Osteogenic differentiation and mineralization in fibre-reinforced tubular scaffolds: theoretical study and experimental evidences

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The development of composite scaffolds with well-organized architecture and multiscale properties (i.e. porosity, degradation) represents a valid approach for achieving a tissue-engineered construct capable of reproducing the medium- and long-term in vitro behaviour of hierarchically complex tissues such as spongy bone. To date, the implementation of scaffold design strategies able to summarize optimal scaffold architecture as well as intrinsic mechanical, chemical and fluid transport properties still remains a challenging issue. In this study, poly e-caprolactone/polyactic acid (PCL/PLA) tubular devices (fibres of PLA in a PCL matrix) obtained by phase inversion/salt leaching and filament winding techniques were proposed as cell instructive scaffold for bone osteogenesis. Continuous fibres embedded in the polymeric matrix drastically improved the mechanical response as confirmed by compression elastic moduli, which vary from 0.214 ± 0.065 to 1.174 ± 0.143 MPa depending on the relative fibre/matrix and polymer/solvent ratios. Moreover, computational fluid dynamic simulations demonstrated the ability of composite structure to transfer hydrodynamic forces during in vitro culture, thus indicating the optimal flow rate conditions that, case by case, enables specific cellular events—i.e. osteoblast differentiation from human mesenchymal stem cells (hMSCs), mineralization, etc. Hence, we demonstrate that the hMSC differentiation preferentially occurs in the case of higher perfusion rates—over 0.05 ml min−1—as confirmed by the expression of alkaline phosphate and osteocalcin markers. In particular, the highest osteopontin values and a massive mineral phase precipitation of bone-like phases detected in the case of intermediate flow rates (i.e. 0.05 ml min−1) allows us to identify the best condition to stimulate the bone extracellular matrix in-growth, in agreement with the hydrodynamic model prediction. All these results concur to prove the successful use of tubular composite as temporary device for long bone treatment.

Keywords: fibre-reinforced scaffolds; hydrodynamic model; human mesenchymal stem cell response; perfusion bioreactor; bone tissue engineering

1. INTRODUCTION

The bone capability of sustaining physiological loads is conferred by tailored hierarchical structure at micrometric/sub-micrometric scale and peculiar chemical composition [1]. The hierarchical structure of bone is the result of the strict combination of collagen fibres and apatite mineral nano-domains that concur to form its extracellular matrix (ECM) substance [1–3]. Hence, an accurate design of three-dimensional scaffolds has to mimic the structural complexity of mineralized ECM (mECM) in order to reproduce its functional response. Moreover, it has also to guarantee chrono-programmed biomechanical properties to improve the stress transfer from the engineered material to the bone cells during the load bearing [4]. At the same time, a tailored microenvironment is desirable to offer, in turn, a myriad of signals able to address relevant biological events (i.e. cell differentiation, mineralization) leading to linear commitment [5].

In this context, biomaterials design is expanding with new material syntheses and processing techniques to create new environment adaptive scaffolds with high
structural complexity in order to direct stem cell lineage towards a pre-ordered cell fate [6]. Currently, several technologies based on templating strategies allow us to optimize the biomechanical response of the scaffolds through correctly balancing porosity and mechanical properties. This is a prerequisite for assuring sufficient porosity and permeability to promote efficient cell colonization, complete vascular invasion and satisfying all the main transport demands - i.e. nutrients and oxygen trafficking - of the remodelling tissue [7,8]. In order to guarantee the desired bio-mechanical response, the highly porous network may be further sustained mechanically through the integration of reinforcement systems - i.e. ceramic particles [9] or continuous fibres [10] to prevent the collapse of the pore architecture during load application. However, the development of functional analogue of hard tissues such as bone has to satisfy several convincing criteria for inducing the correct restoration of the regeneration mechanism [11,12]. It must also be durable, resistant to fatigue and possess appropriate stiffness to immobilize the fracture site without shielding the native tissue from the necessary in situ mechanical stimuli required for bone remodelling [13]. At the same time, it should exhibit bio-compatible properties and degradation or resorption rates that are capable of stimulating tissue growth, without leaving any traces once the tissue is totally formed. In particular, the use of biodegradable-polymer-based scaffolds with continuous degradation favours a gradual load transfer to the healing tissue and prevents bone atrophy by regulating the cell mechanisms involved in bone remodelling, thus overcoming the stress-shielding phenomena associated with the use of traditional rigid metal-based implants [13,14].

