Layer-by-layer approach for a uniformed fabrication of a cell patterned vessel-like construct

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Abstract
Successful tissue engineered small diameter blood vessels (SDBV) require manufacturing systems capable of precisely controlling different key elements, such as material composition, geometry and spatial location of specialized biomaterials and cells types. We report in this work an automated methodology that enables the manufacture of multilayer cylindrical constructs for SDBV fabrication that uses a layer-by-layer deposition approach while controlling variables such as dipping and spinning speed of a rod and biomaterial viscosity. Different biomaterials including methacrylated gelatin, alginate and chitosan were tested using this procedure to build different parts of the constructs. The system was capable of controlling dimensions of lumen from 0.5 mm up to 6 mm diameter and individual layers from 1 μm up to 400 μm thick. A cellular component was successfully added to the biomaterial in the absence of significant cytotoxic effect which was assessed by viability and proliferation assays. Additionally, cells showed a homogenous distribution with well-defined concentric patterns across the multilayer vessel grafts. The challenging generation of inner endothelial cells of approximately 20–30 μm of thickness was achieved. Preliminary experimental evidences of microstructural alignment of the biomaterial were obtained when the dipping approach was combined with the rod rotation. The study demonstrated the wide versatility and scalability of the automated system to easily and rapidly fabricate complex cellularized multilayer vascular grafts with structural configuration that resembles natural blood vessels.

1. Introduction
In 2012 over 15 million people in the United States were diagnosed with coronary heart disease [1] and it is currently the leading cause of death [2]. Coronary arteries are small diameter blood vessels (SDBV), on average 4 mm in diameter [3], and once occluded, they pose a serious risk for myocardial infarction. A stent is typically deployed to open-up the narrowed vessel; however, restenosis may occur, necessitating replacement of the vessel. Whereas large diameter blood vessels are readily substituted with Teflon or other synthetic-based constructs, they fall short of meeting the physiological requirements for SDBV, and are only replaced with an autologous graft. Large diameter vessels benefit from high blood flow velocities, which reduce blood–graft interface contact activation [4], and thereby minimize the potential for thrombus formation. The opposite is true with SDBV; low blood flow velocities increase interface time and the propensity for thrombus formation, which occludes the lumen of vessel grafts. The current standard of care for SDBV bypass surgeries are the internal mammary artery, saphenous vein, radial artery and the right...
gastroepiploic artery [5]. Nevertheless, these alternatives increase the risk of comorbidity and patients can go through several rounds of surgical procedures [6, 7]. These autografts, although mechanically inferior, provide a blood-compatible vessel solution. However, limited availability and donor site co-morbidity are major points of concern for employing an autograft for SDBV. On the other hand, differences in diameter and compliance to the natural vessels in the anastomosis area might lead to intimal hyperplasia and failure of the graft [8, 9].

Tissue engineered SDBV are poised to replace autografts by recapitulating the native structure and function of blood vessels without requiring tissue to be harvested [10–13]. Structurally, blood vessels are composed of three distinct layers: the tunica externa (adventitia), tunica media, and tunica intima. The tunica externa primarily provides a protective coating to the vessel, which doubles as an attachment point to tissues. The tunica media consists mainly of smooth muscle cells and elastic tissue, oriented circumferentially around the vessel, providing compliance and resilience to arterial pressure. Finally, the endothelial lining of the tunica intima provides the blood-compatible, luminal interface. Tissue engineering approaches to construct SDBVs have focused their research on recreating the media and the intima layers, because the non-thrombus forming surface and a mechanical behavior and durability are desirable characteristics in engineered grafts.

Many past attempts for engineering SDBV consisted of variable scaffold compositions and fabrication techniques intended to replicate vessels’ natural layers. Decellularized scaffolds employ the natural structure of allografts to provide the proper extracellular environment for subsequent cell seeding and repopulation [14–16]. Electrospinning is a novel technique that has been widely reported due to the fibrous, durable matrix it can produce [17, 18], which can be deposited in an aligned manner, wrapped around the vessel for recreating the mechanical strength that the tunica media has [19, 20] or as a scaffold for cell seeding [21, 22]. A third approach consists in dipping a thin rod into a hydrogel solution, which upon gelation provides a tubular structure [23]. This is a very promising method as it allows an easy layer-by-layer fabrication, recapitulating the unique structure and function of each vascular layer. However, it has neither been explored to generate more complex multilayer vessel-like structures nor used to fabricate cell-laden concentric layers.

Scalability and reproducibility of results is a major concern in the biomedical field, where reports indicate that up to 90% of studies could not be reproduced [24]. A reason for the lack of reproducibility is the artist-like, nuanced method by which many studies are performed, especially those of tissue engineering, where each implant is a one-off device. A means for overcoming the just-right methodology is to automate processes using simple robotics, removing the human element and standardizing the process. Never before has this prospect been as available as it is now, with simple, inexpensive microprocessors widely available, such as the Arduino platform. This open-source microprocessor is easy to program and supports a number of ‘shields’ which add functionality to the system, such as a stepper motor driver. By automatizing the fabrication of tissue engineered SDBV it is possible to dramatically increase the production volume and decrease sample-to-sample variability, which otherwise could confound results and reduce the project’s success and medical translation.

The objective of the study is to demonstrate the versatility and suitability of a device for automated fabrication of complex multilayer constructs for SDBV manufacture that uses a layer-by-layer rod dipping approach using different biomaterials. The device enables fine control over fabrication parameters, such as ascending/descending speeds, rod rotational velocity, and crosslinking time. All these technologies allow the generation of reproducible concentric patterns. Furthermore, more complex multilayer vessel-like structures were obtained through the cycles of automated dipping approach with different materials. In this work we also describe a paradigm-shifting alternative to cell seeding using a cell-compatible hydrogel that allows cells to be directly incorporated into a matrix instead of post hoc seeding. This system relies on automization of processes assisting in the effort to reduce variability and increase significance of results.

2. Materials and methods

2.1. Robotic device

A computer numeric controlled (CNC) machine that allows the controlled elevation, descent and rotation of a metal mandrel was designed and constructed in our laboratory and it is illustrated in figure 1. The robot consists of several platforms that can slide vertically, guided by steel rods, and moved by pulleys, belts and 2 NEMA 16 stepper motors (SM-42BYG011-25, Mercury Motor, China). The first motor, denominated as Motor X, allows the system to go from rest position (figure 1(a)) to working position (figure 1(b)); whereas the second motor, Motor Y, controls the mandrel dipping and the rotational movement is performed by a third NEMA 16 stepper motor, Motor Z, shown in figure 1(c).

