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Original Research

Volume-by-volume bioprinting of chondrocytes-alginate bioinks in high temperature thermoplastic scaffolds for cartilage regeneration

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Impact statement

3D bioprinting represents a novel advance in the area of regenerative biomedicine and tissue engineering for the treatment of different pathologies, among which are those related to cartilage. Currently, the use of different thermoplastic polymers, such as PLA or PCL, for bioprinting processes presents an important limitation: the high temperatures that are required for extrusion affect the cell viability and the final characteristics of the construct. In this work, we present a novel bioprinting process called volume-by-volume (VbV) that allows us to preserve cell viability after bioprinting. This procedure allows cell injection at a safe thermoplastic temperature, and also allows the cells to be deposited in the desired areas of the construct, without the limitations caused by high temperatures. The VbV process could make it easier to bring 3D bioprinting into the clinic, allowing the generation of tissue constructs with polymers that are currently approved for clinical use.

Abstract

Biofabrication technologies with layer-by-layer simultaneous deposition of a polymeric matrix and cell-laden bioinks (also known as bioprinting) offer an alternative to conventional treatments to regenerate cartilage tissue. Thermoplastic polymers, like poly-lactic acid, are easy to print using fused deposition modeling, and the shape, mesh structure, biodegradation time, and stiffness can be easily controlled. Besides some of them being clinically approved, the high manufacturing temperatures used in bioprinting applications with these clinically available thermoplastics decrease cell viability. Geometric restriction prevents cell contact with the heated printed fibers, increasing cell viability but comprising the mechanical performance and biodegradation time of the printed parts. The objective of this study was to develop a novel volume-by-volume 3D-biofabrication process that divides the printed part into different volumes and injects the cells after each volume has been printed, once the temperature of the printed thermoplastic fibers has decreased. In order to show the suitability of this process, chondrocytes were isolated from osteoarthritic patient samples and after characterization were used to test the feasibility of the process. Human chondrocytes were bioprinted together with poly-lactic acid and apoptosis, proliferation and metabolic activity were analyzed. This novel volume-by-volume 3D-biofabrication procedure prints a mesh structure layer-by-layer with a high adhesion surface/volume ratio, driving a rapid decrease in the temperature, avoiding contact with cells in high temperature zones. In our study, chondrocytes survived the manufacturing process, with 90% of viability, 2 h after printing, and, after seven days in culture, chondrocytes proliferated and totally colonized the scaffold. The use of the volume-by-volume-based biofabrication process presented in this study shows valuable potential in the short-term development of bioprint-based clinical therapies for cartilage injuries.

Keywords: Bioprinting, additive manufacturing, scaffold, cartilage, engineering, regenerative medicine

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Introduction

Bioprinting technologies have emerged as a powerful tool for tissue engineering (TE) due to the ability to mimic the 3D structure of any tissue. The use of biomaterials, cells, and biomolecules combined with this manufacturing technique is gaining increasing interest within the scientific community.[1–3] There is a wide range of different bioprinting technologies available and the selection of an appropriate technology must be based on the characteristics of the tissue you want to regenerate.[4]

Joint cartilage is a non-vascular and non-innervated special connective tissue, composed of a specific extracellular matrix (ECM).[5] The healing process of cartilage tissue is slow and results in a fibrous scar-like tissue that lacks the functional properties of the hyaline cartilage leading to further tissue degeneration.[6] Current surgical treatments are not very effective, and this is motivating the development of new approaches. In this sense, TE and, in particular, bioprinting have emerged as a potential alternative.[7]

Additive manufacturing (AM) techniques are based on the principle of adding material layer-by-layer, allowing the manufacture of complex external and internal shapes with a mesh structure.[8–10] The use of biopolymers for AM technologies has emerged during the last years.[11,12] Parameters of the biopolymer deposition process, and the structure of the mesh, play an important role and can affect the structural, mechanical, biodegradation time, and cellular properties of AM scaffolds.[13] Among different options, thermoplastic polymers can be easily printed using the 3D printing technology known as fused deposition modeling (FDM). This technology consists of a nozzle with a heater that melts a thermoplastic filament and deposits it in a controlled and organized manner, layer-by-layer, on a surface. After one layer has been printed, the z-axis moves and a new layer is printed.[14] In this way, scaffolds are manufactured as a mesh, being rigid enough to ensure the maintenance of the required structure, and at the same time being flexible.

The use of already clinically approved thermoplastic polymers presents a promising approach in treating cartilage defects.[15,16] However, the high manufacturing temperatures of thermoplastic polymers decrease cell viability and also limit the scaffold geometry due to the need to avoid direct cell contact with the printed filaments. In order to solve these problems, low temperature biomaterials and novel printing procedures have been explored,[17] but new biomaterials need a lot of time and many procedures before they can be approved for in-patient use. Therefore, new procedures based on currently approved biomaterials have to be investigated. The aim of this work was to implement a novel bioprinting method for high melting temperature thermoplastics, such as PLA, that allows high cell viability without shape and mesh restrictions. This method consists of a volume-by-volume (VbV) 3D-biofabrication process with an algorithm that divides the printed part into different volumes and injects the cells after each volume has been printed, once the temperature of the printed thermoplastic fibers has decreased. This 3D-bioprinting method has the main objective of avoiding thermal damage to the cells. Moreover, it allows us to manufacture scaffolds with the desired biodegradation time and stiffness to enhance the performance and outcomes of the treatment. The biological feasibility of the printing process was assessed by printing bioinks of human chondrocytes together with PLA fibers and testing cell viability, distribution, and proliferation.

