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Combining electrospinning and fused deposition modeling for the fabrication of a hybrid vascular graft

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Abstract
Tissue engineering of blood vessels is a promising strategy in regenerative medicine with a broad spectrum of potential applications. However, many hurdles for tissue-engineered vascular grafts, such as poor mechanical properties, thrombogenicity and cell over-growth inside the construct, need to be overcome prior to the clinical application. To surmount these shortcomings, we developed a poly-L-lactide (PLLA)/poly-ε-caprolactone (PCL) scaffold releasing heparin by a combination of electrospinning and fused deposition modeling technique. PLLA/heparin scaffolds were produced by electrospinning in tubular shape and then fused deposition modeling was used to armor the tube with a single coil of PCL on the outer layer to improve mechanical properties. Scaffolds were then seeded with human mesenchymal stem cells (hMSCs) and assayed in terms of morphology, mechanical tensile strength, cell viability and differentiation. This particular scaffold design allowed the generation of both a drug delivery system amenable to surmount thrombogenic issues and a microenvironment able to induce endothelial differentiation. At the same time, the PCL external coiling improved mechanical resistance of the microfibrous scaffold. By the combination of two notable techniques in biofabrication—electrospinning and FDM—and exploiting the biological effects of heparin, we developed an ad hoc differentiating device for hMSCs seeding, able to induce differentiation into vascular endothelium.

1. Introduction
Tissue engineering is an emerging area in contemporary human health care, in which applications of bioengineering meet the basic understanding of cellular biology for developing tissue substitutes to aid treatment of many pathological conditions associated with tissue loss or degeneration [1]. In the cardiovascular field, tissue engineered vascular graft (TEVG) constructs, developed by using cultured autologous vascular wall cells, seeded onto a synthetic biodegradable polymeric scaffold [2], represent one of the most encouraging strategies to overcome the shortcomings arising from the surgical replacement with autologous vessels, allografts or xenografts as well as synthetic materials [3]. The main advantages of autologous TEVGs are increased cell growth, construct durability and resistance to infections, but they are still harnessed by limitations regarding thrombogenicity and donor scarcity. Both pre-clinical and clinical studies have demonstrated the feasibility of constructing functional vascular grafts from autologous vascular cells seeded onto a...
biodegradable tubular matrix with large diameter (\( \varnothing > 6\) mm) [4]. In this context, bioengineering is required to produce scaffolds as biological surrogates as similar as possible to the native histoarchitecture of the damage vessel in order to better assist cell engraftment and proliferation. Many different techniques have been developed for the fabrication of such scaffolds with biopolymers [5]. Among these, electrospinning represents a very attractive technique, allowing the production of polymeric fibrillar structures mimicking the extracellular matrix (ECM) organization. This technology allows for control over the morphology of fibers, with diameters ranging from tens of nm to several \( \mu m \) [6]. The small diameter fibers produced by electrospinning have the advantage of a large surface-to-volume ratio, as well as a high permeability and an interconnected pore structure, both of which are desirable in a biological setting [7]. The attractive features of electrospinning are the simple and inexpensive nature of the setup that can easily be adapted to the manufacturing of tubular shapes [6], and the possibility of being combined to the newest methodologies of cell seeding [8].

Nano- and microfibrous electrospun scaffolds, mimicking the arrangement of connective tissue fibrillar proteins, have been demonstrated to allow the remodeling of vascular grafts in both cellular and extracellular content, thus representing an optimal candidate for TEVGs fabrication [9].

However, despite a number of efforts experimenting with various blending systems, different process parameters and post-processing treatments, the mechanical properties of electrospun fibrillar matrices are still poor. The mechanical ability of the scaffold to maintain structural and functional integrity immediately after implantation and during the remodeling phases still represents an issue [10].

With this in mind, we chose to increase the mechanical strength of the scaffold. Fused deposition modeling (FDM) was chosen as the candidate technique. FDM creates models out of heating thermoplastic material, extruded through a nozzle, positioned over a computer-controlled table. In the emerging field of so-called computer aided tissue engineering (CATE) several groups are reporting the use of FDM for the fabrication of 3D constructs [11, 12].

Several types of polymers have been proposed to obtain scaffolds for TEVG, such as collagen, poly-\( \varepsilon \)-caprolactone (PCL), poly-lactide (PLA), poly-glycolide (PGA) and block copolymers thereof [13].

Polymers loaded with growth factors, cytokines, and drugs have also been developed, generating drug-releasing systems capable of localized delivery of biomolecules [14, 15].