Besides, it is well known that all mechanical stimuli transmitted to the scaffold material are strongly perceived by the m(ECM), mainly influencing cellular and molecular events [15]. A programmed mechanical loading can create local strains and pressure gradients that make the interstitial fluid flow able to influence cells residing in bone marrow, including marrow stromal cells (MSCs) [16]. Recently, it has been demonstrated that shear stresses in the range of 0.5–1.5 Pa (5–15 dynes cm⁻²) are able to affect osteoblast proliferation as well as production of alkaline phosphatase, nitric oxide and prostaglandin (PGE2), indicating the relevance of shear stress as an important regulator of cell function [17,18]. In this context, temporary scaffolds with controlled architecture and peculiar material properties play a leading role in transferring mechanical stimuli to cells during in vitro culture [19,20].

In this work, a polymer-based system made of poly e-caprolactone (PCL) and polyactic acid (PLA) was considered to develop composite scaffolds (PLA fibres in a PCL matrix) by the synergistic use of phase inversion/salt leaching and the filament winding technique. Here, a preliminary screening of scaffold architecture and basic mechanical properties has been assessed to optimize the scaffold preparation process. Then, this information has also been collected to implement a predictive model able to describe the fluid velocities and pore pressure profiles within the scaffold. Hence, the evaluation of fluidodynamics and mechanical stresses distribution within the porous construct gave the chance of mapping the local stresses associated with the imposed fluid perfusion condition. Finally, experimental studies upon the osteoblast differentiation and mineralization have been correlated with theoretical data in order to interpret the transfer mechanisms of mechanical cues from scaffold to cells, and, also to offer a valuable guidance regarding the fluidodynamic conditions necessary during in vitro culture to drive osteogenesis.

2. MATERIAL AND METHODS

2.1. Materials and scaffold preparation

PCL (Mw 65 kDa, Tg = −62°C; Tm = 58°C) and PLA fibres (75/24 d-tex; Tg = 52°C; Tm = 165°C) were purchased, respectively, from Sigma-Aldrich and Sofradim. Tetrahydrofuran (THF; J. T. Baker, Italy) and ethanol (Merck, >99.9%, Italy) with reagent grade were used without further purification. Sodium chloride crystals (Fluka, AT >99.9%, Italy) were sieved in a specific size range (300–500 μm) for 3 h. Three different polymer solutions, with 10/90, 15/85 and 20/80 w/w polymer/solvent ratios, were prepared by the dissolution of PCL pellets in THF via gentle stirring for about 3 h at 50°C. Sodium chloride particles were mixed into the polymer solution to give a homogeneous mixture (porogen/polymer weight ratio, 9/1).

Fibre-reinforced composite structures were obtained by the integration of PLA fibres prepared by the filament winding technique by a winding machine (AS LAB 101—T.EL.MEC, Italy). The preparation of the fibre-reinforced composite scaffolds was extensively described elsewhere [21] and is schematically shown here in figure 1a,b. A multi-filament fibre moves across a resin bath, forming a thin surface resin coating, whose characteristics are dependent on the solution viscosity and the fibre-to-matrix chemical adhesion. Accordingly, the coated fibres were wound on the rotating mandrel at different rates. The mandrel rotation was synchronized with the fibre dispensing head moving along the mandrel length. The relative component rates enables the definition of some parameters related to the spatial distribution of the fibre in the composite, such as winding angle (i.e. angle of the fibre with respect to the mandrel axis) or winding pitch (i.e. the distance between adjacent helical plies). In this specific case, PLA fibres impregnated with the PCL-based mixture were rotated by using a winding angle of 45° and a winding pitch of 500 μm on a Teflon-coated steel mandrel for five (5F) and 10 (10F) fibre cycles. Hence, PLA/PCL weight ratios, respectively of (16/84) and (32/68) were calculated by weight measurements and modelling of geometrical parameters in a theoretical model [10]. Once the composite was obtained, the solvent was extracted from the matrix by ethanol dipping (35 ml g⁻¹ of composite) for 24 h. Samples were then totally immersed in bi-distilled water for 7 days in order to leach out salt and any other contaminants. Finally, the porous material was dried under a hood at room temperature for 6 h to obtain the final sample ready to use (figure 1b, right).