Materials and methods

Bioprinter and software configuration

A bioprinter with three syringes and one FDM extruder (REGEMAT 3D, Granada, Spain), consisting of hardware, Designer software (REGEMAT 3D, Granada, Spain) with the algorithms that allow the configuration of VbV, and an electronic control unit (ECU) that connects the software to the hardware, were used for the experiments (Figure 1(a) and (b)).

The bioprinting system was configured for PLA and used two syringes with needles of different diameters to inject embedded chondrocytes in alginate bioink and a calcium solution (Figure 1(b)). The position and distance between the FDM extruder and the syringes were configured to optimize the procedure. The Designer software can be set up using a simple graphical user interface (GUI) to print anatomical structures, selecting how and when the bioinks are deposited.

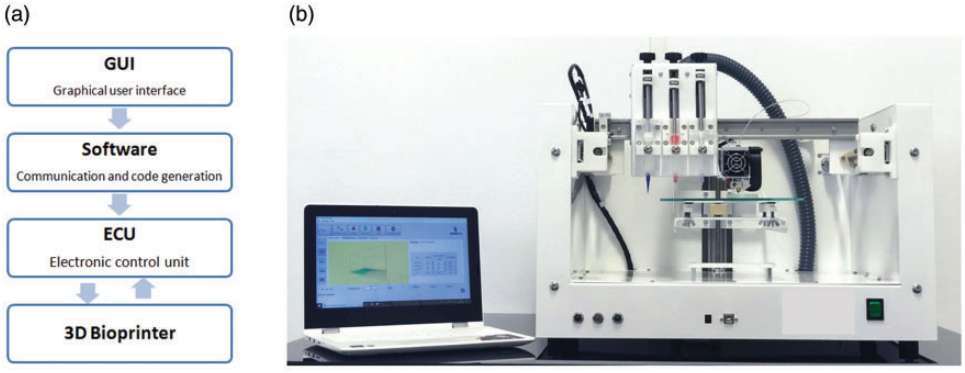


Figure 1. Bioprinter characteristics, hardware and software of the bioprinting system. Process flow of bioprinting system (a). System and bioprinter images (b). (A color version of this figure is available in the online journal.)

In order to avoid cell contact with the high temperature thermoplastic printed parts, VbV allows the deposition of x layers of the thermoplastic and afterwards the filling of the resulting volume ($x \cdot A_n$, being A_n the area of the layer n) with chondrocytes. All the data acquisition parameters have been programmed and integrated into the software's GUI for the configuration of the VbV printing procedure.

The configuration of the VbV process is quite straightforward. The number of layers printed before the resulting volume is filled has to be set. Once the layers have been printed, the syringe containing the cell-loaded hydrogel solution will penetrate into the scaffold in different points (N points), filling it up uniformly. Afterwards, a second syringe will inject the solution that will gel the hydrogel keeping the desired distribution of the cells in the scaffold. After that, the system prints the next volume layers that will be filled in a similar way. Following the configuration of the scaffold and syringe parameters, the next step is to generate the associated G code. The G code with the G commands controls the printer system and will build the geometry from the GUI-defined specifications. Once the G code is generated, the printing can be started.

Chondrocytes isolation and characterization

Articular cartilage was obtained from patients with knee osteoarthritis during joint replacement surgery. Ethical approval for the study was obtained from the Ethics Committee of the Clinical University Hospital of Málaga, Spain. Informed patient consent was obtained for all samples used in this study. None of the patients had a history of inflammatory arthritis or crystal-induced arthritis. Human articular cartilage was obtained from the femoral side, selecting the non-overload compartment: lateral condyle in varus knees and medial condyle in the valgus cases. Only cartilage that macroscopically looked relatively normal was used for this study. Human articular chondrocytes were isolated as previously described.[18] Chondrocytes were grown in Dulbecco's modified eagle's medium (DMEM) – high glucose (Sigma) supplemented with 10% fetal bovine serum (Gibco), 50 μg/μL of l-ascorbic acid 2-phosphate (Sigma), 1% penicillin-streptomycin (Sigma), and 1% ITS (Gibco) in a 25-cm^2 cell culture flask. Cells were incubated at 37°C humidified atmosphere containing 5% CO_2 and expanded in a monolayer for 7–10 days before the experiment.