In particular, to improve the performance of biodegradable TEVGs in terms of blood compatibility, researchers have extensively been working on their surface modification using heparin. Localized heparin release has also been investigated for prevention of cardiovascular thrombosis and vascular repair effects [16]. Heparin is known to specifically bind various angiogenic growth factors, such as VEGF and bFGF. With this in mind, addition of heparin is considered a functionalization strategy that enables the capture and concentration of the circulating angiogenic growth factors released during vascular injury. Finally, heparin itself has an important effect on cell differentiation toward vascular endothelium and homeostasis and has been shown to promote endothelial commitment in bone marrow stem cells even under conditions of organ damage [17].

Human mesenchymal stem cells (hMSCs) from bone marrow have been shown to be a suitable candidate for tissue regeneration including vascular, cardiac [18] and bone tissues [19]. Recently, they exhibited antithrombogenic properties, especially in relation to their cell-surface heparan sulfate proteoglycans [9]. The guided differentiation of an easily recruitable and highly plastic stem cell type, such as hMSCs, could overcome the current difficulties related to the lack of ‘off-the-shelf’ availability, the low cell yields and quality, and the problems in harvesting and propagating autologous mature fully differentiated endothelial cells. These problems are especially daunting in vascular surgery, where treatment of an acutely ischemic lower limb is involved.

The aim of this study was to develop a heparin loaded electrospun PLLA tubular scaffold armored on its outer wall with a coil of PCL by the FDM technique. The obtained scaffold was seeded with hMSCs and assayed in terms of cell viability, proliferation and differentiation in order to produce a device which is able to both guide the differentiation of stem cells toward vascular endothelium and reproduce the histoarchitecture of the native blood vessel.

2. Materials and methods

2.1. Tubular scaffold preparation

The first step of the process consisted in the fabrication of the electrospun tubular scaffold. PLLA loaded with heparin scaffolds (PLLA/Hep) were prepared starting from a 13% w/w PLLA (Sigma, Milwaukee, WI) solution in dichloromethane (Sigma). Unfractioned heparin (sodium salt, 5000 UI mL\(^{-1}\), MSPharma, Gaither, MD) was added to the polymer solution using methanol (Sigma) as a cosolvent, to obtain a final heparin concentration (with respect to PLLA) of 830 \( \mu g \) g\(^{-1}\) (equivalent to 250 UI g\(^{-1}\)). This concentration was chosen in accordance with both the literature and the clinical dosage routinely used [20], in order to create a device suitable for direct clinical application.

An electrospinning equipment was used (DynaSpin, Biomatica, Rome, Italy); the solution was fed through a 28G needle with a feed rate of 1.5 mL h\(^{-1}\) and electrospun onto an earthed collector placed at a distance of 15 cm, using a voltage of 15 kV. In order to achieve a tubular scaffold, a controlled rotating cylindrical AISI 316L mandrel (5 mm in diameter and 6 cm in length, rotating at 100 rpm) was used as the collector target for electrospinning. At the same time, bare PLLA scaffolds (PLLA/ctrl)—without heparin addition—were obtained under the same experimental conditions which were to be used as a control for biological assessments.

The following step consisted in the fabrication of the outer poly-\( \varepsilon \)-caprolactone (PCL, MW 80 000, Sigma, Milwaukee, WI) armor. For this purpose, a prototypical FDM apparatus was...
developed by our group (figure 1). The apparatus comprises an aluminum heated dispensing head ended with a 21G needle, an X–Y motorized stage (miCos GmbH, Eschbach, Germany) for the positioning of the sample and a Z stage (miCos GmbH) for controlling the distance between the dispensing head and the sample holder. The aluminum dispensing head is supplied with two heating cartridges and a resistance thermometer (Pt100), connected to a programmable temperature controller (model 400, Gefran, Brescia, Italy). Therefore, the pellet-form PCL is charged in the dispensing head and melted at 80 °C. The extrusion process is performed by pressure-assisted dispensation, feeding pressurized argon gas by means of a high-pressure line (working pressure about 8 bar) connected to a control electrovalve. Custom-developed control software generates the process toolpath and controls the actuation of all the system components (axes movement, dispensing head temperature and electrovalve). The system was tailored to the fabrication of hybrid grafts by the addition of a rotating mandrel, actuated by a stepper motor (Astrosyn model 17 PM, Minebea Co., USA) driven by the control software. The PLLA/Hep scaffold was mounted on the mandrel. Combining controlled rotation of the mandrel (10 rpm) with linear motion of the Y-axis stage (150 μm s⁻¹), it was possible to accurately wrap the scaffold with an armoring coil of PCL (PLLA/Hep/PCL sample, figure 2(b)).

