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# Tailored star poly( $\epsilon$ -caprolactone) wet-spun scaffolds for in vivo regeneration of long bone critical size defects

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## Abstract

One of the most challenging requirements of a successful bone tissue engineering approach is the development of scaffolds specifically tailored to individual tissue defects. Besides materials chemistry, well-defined scaffold's structural features at the micro- and macro-levels are needed for optimal bone in-growth. In this study, polymeric fibrous scaffolds with a controlled internal network of pores and modelled on the anatomical shape and dimensions of a critical size bone defect in a rabbit's radius model were developed by employing a computer-aided wet-spinning technique. The tailored scaffolds made of star poly( $\epsilon$ -caprolactone) or star poly( $\epsilon$ -caprolactone)–hydroxyapatite composite material were implanted into 20-mm segmental defects created in radial diaphysis of New Zealand white rabbits. Bone regeneration and tissue response were assessed by X-rays and histological analysis at 4, 8 and 12 weeks after surgery. No signs of macroscopic and microscopic inflammatory reactions were detected, and the developed scaffolds showed a good ability to support and promote the bone regeneration process. However, no significant differences in osteoconductivity were observed between star poly( $\epsilon$ -caprolactone) and star poly( $\epsilon$ -caprolactone)–hydroxyapatite scaffolds. Long-term study on implanted star poly( $\epsilon$ -caprolactone) scaffolds confirmed the presence of signs of bone regeneration and remodelling, particularly evident at 24 weeks.

## Keywords

Tissue engineering, critical size defects, additive manufacturing, wet-spinning, star poly( $\epsilon$ -caprolactone)

## Introduction

The last three decades have seen an exponential increase of studies aimed at bone defects repair using the tissue engineering approach to avoid the major drawbacks of conventional clinically used bone grafts and treatments (e.g. donor site morbidity, limited availability, immune rejection and pathogen transfer).<sup>1</sup> The most exploited tissue engineering strategy involves the use of a biodegradable, porous scaffold functioning as structural template to fill the tissue lesion and to support tissue regeneration processes.<sup>2</sup> The structural properties of the scaffold at the macro- and microscale levels affect cells shape and activities related to tissue growth.<sup>3</sup> In order to process materials with different physical–chemical and processing properties for the development of scaffolds with suitable porous architecture, several fabrication techniques (e.g. freeze-drying, solvent casting combined with particulate leaching, phase inversion techniques

and electrospinning) have been investigated.<sup>4</sup> However, most of them do not allow an accurate control over scaffold internal porosity and external shape.<sup>5</sup> Additive manufacturing (AM) techniques represent a promising alternative for scaffold fabrication due to the possibility of obtaining three-dimensional (3D) structures with customized composition, porosity, shape and size.<sup>6</sup> A wide range of AM techniques based on layer-by-layer fabrication of scaffolds from 3D model data, such as fused deposition modelling, selective laser sintering and 3D printing, has been explored for different bone tissue engineering purposes.

Wet-spinning is a well-known industrial technique for the production of continuous polymeric fibres that has been widely investigated for biomedical applications.<sup>7</sup> Thanks to the possibility of processing a wide range of natural and synthetic polymers, different fabrication procedures based on wet-spinning technique have been developed for the preparation of polymeric scaffolds. Indeed, a number of studies have reported on the development of scaffolds composed by wet-spun fibres made of natural<sup>8–14</sup> or synthetic<sup>15,16</sup> polymers, through either a physical bonding of prefabricated fibres or a continuous deposition of the solidifying wet-spun filament into a coagulation bath. However, the proposed methods suffer from lack of structural reproducibility and poor automation degree. In order to overcome these limitations, a computer-controlled wet-spinning technique based on AM principles was recently developed and successfully employed to fabricate 3D scaffolds made of different aliphatic polyesters achieving a good control over internal architecture and external shape.<sup>17–19</sup>

Poly( $\epsilon$ -caprolactone) (PCL) is a semicrystalline polymer that has been widely investigated for tissue regeneration applications because of its good biocompatibility, inexpensive production routes, tuneable biodegradation kinetics and mechanical properties, exceptional blend compatibility, good processing properties and ease of shaping.<sup>20</sup> In addition, various PCL-based drug delivery devices have received FDA approval and CE Mark registration. The combination of PCL with hydroxyapatite (HA), a calcium phosphate ceramic that mimics the natural apatite composition of bones and teeth, to develop bioactive composite scaffolds has been widely investigated as an effective means of improving the osteoconductivity and mechanical properties of bone scaffolds.<sup>21–24</sup>