Chondrocytes seeded on glass coverslips were fixed and stained with 0.4% toluidine blue staining for 15 min and then were rinsed with distilled water. For immunohistochemical analysis, fixed monolayers were blocked for 1 h at room temperature (RT) with 5% BSA, 5% fetal bovine serum (FBS) in phosphate-buffered saline (PBS), and then incubated with Col I (SC25974, St. Cruz) and Col II (SC52658, St. Cruz) overnight at 4°C. The next day, samples were washed three times with PBS and incubated with the secondary antibodies (Alexa) for 1 h at RT, and finally were washed three times with PBS and mounted with mounting medium with DAPI. Photographs were taken with a Leica DM 5500B (Solms, Germany) microscope.

VbV printing of 3D hybrid scaffolds

The biofabrication technique comprises two processes for every volume: first, the fused deposition of the thermoplastic polymer to obtain the scaffold and second, the injection of the bioink and $CaCl_2$ solution to fill the printed scaffold. Firstly, commercial non-medical grade PLA (1.75 mm filament) was used and it was melted at 210°C in a nozzle extruder and deposited, at rate of 1.4 mm/s, in a layer-by-layer manner to generate a previously designed matrix (600 μm pore size, cylinder-type structure of 10 mm diameter and 10 mm height). After printing four layers of the scaffold (0.35 mm thickness), the bioink (25 μL; 1×10^6 cells mL^{-1} encapsulated in alginate, 2% w/v) was injected into the scaffold in the selected points (speed: 8 μL/s; 21 G nozzle). Then, cell-loaded alginate solution was chemically cross-linked by following with an injection of 100 mM $CaCl_2$ (40 μL; speed: 8 μL/s). All these processes were repeated until the target dimensions of the constructs were fulfilled. Finally, all cell-laden scaffolds were cultured in medium, as previously described, at 37°C in 5% CO_2 atmosphere.

Confocal microscopy

Chondrocyte viability after the bioprinting process was assessed at 24 h and seven days using the confocal microscopy. For this purpose, samples were washed twice with PBS and incubated for 15 min at 37°C with Cell Tracker Green/CMFDA (CTG) (Invitrogen). Afterwards, samples were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 20 min at RT, and stained with DAPI. Cells were photographed by confocal microscopy (Nikon Eclipse Ti-E A1, USA) and analyzed using NIS-Elements software. Cell density was quantified using ImageJ software (v1.43, NIH) by counting the DAPI-stained nuclei.

Apoptosis evaluation

Chondrocytes cultured within the printed constructs were evaluated for apoptosis signals just after impression (0 min), 30 min and 2 h after the printing process. Cell apoptosis was checked under four different conditions in order to analyze any effect on the cells: (i) control cells before the printing process, (ii) cells embedded in alginate, (iii) printed cells with alginate, and finally (iv) printed cells with alginate over PLA fibers.

Cells were isolated using ammonium citrate (55 mM) and analyzed with Annexin V-fluorescein isothiocyanate detection kit (eBioscience Inc.) by a FACS CANTO II (BD Biosciences) using the FACS DIVA software, according to the manufacturer's instructions.

Cell proliferation assay

Alamar blue assay (Biorad) was used as a measurement for the determination of cell viability. Cell growth was analyzed at different time points: 0, 1, 2, 5 and 7 days. Scaffolds were incubated with 10 μl of Alamar blue solution per each 100 μL of media and incubated for 3 h. At this time, fluorescence intensity was measured at an excitation

wavelength of 530 nm and emission of 590 nm (Synergy HT, BIO-TEK).

Statistical analysis

Results were processed using GraphPad Prism 5.0 software for Windows (Graphpad Software, La Jolla California USA). All graphical data represent the mean +/- the 95% confidence interval (95% CI) from at least three experiments. Differences between treatments were tested using the two-tailed Student's T test. Assumptions of Student's T test (homocedasticity and normality) were tested and assured by using transformed data sets [log(dependent variable value + 1)] when necessary. P-values < 0.05 (*) and P-values < 0.01 (**) were considered statistically significant in all cases.

Results

VbV 3D bioprinting

The VbV bioprinting process allows us to configure the numbers of scaffold layers and material volume to be printed, before the volume is filled with cells, ensuring that the high temperature of the thermoplastic will not affect cell viability. The first step for printing the cell laden construct will be to upload the desired external geometry and configure the printing process and the characteristics of the scaffolds (Figure 2). These include the configuration of several parameters (Figure 2(a)) related to scaffold porosity, infill trajectories, number of external perimeters, layer height, hot end configuration (as nozzle size, temperature, ...), speed of the printing head, number of first layer "skirts", and fan cooling of the scaffold (Figure 2(b) and (c)).

Cell-laden constructs were generated using the bioprinting system composed of a head and two different syringes, one containing cell-laden pre-gel (bioink) and the other one loaded with $CaCl_2$ solution (Figure 3(a) and (b)). Using VbV, the mesh structure and shape of the construct can be printed as desired to obtain the optimal stiffness and biodegradation times and the expected outcomes for the specific application (Figure 3(c)). This method has no shape or mesh structure restriction in comparison to typical FDM deposition, which uses a zig-zag geometry to avoid cell contact with high temperature zones (Figure 4(a)).

