3D fiber deposition and stereolithography techniques for the design of multifunctional nanocomposite magnetic scaffolds

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Abstract

Magnetic nanocomposite scaffolds based on poly(ε-caprolactone) and poly(ethylene glycol) were fabricated using 3D fiber deposition modeling and stereolitography techniques, and by combining these techniques it has been possible to produce hybrid coaxial and bilayer magnetic scaffolds. The compressive mechanical behavior of these scaffolds were investigated in a wet environment at 37°C, and morphological features were imaged through scanning electron microscopy and X-ray micro computed tomography. Confocal LASER scanning microscopy was used to investigate the *in vitro* biological behavior of human mesenchymal stem cells.

Please provide an abstract of 150 to 250 words

Introduction

In the field of reconstructive surgery, bone and osteochondral bone injuries beyond the self-repair threshold still represents a great challenge. In this scenario, different strategies are currently available for tissue regeneration. Even though the gold standard is represented by autologous bone grafts harvested from healthy bone regions, the amount of tissue required for grafting and the donor site morbidity strongly limits such approach [1]. For this reason, bone tissue engineering may be considered as a promising solution to overcome this limitation [2, 3].

An ideal scaffold for tissue engineering should possess an interconnected porous architecture able to support cell adhesion, proliferation and differentiation, as well as to promote the extracellular matrix (ECM) deposition which is fundamental for tissue regeneration. Taking into account the load-bearing function of bone, a further feature related to capability to withstand forces higher than the body weight during walking must be clearly considered as an important criterion [4]. After the implantation the designed structure must withstand the forces acting on the bone segment and must mimic the mechanical stimulation of bone tissue cells as well as their mechanosensitive response. As consequence, to reproduce a physiological tissue regeneration process mechanical and biological patterns should be properly integrated [5].

Specifically, a scaffold for bone tissue regeneration must possess suitable mechanical properties and must be able to transfer the stress to the hosting tissue. Accordingly, polymer-based composite materials are generally considered to design advanced scaffolds with tailored mechanical and degradation properties which should gradually transfer the

loading function to the newly forming tissue. Thus, the scaffold would be totally replaced by newly formed bone as a results of a tailored degradation process [6-11]. To improve the structural and functional performances of the scaffolds, nanotechnology has allowed the design of nanomaterials, such as particles, grains or fibers which have at least one dimension in the range 1 to 100 nm materials, as well as materials with nano-structured surface features. As a consequence of novel physical properties owing to nanoscale features (i.e., superparamagnetism), nanomaterials may show properties that are better than those of their microstructured counterparts. Benefiting from these basic concepts, it results possible to design materials which may also reproduce the natural nanostructure of human tissues. In particular, as bone is charactterized by a hierarchical structure where the lowest level relies in the nanoscale range, nanocomposite materials can better mimic the constituents of natural bone in comparison to the individual components (polymer and inorganic nanofillers) [10].

A critical feature is generally related to the loading of cells into the scaffolds, and the adopted strategies involve cell seeding before implantation or cell recruitment from the hosting tissue. With regard to the first strategy, the most popular seeding approach is represented by the use of mesenchymal stem cells (MSCs), and the scaffold material must be able to promote MSC differentiation into osteoblasts as well as the vascularization eventually through the incorporation of angiogenic factors [3, 12, 13].

Different techniques have been proposed to manufacture 3D porous scaffolds, each characterized by its own advantages and limitations. Conventional methods such as salt leaching and solvent casting are defined as processes to obtain scaffolds that are characterized by continuous, uninterrupted pore structure. These processing techniques are incapable of precisely controlling pore size, pore geometry, spatial distribution of pores and pore interconnectivity [14, 15]. Conversely, in the field of tissue engineering, solid freeform fabrication, also known as additive manufacturing or 3D printing, represents the key to produce customized scaffolds with reproducible internal morphology. Among these technologies, Fused Deposition Modeling (FDM) represents a challenging method to obtain 3D morphologically controlled scaffolds. It adopts a moving nozzle to extrude a fiber of polymeric material by which the physical model is built layer-by-layer. The pore sizes in tissue engineering scaffolds are sufficiently small enough for the fiber strand to bridge across without additional support structures [14, 16-18].

Stereolithography involves selective polymerization of a liquid photocurable monomer by a ultraviolet (UV) light reflected by a digital light processor, that is a device based on micromirror driven by micro-electro-mechanical technology. This projector provides UV masks according to the CAD cross-sectional data derived from the 3D model. Often, this technique requires support structures to be added to the model, to prevent any over-hanging or unconnected features from falling into the liquid resin filled vat. After printing completion, the model is raised and supports are removed [14, 15, 19-20].