Motors are controlled by an electronic circuit composed by different Open Source elements. The hardware consists of an Arduino UNO R3 microprocessor (50, Adafruit, USA) connected to a gShield v.5 (1750, Adafruit, USA) specially designed to allow easy control of 3 bipolar stepper motors simultaneously. The CNC machine is controlled by the Grbl firmware, which is a high performance program code
for controlling the motion of stepper motors that run on the Arduino-gShield circuit. G Code command inputs are streamed to the circuit using the universal G-Code Sender. Additionally, a moving rack system is included to allow interchange of biomaterial during multi-material based graft fabrication, and a UV lights source (365 nm, OmniCure® S2000, Excelitas Technologies, USA) to induce photo-crosslinking of specialized biomaterials when is needed. Both systems are controlled by a second Arduino UNO R3 microprocessor which is coordinated by inputs from the first Arduino. Rack movement uses a Bigeasydriver using a 5% stepper motor (10735, SparkFun Electronics, USA) to run a fourth stepper motor (see figure 1(a)), and the UV light switching is handled directly through the outputs commanding of the second Arduino. Commands for the rack and UV lights are directed using the Arduino Sketch software.

### 2.2. Hydrogels

Alginate solutions were prepared at different concentration in PBS 1×, without CaCl_2 or MgCl_2, using medium viscosity alginate (A2033, Sigma, USA). After dissolving alginate under vigorous stirring at 70 °C, solutions were filtered through a 0.45 μm syringe filter before use. Alginate hydrogels were prepared using a 5% (w/v) CaCl_2 crosslinking solution (06991, Sigma, USA) prepared fresh before each experiment.

Bovine methacrylated gelatin, or GelMa, was synthesized after mixing methacrylic anhydride with bovine gelatin and allowing methacryloyl groups react with amino groups from gelatin molecules as previously described [25, 26]. Briefly, bovine gelatin (Bloom 220, Rousselot, Netherlands) was dissolved to a final concentration of 10% (w/v) in PBS 1X (pH 7.4) at 60 °C. After fully dissolved, while still stirring, methacrylic anhydride (276685, Sigma, USA) was added slowly to a final concentration of 8% (w/v). After 3 h of reaction, 5X dilution using PBS 1X was performed and the reacted gelatin dialyzed against deionized water at 40 °C for 1 week with daily replacements of fresh deionized water to remove all unreacted methacrylic anhydride. Finally, the dialyzed mixture was filtered and freeze dried before storage.

To promote free radical crosslinking of GelMa in solution, a photoinitiator (PI) was used at different final concentrations between 0.2% (w/v) and 1% (w/v). The 5% (w/v) PI stock solution was prepared after dissolving 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiofenone (410896, Sigma, USA) in sterile PBS 1× and heated to 70 °C until fully dissolved. Fresh pre-crosslinked solutions were prepared for each experiment and the basal GelMa solution was consisted of 10% (w/v) methacrylated gelatin and 0.5% (w/v) PI in PBS 1×. To improve transparency, small precipitants were removed after filtering through a 0.45 μm syringe filter.

Chitosan (CS) solutions were prepared at different concentrations after dissolving chitosan (44887, Sigma, USA) in 1% (v/v) acetic acid solution (537020, Sigma, USA) to a final concentration of 2% or 1% (w/v) and filtered through a 0.45 μm syringe filter before use. The tripolyphosphate (TPP) solution, for chitosan ionic gelation during immersion was prepared by dissolving sodium tripolyphosphate (72061, Sigma, USA) in ddH_2O to a final concentration of 4% (w/v). Thickness of the chitosan layers were calculated as half the difference between the diameter of the alginate mandrel coated with chitosan coating and the average mandrel diameter (Thickness = (D_{mandrel} + CS layer − D_{mandrel}) / 2).

### 2.3. Viscosity measurements

For viscosity measurements, samples were equilibrated for 20–25 min at 37 °C. An Anton Paar MCR 301 rheometer equipped with a cone-plate geometry (plate diameter of 50 mm and cone opening angle of 0.5°) was used to investigate the solutions viscosity at different shear rate. A shear flow test with shear rate ramp from 10 to 1000 s⁻¹ was performed at 37 °C. Viscosity data shown in this paper was obtained at a shear rate of 100 s⁻¹.
2.4. Fabrication of multilayer cylinder structures

2.4.1. Fabrication and analysis of lumen mandrel structures

The strategy used for the hollow lumen formation is through the disposition of layers around an alginate-based mandrel of 2–6 mm in diameter, which can later be dissolved for removal. The remaining cylindrical structure would have an emptied centric region mimicking the luminal part of vascular vessels.

These alginate mandrels with controllable diameter were built after dipping a thin metal mandrel of 0.5 mm in diameter in dissolved alginate, then submerged for 15 s in the CaCl₂ crosslinking solution for ionic gelation, and finally immersed in PBS for 1 min for cleansing. To fabricate structures with larger diameters, successive dipping rounds were performed (see figure S1(A) for further explanation). The first round always consisted of 2 dippings in alginate solution (parameter obtained through optimization), crosslinking and a PBS washing, whereas the successive rounds consisted either of 1, 2, 3 or 4 alginate dippings, followed by ionic crosslinking and PBS washing. Each mandrel structure obtained with different combination of fabrication parameters were repeated 3 times for statistical analysis. In order to observe the structural homogeneity along the construct, the total length was divided in three different sections of equal length, and defined as front, mid and tail. Head section as illustrated in figure 2(a) was removed and not considered for vessel graft fabrication.

For every condition (viscosity/upward-speed), one experiment with 3 replicates was performed, and 7 diameter measurements were obtained per construct section (tail, mid, front) in each replicate.

For multilayer cylindrical construct fabrication, usable length of the alginate mandrel defines the section of the mandrel in which there is an adequate homogeneity of diameters along the construct and is delimited by a front and a tail end. These limits are determined by simple arithmetic comparison between the diameter of a given point and the accumulative average diameter of the previous section. Briefly, starting from the middle of the construct, diameter measurements \( D_n \) are performed every 2.5 mm moving either toward the front or the tail end. The front and tail limit is then set at the point \( n \) in which \( D_n - \frac{1}{n-1} \sum_{i=1}^{n-1} D_i \) \( \geq 0.5 \).