We configured the software to allow us to select in which layer we wanted to inject from a selected syringe, the volume to be injected, and the number of points (Figure 4 (b) and (c)). The printing head has three different syringes (T0, T1, T2) that allow the injection of different types of cells or multi component hydrogels (Figure 4(b) and (c)). After this, x layers volume was printed and the syringe filled it with the cell-loaded bioink, injecting it in the N points selected by the software (Figure 4(c)).

Evaluation of the VbV printing process on cell viability and distribution

In order to demonstrate that VbV is not aggressive, chondrocytes immersed in alginate were printed together with PLA (Figure 5(a) to (c)). Freshly isolated human chondrocytes cultured in a monolayer for 7–10 days were characterized before its use. Chondrocytes displayed a typical polygonal-shape with high expression of collagen 2 and proteoglycans, and a non-detectable expression of collagen 1 (Figure 6(a)).

Apoptosis analysis was performed to quantify live/dead cells after the printing process. First, chondrocytes immersed in alginate (ALG) were bioprinted with the aim of analyzing whether ALG, $CaCl_2$ solutions, or stress induced by the nozzle affect cell viability. As shown in Figure 6(b), printed cell viability was always higher than

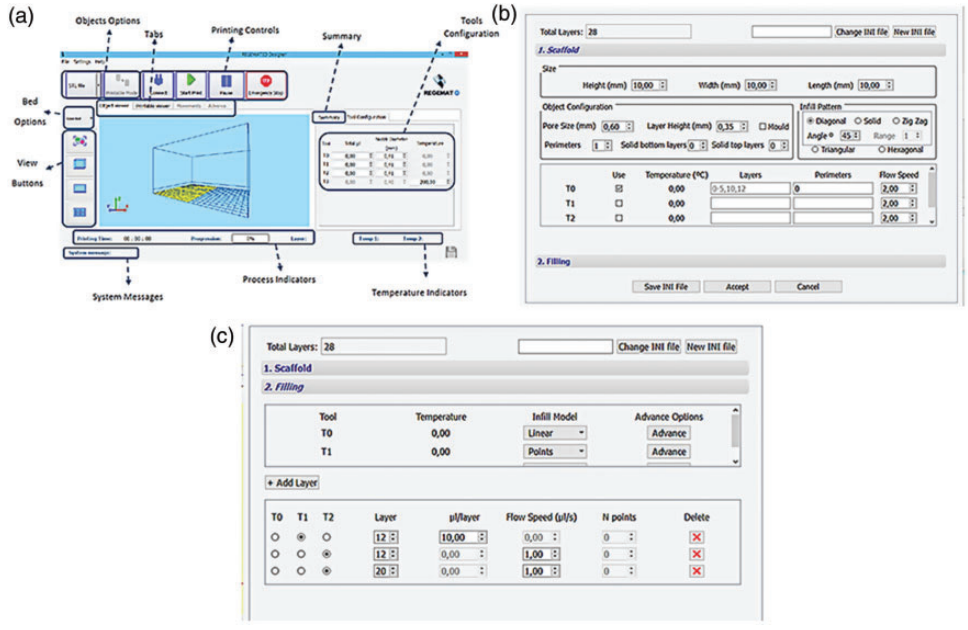


Figure 2. Bioprinter configuration. Layout of the Designer GUI (a) and scaffold parameter configuration (b). Example of VbV configuration process selecting the layer in which VbV will take place, the volume to be injected and the infill model (c). (A color version of this figure is available in the online journal.)

