterized by a highly specific microenvironment in which both cells and extra-cellular matrix (ECM) components are fundamental to maintain its stability and properties. One of the most fascinating and complex challenges for clinicians is represented by the possibility to regenerate bony defects, thus reestablishing its architectural stability and function. Currently, the gold standard in bone regeneration is represented by autologous bone, harvested from a donor site and then fixed to the bone defect region. However, this procedure includes some limitations such as the amount of available bone for transfer without donor site morbidity and an increased risk of infection. [39] In the last years, the possible biomedical applications of 3D bioprinting have significantly attracted different research areas and in particular Bone Tissue Engineering (BTE). One of the major advantages in that field is the possibility to print a tailored implant with dimension and shape identical of those of bone defect, starting from a 3D scan of the site. [40], [41] The ideal 3D printing materials should exhibit six main properties, which are printability, biocompatibility, biodegradability, suitable mechanical properties, non-toxic by-products and the ability to imitate the natural tissue environment. [42]–[49] Moreover, biomaterials must show adequate porosity and viscosity, and also need to well resist to pressure and temperature. Pore size greater than 300 μm have been proven to enhance both angiogenesis and osteogenesis.[50] Thus, one of the primary challenges in 3D bioprinting is the fabrication of an appropriate material which contains all of these features. [51]

3.1. Synthetic polymers

Polymers are macromolecules mainly composed of repeated subunits made of carbon, hydrogen, oxygen and nitrogen, defined as monomers. Those subunits are bonded together through covalent bonds, creating long molecular chains with different weight and properties. The most frequently employed synthetic polymers are Poly(Ɛ-caprolactone) (PCL), Polyhydroxy Acids such as Poly-Lactic Acid (PLA) and Poly (Lactic-co-Glycolic) Acid (PLGA), and Polyurethanes (PU).

3.1.1. Poly(Ɛ-caprolactone)

Poly(Ɛ-caprolactone) (PCL) is a semi-crystalline, non-toxic, aliphatic, thermoplastic polyester, with a relatively low melting point of 60°C – which corresponds to a reduced temperature-related cellular damage [52], obtained through a mechanism of ring opening polymerization of Ɛ-caprolactone in presence of a catalyst. [53] Furthermore, PCL shows the ability to be biodegraded by the organism over time, [52]–[56] with a biological half-life of approximately 3 years.

3.1.2. Polyhydroxy acids

Polyhydroxy acids such as Poly-Lactic Acid (PLA) or Poly (Lactic-co-Glycolic) Acid (PLGA) are biodegradable, synthetic polymers which have been successfully involved in the production of porous scaffolds for tissue engineering.[57] The biodegradation mechanism that occurs after implantation of those materials is characterized by the production of lactic acid and carbon dioxide, which can be easily removed from the organism, but at the same time creates an acidic environment in which inflammation process prevails on healing. PLA is a hydrophobic, aliphatic polyester derived from the polymerization of lactic acid; it showed great mechanical strength other than good chemical and physical properties. PLGA is the copolymer of PLA and Poly-Glycolic Acid (PLG); it is frequently employed in tissue engineering due to its biocompatibility, biodegradability and valid mechanical properties. Nevertheless, PLGA and PLA are characterized by poor cell-scaffold interactions, both for its hydrophobic nature and for the lack of biological binding sites.

3.1.3. Polyurethane

Polyurethane (PU) belongs to the class of synthetic elastomers; it is composed of a main organic molecular chain bonded by urethane linkages. PU have been recently reformulated for biomedical applications to take advantage of its excellent properties such as high mechanical strength, flexibility and good biocompatibility.[58] Thus, the limits in using PUs for tailored 3D printing seems to have been handled in the last years. [51], [59] As an example, in 2019 piperazine (PP)-based polyurethane-urea (P-PUU) scaffolds were 3D printed showing both in vitro and in vivo great biocompatibility and osteoconduction, and consequently an increased bone formation. In a rat tibia model the same scaffold demonstrated good newly formed bone density, volume and trabecular features, which were correlated to the increase in PP content. More recently, Cooke et al. 3D printed a PU scaffold to be investigated both in vitro and in vivo for bone defects repair. To study cells behavior on PU scaffold, dental pulp stem cells have been seeded onto the scaffold, showing the ability to adhere, proliferate and produce mineralized matrix. Moreover, its application in rats mandibular defect leads to an

increased production of mineralized tissue – other than higher trabecular thickness - if compared to a currently used bone putty, as demonstrated by authors through µCT analysis. [59]

3.2. Natural polymers

In order to be printed, natural materials are manipulated in form of hydrogel. In the following paragraph we will briefly introduce Hydrogels in order to better understand the employment of natural polymers in 3D Bioprinting.