2.4.2. Layers

Layers of gelatin were deposited by dipping the metal rod harboring a previously constructed alginate mandrel, in a solution of 10% (w/v) GelMa, 0.5% (w/v) PI, with or without supplemented alginate at different concentrations (see figure S1(B)). Alternatively, HUVEC cells or 1 μg ml⁻¹ BSA-FITC (A9771, Sigma, USA) were added to the pre-crosslinked solution in order to obtain cellularized or fluorescent layers respectively. Solutions were kept in a water bath at 30 °C to avoid spontaneous gelation at room temperature. Crosslinking was achieved by irradiating the gelatin solution with UV light at 365 nm wavelength (261 mW cm⁻²) from a distance of 2 cm while the GelMa coated mandrel emerged rotating from the pre-crosslinked solutions.

Chitosan layers were fabricated by dipping the alginate rod twice in a chitosan solution at a defined dipping speed, once in TPP for 15 s to promote ionic crosslinking and washed in PBS 1X for 1 min afterward.

For every condition (viscosity/number of dippings/cell density), one experiment with 3 replicates were performed, and 7 diameter measurements were obtained per construct section (front, mid) in each replicate, while only one length measurement was performed per replicate.

2.4.3. Removal/dissolution of the alginate mandrel

To remove the alginate mandrel, the cellularized cylindrical construct were submitted to incubation in 2% (w/v) TPP solution for 10 min after fabrication, followed by the gently removal of the thin metal rod. TPP is capable to quellate Ca²⁺ cations and induce de-crosslinking of the alginate hydrogel, then, alginate works as a sacrificial material. An alternative approach, but not used in this work, consisted of immersing the cylindrical construct in a 2% (w/v) CaCl₂ solution, inducing further calcium-driven compaction of the alginate mandrel creating space between the alginate mandrel and the cylindrical layers, allowing gentle mandrel removal from the inside of the construct.

2.5. Cell culture and cell encapsulation

HUVEC-EC-C [HUVEC] (ATCC® CRL1730™) cells were cultured in high glucose Dulbecco’s Modified Eagle Medium (10313, Gibco, USA) supplemented with 2 mM glutamine (25030-081, Gibco, USA), 10% (v/v) fetal bovine serum (16000-044, Gibco, USA) and 1% (v/v) penicillin-streptomycin (15140-122, Gibco, USA). Cultures were kept in an incubator at 37 °C and 96% humidity with 5% CO₂ enriched air atmosphere. Cells were maintained changing fresh media every 2–3 days and passaged when 80% cell confluence was reached. For encapsulation of cells in gelatin-based hydrogels, fresh pre-crosslinked solution (e.g. 10% (w/v) modified gelatin, 0.2% (w/v) PI in PBS 1X) is mixed with cells at the desired ratio, poured on the appropriated container for using in the automated system and maintained at 30 °C irradiated. During multilayer cylinder fabrication cells are encapsulated after irradiation with UV light at 365 nm wavelength (e.g. 261 mW cm⁻²) during 4 s (OmniCure® S2000, Excilites Technologies, USA).

For every condition (UV/PI concentration), one experiment with 3 replicates was performed. The percentage of encapsulated cell was calculated in each replicate by cell counting in 4 separated quadrants at
the different sections of the construct (front and mid) in order to estimate the cells per defined volume.

2.6. Proliferation and viability testing

Cell proliferation assessment of encapsulated HUVEC in the crosslinked biomaterials were performed using the WST Cell Proliferation Colorimetric Assay Kit (K302, Biovision, USA) following the manufacturer instructions. Briefly, this assay quantifies the metabolic cleavage of tetrazolium salt to formazan by cellular mitochondrial dehydrogenase. For this assay 30 μL of a 2 × 10^6 cells mL^-1 in a 10% (w/v) GelMA solution, supplemented with 0.1% (w/v) alginate and PI at different concentrations was deposited in 96 well plates and crosslinked for 4 s; media was changed 3 times a week throughout the experiment and WST incubation lasted 120 min.

On the other hand, cell viability of encapsulated HUVECs was determined using the LIVE/DEAD® Cell Imaging Kit (488/570) (R37601, Thermo Fisher Scientifics, USA). To improve diffusion of reagents the hydrogel with cells was 0.145 mm thick and its area was approximately 3.5 cm². Media was changed 3 times a week throughout the experiment and LIVE/DEAD reagents were incubated for 15 min before fluorescent imaging.

In both assays, 1 experiment per condition and 4 replicates per experiments were performed.

2.7. Image analysis

Image analysis to determine structures’ dimensions, such as length and thickness, were performed using the open source and Java-based imaging program ImageJ (National Institutes of Health). For diameter
and layer thickness measurements, longitudinal phase contrast imaging of the multilayer cylindrical constructs were performed using an inverted microscope (CKX41, Olympus, USA). Image focus was set at the transversal center of the construct (half distance between the bottom and upper part of the graft placed longitudinally at the microscope). This allows clear distance measurement between layer borders for diameter and layer thickness calculation. Measurements were performed at 7 equidistant points along every section of the construct (front, mid, tail), and average and standard deviations were calculated.

2.8. Scanning electron microscopy (SEM)
Cylindrical multilayers constructs were dehydrated through a series of 1 h incubations in ethanol solutions (20%, 40%, 60%, 80%, 100%). Afterwards, samples were submitted to critical point drying for 1 h using a Polaron E3000 CPD apparatus. Dryed constructs were mounted on a copper plate and coated with gold using a sputter coating unit (108 Auto Sputter Coater, Cressington). Imaging was performed using the SEM LEO 1420 VP.

2.9. Statistical analysis
Data are presented as means ± standard deviation. Differences between means were tested by Student’s t-test. A probability level of $P < 0.05$ was considered to be significant.

3. Results
3.1. Parameterization of fabrication of complex cylindrical structures using biomaterials

3.1.1. Lumen mandrel structure
In order to standardize luminal diameter of SDBV using the strategy of sacrificial alginate mandrel and to determine the most suitable parameters that control the diameter and lumen dimensional uniformity, several parameters were optimized. Among those, the effect of alginate concentration, number of dippings and mandrel upward-speed during emersion from the pre-crosslinked alginate solution, in the process of alginate mandrels fabrication were studied. The studied range of alginate concentration spans between 1% and 3% (w/v) with interval of 0.5% increments, giving viscosity values of $84 \pm 5$, $124 \pm 9$, $165 \pm 11$, $198 \pm 11$ and $245 \pm 15$ cP respectively. The number of dippings tested before cross-linking were 1 to 4, and finally, the range of upward-speeds studied goes from 4.6 to 184 mm s$^{-1}$. Figure 2(a) illustrates the morphology and shape variation of the alginate mandrel throughout its different sections considered for dimensional analysis.