Star polymers are synthetic macromolecules constituted of a number of linear polymeric chains attached to a small central moiety.<sup>25</sup> Due to their relatively small size, spherical structure and limited molecular interactions, star polymers usually show different properties in comparison to their linear molecular structure counterparts, such as lower crystallinity, lower solution and melt viscosity, and better control over chain end concentration.<sup>25,26</sup> A three-arm star PCL (\*PCL)<sup>27,28</sup> was investigated in the past years for the development of microfibrinous scaffolds by solution electrospinning,<sup>29</sup> melt electrospinning<sup>30,31</sup> or wet-spinning<sup>15,16,18</sup> techniques showing good compatibility with preosteoblast cells as well as fibroblast and keratinocyte cell lines in co-culture studies.

In particular, by exploiting a computer-aided wet-spinning system based on a layering manufacturing process, the processing conditions for the fabrication of 3D scaffolds made of either \*PCL or \*PCL-HA composite were optimized achieving good control over internal microarchitecture and external shape.<sup>18</sup> In vitro cell culture experiments showed that the developed \*PCL and \*PCL-HA scaffolds were able to support the adhesion, proliferation and differentiation of MC3T3 murine preosteoblast cells.

The aim of this work was to assess in vivo the ability of tailored \*PCL-based scaffolds fabricated by computer-aided wet-spinning to promote bone regeneration in a critical size defect created in a New Zealand white rabbit model. For this purpose, the processing conditions for the fabrication of \*PCL or HA-loaded \*PCL scaffolds with a geometry closely resembling the anatomical shape of a critical size segment of rabbit's radius were investigated. The presence

and quality of the newly regenerated bone were evaluated by means of X-ray and histomorphometric investigations.

## **Materials and methods**

### **Materials**

A three-arm \*PCL ( $M_w = 189,000$  g/mol) was provided by Michigan Biotechnology Institute (Lansing, MI). HA nanoparticles (size < 200 nm) were bought from Sigma–Aldrich (Milan, Italy).

Acetone and ethanol were purchased from Sigma–Aldrich and used as received.

### **Scaffold preparation by computer-aided wet-spinning**

\*PCL-based scaffolds were prepared using a computer-aided wet-spinning apparatus as described elsewhere.<sup>18</sup> Briefly, \*PCL pellets were dissolved in acetone at 35 C for 3 h under gentle stirring to obtain a homogeneous solution (20% w/v). For the production of \*PCL-HA composite scaffolds, HA nanoparticles were added to the polymeric solution and left under vigorous stirring at 35 C for 1 h until a homogeneous dispersion of the nanoparticles was achieved. On the basis of the results achieved in previous studies,<sup>15,18</sup> the weight ratio between HA and \*PCL in the solution was chosen to be 25%. The prepared solution was loaded into a 5-mL syringe, fitted with a blunt tip stainless steel needle (gauge 22) and placed into an in-house modified subtractive Rapid Prototyping system (MDX-40A, ROLAND DG Mid Europe Srl, Italy) equipped with a syringe pump controlling the solution feed rate (1 mL/h). The 3D scaffolds were fabricated with a layer-by-layer process by extruding the solution directly into a glass beaker containing ethanol. The initial distance between the tip of the needle and the bottom of the beaker was 3 mm, and the deposition velocity was 240 mm/min. A deposition trajectory aimed at the production of anatomically shaped scaffolds with a 0–90 lay-down pattern (inter-fibre needle translation distance of 0.5 mm along the X-axis and of 1 mm along the Y-axis, with 0.5-mm staggered fibre spacing between successive layers composed of fibres aligned along the Y-axis) was calculated using an algorithm developed in MATLAB software (The Mathworks, Inc., Natick, MA) and uploaded into the equipment through the software Vpanel for MDX-40A. After fabrication, the scaffolds were removed from the coagulation bath and kept under a fume hood overnight. Residual solvents were removed by keeping the scaffolds in a vacuum chamber for 48 h. The scaffolds were then exposed to ultraviolet (UV) light for 20 min each side and disinfected with 70% ethanol–water solution for 3 h. After ethanol removal, scaffolds were extensively washed with physiological solution.