Novel routes in tissue engineering, involving the use of magnetic nanoparticles (MNP), have been proposed in order to trigger tissue growth through magnetic fields [21, 22]. Iron oxide and iron doped hydroxyapatite MNP have been incorporated into a polymeric matrix as an advanced method for functionalising biomaterials for guiding bone regeneration [23, 24]. Moreover, the superparamagnetic feature of these magnetic nanocomposites has allowed to benefit from the effects of hyperthermia [25, 26]. Furthermore, seeding of magnetic labeled cells into magnetic scaffold showed to be a very efficient *in vitro* method, as well as a remarkable bone regeneration has been observed *in vivo* in a rabbit model [27].

Accordingly, the aim of the current research was to analyze some structural and functional features of 3D magnetic scaffolds obtained using 3D fiber deposition technique and stereolithography as well as of multimaterial scaffolds in the form of coaxial and bi-layer structures obtained by properly integrating such methods.

Materials and Methods

Poly(ε-caprolactone) (PCL) and Poly(ε-caprolactone)/iron oxide (PCL/Fe_3O_4) (PCL/ MNPs) were manufactured through FDM technique, poly(ethylene glycol) diacrylate (PEGDA) and PEGDA/MNPs were fabricated using stereolithography. Multimaterial 3D scaffold were also fabricated in the form of coaxial and bi-layer structures by properly combining the above reported techniques.

With regard to PCL-based nanocomposite scaffolds, PCL/MNPs nanocomposite pellets were first prepared and then properly processed to fabricate scaffolds using FDM. PCL pellets (average molecular weight of 65,000, Sigma-Aldrich, St. Louis, MO) were dissolved in tetrahydrofuran (THF, Sigma-Aldrich, St. Louis, MO). MNPs and then ethanol were added to the PCL/THF solution during stirring. A polymer/filler weight ratio (w/w) of 80/20 was considered. To optimize the dispersion of the nanoparticles in the solution, an ultrasonic bath (Branson 1510 MT, Danbury, CT) was also used. The obtained PCL/MNPs pellets were then processed using a 3D bioplotter dispensing machine (Envisiontec GmbH, Gladbeck, Germany) to fabricate 3D cylindrical scaffolds (6 mm in diameter, 8 mm in height) with a 0/0/90/90° lay-down pattern. In particular, PCL and PCL/MNPs scaffolds were built by extruding and depositing the fibers along specific directions according to the properly selected lay-down pattern (Fig. 1a, 1b). The PCL/MNPs pellets were placed in a stainless steel syringe and heated to a temperature of 90°C and 12°C for PCL and PCL/MNP scaffolds, respectively. A nitrogen pressure of 8.5bar was applied to the syringe through a cap. The material was injected/extruded through a nozzle with an inner diameter of 600 μm, and the continuous filament was deposited at a speed of approximately 35 mm/min. 3D fiber-deposited PCL and PCL/MNPs scaffolds were characterized by a fiber diameter of 400-500 μm, a layer thickness of 400 μm and a strand distance (i.e., center-to-center fiber distance) of approximately 1600 μm.

Nanosized MNP powder (particle size <200 nm) was used to prepare PEGDA/MNP resins for stereolithography apparatus. Homogeneous nanocomposite resins were prepared by mixing 15 g of PEGDA with MNP (10 wt% with respect to PEGDA), the dispersions were sonicated for 15 min before use in order to ensure homogeneity. 4 wt% Lucirin-TPO photoinitiator were added to the PEGDA to 3D print PEGDA/MNP composite porous scaffolds through stereolithography. Periodic surfaces in three independent directions were generated using K3DSurf v0.6.2 software in order to generate CAD-files that describe the surfaces of diamond like architectures. The following trigonometric functions with boundary conditions x2+y2: $[-4\pi,4\pi]$ and z: $[-8\pi,8\pi]$ were used [28, 29]. The following equation was used to generate porous structures with porosities of about 70%:

$$\sin(x)\cdot\sin(y)\cdot\sin(z)+\sin(x)\cdot\cos(y)\cdot\cos(z)+\cos(x)\cdot\sin(y)\cdot\cos(z)+\cos(x)\cdot\cos(y)\cdot\sin(z)-0.4=C$$

where the value of -0.4 was the required offset values. A commercial stereolithography apparatus (Envisiontec Perfactory Mini Multilens SLA) was employed to build designed structures. The building process involves subsequent projections of 1280*1024 pixels, each 32*32 μm2 in size. Layers with a thickness of 25 μm were cured by irradiating composite resin with UV light (intensity 17 mW/cm2). The resins formulated with different amount of MNP have been previous characterized on the basis of their working parameters with the STL apparatus. Exposure time depends on the resin composition and it has to be increased by increasing the amount of MNP. The CAD-file reproducing a porous cylinder (D=6mm, h=8mm) according to the periodic surface is converted into STL format and virtually sliced in the Z direction into the layers used in the layer-by-layer fabrication process. Once the building process was terminated uncured excess resin, non-reacted macromer and photo-initiator were extracted from the structures with acetone. The extracted structures were then post-cured at 90 °C for 1 d under vacuum. PEG, PEG/MNP 95/5 and PEG/MNP 90/10 scaffolds (Fig. 1f, 1g, 1h, respectively) were 3D printed.