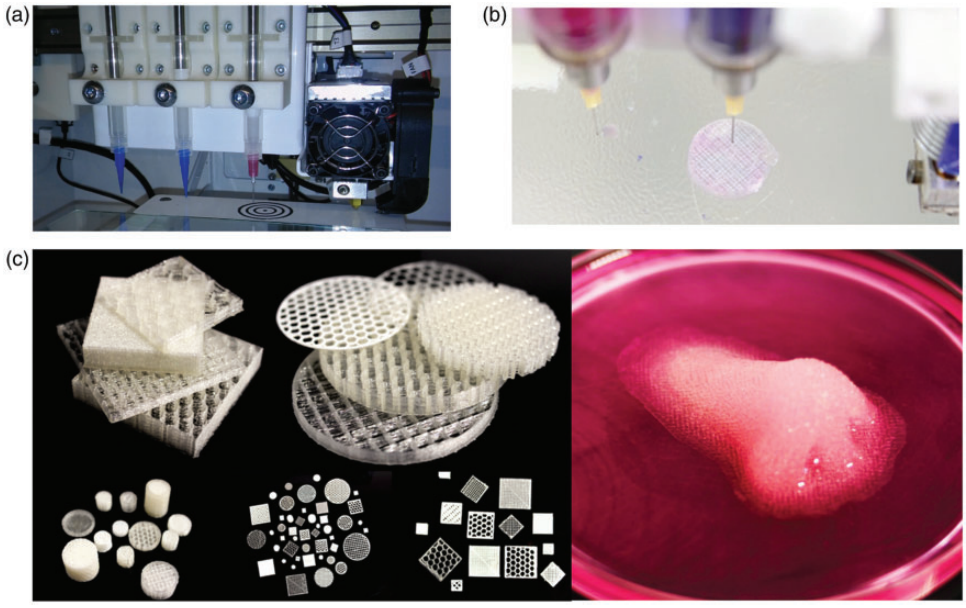


Figure 3. Bioprinter system. Configuration of the printing head, with the FDM unit (right), a syringe with a pink needle (T0) and two syringes with a blue tip (T1,T2) (left) (a). Injection volume filling after a few layers in a cylindrical shape (b). Representative examples of shapes with different mesh structures that can be printed (c). (A color version of this figure is available in the online journal.)

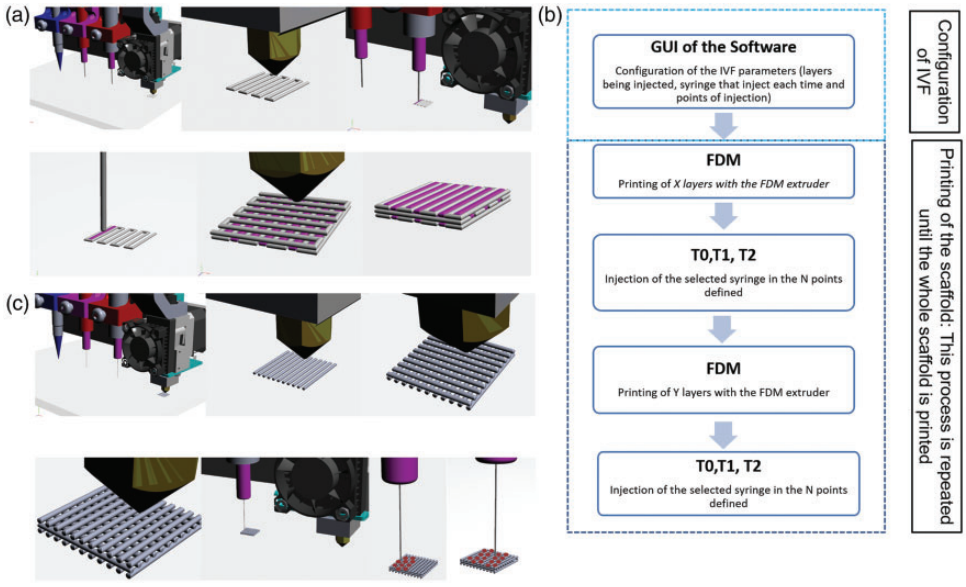


Figure 4. Comparison between conventional FDM deposition and VbV printing procedure. Schematic representation of the printing procedures found in the literature with restricted geometries to avoid contact of the cell-laden material (pink) with the high temperature parts of the printed thermoplastics (grey). FDM deposition of the first layer and filling the spaces with the cell-laden materials. Zoom of the space filling, FDM of next layer, and restricted multimaterial scaffold (a).Working process diagram represented by a schematic representation of the VbV printing procedure (b). FDM deposition of the *x* layers without restrictions in the mesh geometry. Zoom of the volume to be filled with the cell-loaded bioink, and injection in the N points selected by the software (c). (A color version of this figure is available in the online journal.)

80%, and the values of apoptosis and necrosis were similar to control cells (CTL) not exposed to the printing process. Furthermore, PLA constructs (alginate+PLA) were analyzed to determine if PLA layers had induced thermal damage. Results showed that only immediately after the printing process (0 min), live cells number decrease, and apoptotic and necrotic cells significantly increase ($P < 0.05$). However, after 30 min or 2 h in culture, chondrocytes seem to recover from the stress of the printing procedure and no significant differences in apoptosis, necrosis, or live cells when compared with CTL cells were found.

In addition, proliferation and metabolic activity of chondrocytes were quantified by Alamar blue assay. Results showed an incremental growth in the number of cells from day 0 until day 5, with a stabilization of growth between day 5 and day 7 (Figure 6(c)), which was maintained beyond seven days (data not shown). Also, cell viability and cell distribution were checked at 24 h and at