Structures fabricated with 1% and 1.5% alginate solutions, or 84 and 124 cP respectively, at any upward-speed and with 2% alginate (165 cP) with upward-speeds below $23$ mm s$^{-1}$ were highly irregular and not suitable for analysis. Visual inspection of these constructs indicated that when working under those parameters, the slight vibration of the CNC machine has a negative effect on the structural regularity inducing a rippled surface pattern.

Figures 2(b)–(d) show the diameter of alginate structures at the front, mid and tail sections after a first round of alginate deposition at different concentrations of alginate and mandrel’s upward-speed. An overall analysis of the graph indicates that diameter expands along with upward-speed increments, reaching its maximum value at a speed of $92$ mm s$^{-1}$. At higher speeds the diameters get smaller. Examining the results for each alginate concentration separately (see figures 2(e)–(g)), it is possible to observe that mandrel structures fabricated with 2% (w/v) alginate (165 cP) are the most uniform. This was concluded because structures fabricated with these parameters had the most similar diameter values comparing front, mid and tail sections, with no significant difference between those values at any speed. Whereas most 2.5% alginate (198 ± 11 cP) constructs show diameters increment going from mid to tail, presenting statistical differences in average diameters between these two sections. For alginate structures using an alginate solution at 245 ± 15 cP in viscosity, which appear to be the most irregular ones, significant differences are observed when comparing all different sections. In conclusion, results indicate that the combination of alginate at 2% concentration, viscosity of 165 ± 11 cP and 138 mm s$^{-1}$ upward-speed are the most suitable parameters to obtain uniform structures after 1 round of material deposition.

Given the parameter optimization described above, the alginate mandrel diameter had to be increased with additional alginate layers. Different alginate concentrations were tested, however, and considering the previous findings, upward-speed was set only at 138 mm s$^{-1}$ and the results are exhibited in figures 2(h)–(j).

Concentric layers were added by dipping the alginate mandrel, previously fabricated with the optimized parameters, 1–4 additional times in alginate solutions of 1%, 1.5%, 2%, 2.5% and 3%, or 84 ± 5, 124 ± 9, 165 ± 11, 198 ± 11 and 245 ± 15 cP respectively, before crosslinking. Constructs with 2.5% and 3% alginate were irregular and therefore excluded from the analysis. Overall, the obtained structures with 1%, 1.5%, and 2% alginate presented small differences in diameter between front, mid and tail sections, therefore the observed diameter values in figures 2(h)–(k) correspond to the mean diameter considering all different sections. It is possible to observe that additional dipping before crosslinking did not necessarily increase the structures’ average diameter or length but had an impact on reducing the mean length’s standard deviation. Additionally, there is no significant difference in the diameter of constructed mandrels using 3 and 4 dips with 84 cP and
124 cP alginate before crosslinking. In contrast, structures obtained after using 1, 2, 3 or 4 dips in 165 cP alginate solution are significantly thicker than those with alginate at lower viscosity, reaching a maximum of 4.3 mm with 1 dip. Lastly, 4 dips of alginate at 165 cP before crosslinking appear to be the best setting because of the small standard deviation in diameter and length.

To achieve a final diameter of 6 mm, which is the upper range limit for coronary arteries, a third round of alginate dipping and ionic crosslinking was performed. Considering the previous results, testing of the dipping number before crosslinking was done using only alginate at 165 cP and 138 mm s\(^{-1}\) upward-speed. Interestingly, the double dipped structures in the third round of dipping/crosslinking showed an average diameter of 6 and 50 mm in length as depicted in figure 2(k). As discussed above, this data does not reveal any correlation between structure diameter or length and the number of dipping before crosslinking, but as dipping number increases, the structures’ dimensions have lower standard deviation, especially in length.

3.1.2. Layers
Natural blood vessel’s structure consists of a series of layers with different thickness, cellular content and extracellular matrix composition, therefore in this work; we explored the control in thickness for layers generated with different biomaterials and cellular content.

3.1.2.1. GelMa-alginate layers
10% (w/v) GelMa solutions supplemented with variable concentrations of alginate and 0.5% (w/v) PI were prepared as described in the materials and methods section, and layers were deposited over an alginate mandrel structure obtained from one round of dippings and crosslinking (2 dippings, 165 cP alginate and 138 mm s\(^{-1}\) upward-speed). Variable concentrations of alginate were tested in order to obtain a GelMa solutions with a wide viscosity range, and to test the effect of viscosity in the control of layer thickness. Average thickness of layers obtained at different concentration of supplemented alginate at the front and mid sections are shown in figure 3(a), deposition of layers on the tail were uneven and therefore excluded from the analysis. Within a low range of alginate concentrations, ranging from 0.025% up to 0.075% (w/v), with corresponding viscosities values from 5.8 to 9.8 cP, no statistical differences in thickness were observed (~0.04 mm), and the standard deviations were maintained at lower values. However, a rapid increment in thickness is observed (up to ~0.3 mm) when gelatin solutions with viscosities from 9.8 to 15.8 cP (0.1%–0.15% (w/v)) were used. In contrast, statistical differences were not observed at different viscosities ranging from 15.8 to 125 cP (0.15%–1.5% (w/v)). In summary, thicknesses at low viscosities are statistically different from those using higher viscosity solutions, however the fine tuning in vessel thickness is only possible within the short range from 9.8 to 15.8 cP when 10% (w/v) GelMa solution is supplemented with different concentrations of alginate. Due to the crosslinking of layers are induced simultaneously during emersion, and immediately after every section of the cylindrical graft is egressing from the pre-crosslinked solution, upward-speed was fixed at 6.9 mm s\(^{-1}\) and rotational speed of the mandrel at 42 rpm for homogeneous UV irradiation.

3.1.2.2. Chitosan coating
The effect of chitosan concentrations and the upward-speed on the final deposition of crosslinked chitosan were assessed by measuring the thickness of concentric chitosan layers encircling the alginate mandrel fabricated previously. To describe the chitosan concentration effect, 1%, 1.5% and 2% (w/v) chitosan solutions were used at a fixed upward-speed of 138 mm s\(^{-1}\). These concentrations resulted in solutions at 100, 141 and 182 cP respectively. The effect of upward-speed over thickness was evaluated fabricating chitosan layers using a solution of 1% (w/v) chitosan with 100 cP in viscosity and two different upward-speeds ranging from 23 to 138 mm s\(^{-1}\). Finally, due to lack of transparency of chitosan layer, thickness was calculated as half the difference between the diameter of the alginate mandrel and the resulting diameter of the whole construct including the chitosan layer (see figure 3(b)), as explained in the Material and Methods section.