Microstructure of these obtained materials was evaluated by field emission scanning electron microscopy (FE-SEM, Supra 1535, Leo Electron Microscopy, Cambridge, UK).

2.2. Drug release study

Heparin release rate was assayed using the Azure A spectrofluorimetric method developed by Jacques et al [21]. Samples of the PLLA/Hep/PCL scaffold (weighing 40 mg) were separately placed in a 15 mL sealed tube with 800 μL of pre-warmed phosphate buffered saline (PBS) containing 0.03% w/v sodium azide (NaN₃) and incubated at 37 °C. Aliquots (60 μL) of supernatant were timely collected.
and quantified according to the above-mentioned assay. Experiments were performed in quintuplicate. The same experiments were also performed on PLLA/Hep samples, to assess whether the presence of PCL layer affects the heparin release profile.

2.3. Mechanical properties

Mechanical tests were performed both on electrospun grafts and on armored tubes (PLLA/Hep and PLLA/Hep/PCL samples). The great saphenous vein from an adult male patient was also tested as a reference (three samples for each experimental group). To provide a complete mechanical characterization, measurements included static compliance, stiffness index, ring and longitudinal uniaxial tensile parameters (including burst pressure), and suture retention [22–24].

In the hybrid structure PLLA/Hep/PCL, the outer helix could not be assumed as a homogeneous layer, undergoing the deformation of the underlying electrospun PLLA layer. Moreover, with the aim of evaluating the strengthening effect provided by this PCL coil with respect to the unarmored sample PLLA/Hep, the authors chose to calculate stress and strain values from measured load–displacement data referring to the section of the electrospun layer, that is identical for the two samples. In this assumption, the contribution of the PCL coil reflects in a modification of the mechanical properties of the electrospun layer in the PLLA/Hep/PCL sample.

Regarding circumferential characterization, each construct was cut into rings that were measured by means of a dial caliper. Rings were inserted with two stainless steel flat hooks that were subsequently fixed to the clamps of a testing device (TH2730, Thumler, Nurnberg, Germany) equipped with a force transducer (Nordisk Transducer Teknik, Hadsund, Denmark), and pulled at 10 mm min\(^{-1}\) crosshead speed until sample failure. Prior to testing, all samples were preconditioned with cycles at 5% circumferential stretch. Load–displacement data were computed to obtain stress–strain curves starting from specimen geometrical parameters.

According to Nieponice et al [25], ultimate tensile stress (UTS) and strain to failure (STF) were considered as the maximum stress value before failure and its corresponding value of strain, respectively. Burst pressure (\(P_b\)) was also estimated from UTS using the following law:

\[
P_b = \frac{2 \cdot \text{UTS} \cdot r}{D_0},
\]

where \(D_0\) is the initial diameter of the samples and \(r\) is the initial thickness of the tested scaffolds. From this stress–strain relationship, the circumferential elastic modulus (CEM) was calculated as the first derivative curve in the linear segment. The maximum elastic modulus (MEM) and the stretch at maximum elastic modulus (SMEM) were, respectively, the maximum value of the derivative curve and its corresponding strain value [22].

A similar approach has been followed for the longitudinal uniaxial tests. Given the peculiar arrangement of the hybrid graft, it was not possible to perform the test using the standard ‘dog bone’ shaped sample, described by Sell et al [23]. Hence, two cylindrical aluminum terminations were inserted at the ends of each construct segment and fixed with epoxy resin. Such terminations were connected to the clamps of the tensile tester. Test was performed at an extension rate of 10 mm min\(^{-1}\). Longitudinal elastic modulus (LEM), peak stress (PS) and longitudinal strain at failure (LSTF) were calculated starting from load–displacement data collected in the tests.