3.2.1. Hydrogel

Hydrogels are natural or synthetic polymers able to absorb and hold a great amount of water in their structure; [60] they can absorb approximately a thousand times their weight in aqueous environment without dissolving.[61] They show similar properties to the ECM, such as biodegradability, non-immunogenicity and biocompatibility. Therefore, those materials are widely used in tissue engineering for loading cells on biomaterials. [62] As opposed to other polymeric scaffolds in which cells and biomolecules are mainly seeded on the biomaterial after printing, hydrogels furnish a complex environment in which cells can migrate and interact.[63] Basing on their origin, hydrogels can be grouped in natively and synthetically derived hydrogels. The first group includes biomolecules frequently derived from ECM components and widely employed in tissue engineering, such as collagen or hyaluronic acid (HY), which show low toxicity other than high biocompatibility and cells affinity.[64] Nevertheless, the possible uncontrolled degradation of those polymers could undermine the mechanical properties of printed construct, and thus the in vivo applications of hydrogel- based load-bearing scaffolds are limited.[65] As opposed, synthetically-derived hydrogels provide good mechanical strength and long-term stability, although they lack biological cues.

3.2.1.1. Collagen

Collagen is the main structural protein of the ECM of bone tissue; it is a triple-helical molecule of natural source with high biocompatibility, and thus it has been frequently employed for biomedical applications. [66] Among the 29 known different types of collagens, type I collagen demonstrated a lack of immunogenic response associated with its use, thus promoting its usage for bone tissue engineering. [67] As a component of the native ECM, collagen is also biodegradable and biocompatible, other than able to stimulate cells behavior. [68] Actually, collagen is rich in Arginine-Glycine-Aspartic (Arg-Gly-Asp; RGD) sequences which promote cell adhesion and proliferation via integrin-RGD interactions. [54]

3.2.1.2. Gelatin

Gelatin is a natural protein obtained from collagen hydrolysis. [69] It is mainly composed of RGD sequences and thus extremely biocompatible, biodegradable and promote cell migration, adhesion, differentiation and proliferation. Because of its high solubility in physiological environment, gelatin presents high enzymatic degradation rates and poor mechanical features, thus limiting its applications as a load-bearing scaffold.

3.2.1.3. Hyaluronic acid

Hyaluronic acid (HY) is a linear, non-sulphated glycosaminoglycan (GAG) widely employed in tissue engineering because of its highly desirable properties such as bio-degradability and biocompatibility, other than its non-adhesive, non-thrombogenic and non-immunogenic nature. As a main component of the ECM, HY is significantly involved in cellular proliferation and migration. To employ HY in 3D printing applications it is necessary to submit the molecule to chemical modifications or mixing with other polymers to improve its rheological and mechanical properties.[68]

3.2.1.4. Chitosan

Chitosan (CS) is a natural polymer frequently used for tissue engineering applications. Its favorable properties such as biocompatibility, biodegradability, antibacterial, bio-adhesive and low price make it a desirable material for tissue engineering. [70], [71] As reported in a recent review, the applications of CS for BTE both in vitro and in vivo studies evidenced an enhancement of cellular bioactivity and mineralization of newly formed bone, other than the promotion of osteogenesis process.[72] As an example, the incorporation of CS hydrogel onto a 3D printed PCL scaffold seeded with rabbit bone marrow mesenchymal stem cells showed good mechanical strength, promising osteogenic properties and a high bone-matrix formation after 2 weeks in osteogenic culture. [72] Moreover, 3D printed PLA scaffold has been modified by coating with CS hydrogel and HA to improve its features for tissue engineering applications. In vitro bone cell seeding showed higher attachment and proliferation in PLA/CS/HA scaffold if compared with bare PLA scaffold.