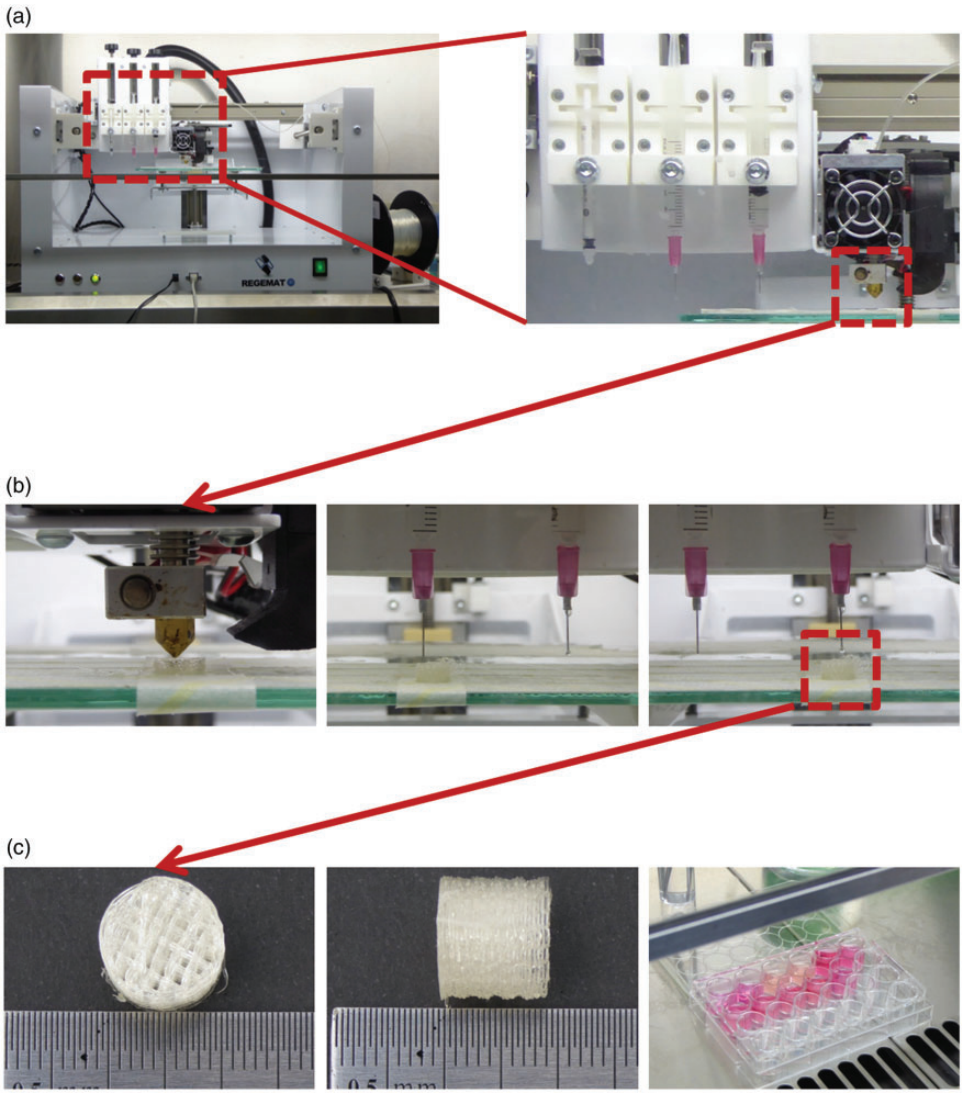


Figure 5. IVF printing process. Schematic images of the bioprinter with the FDM extruder (right) and the three syringes (T0,T1 and T2) (a) and the bioprinting process with the FDM deposition of four layers of PLA by the nozzle, and the injection of alginate with chondrocytes (T0) and calcium chloride (T1) in the selected points (b). Images of representative 3D printed scaffolds with cells embedded in alginate and cultured during seven days (c). (A color version of this figure is available in the online journal.)

seven days using CTG (Figure 6(d)). After 24 h in culture, individual cells with rounded shape appeared; however, seven days later, chondrocytes were able to migrate and proliferate throughout the scaffolds, completely colonizing the PLA fibers, and forming a homogeneous surface. The black areas in the images are the pores made when PLA fibers are crisscrossed. Also, cell density, defined as the number of DAPI positive cells per image (W: 1.27 mm, H: 1.27 mm and Z: 0.68 mm), was calculated over volume of the scaffold. At seven days, 1.423 ± 403.8 DAPI positive cells were found per 1 mm^3 of scaffold, being a total of 1,423,000 of cells per 1000 mm^3 (total volume of the scaffold).

Discussion

Many thermoplastic polymers with optimal mechanical properties for the application of cartilage regeneration, that are already approved for clinical use, have a high melting temperature that decreases the cell viability in a normal FDM bioprinting process. Low temperature biomaterials[19–21] and printing procedures[22] have been reported in the literature, but these constructs present a lower mechanical and biodegradability behavior. The optimal mechanical stiffness and biodegradation time of the construct depend on the rehabilitation procedure, and higher joint loading after surgery will demand higher stiffness of the constructs and a higher biodegradation time. These main characteristics can be tuned using a specific scaffold architecture and printed thermoplastic volume. The biodegradation time is highly dependent on the PLA composition (higher L lactic monomer, higher biodegradation time), the anatomical part inserted, the ratio of contact area/volume and the rehabilitation procedure. Different compositions of PLA will also have a big influence on the mechanical behavior. The alginate used as a cell carrier has no relevant influence on the