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2.2. Scaffold characterization

2.2.1. Morphology

The morphology of the scaffold was preliminarily investigated by scanning electron microscopy (SEM) (Stereoscan 440, Leica Oxford, UK) along the longitudinal cross section (figures 2 and 3). The samples were gold-coated by using a sputter coated set at 15 mA for ca 20 min. All porosity features were investigated in terms of pore size, shape and spatial distribution by images at different magnifications. A quantitative estimation of porosity degree has been carried out by using computer-aided tomography micro-computed tomography (μCT) (SKYSCAN 1072 μCT, Belgium) able to provide the three-dimensional reconstruction with the support of dedicated software (SKYSCAN pack, Belgium).

2.3. Mechanical response under uni-axial compression

Compressive mechanical properties have been analysed at room temperature (50% of relative humidity) on a dynamometric machine (Instron 4204) equipped with a 1 kN load cell and requiring a crosshead speed of 1 mm min⁻¹. Tubular-shaped specimens (internal diameter of 3 mm and external diameter of 7 mm) with width/diameter ratio equal to 2:1 were tested according to the guidelines specified in the standard American Society for Testing and Materials (ASTM 695-2a) for compressive tests. The cross-sectional geometry of the scaffolds was assumed approximately circular with slight irregularities owing to sample processing. Effective stresses were computed on the basis of the macroscopic scaffold cross-sectional area. The initial compressive modulus was determined from the slope of the linear part of the stress–strain curves by a linear regression over a range of 0.01 mm mm⁻¹ [22,23], whereas the compressive stress was estimated at the end of the linear region (ε = 0.1) (table 1). A minimum of six samples were tested in dry conditions.

2.4. Computational fluid dynamics simulation

2.4.1. Fluid mesh reconstruction

The three-dimensional architecture of the porous scaffold, from specimens used for mechanical testing, was acquired by μCT analysis. Three-dimensional rendering was obtained by imposing a pixel size of 18 μm (horizontal resolution) and a ‘slice-to-slice’ gap of 10 μm (vertical resolution). In detail, planar images outputted by μCT analyses have been processed by using a commercial available software, MIMICS v. 7.0 (Materialise, Belgium) to obtain the three-dimensional fluid mesh. Upper and lower thresholds (image windowing) have been applied to distinguish the ‘solid’ from ‘fluid’ phase. In this case, threshold values were adjusted up to match the porosity value previously obtained experimentally by μCT analysis (table 2). Then, the fluid phase has been exported as
three-dimensional computer aided grafting model and elaborated by GAMBIT (Fluent Inc.) to generate three-dimensional fluid mesh. Finally, the mesh was elaborated by FLUENT to perform computational fluid dynamics (CFD) analysis.

2.4.2. Governing equations and simulation parameters

A commercial software, FLUENT, has been used to perform the simulations. The software is based on a classical finite-volume code for CFD applications. It resolves the Navier–Stokes equations in their different formulations. The aim of these CFD simulations was to analyse water flow in porous zones via microscopic approach by using a laminar incompressible formulation of RANS (Reynolds Averaged Navier–Stokes) equations. The vector form of RANS equations were reported:

$$\frac{\partial}{\partial t} \rho + \nabla \cdot (\rho \mathbf{V})^T = 0,$$

$$\frac{\partial}{\partial t} (\mathbf{p} \mathbf{V}) + \nabla \cdot (\mathbf{p} \mathbf{V} \mathbf{V} + \mathbf{p} \mathbf{I} - \mathbf{\tau}) + \mathbf{p} \mathbf{g} = \mathbf{S q}$$

and

$$\frac{\partial}{\partial t} (\rho \mathbf{E}) + \nabla \cdot (\rho \mathbf{H} \mathbf{V} - \mathbf{\tau} \cdot \mathbf{V} + \mathbf{q})^T = \mathbf{S e},$$

where $\rho$ is thermodynamic pressure, $\mathbf{\tau}$ is the dissipative part of the stress tensor, $\mathbf{I}$ unitary tensor, $\mathbf{H} = \mathbf{E} + p/\rho = h$, total enthalpy and $\mathbf{q}$ diffusive thermal flux vector. $\mathbf{S q}$ and $\mathbf{S e}$ are momentum and energy source terms; $\mathbf{E}$ is internal energy, respectively. In the case of laminar incompressible approach, density can be assumed as constant, while turbulent stress tensor terms are not considered.

In order to perform an efficient and accurate CFD simulation, only a representative portion, 300 $\mu$m as thickness, at the middle of longitudinal axis of the scaffold was considered to build a three-dimensional mesh (figure 4). Computational grid counts show approximately $8 \times 10^5$ cells, and it satisfies all needed quality parameters for an accurate and stable CFD simulation. The average cell size range is around $10^{-3}$ mm$^3$ for the finest grid level. A convergence study has been performed; in particular, it has been verified that the solution was convergent to zero mesh size.