3.3. Decellularized Extracellular Matrix Bioinks

Bone tissue ECM is mainly composed of type I collagen (approximately 95%) and different non-collagen proteins such as osteopontin, osteonectin, bone sialoprotein, fibronectin and alkaline phosphatase which are mainly involved in the nucleation process of HA crystals and in the composition of the ossified ECM. One of the possible applications of bone ECM in BTE is the removal of cells from tissue through chemical, physical or enzymatic processes and simultaneously the preservation of the ECM and its com-ponent [73] the result of that mechanism is known as decellularized extracellular matrix (dECM). The ideal source of dECM is human-derived ECM since xenogenic dECM may result in a massive stimulation of immunogenic response. [74] However, ethical issues are a disadvantage in the use of human-derived ECM, due to its human derivation. [51]

DISCUSSION

To create a bio-printed tissue many steps are required. The printing geometry of the grafts has to be designed. Based upon that geometry and the nature of the receiving site, the appropriate cell culture and hydrogel need to be selected, loaded onto the bioprinting device and, through the appropriate software, a set of printing instructions must be established. At this stage the quality of the bio-printed tissue is microscopically checked and sent to an incubator for culturing.

This process is operator based and time-consuming, therefore the focus of the research has been to include Bio-CAM and Bio-CAD techniques in order to automatize and speed up the bioprinting protocols.

Bio-CAM can provide virtual models of the engineered tissue with known physical characteristics and production patterns.

Combining 3D imaging and stereolithographic files with Bio-CAD technologies might lead to an affordable case-by-case production of the required tissue. Graft morphology can be modeled starting from MRI or CT scans converted to STL files. The STL file gets sliced in layers with the desired thickness and, thanks to the bio-printer's software, transformed into instructions for the bio-printer.

Has discussed in the introduction, there are multiple 3D bioprinting protocols available today, all of which presenting streanghts and weaknesses.

Material extrusion is the most adopted protocol. [75] The cellular density achievable with this procedure is high and, compared to the other 3D printing techniques, printing of different cellular types on larger scales is relatively easier also thanks to the large amount of bio-inks compatible with the protocol. [20], [76] Through extrusion, thanks to the high viscosity and the wide variety of compatible bio-inks, it is possible to achieve a cells density similar to the physiological counterpart, with highly heterogeneous substrates and resilient 3D structure of the grafts. [77], [78] With these techniques aortic valves and vascular tissue were printed. [79]

Inkjet 3D bioprinting is the least expensive of the group because its components are derived from the commonly used 2D inkjet printers. It presents high printing speeds and resolutions, but cellular density and overall quality of the products are poor compared to extrusion.[80] This technique is plagued by nozzle clogging and the variety of compatible bioinks is limited. [81] To avoid nozzle clogging is necessary to adopt low viscosity inks, making the mechanical strength of the final product not ideal for most applications. Widening the nozzle makes possible to have higher viscosities along with a reduction in resolution.

To obtain dimensional stability with good resolution, it is mandatory to select crosslinked scaffolds. These scaffolds, however, slow down the printing process and lower viability and number of the cells due to the cytotoxic environment required for the crosslink process. The nozzle is also responsible for a survival rate that ranges from 40 to 80%, which is low compared to the other techniques.[82]

In comparison with inkjet, LAB, does not present issue with clogging since a nozzle is not required. For this reason, the viscosity range is wider (1-300 mPa s-1) and has no impact on viability and proliferation of the cells, leading to a survival rate that can reach 100% in specific cases.[83], [84]

Various parameters such as the energy density of the laser, the gap between ribbon and substrate and its wettability concur to a resolution that can achieve a few µm,[85] allowing fast and high-density cell deposition.

Cellular density and proliferation in the substrate are difficult to manage since a density that is too high can lead to apoptosis and too low proliferation may lead to cellular senescence; therefore, finding the balance between these aspects can be challenging in LAB substrates.