Coaxial scaffolds (Fig. 1c) were obtained using both the FDM and the stereolitagraphy techniques. The inner porous cilynder (D=6mm and h=12mm) composed of PEG/MNP 95/5 was first obtained through sterelitography as previously described. This cylinder was stucked with a biadesive tape to a rotating mandrel driven by a stepper motor. Hence, a PCL porous outer tube was realised using the 3D bioplotter system in the same fashion of the filament winding process. Bilayer scaffolds (Fig. 1d, 1e) were manufactured using the FDM and the stereolitagraphy techniques. The first layer is a porous cylinder (D=6mm, h=3mm), composed of PEG/MNP 95/5 (Fig. 1d) or PEG/MNP 90/10 (Fig. 1e) magnetic nanocomposites. This layer was realised through steriolitography as previously described. Hence, a second porous layer (D=6mm, h=5mm) composed of PCL (Fig. 1d) or PCL/MNP 80/20 (Fig. 1e) was realised through FDM.

Compression tests were performed on the different kinds of scaffolds in physiological conditions. All the tests were carried out at a rate of 1 mm/min using an INSTRON 5566 testing machine. The stress was computed as the force *F* divided by the total area (A) of the apparent cross section of the porous structure, while the strain was evaluated as the ratio between the scaffold height variation (h) and the initial height (h_0). Mechanical tests were performed in a wet environment at 37°C.

Scanning electron microscopy (SEM) was carried out to assess morphology, pore shape and size of the different kinds of 3D scaffolds. The specimens were gold sputtered and analyzed through a FEI QUANTA 200 FEG scanning electron microscope. Micro-computed tomography (μCT) was also performed using a Skyscan 1072 system (Skyscan, Belgium), and 3D reconstructions were obtained using Mimics 16.0 software (Materialise, Belgium).

Human Mesenchymal Stem Cells (hMSCs, $5.0*10^4$ cells/sample) were seeded on the fabricated 3D scaffolds and grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, BioWhittaker, Walkersville, MD). Cell adhesion and spreading were studied at 7 days after seeding through Confocal Laser Scanning Microscopy (CLSM, Zeiss LSM 510/Confocor 2, Oberkochen, Germany). Actin microfilaments were stained with phalloidin-tetramethylrhodamine B isothiocyanate (Sigma Aldrich) to analyze the different kinds of cell-constructs. The microscope was equipped with helium–neon laser sources at a wavelength of 543 nm and with a 20x objective.

Results

Magnetic nanocoposite cylindrical scaffolds have been successfully processed through 3D printing techniques, and by combining the 3D fiber deposition and stereolitography techniques, it has beed possible to taylor the stiffness, the strength and the ductility of nanocomposite scaffolds over a wide range (Table 1).

Typical stress–strain curves obtained for the 3D scaffolds are reported in figure 2, which evidences the effect of the different materials and of the architecture as a result of technology combination on the mechanical performances of the structures.

The mechanical behaviour of scaffolds obtained through FDM is characterized by a linear region which is initially well evident and suggests a stiff mechanical response at the onset. Such linear zone is then followed by a region with lower stiffness, another stiff region of the stress–strain curve can be finally observed resembling the densification region usually reported for flexible foams. Anyway, unlike the typical behaviour of flexible foams the central region does not show a plateau (i.e., a zero slope region). The compressive modulus (E) has been evaluated as the slope of the initial linear region of the stress–strain curve. Compressive modulus, maximum stress (σ_{max}), maximum strain (ε_{max}), yield stress (σ_y) and yield strain (ε_y) are reported in Table1 as mean value ± standard deviation. Mechanical properties suggest that Young's modulus and strength of PEG based magnetic scaffolds are about 2 order of magnitude lower than PCL based scaffolds, while the combination (i.e. coaxial and bilayer structures) of these materials provides values in between PEG and PCL based nanocomposites (Table 1). It is interesting to note that bilayer magnetic scaffolds (Fig. 2