As stated before, CFD simulations are laminar stationary and the fluid is water. The inflow boundary condition was a mass flow inlet type. Three flow rates, 0.005, 0.05 and 0.5 ml min$^{-1}$, respectively, were analysed (figure 5). The pressure outlet boundary conditions were applied at all faces delimiting the domain. In particular, symmetric boundary conditions were specifically applied along faces at the symmetry surface, whereas solid walls were treated with
no-slip boundary conditions. For greater accuracy, all simulations have been performed using a double precision and second order numerical scheme solver.

2.5. Biological validation in radial perfusion system

2.5.1. Cell culture

Biological assays were performed using bone-marrow-derived human mesenchymal stem cells line (hMSC, PT-2501) obtained from LONZA. hMSC were cultured in a 75 cm² cell culture flask in Eagle’s alpha minimum essential medium (α-MEM) supplemented with 10% foetal bovine serum, antibiotic solution (streptomycin 100 µg ml⁻¹ and penicillin 100 U ml⁻¹, Sigma Chem. Co) and 2 mM l-glutamin. For in vitro osteogenic differentiation, hMSC cells were cultured in ‘osteogenic medium’ composing of α-MEM medium supplemented with 50 µg ml⁻¹ of ascorbic acid, 10 mM glycerol-2-phosphate and

Table 1. Summary of elastic moduli and compressive stresses at ε = 10% of fibre-reinforced composite scaffolds.

<table>
<thead>
<tr>
<th>PCL/THF (w/w)</th>
<th>elastic modulus (MPa)</th>
<th>compressive stress (ε = 10%) (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10/90</td>
<td>0.214 ± 0.065</td>
<td>0.922 ± 0.099 0.021 ± 0.002 0.067 ± 0.005</td>
</tr>
<tr>
<td>15/85</td>
<td>0.320 ± 0.012</td>
<td>1.082 ± 0.150 0.034 ± 0.004 0.096 ± 0.006</td>
</tr>
<tr>
<td>20/80</td>
<td>0.485 ± 0.041</td>
<td>1.174 ± 0.143 0.062 ± 0.004 0.124 ± 0.012</td>
</tr>
<tr>
<td>16/84</td>
<td>0.021 ± 0.002 0.067 ± 0.005</td>
<td></td>
</tr>
<tr>
<td>32/68</td>
<td>0.124 ± 0.012</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Summary of porosity features calculated by µCT analysis in the PLA fibre-reinforced PCL composite scaffolds selected for CFD simulation.

<table>
<thead>
<tr>
<th>PCL/THF 16/84 (w/w); PCL/THF 20/80 (w/w)</th>
<th>total porosity (%)</th>
<th>average macropore size (µm)</th>
<th>interconnection degree (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCL/THF 16/84 (w/w)</td>
<td>54.38 ± 4.54</td>
<td>247.2 ± 13.2</td>
<td>94</td>
</tr>
<tr>
<td>PCL/THF 20/80 (w/w)</td>
<td></td>
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Differentiation medium in perfusion was refreshed 5 per cent CO2 and 95 per cent air. Then, the scaffolds to scaffolds at 37°C with cells were incubated for 1 h to let cells adhere at 37°C used for all the experimental procedures and incubated 10 minutes with 0.05 and 0.5 ml min⁻¹, respectively. The osteogenic differentiation medium was 7 M of dexamethasone. hMSC at fifth passage were seeded at a concentration of 10⁵ cell ml⁻¹ of hMSC. After seeding, the scaffolds with cells were transferred to a home-made system for all the experimental procedures and incubated 21 days of culture was characterized by the analysis of gene expression of bone-related molecules.

PCL/PLA tubular scaffolds placed onto 24 well culture tissue plate were seeded at a concentration of 4 x 10⁵ cell ml⁻¹ of hMSC. After seeding, the scaffolds with cells were incubated for 1 h to let cells adhere to scaffolds at 37°C in a humidified atmosphere with 5 per cent CO2 and 95 per cent air. Then, the scaffolds with cells were transferred to a home-made system (figure 6) able to stimulate the radial perfusion of medium within the scaffold system with 50 ml of osteogenic differentiation medium. Three tubular scaffolds were fixed onto a 11 gauge metallic needle without any chemical agent. The bottom part of the tube has been filled with a thin layer of silicon glue to prevent any chemical agent. The bottom part of the tube has been filled with a thin layer of silicon glue to prevent any chemical agent. The bottom part of the tube has been filled with a thin layer of silicon glue to prevent any chemical agent. The bottom part of the tube has been filled with a thin layer of silicon glue to prevent any chemical agent. The bottom part of the tube has been filled with a thin layer of silicon glue to prevent any chemical agent.