Static compliance tests were performed according to Stankus et al [22]. Constructs were mounted in a hydraulic circuit and subjected to a pressure ramp (0–250 mmHg) by means of a syringe pump (KD Scientific, Holliston, MA). The circuit was clamped downstream while the pump infused PBS at a constant rate (4 mL min\(^{-1}\)). Pressure was monitored using a pressure transducer (40PC100G1A, Honeywell, Freeport, IL) connected to the hydraulic circuit, while the outer diameter was measured by using a high-resolution camera and by processing the acquired images with an analysis tool (ImageTool 3.0, UTHSCSA, San Antonio, TX). Samples were preconditioned by clogging the porosities with a high-viscosity freezing medium (OCT Embedding Matrix, CellPath, Newton Pawys, SY). Assuming an incompressible wall, static compliance (SC) was calculated as follows, and reported as % per 100 mmHg as specified in the ANSI 7198 standard [23, 26]:

\[
\%C/100 \text{ mmHg} = \frac{D_{in}^n - D_{in}^m}{D_{in}^m} \cdot \frac{1}{A} \cdot 10^4,
\]

where the inner diameter was estimated starting from the measured outer diameter and using the assumption of incompressibility of cross-sectional area (A):

\[
D_{in} = 2 \cdot \sqrt{\left(\frac{D_{ext}}{2}\right)^2 - \frac{A}{\pi}}.
\]

The pressure–diameter relationship during static compliance measurements were also used to compute the stiffness index, \(\beta\), via [22]:

\[
\beta = \ln \left(\frac{P_{fin}}{P_{f0}}\right) \div \frac{D_{in}^m - D_{in}^n}{P_{fin} - P_{f0}}.
\]

Finally, suture retention tests were performed on 7 mm long samples by using the previously described tensile testing equipment at an extension rate of 150 mm min\(^{-1}\) in accordance with ANSI/AAMI VP20:1994 [23]. Geometrical characteristics were measured by means of a dial caliper. A single commercial 6.0 prolene loop (Ethicon Inc., Somerville, NJ) was placed 2 mm from the end of the sample, and secured to a hook connected to the upper clamp of the testing device. Suture retention force (SRF) was considered as the maximum force recorded prior to failure, while the suture retention tension (SRT) was obtained by normalizing the retention force by sample thickness [22].

2.4. Cell seeding on scaffolds

Human adult bone marrow mesenchymal stem cells (hMSCs), obtained from a 22 year old female, were provided by a commercial source (Lonza, Basel, Switzerland) and used at...
passage 4 for this study. hMSCs were seeded both on tubular PLLA/Hep/PCL scaffolds and on PLLA/ctrl tubular devices used as a control for differentiation induction. Prior to cell culture, both PLLA/ctrl and PLLA/Hep/PCL scaffolds were sterilized by soaking in absolute ethanol [14] and rinsing in sterile PBS. Scaffolds were seeded with hMSCs under rotating dynamic conditions (12 g) to guarantee uniform cell distribution, at a density of \(5 \times 10^5\) cells cm\(^{-2}\) in Dulbecco’s modified eagle medium (DMEM, Lonza) supplemented with 10% fetal bovine serum (FBS, Lonza, Lot no 8SB013), 100 units mL\(^{-1}\) penicillin, 100 \(\mu\)g mL\(^{-1}\) streptomycin (Lonza) and incubated for 48 h in 25 mL culture media [27].

2.5. Cell engraftment, viability and proliferation assays

Cell viability, attachment and proliferation within the construct were evaluated by measuring the total cellular DNA content, immunohistochemistry and confocal microscopy. For cell viability assessment, the Live/Dead assay (Invitrogen, San Diego, CA), based on cellular membrane intactness, was used. To evaluate cell proliferation, a semiquantitative analysis by immunostaining for Ki67 using confocal microscopy and the measurement of the total cellular DNA content were performed. Cellular DNA content was obtained as previously described [28]. Briefly, constructs were lysed in RIPA buffer with added protease inhibitor cocktail Halt (Thermo Fisher Scientific, Rockford, IL) and cells were sonicated using a pulsed ultrasonic disruptor (Thermo Fisher Scientific) three times for 10 s. DNA content was measured using a Quant-iT ds-DNA assay kit (Invitrogen) on a Qubit fluorimeter (Invitrogen). DNA content measurement was also performed soon after seeding to assess seeding efficacy.

Immunohistochemical analysis was performed on seeded constructs in order to obtain a cell morphology evaluation. Tubular scaffolds were fixed with 4% paraformaldehyde (PFA) for 15 min, embedded in OCT cryomatrix, snap frozen and cut in 15 \(\mu\)m slices. Slides were further processed for standard Hematoxilin-Eosin (Dako, Carpinteria, CA). Finally, to assess cell attachment and engraftment confocal microscopy was performed, staining cells for F-Actin with Rhodamine Phalloidin (Invitrogen). Samples were then mounted in Prolong antifade medium (Invitrogen) and viewed under an Olympus Fluoview F1000 confocal microscope by two independent blinded observers.