Preparing the ribbon and substrate can be time consuming and expensive especially if the grafts need multiple cellular types.[86], [87]

Vat Polymerization techniques (SLA DLP and Direct Laser Writing Bioprinting) present high resolutions, good survivability rates, high printing speeds and good quality of the final product.[88] The highest resolution is obtainable by Direct Laser Writing Bioprinting with SLA and DLP following closely.[89] SLA and Direct Laser Writing Bioprinting have a point-by-point printing process, while DLP is layer-by-layer allowing faster printing speeds.[90] Cellular survival in Vat polymerization is close to Laser-assisted jetting even if the UV, IR or visible light involved in the Vat might be toxic; moreover, both Vat and Laser-assisted jetting have values higher than inkjet but lower than extrusion.

High costs (except for SLA) and time-consuming bio-inks preparation are the major concerns about Vat Polymerization printing.

After dealing with the various printing methods, attention must be paid to the Bio-inks involved in Bone Tissue Engeneering.

During the last decade, synthetic polymers have become the most employed materials in 3D bioprinting applications due to their biological, chemical and mechanical properties which render them versatile and easy to manipulate. Their main limitations are that high extrusion temperatures - ranging from 608°C to 2008°C, [91]- toxic organic solvents and crosslinking agents, are required during the printing process.[54] To maintain cells viability and molecules bioactivity, the synchronous bioprinting of cells and polymers is typically avoided [91] and a post-extrusion approach is preferred.

Syntethic-Polymer scaffolds aroused more interest, concerning BTE, than the natural-based alternatives due to their longer degradation time and higher porosity. In addition, their mechanical strength makes them ideal candidates for the reconstruction of tissues that require high levels of resistance such as bone. [91]

For instance, PLC, is widely employed as a scaffold for tissue engineering. However, PCL is highly hydrophobic - thus disfavoring cells attachment and growth [42] - and lacks natural peptide sequences that provide specific sites for cells-biomaterial interactions. Because of the lack of biological cues, PCL is frequently combined with other biomaterials. [54] PCL functionalization has been investigated using different biological compounds such as Hydroxyapatite (HA), β-tricalcium phosphate (TCP) or growth factors. [92], [93] Oberdiek et al. recently evaluated the properties of a composite scaffold made from PCL and biphasic calcium phosphate (BCP), both in vitro and in vivo, finding biocompatibility and good mechanical strength other than osteoconductive properties and a reduced swelling.[94] Even the combination of PCL and HA with other synthetic polymers such as PLA or PLGA has been proved to be promising for bone tissue engineering applications. [95]

PLA and PLGA share similar proprieties with PCL, such as great mechanical strength paired with lacking biocompatibility.

PLA's release over time of acidic compounds reduces its biocompatibility. To overcome that disadvantage, composite scaffolds made from PLA and calcium phosphate have been produced; those materials positively influenced new bone apposition and reduced the formation of acidic molecules. [35]–[38], [42], [50], [96] Other authors combined PLA and ceramic to take advantage of PLA mechanical properties, thus overcoming ceramic brittleness.[42]A recent in vitro analysis of porous, human femur shaped PLA and PLA-HA scaffolds assessed good biocompatibility with pre-osteoblasts, other than high cell viability and proliferation. Therefore, PLA-HA scaffold showed significative higher compressive strength and levels of alkaline phosphatase activity and calcium deposition, evidencing higher osteoconductive feature. [97] Diomede et al. evaluated a composite scaffold made from PLA and human gingival mesenchymal stem cells (hGMSCs), founding great osteoinductive features in vitro and promising osteogenic properties in rats calvaria bone defects.[98]

As for PLGA, in order to overcome the biocompatibility issues, different biological materials such as collagen, gelatin, elastin,[99] chitosan, alginate,[100] hydroxyapatite, and hyaluronic acid [101] have been incorporated in the PLGA matrix. Recently, both in vitro and in vivo evaluation of TCP-PLGA scaffold loaded with the osteogenesis-promoting drug HA15 showed promising results for the treatment of bone defects. In particular, TCP-PLGA-HA15 scaffold revealed biomechanical properties overlapping those of cancellous bone tissue other than the ability to promote cell differentiation into osteoblast lineage.