Figure 4. Modelling based on computational fluid dynamics (CFD) simulation. (a) Three-dimensional images of the rendered scaffold obtained by µCT elaboration; three-dimensional mesh by a portion of the scaffold located at middle of the longitudinal axis, (b) 300 μm as thickness. Fluid velocity field (c) and (d) wall shear stresses in the scaffold at a flow rate of 0.5 ml min⁻¹.

Every 3 days. After 21 days of culture, the scaffolds from each group were measured for calcium deposits and bone-related molecules.

2.5.2. Detection of osteoblast markers
The analysis of gene expression of bone-related molecules of hMSC cultured onto perfusion bioreactor scaffolds at 21 days of culture was characterized by reverse transcription-polymerase chain reaction (RT-PCR; figure 7). For extraction of total RNA; each group of experimental scaffolds were transferred into a 1.5 ml microcentrifuge tube and incubated with 1 ml of total RNA isolation-reagent (SIGMA) for 1 h. After the period of incubation, the RNA isolation was performed according to the manufacturer’s protocol with final step of DNAse I digested (Invitrogen Carlsbad, CA, USA) to remove contamination of genomic DNA. The absorbency at 260/280 nm was measured to determine the RNA concentration. A total of 500 nanograms of total RNA was used to perform a one-step RT-PCR reaction (Invitrogen Carlsbad, CA) according to the manufacturer’s protocol. Briedly, the cDNA synthesis programme was 1 cycle at 60°C for 30 min followed by a denaturation cycle of 94°C for 2 min. cDNA was amplified at 94°C for 15 s, 55°C for 30 s and 68°C for 1 min for 35 cycles in a thermal cycler (Applied Biosystem). Primers used for amplification of bone-related molecules were osteocalcin 5'-gg caaggggaagaggaagaag-3; 5'-ggcagcaggtagtgagaagagc-3.
3′; osteopontin 5′-ttcggatgatcgagagacc-3′; 5′-ggacagctgagagAGTCGGATGAGTCTGAGAGACC-3′ and alkaline phosphate 5′-ggaagaacagaagaagcta-3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers used as a positive control were as follows: 5′-ccacccatggcaaatccatggca-3′ and 5′-tctagactggcaggtcaggtccacc-3′. Reaction products were separated using gel electrophoresis on 1.2 per cent agarose gel stained with ethidium-bromide. Bands were visualized using ultraviolet illumination and captured with a BioRad Imaging System (BIORAD). Image density of amplified bone-related products was quantified and reported as a ratio of the respective PCR product/GAPDH PCR product.

2.5.3. Alizarin Red staining

The evaluation of mineralization due to the calcium deposition during the hMSC cultured under perfusion conditions was assessed by Alizarin Red-S (ARS) staining dye able to selectively bind calcium salts (figure 8). Scaffolds were washed three times with phosphate buffer solution and fixed with 4 per cent formaldehyde for 1 h, washed five times carefully with distilled H2O and stained with ARS (40 mM) for 30 min at room temperature. After several washes with distilled H2O to remove excess dye, the scaffolds were observed under an optical microscope. For a quantitative analysis of ARS staining, scaffolds were centrifuged into a 1.5 ml tube while the extraction of ARS was performed according to the manufacturer’s protocol (Osteogenesis Assay Kit Milipore).

2.6. Statistics

Statistical analysis was performed using the Sigma Stat software package v. 3.1 by student’s t-test, and data represented as mean ± s.d. As for the mechanical tests, differences among samples from different groups (i.e. composition, treatments) were examined by an ANOVA test. Results were considered significant at p < 0.05.