Flow cytometry was also performed for CD31 expression, using CD29 (Neomarkers) as a positive control [30]. Cells were detached from scaffolds by mild trypsinization and run through a flow cytometer (Accuri Cytometers, Ann Arbor, MI); data were analyzed using CellQuest software (BD Biosciences, San Jose, CA).

Finally, total protein content was extracted from constructs and evaluated by Western blotting analysis and densitometry analysis, as previously described [29], using a CD31 monoclonal antibody. Briefly, protein concentration was determined by the BCA method and total protein amounts of 25 \(\mu\)g of the lysates were subjected to 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Sigma) and transferred onto 0.20 \(\mu\)m Nitrocellulose P Membranes (Pierce Biotechnology, Rockford, IL). Membranes were blocked in a TBS protein-free solution (Tris Buffered Saline pH 7.4 + 0.05% Tween 20 + Kathon Antimicrobicidal agent, Pierce Biotechnology) and were immunoblotted with the primary antibody overnight, followed by horseradish peroxidase-conjugated secondary antibodies antimonious IgG (Zymed Laboratories, South San Francisco, CA). Membranes were exposed on radiographic film using an enhanced chemiluminescent substrate SuperSignal West Pico Trial Kit (Pierce Biotechnology). Densitometry analysis was performed using Image-Pro 6.0 (Media Cybernetics, Silver Spring, MD).

2.7. Statistical analysis

Data from biological investigations were processed using SPSS (Statistical Package for Social Sciences) release 13.0 for Windows (SPSS, Chicago, IL). Data are presented as mean \(\pm\) SD. One-way ANOVA was performed to compare groups with different treatments, followed by multiple pairwise comparison procedure (Tukey test). Significance was at the 0.05/0.01 levels.

Student’s \(t\)-test was used for analyzing average fiber diameter, heparin elution profile and mechanical properties, with a significance at 0.05 levels.

3. Results

3.1. Scaffold morphology

Electrospinning on a rotating mandrel was performed to obtain PLLA/ctrl and PLLA/Hep synthetic grafts (figure 2(a), 5 mm diameter, 0.3 mm wall thickness, 6 cm length). Then, these tubular grafts were reinforced with a single-layer helix of PCL coil by means of FDM obtaining a PLLA/Hep/PCL scaffold (figure 2(b)). Pictures and FE-SEM micrographs of the obtained tubular PLLA/Hep scaffolds are presented in figures 2(a)–(c). An electrospun layer was formed by a homogeneous distribution of fibers with average diameter of 450 \(\pm\) 150 nm (\(t\)-test, \(P = 0.05\)) as calculated using ImageTool 3.0 (UTHSCSA) starting from electron micrographs.
This fibrillar arrangement could represent a suitable environment for cell culture as it closely mimics the structure of native extracellular matrix, and can potentially provide similar support to cell growth and differentiation.

The outer PCL armor was composed of a single filament wrapping the scaffold in a helix arrangement, showing a mean diameter of 0.3 mm, and a pitch of 0.9 mm (figures 2(d) and (e)).

3.2. Drug release study

The cumulative release of heparin from the PLLA/Hep/PCL scaffold was assessed by a colorimetric assay, originally developed by Jaques et al [31] and modified by Yang et al [32]. Figure 3 shows the heparin release profile that results, very similar to other drug release profiles from a biodegradable polymeric matrix [33, 34]. This assay is based on the metachromatic behavior of Azure A dye, which presents a shift toward shorter wavelengths (from 620 to 520 nm) of the absorption band when reacting with heparin. This reaction is primarily dependent upon the formation of a bond between amino groups of the dye and carboxylic groups of heparin.

An initial releasing burst in the first 24 h, around 5.75% of the total drug load, could be detected in the PLLA/Hep/PCL sample, followed by a sustained release. A cumulative release of about 10% of the total drug load was assessed after 4 weeks (t-test, P = 0.05). An analogous profile was reported for the PLLA/Hep sample, showing no influence of the PCL coil on drug elution kinetics.