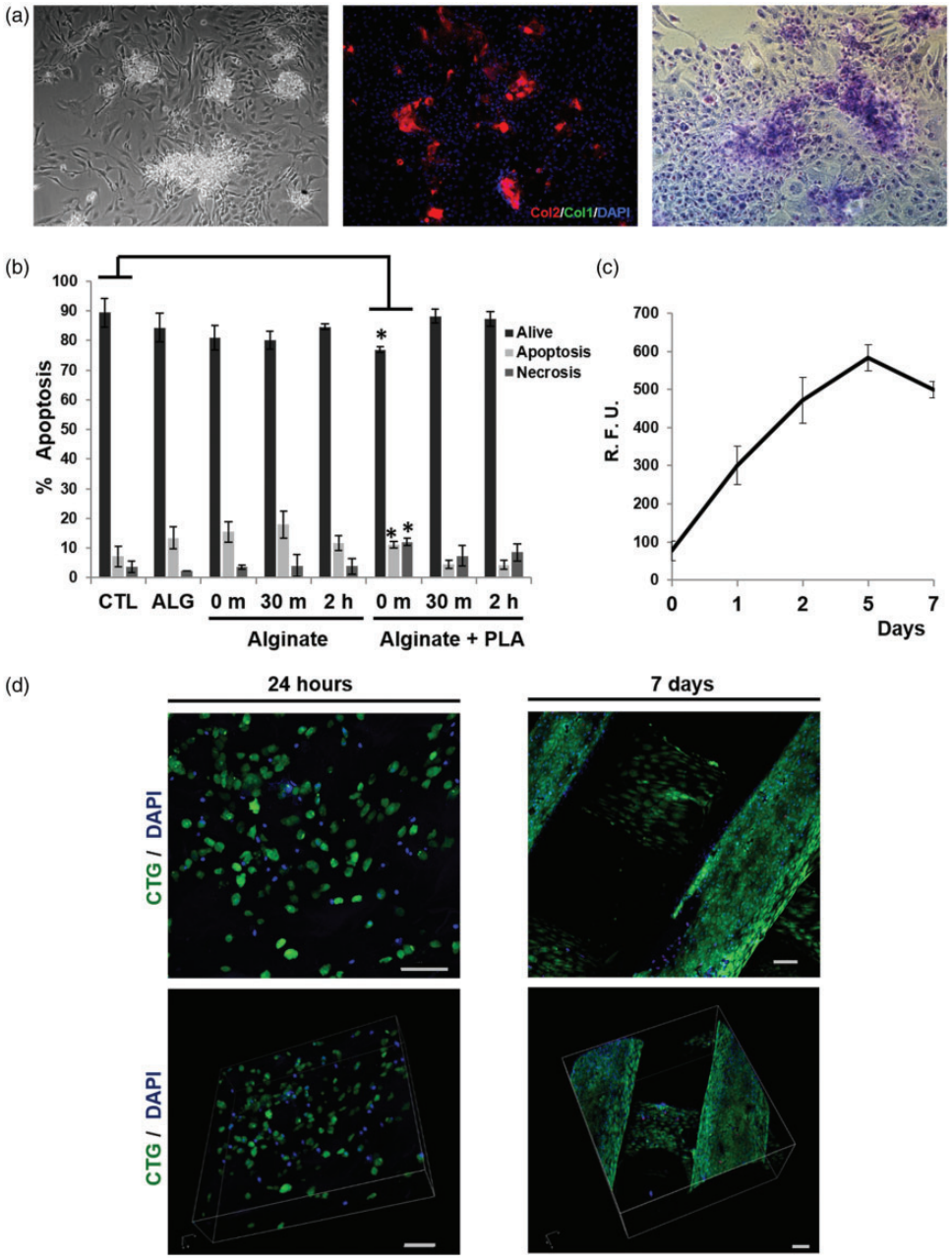


Figure 6. Effect of the VbV bioprinting process on cell viability. Characterization of freshly isolated chondrocytes that displayed a typical polygonal shape, high expression of collagen 2 (Col2) and proteoglycans. Original magnification: 10× (a). Cell viability values of chondrocytes manually printed with alginate (ALG) and bioprinted using the VbV method with alginate and alginate in combination with PLA. *$P < 0.05$ compared with control cells before the printing process (b). Cell proliferation using Alamar blue assay at different time points (c). Confocal laser scanning microscopy images of bioprinted human chondrocytes stained with CTG (green) and DAPI (blue) after 24 h and seven days. Scale bars indicate 100 μm (d). (A color version of this figure is available in the online journal.)