3. RESULTS

An optimization of the preparation process of PCL/PLA scaffolds was preliminary assessed in terms of morphology and mechanical properties. An initial investigation of the scaffold morphology was performed by SEM images at different magnifications (figures 2 and 3). Fibrous scaffolds obtained by using 20/80 w/w polymer/solvent ratio and five fibre winding cycles (5FC) show a spatially ordered porous structure, characterized by PLA fibre bundles strictly arranged between adjacent pores ranging from 100 to 400 μm in size (figure 2b). By contrast, the use of lower polymer concentration (10/90 w/w) as well as higher fibre cycles (10FC), and consequently higher PLA/PCL ratios, seems to severely compromise the average structural order. In particular, scaffolds obtained by 10/90 w/w polymer/solvent ratio show a heterogeneous distribution of fibres that tend to form highly dense layers, preferentially arranged along the inner surfaces (figure 2a). At the same
scaffold architecture with the equivalent pore volume fraction and PLA/PCL ratio [7]. This variation of elastic moduli is statistically different ($p = 0.002$ and $F = 7.71$) by the ANOVA test. Compressive stress at the end of the linear region ($\varepsilon = 10\%$) show similar trends, varying from $0.021 \pm 0.002$ to $0.124 \pm 0.012$ MPa ($p = 0.002$ and $F = 7.3$). However, these values do not match the mechanical response of native bone, which shows an elastic moduli of two/three orders of magnitude higher. Statistical analysis performed by t-student tests demonstrated that reported data were significant at the level $p < 0.05$ (table 1).

Starting from all the previous evidences, tubular structures with a 16/84 (w/w) PCL/PLA ratio obtained from 20/80 (w/w) polymer solution represent the best compromise between morphology and mechanical properties for three-dimensional scaffolds modelling: for this scaffold typology, we have performed a CFD simulation to investigate the hydrodynamic shear stresses that appear at the cell/scaffold interface during the in vitro culture (figure 4). The computational study has been optimized in order to simulate an ideal process of radial perfusion. Proposed analysis has been performed on the assumption that cell sizes were negligible in comparison with average pore size, according to previous studies [25]. Fluid-induced shear stresses that act along the inner pore walls have been calculated; providing an estimation of the shear stresses acting on the cell membranes. shear stresses within the scaffold volume have been estimated by imposing a flow through the scaffolds with a faithfully reproduced pore structure with proper pore architecture (i.e. porosity, pore size, tortuosity) outputted by µCT reconstruction (figure 4a). Porosity data calculated from µCT analysis were reported in table 2. Therefore, the fluid mesh (figure 4b) has been obtained via MIMICS software on the basis of the three-dimensional scaffold volume reconstruction. The fluid moves from the inner of the scaffold to the outer through the annular thickness (2 nm as thickness). Boundary conditions have been applied, as shown in figure 4c.

By imposing three different flow rates as boundary conditions ($Q = 0.005, 0.05, 0.5$ ml min$^{-1}$), the distribution of three-dimensional velocities profiles as well as shear stresses distribution have been calculated into the whole porous structure (figure 4c,d). In detail, figure 4c shows an homogeneous distribution of flow directions into the whole scaffold volume, with preferential escape routes along the smaller pores (cyan regions). By contrast, a higher concentration of shear stresses (figure 4d) is located in a well localized region near the inlet part (red spots). This may be also quantitatively estimated by the wall shear stress distributions, reported in figure 5a–c. A shift of the shear stress distribution occurs as the flow rate increases, from 0.005 to 1 ml min$^{-1}$, rising up the shear stress maximum peak from $10^{-2}$ to 1 Pa (figure 5d). Moreover, we have calculated the summation of shear stresses into the porous structure at each assigned flow rate (figure 5d), falling into the range from $10^{-3}$ to 1 Pa, which collects the characteristic stress values able to stimulate a cell differentiation [26–28]. The summation values have been reported as a function of the applied perfusion rate.

Figure 6. Radial perfusion bioreactor system. (a) Scheme of basic components and (b) particular of tube accommodation into the bioreactor chamber.
A maximum peak can be recognized in the case of flow rate equal to 0.05 ml min$^{-1}$, indicating a preferential operative condition to be applied during the in vitro culture under perfusion. To confirm the model prediction, an evaluation of bone molecules markers and mineralization has been performed on scaffolds with primary hMSCs undergoing dynamic culture in a home-made bioreactor system by imposing different flow rates (figure 6). After 21 days of culture, the analysis of gene expression of proteins related to stage marker of osteogenic lineage differentiation of hMSC—alkaline phosphate (ALP) and osteocalcin (OC)—show a brighter signal (figure 7) for higher flow rates—above 0.05 ml min$^{-1}$—so proving the effect of perfusion condition on the MSC differentiation in the osteogenic way. In particular, osteopontin (OPN) revealed a higher expression at 0.05 ml min$^{-1}$, thus indicating a more efficient tendency to bone ingrowth for intermediate flow rates of perfusion, where higher shear stresses were applied (figure 5). Accordingly, ARS staining identified a higher presence of calcium mineral deposits at the same perfusion conditions (figure 8). Light microscope images from the scaffold cross sections clearly show the presence of red rounded agglomerates, which preferentially occur in the case of intermediate flow rates (see quantitative values on figure 8), thus confirming the significant role of perfusion stimuli dose on the production of new mECM by hMSC cells.