The concentration effect (figure 3(c)) and the influence of upward-speed on the layer’s thickness in the front and middle sections measurements (figure 3(d)) show no obvious correlation between the studied parameters and the level of deposited material at any point of the construct. Nevertheless, layers of chitosan at 100 cP showed more dimensional similarity between the front and mid sections, making this concentration more appropriate to construct multilayer structures with uniform metrical properties along its length (figure 3(d)). A similar analysis in figure 3(d), indicates 23 and 138 mm s\(^{-1}\) upward-speeds as right fabrication parameter since the resulting structure in both cases does not show any dimensionally significant difference at the front and middle section.

Similarly to alginate, viscosity increments have the tendency to generate thicker concentric layers, however at 182 cP this correlation is lost. On the other side, and similarly to alginate, there is a positive proportionality between upward-speed and layer thickness. Hence, the introduction of a different biomaterial such as chitosan can be also adapted to generate concentric multilayers with controllable thickness as shown for alginate and gelatin.
3.2. Cell encapsulation

Functional SDBV grafts can only be achieved with the appropriate cellular contents that are commonly found in natural vessel structures. Therefore, we investigated the feasibility of adding concentric multi-layers of cell-laden hydrogel using the automated system. In order to achieve this, HUVECs (endothelial cells) were added to a final concentration of $4 \times 10^6$, $6 \times 10^6$, and $8 \times 10^6$ cells ml$^{-1}$ to a 10\% (w/v) GelMa, 0.5\% (w/v) PI and 0.5\% (w/v) alginate solution; the layer was formed after round material deposition over an alginate mandrel. UV crosslinking was performed using an emersion speed of 6.9 mm s$^{-1}$ and a rotational speed of the mandrel of 42 rpm. A photograph of the resulting layer can be seen in figure 4(a) as well as a zoom in in figure 4(b). These results evidence the capability of the dipping technology to allows the fabrication of a complete layer of encapsulated cells that are evenly distributed along the axial axis.

The effect of cell concentration on the thickness of the cellularized layer is shown in figure 4(c). As expected, no significant difference in thickness of the layers was found at different cell concentrations. The front and middle section are very similar when cells are seeded at $4 \times 10^6$ and $6 \times 10^6$ cells ml$^{-1}$ and it is slightly wider at $8 \times 10^6$ cells ml$^{-1}$, but under this last condition the values of layer’s thickness has a broader distributions.

Figure 4(d) shows how cell concentration in the pre-crosslinked GelMA solution affects the concentration of cells encapsulated in the layer. Concentration of seeded cells showed no significant effect on the concentration of encapsulated cells, but it is possible to observe a tendency for higher amounts of encapsulated cells as the concentration of cells mixed in the pre-crosslinked solution increases. Percentage of encapsulated cells was determined after counting the total cell number in a determined volume of the cylindrical section, and dividing that number by the cell number contained in the same volume of the original cellularized dipping solution. Percentage of encapsulated cells at the front and mid sections of the construct is shown in figure 4(e). Finally, it is possible to conclude from the figures that when cells are seeded at $8 \times 10^6$ cells ml$^{-1}$ constructs appear to have a more even distribution of cells across its axial axis, as evidences by the similar number of encapsulated cells at the front and mid sections.

3.2.1. Cell recovery after encapsulation

Compared to non-biological fabrication, biofabrication with cell content involves additional complexities related to the sensitivities of living cells and the
construction of tissues. Mechanical stress, UV treatment required for chemical crosslinking and cell handling in non-physiological conditions are some of the many insults that can either alter the cell function or result in their death. For this, it is relevant to adapt the used technologies to the cellular requirements, making relevant the choice of materials devoid of cytotoxic effect, stress-resistant cell types, and appropriate growth and differentiation factors. In order to determine the effect of PI, UV light and the encapsulation process on cells different experiments were performed.

Figure 4. Cellularized layer experiments. (a) Side view of a construct with an outer layer of 10% (w/v) GelMa, 0.5% (w/v) alginate and $6 \times 10^6$ HUVEC cells ml$^{-1}$. (b) Zoom in of the previous construct. (c) Effect of the cell concentration in the pre-crosslinked solution on the layers’ thickness, (d) cell density in the cross-linked layer as a function of cell concentration in the pre-crosslinked solution, (e) Percentage of cells of the pre-crosslinked solution encapsulated in the layer; Proliferation and viability assessment of encapsulated cells. (f) Effect of UV and PI on HUVEC 2D cultures, (g) Time-dependent viability of encapsulated cells with different UV conditions, (h) LIVE/DEAD images of encapsulated cells at different time intervals and different conditions, (i)–(k) Effect of different PI concentrations on cell proliferation when using UV irradiation at 121 mW cm$^{-2}$, 261 mW cm$^{-2}$ and 323 mW cm$^{-2}$ respectively.
The first experiment consisted in exposing cells to PI (0.2%, 0.5% and 1% (w/v)), UV light (261 and 323 mW cm\(^{-2}\), 3 s) and PI and UV light simultaneously (0.2% (w/v) PI + 261 mW cm\(^{-2}\), 0.5% (w/v) PI + 261 mW cm\(^{-2}\) and 1% (w/v) PI + 323 mW cm\(^{-2}\), UV light for 3 s). 100 cells were seeded in 96 well plates and exposed to the different conditions; afterwards proliferation was determined using the WST assay on days 1, 4, 7, 11 and 14. As seen conditions; afterwards proliferation was determined using the WST assay on days 1, 4, 7, 11 and 14. As seen in figure 4(f) the solely presence of PI does not affect significantly the viability or proliferation of cells, especially at lower concentrations (0.2%) where no effect could be observed. Although concerns exist regarding UV light, cultures were able to recover and match the metabolic activity of cells.