and Table 1) show a behaviour similar to PEG based scaffolds, while the coaxial scaffold shows a behaviour closer to PCL based scaffolds. During compression of PCL and PCL/MNP scaffolds, a linear region has been detected up to a strain of approximately 5%, and the yield strain of PCL/MNP scaffold was lower than PCL scaffold, while higher values of yield stress have been recorded for PCL/MNP scaffolds (Table 1). A hardening region has been observed for both the PCL and PCL/MNP scaffolds. This region can be interpreted through a densification accuring for pore collapse. In particular, for the PCL scaffold, the hardening region extended up to a strain of about 50%. In contrast, for the PCL/MNP scaffold, this hardening region occurred up to a maximum load at about 40% of strain (Figure 2) followed by a second plateau-like region. The introduction of magnetic nanoparticles strongly influences mechnical properties of both PCL and PEG based scaffolds (Table 1). The Young's modulus increases while ductility (e.g. the strain at failure) decreases. This result can be ascribed to to the difference in the ductility between the polymeric matrix and purely inorganic nanoparticles, and to the discontinuities at the nanoparticle/matrix interface. Hence, the different ductility of the polymers and inorganic nanoparticles are responsible for the distinct mechanical behavior during compression.

SEM images (Fig. 3) and μCT reconstructions (Fig. 4) has allowed to evaluate the physical integrity of the filaments/fibers and layers and to check if the pore shape and size obtained were consistent with the theoretical values defined during the design process. The obtained results have demonstrated that all the designed scaffolds show a fully interconnected pore network as well as a defined architecture, highlighting the different morphology of the structures which should influence the mechanical and functional properties. In particular, SEM image of PEG based scaffolds (Fig. 3b) clearly shows the periodic surface that generated the scaffold. Also, the 25 μm layer stacking sequence can be easily detected. Figure 3c illustrates the quality of the interface between PEG and PCL based magnetic scaffolds obtained combining the stereolitography and the FDM techniques. The good quality of this interface is highlighted by the penetration into the porosity of the PEG based scaffold obtained through stereolitography.

CLSM analyses performed on the different cell constructs have provided interesting results in terms of human hMSC adhesion and spreading over time. As an example, figure 5 reports typical images of the cytoskeleton organization at 7 days after cell seeding for 3D fiber deposited PCL and PCL/MNPs scaffolds. In particular, the results suggested that hMSCs were well spread and better adhered on PCL/MNPs nanocomposites. Furthermore, an increase in the adhered cell number of hMSCs was also evident in the case of the nanocomposite scaffolds.

Discussions

By combining the 3D fiber deposition and stereolitography techniques, it has been possible to taylor the stiffness and the strength of magnetic nanocomposite scaffolds over a wide range, thus covering properties of soft and hard biological tissues [30]. All the implemented 3D printing processes and their combination allowed to manufacture fully porous interconnected scaffolds defined by a precise and repetitive architecture.

Magnetic nanocomposite materials offer the unique mechanism to functionalise a scaffold by switching an external magnetic field on and off. This feature can be incorporated into the scaffold in order to provide cue signals that can be activated on demand, thus triggering the sequential biological events that occur during tissue regeneration [22, 24, 27].

The different mechanical behaviour observed for PCL and PEG based scaffolds tested at 37°C in a wet environment (Figure 2) can be ascribed to the different nature of these two polymers. PCL is a thermoplastic polymer with a glass transition temperature lower than 37°C, thus it is a polymer in the rubbery state at the testing temperature condition. On the other hand, PEG based scaffolds are diacrylates crosslinked through photo-polymerization. The Young's modulus in compression measured at 37°C for PCL and PCL/MNP 80/20 scaffold is slightly lower than those reported for the same

samples tested at room temperature [24]. The though and a brittle behaviour of these polymers can be related to the rubbery and to the crosslinked condition of PCL and PEG, respectively. On the other hand, the bilayer magnetic scaffolds (Fig. 2 and Table 1) show a behaviour similar to PEG based scaffolds, while the coaxial scaffold shows a behaviour closer to PCL based scaffolds. This result can be interpreted through the series and parallel models of the belayer and coaxial scaffold, respectively. In compression, for the series model, the softer material controls the mechanical behaviour, insted in the parallel model the stiffer material mainly transfers the compression stress. Therefore, the methods by which the 3D printing processes are combined strongly influence the mechanical behaviour of the scaffold. The layer upon layer printing along a straight axis (bilayer scaffolds, figures 1d and 1e) and the layer deposition through the winding process (Fig. 1c) allow to obtain two types of hybrid scaffolds characterised by completely different mechanical behaviour (Fig. 2, Table 1). Figure 3c illustrates the good quality of the interface between PEG and PCL based magnetic scaffolds obtained combining the stereolitography and the FDM techniques. The fiber deposition of PCL nanocomposite from the fused state upon (bilayer scaffold) or around (coaxial scaffold) PEG magnetic nanocomposite penetrates into the porosity of the scaffold obtained through stereolitography, thus suggesting a strong interface at least based on a micromechanical adhesion.

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