4. DISCUSSION

The growing scientific interest in scaffold-aided tissue engineering has been encouraged by the advances achieved in scaffold design over the last few years. Despite various methods generating materials and scaffold properties that have been lately developed, an universally recognized gold standard for the generation of ‘ex novo’ tissues such as bone is not yet available. It is well known that an ideal scaffold has to assure an optimal biomechanical environment able to address the cell fate, supply all the needed signals and nutrition to the cells and, thus, more closely mimic the natural healing cascade [29]. However, current limitations in biomaterials and scaffold-aided regeneration strategies for bone increasingly impose a drastic revision of manufacturing processes so that a more fine tuning of basic scaffold features, i.e. porosity, mechanical properties—might satisfy the structural and functional requirements of natural tissue [30]. First of all, high porosity is often coupled with a scarce mechanical response, which prevents sufficient support to cope with the stresses encountered in hard tissue regeneration. In this context, processing strategies typically used for composite materials providing the addition of reinforcement systems [31,32] and/or polymer blending [33,34] may be
adapted to improve mechanical and transport properties for \textit{in vitro} culture.

Recently, PCL/PLA composite scaffolds (fibres of PLA in a PCL matrix) with different degradation kinetics are emerging as a robust model of a microstructured scaffold with time-controlled mechanical and biological properties, able to promote all the basic mechanisms of \textit{in vitro} regeneration \cite{11,12}. The successful idea consists of the judicious combination of the phase inversion/salt leaching technique—a widely employed technique in the design of porous scaffolds—with the filament winding technology traditionally used in the manufacture of composite materials. The resultant scaffold offers an interesting compromise between morphological requirements and mechanical properties, both of which are crucial to assure adequate and suitable spaces to accommodate cells for bone in-growth. The integration of a solid porogen (i.e. sodium chloride crystals) within a three-dimensional polymer matrix enables the creation of a porous network with well-defined pore sizes, consistent with the sizes of porogen used \cite{21} and peculiar shapes able to create a hospitable environment for cells. Also, the use of constituent materials (i.e. PCL or PLA) with tailored degradation properties allows one to modulate the mechanical response of the scaffolds so that they may attain the status of long-term successful platforms for bone tissue engineering. As for the scaffold design, we demonstrated that the use of more concentrated polymer solutions (20/80 w/w) and less fibre cycles (figure 2) assure a more homogeneous spatial distribution of fibres, uniformly packed within the porous structure. Indeed, more viscous solutions inhibit the tendency to form highly fibre-packed layers during the winding procedure.

The latter frequently occurs in the case of less concentrated solutions, where fibres tend to cover the mandrel surface (figure 2b). Meanwhile, the interconnection of pore architecture is mainly guaranteed by the arrangement of single pores between the fibres through appropriate selection of the fibre step during the winding process, which must be larger than the NaCl crystal size \cite{10}. In this context, another contributory factor to obtain fully porous interconnection is the presence of micropores—few micrometres in size—induced by a controlled solvent removal via thermodynamically driven phase inversion mechanisms that adequately support the transport mechanisms involving the nutrient supply and waste removal \cite{35}. These features concur to define the bimodal pore architecture that is necessary to create a favourable environment for culturing MSCs and human osteoblasts, as recently demonstrated elsewhere \cite{21}. Moreover, the selection of appropriate PLA fibre cycle number plays a predominant role on the mechanical response (figures 2 and 3c,d). In particular, the increase of elastic moduli and compressive stresses—calculated at the end of the elastic region (table 1)—appear to be directly ascribable to the reinforcing action of the continuous fibres, firmly embedded in the polymer matrix, which are capable of absorbing the applied cyclic stresses, thus reducing their transfer to the adjacent matrix.

It is noteworthy that the reinforcing contribution of fibres to the mechanical response also contributes to the capability of the stresses to transfer from the polymer matrix to the cells and/or mECM during the culture, absolutely required during the initial stages of bone in-growth.