In summary, one of the advantages of such composite materials is the possibility to take advantage of the most desirable properties of each one of its components.

Contrary to other synthetic polymers, PU, shows in vitro and in vivo great biocompatibility and osteoconduction. As an example, in 2019 piperazine (PP)-based polyurethane-urea (P-PUU) proved to be effective in bone formation. In a rat tibia model the same scaffold demonstrated good newly formed bone density, volume and trabecular features, which were correlated to the increase in PP content. More recently, Cooke et al. 3D-printed a PU scaffold to be investigated both in vitro and in vivo for bone defects repair. To study cells behavior on PU scaffold, dental pulp stem cells have been seeded onto the scaffold, showing the ability to adhere, proliferate and produce mineralized matrix. Moreover, its application in rats mandibular defect leads to an increased pro-duction of mineralized tissue – other than higher trabecular thickness - if compared to a currently used bone putty, as demonstrated by authors through µCT analysis.[59]

Natural polymers are characterized by features such as biocompatibility and cellular affinity that make them unique. That being said, rapid degradation and the lack of mechanical strength makes them hardly usable in BTE if not in association with synthetic polymers as previously discussed. As an example, Polyethylene Glycol (PEG) is a synthetic polymer synthesized by ethylene oxide polymerization which has been widely investigated in biomedical field; in particular, PEG hydrogels showed suitable properties for tissue engineering such as hydrophilicity, low toxicity and biocompatibility.[102] Moreover, the addition of lactate or glycolate chemical groups improves both its biodegradability and bio-absorption features. Combined applications of synthetic and natural polymers seem to be effective to overcome limits due to each material disadvantage (e.g., mechanical strength [103]), although more studies are needed to identify new materials and printing strategies.

To transform natural polymers into viable Bio-inks (in association with a synthetic scaffold) the preferred formulation is hydrogel: in this form a favorable environment in which cells can migrate and interact is achievable.

Collagen, Gelatin, Hyaluronic Acid and Chitosan are the most studied natural polymers in tissue engineering.

Collagen has a limitation due to its slow gelling process, which does not guarantee sufficient stability of the structure after printing.[68] Therefore, similarly to the other naturally derived polymers, collagen present inadequate mechanical properties for creating a load-bearing scaffold,[104] and thus is often combined with more resistant materials to form composite scaffolds for BTE applications. The in vitro applications of composite scaffolds made of collagen (Col) and hydroxyapatite (HA) (Col-HA), seeded with different stem cells populations such as bone marrow derived mesenchymal stem cells (BMSCs),[105] human mesenchymal stem cells (hMSCs)[67] or murine adipose derived stromal cells (mASCs),[106] showed promising osteogenic, osteoinductive and angiogenic potentials for BTE applications.

Gelatin is obtained from the collagen's hydrolysis, thus the molecules share similar proprieties, nevertheless, 3D printing of a scaffold composed of gelatin and different, increasing percentages of TCP revealed high affinity with pre-osteoblasts, as evidenced in terms of cell viability, migration, proliferation and differentiation in an in vitro study. The in vivo application on rats calvaria defects resulted in higher newly formed bone values in gelatin/TCP if compared to those obtained from gelatin alone. The authors concluded indicating that gelatin/TCP scaffold has a great potential as a bone substitute for BTE.[107]

In early 2000 Schacht et al. firstly introduced a new suitable material for biomedical applications known as Gelatin Methacrylamide, characterized by the addition to gelatin structure of functional groups which improved gelatin features. The substitution of amine groups can be achieved by exploiting different methacrylate molecules, of which methacrylic anhydride is the most efficient;[108] the final product of the reaction is widely known as GelMA. It has been widely employed in biomedical applications, revealing promising properties.[22], [109] The main limitation of GelMA hydrogel is its poor mechanical properties, which affects the applications in tissues undergoing high mechanical forces such as cartilage and bone; to overcome this limitation, several methods have been tested and the combination of GelMA with PCL showed promising results. As an example, Buyuksungur et al. printed a 3D hybrid scaffold made from PCL and GelMA carrying dental pulp stem cells (DPSCs); cell loaded GelMA supported osteoinductivity, while PCL provided structural and mechanical features. DPSCs present onto PCL/GelMA scaffold showed in vitro high cell viability, osteogenic differentiation and high-rate mineralization.[110]