The effect of UV light (121, 261 and 323 mW cm\(^{-2}\)) on viability on day 0, 1, 3 and 7 after encapsulation was also studied and results are shown in figures 4(g) and (h). These showed important robustness of the crosslinking strategy when using different irradiation levels with 0.5% PI, observing similar results on day 0, 1 and 3. However, on day 7, viability at irradiation of 121 mW cm\(^{-2}\) was 60%, whereas the higher irradiation level showed decreased to 40% of viable cells, indicating a postponed cellular death possibly due to prolonged exposure of cells to higher contents of free radicals generated from the exposure of PI to higher levels of UV. Additionally, because cells were able to maintain higher levels of viability, although using toxic concentration of PI according to the experiments in absence of photosensitive biomaterial, it is suggested that the methacryloyl groups present in GelMa might have a protective effect against the free radicals delivered by the PI after photoinduction.

Fabrication conditions can still be adjusted in order to improved cell viability, especially by the reduction in the concentration of PI, for example up to 0.2%. If the PI concentration is too low, crosslinking reactivity of the polymer solution is low as well, making the formation of hydrogel layers difficult. PI concentration of 0.05% was insufficient to fabrication hydrogel layers, whereas above 0.1% PI layers were formed under the chosen conditions and UV irradiation. However, thickness control based on viscosity was lost or difficult to reproduce at PI concentration lower than 0.2%, requiring in general a higher viscosity to form hydrogel layers. The explanation behind this effect is related to the require higher retention time of the pre-crosslinking solution on the alginate mandrel to allow polymerization when photo-initiator is less concentrated, and this is obtain through increments in viscosity. Otherwise, low viscous solutions rapidly flow down the alginate mandrel during emersion without allowing sufficient UV exposure in order to form the hydrogel from less reactive solutions.

Figures 4(i)–(k) show the combined effect of different PI concentrations and levels of UV irradiation on cell proliferation under encapsulated conditions. Results of proliferation of cell samples at different time intervals with equal amounts of PI, but crosslinked using different doses of UV irradiation, showed no statistical differences as expected. However, when comparisons of time-dependent proliferation of cells samples crosslinked using the same UV irradiation but with different content of PI, significant differences were observed, revealing a dose dependent toxic effect of the PI, which is consistent with results reported above. This observation is in line with previous results published by other authors who established a broader deleterious effect of PI compared to the effect of UV alone [27, 28].

### 3.3. Biomaterial micro-deposition

In the typical structural configuration of natural SDBV, intercalated thin elastin laminas and elastin fibers across the vessel layers are major components responsible for the mechanical properties of vessels. A sub-endothelial internal elastic membrane separates the endothelial layer from the layer that contains the smooth muscles cells (tunica media), which contains as well interpenetrated but few elastin fibers. Between the tunica media and the adventitia layer a second elastin membrane is locate and it constitutes a second mechanically important element of vascular tissues. Fabrication of tissue engineered SDBV capable of mimicking the natural configuration of important thin layers, such as endothelial layer and elastin membranes, would ideally require a high precision method for micro-deposition of thin biomaterial layers and cells.

In order to explore the feasibility of controlling micro-deposition of biomaterial with our automated system, low viscous 10% (w/v) GelMa with 0.5% (w/v) PI solution was used to deposit thin gelatin layers over a previously fabricated alginate mandrel. Figure 5(a) shows the resulting a cylindrical structure composed of intercalated layers of crosslinked GelMa with and without BSA-FITC. Each layer is approximately 25 μm thick and was fabricated with 25 rounds of GelMa deposition which indicates that every deposited micro-layer in a single round is of about 1 μm thick.

In a similar way, the system was subjected to different testing in order to get a consistent and very thin layer with encapsulated HUVEC cells, with the goal of forming an endothelial-like layer. Using a programed protocol of 3 rounds of GelMa deposition using a solution of 10% (w/v) GelMa, 0.5% (w/v) PI, 0.1% (w/v) alginate and 5 × 10^6 cells ml\(^{-1}\), where UV irradiation
was 261 mW cm$^{-2}$, upward-speed 6.9 mm s$^{-1}$ and a rotational speed of the mandrel 42 rpm. The dipping-spinning machine was able to obtain a highly cellularized layer of approximately 20–30 μm thick (see figure 5(b)). A future perspective is that this HUVEC-laden hydrogel would constitute a confluent monolayer after appropriated hydrodynamic stimulation during in vitro or in vivo maturation, layer that could derived from well differentiated mesenchymal stem cells as well [29, 30].

3.4. Control of microstructural alignment of gelatin molecules

It has been well described how micro- and nanostructural alignment of biomaterials impact cell alignment [31–33]. This is a key issue in tissue engineering because cells alignment has a fundamental role in the functionality of tissues, and blood vessels are not an exception. Circumferential aligning of smooth muscle cells confers the ability of contraction/dilation and blood pressure control to the arteries, whereas the alignment of endothelial cells in the direction of the flow reduces the presence of cell adhesion molecules, hence decreasing platelet and leukocyte attachment phenomenon [34].

In the present automated device, the rotational movement of Motor Z is not only used for homogeneous UV exposure of the construct from a lateral fixed UV source, but it allows the controlling matrix alignment of crosslinked hydrogels by modifying the rotational speed of motor Z. We tested two biomaterial composites, one comprised of 10% (w/v) GelMa concentration and 0.1% (w/v) alginate with 0.5% (w/v) PI, and the second was based on 10% (w/v) GelMa with 0.5% (w/v) PI. Graft fabrication was performed with 50 rounds of material deposition using 3 different rotational speeds (14, 98 and 210 rpm).

Correlation between rotational speed and matrix alignment can be observed in figure 6, showing a more evident circumferential alignment at higher speeds. Notably, by controlling rotational speed and therefore the alignment of the matrix, it could be expected the deposition of a more adequate functional media layer when the pre-crosslinked solution is mixed, for example, with smooth muscle cells. This effect has been previously described by other researchers who used smooth muscle cells or others cells in different systems, generating scaffolds with circumferential alignments [35–37]. Although further analytical studies are required, the observation of oriented collagen-like fibers at higher rotational speed, especially at 98 rpm with only gelatin (figure 6(e)), could be the consequence of mechanical stresses generated at the interface between the rotating cylindrical structure and the pre-crosslinked solution, where abundant formation of helix-coil could be stimulated by mechanical means as suggested by the reported evidence in other systems by Courty and collaborators [38].