3.3. Mechanical properties

Table 1 reports the results derived from circumferential, longitudinal, compliance and suture retention mechanical testing for PLLA/Hep, PLLA/Hep/PCL and a human saphenous vein sample (SV), together with data for internal mammary artery (IMA) that were taken from the literature [26]. Stress–strain profiles for PLLA/Hep, PLLA/Hep/PCL and for the reference saphenous vein sample, as recorded during circumferential (figures 4(a)–(c)) and longitudinal (figures 4(d)–(f)) tensile tests are also reported.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PLLA/Hep</th>
<th>PLLA/Hep/PCL</th>
<th>SV</th>
<th>IMAa</th>
</tr>
</thead>
<tbody>
<tr>
<td>UTS (MPa)</td>
<td>0.72 ± 0.03</td>
<td>1.58 ± 0.07</td>
<td>1.15 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>STF (mm mm⁻¹)</td>
<td>0.80 ± 0.05</td>
<td>1.78 ± 0.10</td>
<td>1.30 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Pₛ (MPa)</td>
<td>0.08 ± 0.01</td>
<td>0.30 ± 0.03</td>
<td>0.16 ± 0.06</td>
<td>0.42 ± 0.17</td>
</tr>
<tr>
<td>MEM (MPa)</td>
<td>2.66 ± 0.16</td>
<td>3.37 ± 0.18</td>
<td>3.73 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>SMEM (mm mm⁻¹)</td>
<td>0.77 ± 0.08</td>
<td>1.77 ± 0.16</td>
<td>1.30 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>CEM (MPa)</td>
<td>0.54 ± 0.05</td>
<td>0.99 ± 0.08</td>
<td>0.88 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>PS (MPa)</td>
<td>0.66 ± 0.04</td>
<td>2.84 ± 0.15</td>
<td>1.20 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>LSTF (mm mm⁻¹)</td>
<td>0.31 ± 0.02</td>
<td>0.92 ± 0.05</td>
<td>0.57 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>LEM (MPa)</td>
<td>2.12 ± 0.13</td>
<td>3.08 ± 0.16</td>
<td>2.11 ± 0.12</td>
<td></td>
</tr>
<tr>
<td>SRF (N)</td>
<td>0.72 ± 0.04</td>
<td>1.31 ± 0.07</td>
<td>4.3 ± 0.23</td>
<td>1.38 ± 0.5</td>
</tr>
<tr>
<td>SRT (N m⁻¹)</td>
<td>2.67 ± 0.14</td>
<td>4.85 ± 0.26</td>
<td>12.29 ± 0.89</td>
<td></td>
</tr>
<tr>
<td>SC (%/100 mmHg)</td>
<td>65.9 ± 2.7</td>
<td>9.72 ± 0.31</td>
<td>25.60 ± 1.13</td>
<td>11.5 ± 3.9</td>
</tr>
<tr>
<td>β (adimensional)</td>
<td>2.32 ± 0.09</td>
<td>12.93 ± 0.52</td>
<td>5.42 ± 0.25</td>
<td></td>
</tr>
</tbody>
</table>

3.4. Cell morphology and differentiation

Scaffold biocompatibility was assessed in terms of cytotoxicity and proliferation of hMSCs. Live/Dead assay demonstrated >90% cell viability both in PLLA/Hep/PCL and in PLLA/ctrl scaffolds (figure 5(a)). Initial scaffold population was assayed by measuring the total DNA content per construct on sacrificial samples soon after seeding, showing no significant differences between PLLA/ctrl and PLLA/Hep/PCL. Cell proliferation was also evaluated by total DNA content measurement, showing a statistical significant increase (one-way ANOVA, P < 0.01) in the PLLA/Hep/PCL scaffold with respect to the control (figure 5(b)). In PLLA/Hep/PCL constructs, hMSCs exhibited changes in both cell morphology and protein markers expression, in comparison with controls. Confocal microscopy for F-Actin and nuclear counterstain showed elongated cells with rare cytoplasm and a high
nucleus/cytoplasm ratio (figure 5(c)). Nuclei appeared enlarged with loose chromatin and several nucleoli, indicating conditions of non-quiescence. Moreover, membranes showed characteristic indentations and invaginations that resemble caveolae, typical of endothelial cells. Confocal microscopy 3D rendering was also performed on a central portion of the constructs (figure 5(d)). Standard hematoxylin/eosin staining confirmed engrafting, showing elongated cells attached on PLLA fibers in PLLA/Hep/PCL (figures 5(e) and (f)), and confirming cell migration and engraftment within the scaffold.

Immunofluorescence analysis on the PLLA/Hep/PCL scaffold revealed CD31 expression positive cells, localized on the luminal surface of the construct (figure 6(a)). Flow cytometry confirmed and quantified CD31 positivity, showing a statistically significant higher level of expression (one-way ANOVA, \( P < 0.01 \)) in the PLLA/Hep/PCL scaffolds (figure 6(b)). These findings were confirmed by Western blot analysis, followed by a semiquantitative densitometric evaluation (figure 6(c)).