Modified Hyaluronic Acid chains have been studied by Poldervaart et al. to obtain methacrylated hyaluronic acid (MeHA) scaffolds, thus improving its mechanical features and stability over time.[111] The in vitro analysis was carried out after seeding human bone marrow derived mesenchymal stem cells and revealed good cell viability even after 21 days of culture, other than spontaneous osteogenic differentiation. Furthermore, the addition of Bone Morphogenetic Protein-2 evidenced higher mineralized matrix formation, suggesting improved osteogenic features; thus, authors concluded indicating that MeHA properties seem to be adequate for a possible application in BTE. Moreover, Hamlet et al. evaluated alveolar bone regeneration through a 3D osteoblast-containing hyaluronic acid hydrogel, evidencing a favorable environment for osteogenic gene ex-pressions in vitro. Basing on their findings, the authors concluded indicating that osteoblast-HY hydrogel could be a useful tool for future 3D bioprinting applications.[112]

Although combining the physical strength of synthetic polymers with the biological proprieties of these hydrogels is showing promising results, Decellularized extracellular matrix bio-inks are widely investigated in BTE because of their ability to provide an environment that optimally mimics native bone architecture, thus promoting osteogenic cells behavior and activity. Compared to hydrogels alone, which cannot imitate the highly complex native ECM, the presence of dECM provides the right features necessary to incorporate cells.[51] The increased expression of osteogenic genes by human adi-pose-derived stem cells seeded onto a PCL-dECM scaffold, compared to those obtained from bare PCL scaffold, indicates an effective potential of dECM in bone regeneration field.[113]

Kim et al. fabricated a scaffold made from PCL, TCP and bone dECM, and evaluated its features both in vitro and in vivo, comparing them to those obtained from bare PCL-TCP scaffold. In vitro analysis revealed higher early and late osteogenic differentiation ability in PCL-TCP-bone dECM scaffold, other than better cell proliferation. Furthermore, the in vivo test performed in a rabbit calvaria defect model showed significant bone regeneration results in PCL-TCP-bone-dECM scaffold.[114]

Once isolated, bone dECM can be submitted to a specific mechanism of pepsin di-gestion, producing a solution that can undergo thermal gelation in conditions of physiological temperature and pH. However, as consequence of that treatment, dECM does not preserve the geometrical structure and architecture of native ECM.

Another fascinating application of bone dECM is the ECM ornamentation, a three-steps process in which a scaffold material (e.g., PCL) is primarily seeded with cells to promote ECM deposition, then it undergoes decellularization – leaving intact ECM – and finally it is again seeded with new cells and employed for tissue regeneration.[115] Pati et al. studied the in vitro behavior of a 3D printed scaffold made from PCL, PLGA and TCP, which was seeded with mesenchymal cells, then decellularized and after recolonized with new cells. The authors showed that the presence of a previously produced bone ECM promoted both the deposition of mineralized matrix and the osteogenic differentiations of cells. Moreover, the in vivo ectopic application on a rat model revealed a higher bone formation compared to the same, ECM-free scaffold. A similar study evaluated a PCL-HA scaffold seeded with osteoblasts, then decellularized and finally recolonized with new osteoblasts; enhanced expression of actin and vinculin, other than improved cells-scaffold and cell-cell interactions were registered by the authors. In particular, the increased interactions suggest better cell motility, adhesion and growth if compared with ECM-free PCL-HA.[115]

CONCLUSION

In the field of Dentistry, bioprinting promises to lead to a new gold standard in bone regeneration techniques. The technologies to produce bone grafts tailor made on the patient are up and coming, but Literature doesn't focus on Dentistry at the moment and it results in a lack of clinical protocols. New studies and techniques are required not only to create these protocols, but to contextualize them to the needs of the average dental practice in terms of predictability of the clinical outcome, time/cost efficiency and availability of the bio-inks.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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