3.5. Complex multilayer structure fabrication

Even though the fabrication system proposed in this work follows relatively simple procedures, complex structures with precise positioning of layers can be constructed with this automated method. The precision level of encapsulated cells positioning was evaluated in structures built over an alginate mandrel structure, and consist of four hydrogel layers of 10% (w/v) GelMa with 0.2% (w/v) alginate, where only 1
Figure 6. Micro-structural alignment of 10% (w/v) GelMa supplemented with 0.1% (w/v) alginate (a)–(c) and 10% (w/v) GelMa hydrogels (d)–(f). Scanning electron microscopy of cylindrical hydrogel structures fabricated at a rotational speed of 14 (a), (d), 98 (b), (e) and 210 (c), (f) rpm respectively. Orientation of micro and nano structures in the transversal cut of walls in constructs fabricated at different rotational speeds are shown with an amplification of 500X, 5000X and 30 000X.

Figure 7. Concentric positioning of cellularized layers. Four different 4 layers constructs harboring a single cellularized layer at different concentric positions were fabricated to show precise localization of cell content in a fabrication process of a vessel-like graft. Location order goes from the inner layers to outer layers (a)–(d), respectively. Figure shows transversal and sagittal microscopy view of the fabricated grafts. In each sections of the figure, zoom-out and zoom-in images of the cylindrical constructs are shown. The cells at the cellularized layer of the zoom-out images in each section of the figures, were treated digitally using the Image J program in order to facilitate visualization. Additionally, the five double-headed arrows indicate the borders of the 4 different layers. Two replicates of cellularized graft construct were performed and analyzed per every cell patterning condition.
of these layers contained encapsulated cells (figures 7(a)–(d)). The hydrogel layer with encapsulated cells was first positioned beside the luminal area (figure 7(a)) followed by the subsequent non-cellulared layers. In the consecutive pictures the cell-laden layer is positioned at the second, third and fourth layer respectively (figures 7(b)–(d)). These results reflect a well-controlled positioning of cellularized layers, achieved by the proposed technique and device. Finally, it is important to highlight that the tight bonding between layers due to the crosslinking of methacryloyl groups, allowed the maintenance of the structure, geometry and integrity even after the removal of the alginate mandrel.

A second construct, with higher complexity and multiple materials, was fabricated to prove the versatility of the technique (see figure 8). The constructed graft was obtained after coating an alginate mandrel with encapsulated HUVEC cells in 10% (w/v) GelMa, 0.2% (w/v) alginate and supplemented with BSA-FITC (1 mg ml⁻¹) for fluorescent visualization, followed by 25 micro-layers derived from dipping/crosslinking in 10% (w/v) GelMa solution, 1 layer of 10% (w/v) GelMa, 0.2% (w/v) alginate and BSA-FITC, 25 micro-layers of 10% (w/v) GelMa, 1 layer of 10% (w/v) GelMa with 0.2% (w/v) alginate and BSA-FITC, and an outer layer composed of 25 micro-layers of 10% (w/v) GelMa. In figure 8(a), it is possible to distinguish all 6 layers of the construct, and by examining FITC’s fluorescence of intercalated layers deposited with this technology (see figure 8(b)), we can deduced that layer did not mix or were not ripped during the production process. Additionally, figure 8(c) shows a close-up of the first layer were the encapsulated cells at the luminal region can be appreciated more clearly. In this last experiment, the feasibility of using different materials and the control of deposition and micro-deposition of several layers assembled together is proven, leading to the fabrication of a structurally similar blood vessel configuration (tunica intima, media and externa intercalated with elastin layers).

4. Discussion

Basic research, pre-clinical and clinical evidences bring to the conclusion that successful tissue engineered SDVB require a fine tuning of biological and mechanical properties coordinated through the precise concentric positioning of different layers in order to mimic the functionality and long term patency of natural blood vessels [5, 39]. Thrombogenicity and hyperplasic phenomenon, that rapidly worsen tissue engineered SDVB functionality, can be triggered even by slight disruption of the endothelial layer confluence or unmatched mechanical properties between grafts and natural vessels [40, 41]. The necessary control of these elements can only be obtained through the optimization of construct’s formula that comprises number, position order, dimension, mechanical nature and biological nature of cellularized and
reinforcing layers. A successful execution of such optimization process and final generation of complex multilayer grafts would demand for high fabrication skills and versatility. Although the mechanical properties or complete functionality of our multilayer constructs has not been proven yet, this work has demonstrated that the automatization of a method based on a conceptually simple fabrication strategy, such as dipping, rotation and crosslinking, can reach impressive control of lumen diameter, deposition and micro-deposition of multilayers in a rapid and versatile manner, allowing for complexity and scalable fabrication of vascular grafts.

The automated dipping system is based on the control of four stepper motors using a programmable microprocessor. Two motors control the dipping process while a third motor the rotational movement of the graft during the process of fabrication, allowing a homogeneous crosslinking from a fixed source of lateral UV light. This rotational movement was designed with the perspective of controlling as well the micro-structural alignment of the deposited biomaterial by meaning of variations in the rotational speed as evidenced in figure 6, with strain-induction of coil to triple helix transition. Although preliminary data suggest that, it requires further experimental and analytical inspection of the constructs to prove this concept. The fourth motor controls the rack placement in order to switch from one material to another, and its programmed movements define the order of positioning of the different concentric layers across the vessel graft. A vascular construct composed of an endothelial layer, two additional cell-laden layers and two intercalated reinforcing layers, mimicking the arterial configuration (tunica intima, tunica media and tunica externa intercalated by two elastin layers), would require only 18 min program operated for complete fabrication (see figure 8).

Concerning the control of luminal diameter, an easy strategy would use a metal rods with different diameters, however, the use of mandrels based on sacrificial material such as alginate, allows the delicate removal of vessel-like grafts keeping uncathed luminal cellularized thin layers, necessary to develop a mature endothelial layer. In this case, removal can be accomplished using calcium chelating agents for alginate de-crosslinking or through calcium-driven compaction of the alginate mandrel and gentle graft displacement.

Fabrication of the luminal alginate mandrel based on two variables, upward-speed and alginate concentration or viscosity, describe a three-dimensional function, showing a positive correlation between viscosities or upward-speed and the final alginate mandrel diameter (see figure 2(b)). Therefore, changes in viscosity and upward-speed parameters during fabrication can be utilized to control diameter sizes of vascular grafts using the automated system, which will be the tools of control for later layer deposition too. Our results indicate that up to 46 mm s⁻¹ upward-speed, there is a direct correlation between the emersion speed and the obtained alginate mandrel diameter when using alginate solutions with viscosity above 198 cP (see figures 2(b)–(d)). However, using 165 cP alginate at 138 mm s⁻¹ upward-speed, the diameter variation along different section of the alginate mandrel is minimal, becoming anyway the setting of choice to obtain longer and dimensionally homogeneous vascular grafts (see figures 2(e)–(g)).