### **Scaffold morphological evaluation**

The scaffold morphology was assessed using scanning electron microscopy (SEM; JEOL LSM 5600LV, Tokyo, Japan) under backscattered electron imaging. SEM micrographs were acquired from the top view and the cross section (obtained by fracture in liquid nitrogen) of the scaffolds at different magnifications. Average fibre diameter ( $d_1$ ), XY pore size and Z pore size were determined by means of ImageJ 1.43u software (National Institutes of Health, Bethesda, MD) on SEM micrographs with a 50Å~ magnification. Data were calculated over 20 measurements per scaffold.

### **Animal groups and surgical procedure**

Forty-two 4-month-old male New Zealand white rabbits, weighing between 2.0 and 3.0 kg, were enrolled in the study. Animals were kept into separate cages, fed with a standard diet and

allowed free mobilization during the study. All the procedures were performed under a protocol approved by the local ethic committee of Pisa University. National Institutes of Health (NIH) Guidelines for Care and Use of Laboratory Animals were observed. All animals were screened for physical conditions.

Each surgical procedure was performed under general anaesthesia that was induced by an intramuscular injection of medetomidine (20 µg/kg), ketamine (5 mg/kg) and fentanyl (0.1 mg/2 mL, 10 µg/kg). A venous catheter was inserted into the ear vein for the administration of propofol (4–6 mg/kg in bolus; 0.7–0.9 mg/kg/min in continuous infusion), and every rabbit was maintained with oxygen in mask. The right limb of each animal was shaved and the skin prepared in the standard method with antiseptic solution (10% povidone-iodine solution). A loco-regional anaesthesia with ropivacaine 0.5% was performed in the brachial plexus to optimize the intra- and post-operative analgesia. A constant monitoring of heart rate, breathing, blood pressure and temperature was performed in each animal. In order to reduce peri-operative infectious risk, antibiotic prophylaxis was subcutaneously given (enrofloxacin 10 mg/kg). The in vivo study was performed in two different phases. In the first phase, two different scaffolds (\*PCL and \*PCL-HA) for three different observation times (4, 8 and 12 weeks) were tested. Six groups (six animals each) were created (\*PCL 4w, \*PCL 8w, \*PCL 12w, \*PCL-HA 4w, \*PCL-HA8w and \*PCL-HA 12w). After the first evaluation in terms of bone regeneration, a group of six animals was created to evaluate bone regeneration in a longer observation time (24 weeks). Each animal was positioned in right lateral recumbency; a vertical skin incision 40 mm long was performed over the middle of the antero-medial aspect of the right radius. After muscle dissection, a segmental defect of 20 mm of radial diaphysis with periosteum was created through an osteotomy under continuous saline cooling. Each segmental bone removed was measured in terms of length, width and thickness. A \*PCL or \*PCL-HA scaffold with similar dimensions was filled into the defect.

No additional fixation was used for the implants. Soft tissues were closed in separate layers using monofilament absorbable sutures (polydioxanon 4-0). A protective bandage was applied for 4–5 days. To minimize post-operative discomfort, buprenorphine (30 µg/kg q8h for 2 days) and carprofen (4 mg/kg q24h for 7 days) were administered intramuscularly after surgery. The antibiotic prophylaxis was extended to 5 days after surgery. The animals were housed in single cages and were observed for signs of infections, pain and proper activity. Rabbits of each group were humanely sacrificed at 4, 8, 12 and 24 weeks after scaffold implantation by an intravenous injection of pentobarbital. After euthanasia, surgical skeletization of treated limbs was immediately executed, and macroscopical evaluation of the regeneration site was performed in order to assess the characteristics of peri-implant tissues and newly formed bones.

## **Radiological evaluation**

After surgery, digital X-ray (FUJIFILM FCR Capsule X – FUJIFILM Medical System Italia spa,

software Windows MicroDicom-free DICOM viewer version 0.5.4) of treated forelimb was performed to evaluate the length of osteotomy and radiopacity of each scaffolds. Medio-lateral and antero-posterior X-rays of treated forelimbs were executed under general anaesthesia every 4 weeks to compare the serial changes of callus in the bone defect site. Bone regeneration was evaluated using a modified system to score defect bridging and bone formation<sup>32,33</sup>.

Three features were considered: degree of defect bridging (percentage of the total defect), new bone union with host bone (percentage of bridged bone at each side of the defect) and amount of bone remodelling (formation of cortical bone and/or bone marrow cavity).