mechanical behavior of the construct, but is needed in order to keep the cells attached to the matrix and to promote the cell growth and ECM formation. Polycaprolactone (PCL) is one of the most representative mid-temperature thermoplastics that is already approved by the Food and Drug Administration (FDA); however, despite its very interesting properties, such as its lower stiffness and tunable biomechanical properties and degradation time, its melting temperature of around 60°C still damages cells, a restriction in the printing procedure is necessary in order to avoid the contact of the cell-laden material with the high temperature parts of the printed thermoplastics.[23,24] Also, nanocelluloses represent a material with strong potential for 3D bioprinting, with the ability to adapt to different applications including scaffolds for regenerative medicine, skin injuries and drug delivery among others, and possess suitable rheological properties.[25] Another approach to obtain biomimetic structures is the use of a decellularized matrix from original tissues, which can be filled with new cells and biomolecules. However, decellularization techniques can affect the mechanical and biological properties of the remaining ECM, and the recellularization process is

complicated as internal zones are not easily accessible and the pressure needed to inject the cells to fill up all the ECM volume may damage the cells.[25,26]

Here, we present a novel printing process named VbV that helps to overcome the problems that arise when working with high temperature thermoplastics and combine the advantages of bioprinting and the filling up of decellularized ECM with desirable geometry. VbV allows the researcher to configure the numbers of scaffold layers, shape and mesh structure of each layer or volume to be filled up with the bioink. The geometric restrictions in thermoplastic polymer scaffolds are based on the fact that in order to avoid cell damage due to high temperatures, zigzag meshes are printed so the cells are placed in the spaces in between and the next layer is built on and rests on the thermoplastic of the previous layer, and in this way cells do not touch the recently printed thermoplastic.[23,24] This is a clear geometric limitation of the mesh, and presents restrictions to the final properties of the scaffold (compromising the biodegradation time and mechanical behavior of the construct), and limits the internal contact area for cell adhesion.[27,28] Our results show that the developed procedure avoids cell damage without the geometrical restrictions that may compromise the performance of the tissue. This method has advantages when working on the regeneration of a high weight-bearing tissue such as cartilage and can also be used as a strategy to print more complex and larger sized tissues and organs.

Furthermore, scaffolds need to allow cell and biomolecule incorporation in order to promote the formation of new functional tissue.[29] Apart from the thermal damage described above, it has been shown that the long bioprinting time and the high shear stress at the nozzle decrease cell viability after extrusion, being in the range of 40–86%.[30] With the method presented here, we can significantly reduce these drawbacks and have a higher cell viability and proliferation. Therefore, hybrid-polymer constructs have been made mixing high- and low-melting temperature thermoplastics with the aim of avoiding cell death and achieving optimum mechanical, biological, and degradation properties. For example, PCL is used as a protective layer to prevent thermal damage caused by the high temperature of PLA or PLGA.[17,31] In our study, the cell viability of chondrocytes after bioprinting was close to 90% of that of cells before printing, and thus the VbV-based bioprinting process did not induce cell death with PLA material, which is one of the thermoplastics with the highest and most restrictive melting temperature.

Furthermore, adequate cell proliferation and metabolic activity together with the need to maintain fully differentiated chondrocytes after bioprinting are essential factors that determine the success of the tissue substitute.[32] VbV procedure allows chondrocytes to maintain their proliferation capacity after bioprinting, being able to homogenously colonize the scaffold in its totality. It is also well known that cell proliferation increases in constructs over time[33] and the proliferative capacity is correlated to an *in vitro* avoidance of senescence and phenotypic instability.[34] This proliferation allows us to establish cell-to-cell connections that lead to the secretion of a specific ECM and the formation of a 3D structure that mimics the natural biological function of the cartilage.[35] Our results support the advantage of the VbV bioprinting process using higher temperature thermoplastic polymers such as PLA, since cells showed high viability and proliferative capacity and low apoptosis levels after bioprinting. Moreover, this technology represents an appropriate therapeutic advance for cartilage TE, because 3D constructs mimic the natural microenvironment of cartilage tissue allowing the maintenance of chondrocyte phenotype and ECM production.[36,37]

Conclusion

In conclusion, we have shown that a novel VbV-based bioprinting method enhances chondrocyte survival and distribution within the bioprinted scaffolds, using high temperature thermoplastic without scaffold mesh geometry limitations. This new procedure avoids the contact between the thermoplastic and the cells at a higher temperature than is normally physiologically viable. VbV solves the two main complications of common bioprinting techniques: (i) it can be used with already clinically approved biomaterials and, (ii) this process does not have restrictions in geometries that could limit the clinical application of 3D bioprinting in cartilage TE. The use of a VbV 3D-biofabrication process might accelerate the clinical application of the technology and overcome the current limitation when using thermoplastics as scaffolds.

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Authors' contributions: JMB, GJ, EL-R: conception and design study, data acquisition, data analysis and interpretation, drafting the article. CA, CG-L: design study, data acquisition and drafting the article. MP: conception and design of the study, data analysis and interpretation, drafting the article. PG-M: drafting the article. JAM: conception and design of the study, data analysis and interpretation, obtaining of funding, drafting the article. All authors: final approval of submitted manuscript.

DECLARATION OF CONFLICTING INTERESTS

JMB founded the company REGEMAT 3D to transfer the results of the project to the society with the same name. The present work has been developed within the framework of the REGEMAT 3D research project. JAM is a scientific advisor to the Regemat 3D company.

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