Dipping of a thin metal rod into an alginate solution proved to be a feasible way to customized luminal diameter of vascular grafts, where by simply repetition of dipping rounds, it could permit larger vessel diameter above 6 mm (see figures 2(h)–(k)), adding a major level of versatility to the fabrication system. In order to limit the number of experiments, we explored the control of our system over the layer thickness during fabrication by changing the viscosity value and keeping the upward-speed unperturbed. 10% GelMa solutions with different viscosity values were obtained after mixing with variable amounts of alginate. Positive correlation between viscosity and the layer thickness where only observed within a restricted range (9.8–15.8 cP). Although it is possible to control the thickness of deposited biomaterial using this settings and modifying alginate concentration in order to move between 9.8 and 15.8 cP, wider range of alginate concentration for a better thickness control could be obtained if a different upward-speed is used, as it can be deduced from diameter results in figure 2, where 4.6 mm s⁻¹ upward-speed shows a slow increment in the diameter as the viscosity increases from 165 to 245 cP, whereas at 23 mm s⁻¹ upward-speed the diameter increment is very high already from 165 to 198 cP in viscosity.

One general challenging aspect in tissue engineering and tissue engineered SDBV using acellularized scaffolds is the reduce capacity of cell repopulation or invasion post-fabrication [42, 43]. Our system overcomes this issue by mixing cells in the pre-crosslinked solution previous to fabrication of layers. In this way the fabrication succeed in homogeneously distributing cells in the whole construct (see figure 4), furthermore localizing specific cells and biomaterials in concentric zone across the wall of vessel graft (see figure 7). The automated methodology allows a high level of complexity in fabricating multilayer and cellularized blood vessel grafts.

Functionality of blood vessel grafts depends greatly on the integrity of confluent endothelial monolayer, especially to avoid thrombogenic phenomenon. In order to test the abilities of our system in generating a very thin and stable layer of encapsulated cells, given origin to a possible well-integrated endothelial layer after in vitro or in vivo maturation, we tested different conditions until getting a cellularized layer of HUVECs not wider than the size of 2–3 cells (20 μm thick) as it shown in figure 5.
Regarding cell encapsulation and viability, lower concentrations of PI during layers fabrication will possibly improve the viability of cells, but would change as well the conditions at which certain thicknesses are obtained. In figures 4(a)–(e), layers where fabricated using 0.5% (w/v) of photoinitiator, as well as those shown in figure 3(a), however those results are expected to change with other PI concentrations. In this sense it is important to evaluate the balance between the resulting cell functionality and the necessary layer configuration of a vascular graft. Higher cell functionality would eventually require higher viscosity of dipping solutions to obtain certain thickness of layers using a reduced concentration of PI.

In this work, we have not tested yet other available initiators, such as APS/TEMED, VA-086 or LAP, some of which are less cytotoxic than the one use in this work, or they can be used under induction with visible light [44–46]. Screening of different fabrication methods applying different available photo-initiators and possibly more reactive and less cytotoxic functional groups in the biomaterials for crosslinking could derived in optimized protocols for improved viability, cell fitness and functionality in this type of constructs, reducing for example the risk of DNA mutations. Nevertheless, it is important to remark that little concern has been evidenced from DNA analysis after UV exposure of cells [47, 48], especially in a previous report published by Wong and collaborators, who studied changes in gene expression in presence of UV irradiation at 365 nm using doses similar to the present in our study, showing no significant differences compared to non-irradiated cells [49].

Following recommendations extracted from the experiments shown in figures 4(f)–(k), future endeavor in creating implantable vascular grafts will required a previous incubation in specialized bioreactor for recovery of encapsulated cells, in order to get cell with appropriated functional responsiveness at the time when they are submitted to natural system after implantation.

A final experiment was performed to test the versatility of constructing complex multi-material and multi-layers vascular grafts. Figure 8 shows a multi-layer cylindrical construct comprising a cellularized internal layer, two gelatin/alginate based layer and three intercalated gelatin layer that serve as structural reinforcing elements. All layer to layer interfaces did not show any sign of detachment, most likely due to the presence of methacrylic groups in the gelatin that form covalent bonds during UV exposition, which forms not only in the layer, but as well at the interface between layer. This confers more structural integrity to the cylindrical construct. Other biomaterials such as chitosan, alginate, gellan gum, collagen, elastin, and cellulose can as well undergo methacryloyl functionalization [50–55], therefore be included in a structural stable manner into multilayer constructs using this automated system.

In comparison with other methods, layer-by-layer approach has been applied either in microfluidic systems, hence, not clinical application as small-diameter vascular graft could be expected [56], or in acellularized and simplified cylindrical constructs, lacking the advantage of a highly versatile system coupling fabrication of complex configurations and cell patterning capability [19], beside the recurrent inability of dimensions control.

At this point, it is important to mention that hydrogels are known to be mechanically inferior compared to naturally structured extracellular matrices in vascular tissues, therefore, although the present strategy appears as a unique and versatile automated method for fabrication of complex and cellularized multilayer vascular grafts, additional approaches of mechanical reinforcement through the application of fibers, meshes, hydrogel fillers or extend in vitro maturation need to be integrated to our system in order to reach appropriated mechanical parameters. This aspect of research has been developed in a coming works in our Lab.

5. Conclusions

Although tissue engineering in pre-clinical testing has been showing a great potential in providing adequate and functional SDBV to treat obstructive coronary diseases, to date, actual strategies has failed in fabricating vessel grafts for patients that match functionally and the mechanics of the natural blood vessels. There are many technical and economic reasons why, however durability, tuning of mechanical properties, functionality and scalability appears as the still uncovered problems in the field. Appropriate vessel grafts clearly requires fabrication methodologies capable of adjusting with high precision an in a scalable manner different elements, that goes from material composition, geometry and spatial location of different biomaterials and cells types [39]. Our automated methodology has proven to have a great control over the thickness of the multiple layers, control of micro-deposition, cell viability and spatial control of components such as biomaterials and cells in the fabrication of blood vessel-like structures. This provides an excellent system to explore the optimization, generation, customization and scaling up for the production of complex multilayer constructs capable to match properties and functionality of natural SDBVs.

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**Conflict of interest**

Maroun Khoury is the CSO of Cells for Cells and Regenero, Juan Pablo Acevedo received stipends from Cells for Cells. The other authors declare no conflicts of interest.

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