4. Discussion

An ideal tissue-engineered vascular graft has to fulfill several requirements. The scaffold needs to have antithrombotic properties, should avoid restenosis after in vivo implantation, and have comparable biomechanical properties. Thus, the prevention of thrombosis, vascular intimal thickening and aneurysm is essential for the successful post-implantational functioning and long-term patency of a TEVG [35–37].

Aiming at tissue regeneration or replacement, a good part of the literature currently focuses on developing 3D bioabsorbable scaffolds, to reproduce the ECM structure and topography and to provide a biological support guiding cell attachment and proliferation [13]. To this extent, research is focused on the obtaining of structures able to mimic the organization of a natural vascular ECM and thus improve cell attachment. Vascular scaffolds can be fabricated by phase separation, electrospinning and self-assembly of peptides of structural proteins, such as collagen and elastin [38].

In particular, the electrospinning technique was reported to give excellent results in the fabrication of extracellular matrix-like scaffolds for cardiovascular tissue engineering using both stem and non-stem cells. However, the simulation of the native ECM architecture and the nonlinear biomechanical behavior of the natural vascular wall are actually open challenges [38–41]. Despite a number of efforts experimenting with various blending systems, different process parameters, and post-processing treatments, the mechanical properties of electrospun fibrillar matrices are still poor. The mechanical ability of the scaffold to maintain structural and functional integrity immediately after implantation and during the remodeling phases still represents an issue [10].

With the aim to improve mechanical properties of an electrospun material, we coupled electrospinning of a tubular scaffold to the deposition of a single, continuous biopolymeric coil performed by fused deposition modeling. Mechanical testing was performed, with the aim to assess the contribution of the outer coil to the overall mechanical properties of the scaffold.

The tensile behavior of bare electrospun material was compatible with the model of a fibrillar material; according to the model, fibers are progressively recruited by imposed
strain, resulting in an increase in the steepness of the stress–strain curve. At high stress values, progressive failure of fiber elements is experienced. These phenomena result in a bell-shaped curve. Ultimate tensile stress for the electrospun layer
was found significantly lower than the saphenous vein tissue chosen as a reference.

On account of the fact that only the PLLA electrospin layer of the hybrid construct is designed to work as a scaffold for tissue regeneration, the authors decided to evaluate mechanical properties for PLLA/Hep/PCL constructs as referred to the geometry of the inner electrospun layer, thus providing a more suitable comparison with the unarmored PLLA/Hep samples that have similar dimensions. The resulting specific force values do not have a direct mechanical meaning, since these values were not calculated on the real section of the construct, but still provide an ‘equivalent stress’ value useful for comparison, and immediately pointing out the strengthening effect of the PCL coil. In particular, static compliance and stiffness index were found to be close to the values reported in the literature for human IMA [26]. Burst pressure, as calculated from UTS according to Nieponice et al [25], was still significantly lower than IMA, as well as suture retention force. Authors acknowledge that further optimization is still needed to improve mechanical properties to closely match the artery tissue. This could possibly be obtained by tailoring the thickness of the electrospin layer and/or the thickness and pitch of the outer armor coil. Moreover, the evaluation of mechanical properties for cellularized scaffolds throughout engineered tissue formation has to be performed, and will be the focus of further investigations.

At the same time, however, the obtained hybrid materials succeeded in enhancing the mechanical behavior of bare electrospin materials. The combination of electrospinning for the preparation of nanofibrous scaffolds apt to host cells, with the precise and controlled deposition of a reinforcement layer via fusion deposition modeling resulted in increased mechanical properties, while preserving the optimal fibrillar arrangement for cell attachment and tissue formation.

The incorporation of heparin for sustained release was also pursued. Besides the well-known antithrombotic properties of this drug, which is useful in the management of the peri-implantation phase, the local delivery of heparin to the site of vascular injury could be used to prevent the myoproliferative response [43]. Moreover, heparin plays a crucial role in endothelial cell adhesion and homeostasis, improves cell attachment to the scaffold, providing trophic and differentiating signaling [44], and prevents early thrombosis [45].

Our aim was to exploit these properties and to develop an ad hoc differentiating device for autologous stem cells, able—at the same time—to facilitate and optimize the management of the construct once in the clinical setting. The concept of fabricating a scaffold containing factors—such as heparin—able to induce stem cells differentiation and to exert important systemic effects, could represent an alternative to the mostly used TEVGs with mature, fully differentiated autologous endothelial cells, in order to improve their clinical management.