Besides, the scaffold matrix is the essential element that transfers the mechanical boundary conditions—i.e. mechanical signals—to the cells because it defines their mechanical microenvironment. In this context, fluidodynamic stimuli induced by dynamic culture procedures may contribute to specifically address the biological processes that involve the formation of new bone matrix \cite{29}. Recent studies indicate that different flow rates through scaffold pore architectures that differ in porosity, pore size and pore anisotropy can impart different shear stresses to host cells \cite{36}. In particular, two- and three-dimensional fluidodynamic studies suggest that flow-induced shear stresses can modulate the function of bone cells seeded into three-dimensional scaffolds under perfusion stimuli for long-term culture periods \cite{37}. As a consequence, it is reasonable that the modulation of shear stresses mediated by the use of flow-controlled perfusion systems might successfully influence the differentiation of human mesenchymal stem cells (hMSC) in an osteogenic way. Indeed, applied perfusion flow conditions generally concur to efficient molecular transport and also provides simulation of the complex biomechanical environment that bone cells experience in vivo \cite{38}. Commonly, the medium was directly conveyed throughout the interconnected pores to continuously introduce nutrients and remove waste, for the support of early cell activities (adhesion, proliferation), osteogenic differentiation and mineralized matrix production \cite{39–41}.

In this study, the perfusion chamber (figure 6) has been properly designed on the PCL/PLA tubular scaffolds in order to impose a radial flow along the scaffold walls to recreate the fluidodynamic conditions used in the modelling study. The experimental data confirm the existence of an optimal shear stress range able to stimulate specific cell activities such as osteoblast differentiation and mineralization. In the past, several studies have been carried out to identify a precise stress window associated with specific biological events \cite{26–29}. However, the regulatory effects of shear stress on cellular behaviours have been mainly investigated in planar model systems under a well-defined flow field \cite{42}. Indeed, host cells in three-dimensional constructs tend to develop microenvironments that significantly differ from two-dimensional surface cultures, while information outputted from planar culture systems often are inadequate to be extrapolated to the three-dimensional constructs \cite{43,44}. Recently, Liu \textit{et al.} \cite{45} proposed a novel approach based on the modelling of flow-induced shear stress fields, commonly related to dynamic perfusion procedures, via fluidodynamic simulations. Similarly, we have correlated a predictive model based on the CFD simulations with \textit{in vitro} results to verify the effect of flow rate on the osteogenic activity of hMSC in PCL/PLA scaffolds. In particular, stress distribution data taken from CFD simulations has been compared with the data reported in the literature \cite{26–29} in order to identify the optimal perfusion conditions required for each step of the bone regeneration process.

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Experimental in vitro studies demonstrate that the applied perfusion conditions—in terms of flow rate—significantly influence, in turn, differentiation and mineralization of hMSC in an osteogenic way. These results are in agreement with those of similar studies that extensively show the increase of ALP activity [46] and the over-expression of OC [47], OPN [17] and bone sialoprotein [18] under equivalent conditions. In particular, in vitro results evidently show that intermediate perfusion rates—i.e. 0.05 ml min⁻¹—induce the highest differentiation of hMSC cells to osteoblasts also promoting a calcific massive deposit precipitation to form new mECM. Meanwhile, the further rise of expression level of OC and ALP activity above 0.05 ml min⁻¹ further confirms the ability of selective fluidodynamic features to trigger specific bone-related activities, thus motivating the need of modulating perfusion conditions by chronoprogrammed steps. From this point of view, our approach introduces an efficient operating procedure that allows us to easily identify the proper input parameters (i.e. flow rate) to assign into a perfusion bioreactor during the dynamic culture. In perspective, the implementation of more complex models that further integrate degradation kinetic equations to the proposed model will offer the chance to estimate ab initio changes in local stresses and fluid transport as the polymer phases resorb by a pre-ordered kinetic, so ultimately predicting the evolution of tissue formation in vitro.

5. CONCLUSION
Composite scaffold design represents a new frontier in the development of smart materials that can direct biological events through the guidance offered by morphological, biophysical and biochemical signals, naturally triggered by the extracellular microenvironment. Here, we have studied fibre-reinforced composite scaffolds with porous architecture and functional properties suitable for bone regeneration. The PLA fibres confer a significant improvement of mechanical properties to the PCL matrix that can vary as a function of relative polymer and fibre amount. The matrix capability to transfer hydrodynamic shear stresses to cells allows us to modulate the stimuli at the cell/scaffold interface during in vitro culture, thus directing the cell differentiation to the osteogenic way. The modulation of oscillatory flow conditions in perfusion chambers allows us to promote selectively osteogenic differentiation of hMSC in osteoblasts and mineralization. This suggests that programmed flow rate conditions combined with tailor-made pore architecture should be a valuable and convenient tool for the development of optimization strategies in bone tissue engineering.

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