## **Histological procedures**

At 4, 8, 12 and 24 weeks after surgery, specimens of 40 mm were explanted (scaffold, 10-mm proximal and distal radius bone and ulna) and placed in 4% neutral buffered formaldehyde solution for 48–72 h at room temperature. After which they were transferred to a commercial decalcifying solution (Histo-Line laboratories cat. 02-101) for 1 week at room temperature or until the bone became mechanically flexible. After washing in tap water for 12 h, the specimens were divided in three portions (proximal, central and distal) and processed for paraffin embedding by Leica TP 1020 automatic tissue processor (dehydration with a creasing series of alcohol and clearing with xylene). Either transversal or longitudinal sections of 6 µm were cleared in xylene, dehydrated with a decreasing series of alcohol and stained with haematoxylin–eosin, Mallory trichrome, toluidine blue and Congo red. For light microscopy examination (Leitz DIAPLAN), a limited number of samples selected on the basis of their radiological score (high (>11 points), medium (from 6 to 10 points) and low (<5 points)) were chosen.

### **Statistical analysis**

Quantitative data were presented as mean ± standard deviation (SD). Data regarding the radiological assessment of regeneration were evaluated with the one-way analysis of variance (ANOVA) test, and the Tukey test was used for post hoc analysis. Significance was defined for  $p < 0.05$ . Student's t-test for unpaired data was used to evaluate the difference between the two groups (\*PCL and \*PCL-HA) with significant values fixed for  $p < 0.05$ .

## **Results**

### **Development of anatomically shaped scaffolds**

Anatomically shaped \*PCL and \*PCL-HA composite scaffolds with external shape and size resembling a critical size defect in rabbit's radius were successfully fabricated by employing an AM technique based on the computer-controlled wet-spinning of a polymeric solution. A 3D porous network was built up with a predefined 0–90 lay-down pattern by alternatively fabricating one on top of the other layers obtained with an inter-fibre needle translation distance (dF) of 0.5 or 1 mm. SEM image analysis showed good reproducibility of the internal architecture of the scaffolds in terms of fibre morphology and size, fibre alignment and layered structure. The scaffolds were composed of overlapping layers of oriented \*PCL or \*PCL-HA fibres with a highly porous morphology of the single fibre characterized by a pore size of few micrometres. Fibre diameter was in the range 200–250 µm, while inter-fibre pore size was in the range 300–800 µm in the XY-axes and in the range 200–300 µm in the Z-axis. HA-loaded scaffolds showed significantly smaller fibre diameter with respect to the plain \*PCL scaffolds, together with a lower porosity of the single fibre.<sup>18</sup> As a consequence, the XY and Z pore sizes of \*PCL-HA scaffolds were significantly larger when compared to plain \*PCL scaffolds.

### **In vivo study**

**Macroscopical evaluation.** All treated rabbits showed a good recovery of forelimb function, and in general, no complications were observed. Only one rabbit exhibited signs of discomfort in the first 24 h after surgery, showing resting on the back of the phalanges, self-trauma and mutilation of a phalanx of the treated limb. This complication was pharmacologically treated with systemic glucocorticoid (methylprednisolone hemisuccinate 1 mg/kg q12h for 3 days) and clinical signs solved in 3 days with complete recovery of function of the limb. This animal was not excluded from the study.

**Radiographs.** The X-rays performed immediately after surgery confirmed that the defects were indeed a critical size defect ( $20 \pm 0.89$  mm). The epiphyseal plates at this time point were not completely closed. Results from final X-ray images before the removal of the scaffolds are described below. In \*PCL 4w group, no signs of bone remodelling were detected, proximal and

distal unions were greater than 51% in three rabbits (50%) and new bone regeneration was greater than 51% in four animals (66.7%). The mean total score was 7.75 ( $\pm 3.8$ ). The \*PCL-HA 4w group also showed no signs of bone remodelling, proximal and distal union were judged good (>50%) in a single case and sufficient in four animals (66.7%). New bone bridging was considered good in four animals.