Recently, our group developed a hydroxyapatite functionalized electrospin scaffold with the aim of recapitulating the native histoarchitecture and the molecular signaling of osteochondral tissue to facilitate cell differentiation toward chondrocyte. Poly-L-lactide/hydroxyapatite nanocomposites induced differentiation of hMSCs in a chondrocyte-like phenotype with the generation of a proteoglycan-based matrix [19]. Moreover, we produced preliminary data on scaffolds tailored for cardiovascular structures [18]. These data represent a proof of principle of the possibility of producing a scaffold suitable for stem cells seeding, containing the appropriate factors to induce a guided differentiation toward the desired phenotype. In these settings, differentiation would be realized within a three-dimensional ECM-like environment closely mimicking the tissue native architecture and allowing a harmonious ongoing cell growth and differentiation for tissue regeneration. In the present study, FE-SEM analysis of electrospin scaffolds revealed an arrangement that could provide a favorable environment for cell attachment, growth and differentiation, as closely mimicking the structure of native ECM recapitulating the arrangement of connective tissue fibrillar proteins [9].

**Figure 6.** (a) Confocal microscopy with immunofluorescence staining for CD31, Ki67 and F-Actin of PLLA/Hep/PCL seeded with hMSCs for 48 h (400×). A CD31 positive cell can be detected in an interesting luminal location. Scale bar: 25 μm. (b) Cytofluorimetry analysis of CD31 expression of cells on PLLA/ctrl and PLLA/Hep/PCL scaffolds. (c) Top: Western Blot for CD31 (83 kDa). MW: molecular weight marker. Lane 1: PLLA/ctrl seeded with hMSC for 48 h; Lane 3: PLLA/heparin armored with the PCL coil scaffold seeded with hMSCs for 48 h. Bottom: densitometry analysis of Western blot expressed in optical density.
Regarding the reported two-phase release profile, consisting of an initial burst followed by sustained release, similar behavior is reported in the literature [33, 34, 42]. In the proposed models, release is governed by a combination of diffusion and degradation phenomena. In the earlier stages of the process, the initial burst is driven by Fickian diffusion and sustained by drug gradient concentration. Then, drug release is reliably due to the scaffold degradation, which, in turn, induces a decrease of polymer molecular mass resulting in an increase of permeability and hydrophilicity. The diffusion coefficient increases with the matrix degradation, while, on the other hand, the drug concentration in the biopolymer decreases, increases with the matrix degradation, while, on the other hand, the drug concentration in the biopolymer decreases, resulting in an almost constant release rate. This kinetics could represent an advantage for eventual clinical applications as providing an immediate high-dose heparin release, potentially able to counteract the well-known initial thrombotic tendency soon after surgical vascular anastomosis, which is at the basis of early graft failure. Further calculation of the effective amount of heparin released in the PBS solution during the first 48 h following the seeding, revealed a final concentration of 12 μg mL⁻¹. It is noteworthy that this concentration has been used in previous studies that showed endothelial differentiation induced by addition of heparin to culture media [17].

Results of light and confocal microscopy of scaffolds seeded with hMSCs, together with proliferation assay, confirmed the generation of a non-hostile microenvironment for cell culturing, with an additional proliferative drive reliably induced by the functionalization with heparin. In functionalized constructs, morpohstructural changes were observed, resulting in cell elongation, reduction in cytoplasm with nuclear enlargement, increase in nucleoli number and appearance of membrane invaginations resembling typical endothelial caveolae.

Along with these findings, in the heparin releasing scaffolds a shift toward CD31 positivity could be detected in comparison to bare PLLA, thus indicating a possible endothelial commitment. This finding was confirmed by CD31 expression, flow cytometry, and is consistent with the previous reports describing increased presence of CD31 positive microvessels following subcutaneous application of a covalently-bound heparin-modified porous polyurethane scaffold [46]. All these findings support our data concerning the ability of the heparinized scaffold to induce stem cell differentiation.

5. Conclusions

In the present work, we show the application of a hybrid technique, combining electrospinning and fused deposition modeling technique to fabricate a biopolymeric scaffold for vascular tissue engineering. We developed a tubular electrospun scaffold reinforced with a single-layer helical PCL coil to ameliorate mechanical properties of the TEVG. Such scaffolds showed better mechanical properties with respect to electrospun grafts, preserving the optimal fibrillar arrangement for initial cell attachment. Biological characterization of such scaffolds after seeding with human mesenchymal stem cells demonstrated the ability of the scaffold to drive a differentiation process toward vascular endothelium.

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