The mean total score was 7.41 ( $\pm 1.39$ ). The analysis of the radiographs collected from \*PCL 8w group highlighted signs of remodelling with the formation of an intramedullary canal in two rabbits; proximal and distal unions were considered good in five animals (83.3%), and new bone bridged more than 51% of the scaffold surface in four animals (66.7%). The mean total score was 9.33 ( $\pm 2.71$ ). In \*PCL-HA 8w group, an intramedullary canal was partially detected in three animals, and only one rabbit showed a good remodelling of cortex. Proximal and distal unions and new bone formation were considered greater than 50% in four animals. The mean total score was 9.58 ( $\pm 2.45$ ). In \*PCL 12w group, bone remodelling was not evident in one rabbit (16.6%); a good proximal and distal osteointegration with the host bone was detected in three rabbits (50%). Bone formation occupying greater than 75% was observed in two animals (33.3%). The mean total score was 7.66 ( $\pm 4.72$ ).

In \*PCL-HA 12w group, signs of bone remodelling were detected in three animals (50%), one of which showed a full cortex remodelling. Good union at the proximal and distal sides (>75%) was observed in three rabbits. New bone formation occupying greater than 75% of the defect was observed in four cases (66.7%). The mean total score was 10.08 ( $\pm 3.92$ ).

No significant differences in radiological scores between the two types of scaffolds (\*PCL and \*PCL-HA) at all observation times were detected. Finally, in \*PCL 24w group, a good bone regeneration was detected in five animals (83.4%), with a mean total score of 12.6 ( $\pm 4.5$ ). Only one animal (16.6%) showed poor regeneration with a total score of 3.5.

The comparison of X-rays at different observation times in animals with medium and high radiological scores showed the tendency of the newly formed tissue to fill the scaffolds at 4 weeks. A subsequent bone remodelling with the formation of a medullary canal was also observed. In animals with low radiological scores at 4 weeks, no signs of improvement were detected at all subsequent observation times.

Visual assessment. Macroscopically, inflammatory reaction signs were not evident in soft tissues around the scaffolds in all treated animals. A newly formed bone, on the lateral side of the scaffold near the ulna, was observed in all cases in which high and medium radiological scores were present. In animals showing a low radiological score, no evidence of newly formed bone was macroscopically observed.

Histology. Qualitative histological examination generally confirmed radiological observations, and there were no significant differences between \*PCL and \*PCL-HA groups at all observation intervals considered. Sections obtained from samples with a low radiological score showed small quantities of newly formed bone tissue that did not ever fill the surgical gap; connective or granulation tissue was present between the scaffold fibres together with some areas of mononucleated inflammatory infiltrate and fat cells. Mallory trichrome staining was useful to assess the interface among the biomaterial, the immature bone growing into mature lamellar type and the connective tissue layer around the bone defect.

In sections obtained from samples with a medium and high radiological score, the surgical gap was filled with a new bone segment appearing either as a bridge projecting from ulna to the junction between radius and the scaffold or as ossification cores between the fibres of the scaffold. The newly formed bone progressively invaded the scaffold resembling the radius diaphysis, and in most cases, it was fused with the ulnar diaphysis forming together the specie's physiological angle. In a few sections, two single sprouts were found to be coalescing, thus resembling a single diaphysis. Many small cavities surrounded by

mature bone confluent to a single medullary cavity were observed. Cavities were sometimes occupied by scaffold fibres, fat cells and mononucleated elements such as bone marrow cells. Even at 24 weeks, the scaffolds were not completely invaded or replaced by bone tissue, and the fibrous structure was clearly visible although the layered architecture was less ordered and the fibre morphology less uniform.

## Discussion

A number of AM techniques have been proposed in the past decade for the layered fabrication of PCL-based scaffolds, such as those based on laser and UV light sources (e.g. stereolithography and selective laser sintering), 3D printing, melt extrusion-based techniques (e.g. fused deposition modelling) and direct writing techniques.<sup>20</sup> In addition, the computer-aided wet-spinning technique exploited in the present research activity was recently developed to design and manufacture with AM principles 3D constructs constituted of polymeric microfibres.<sup>17–19</sup> In comparison with other techniques for wet-spun fibre scaffold fabrication, this computer-aided technique enables the design of customized scaffolds that can meet specific requirements in terms of accurate anatomical geometry, tuneable pore size and controllable pores interconnectivity. The spongy morphology of the fibres constituting the scaffolds is the consequence of the phase separation-based solidification mechanism of the spun solution, resulting in the formation of a polymer-rich phase surrounding polymer-lean droplets that will form the pores after solvent removal.<sup>18</sup> In comparison with dense strands obtained by other AM techniques, such highly porous morphology of the single fibres can present some advantages in influencing the biodegradation rate, the mass transfer phenomena associated to cell activities and the mechanisms regulating cell adhesion and proliferation.

Differences in porosity and structural parameter size of HA-loaded scaffolds in comparison to plain \*PCL scaffolds could be attributed to an effect of the bioceramic on polymer solvation and solidification kinetics, as suggested by previous studies reporting on the development of wet-spun \*PCL-HA composite fibrous structures.<sup>15,18</sup> The defect created in the radial diaphysis represents a critical size bone defect since its size (20 mm) is greater than two times the diameter of the radius segment.<sup>34</sup> Different studies regarding bone regeneration in radial diaphysis of New Zealand white rabbit demonstrated that no-treated 15-mm-long bone gaps did not show signs of regeneration.<sup>32,33,35–38</sup> Considering these data, it is possible to assert that \*PCL-based scaffolds seem to show a good ability to support and promote the bone regeneration process. The X-ray data showed that in all cases, bone regeneration along the scaffold was present. There were no differences in radiological scoring results considering the two types of scaffolds (\*PCL and \*PCL-HA). HA is a bioactive synthetic ceramic similar to the natural bone apatite that represents the main component of bone matrix.<sup>39</sup> HA has been extensively studied for bone regeneration and is defined as bioactive for its capacity to support bone growth and osteointegration of orthopaedic, dental or maxillofacial implants.<sup>40</sup> Like other CaP ceramics, HA is degraded *in vivo* through an osteoclast-mediated mechanism by simultaneous resorption and phagocytosis.<sup>41</sup> Usually, HA has a slow degradation that depends on several factors, such as purity, porosity, microstructure, granule size, protein adsorption capability, implant site and body conditions. The commercial synthetic HA nanoparticles employed as scaffold filler in this study were previously investigated by Lee et al.<sup>42</sup> in comparison to natural HA from eggshells. When implanted in a rabbit calvarial defect model, most of the synthetic HA nanoparticles were still present after 8 weeks and appeared as bubble-like voids after the decalcification process. A great number of studies on bone tissue engineering have reported on the development of composite materials by means of inclusion of HA particles in a biodegradable polymeric matrix in order to improve scaffold mechanical properties.<sup>15,43–45</sup> In addition, the inclusion of HA particles in a polymeric matrix can increase



the osteoconductivity and create a basic pH that can contrast the products of the acid degradation of the polymer matrix.<sup>21,24,46,47</sup>

Although previous studies have shown that HA inclusion into a polymeric matrix can lead to increased scaffold osteoconductivity *in vitro*,<sup>2</sup> the \*PCL-HA scaffolds developed in this study showed no significant differences ( $p > 0.05$ ) in terms of bioactivity in comparison to plain \*PCL scaffolds. These results are in line with those of a previous *in vitro* investigation showing that HA loading did not lead to any significant enhancement of proliferation, differentiation and extracellular matrix production of preosteoblast cells grown on \*PCL-based scaffolds by computer-aided wet-spinning.<sup>18</sup> A possible explanation for these results could be that the wet-spinning process does not allow for having a proper concentration of HA on the outer surface of the fibres necessary to achieve an enhancement of scaffold osteoconductivity. However, although a considerable amount of literature reports on the *in vitro* HA-induced osteoconductivity of composite scaffolds, to the best of our knowledge, most of the studies regarding the *in vivo* ability of PCL-HA composite scaffolds to support bone formation do not involve the employment of plain PCL as a reference scaffolding material.<sup>48-51</sup> Further investigations on this topic are clearly needed and should be addressed by performing comparison studies of the bioactivity of both plain PCL and PCL-HA scaffolds in a complex *in vivo* environment. In medium and high radiological score samples, the newly formed bone filled completely the gap at 4 weeks. The subsequent X-ray analysis at 8, 12 and 24 weeks showed signs of bone remodelling.

However, in samples with a low radiological score, signs of radiological bone regeneration was not evident at 4 weeks, and no other signs of bone regeneration was detected in successive X-ray images at 8, 12 or 24 weeks.

Macroscopical analysis of samples confirmed the radiological evidence of absence of periimplant inflammatory reaction. At the time of explantation, the polymer was macroscopically visible in all treated forelimbs after their surgical skeletization. The amount of visible scaffold depended on the degree of bone regeneration. However, a part of the polymer was always evident, also in those rabbits in which bone bridging was complete, including animals belonging to \*PCL 24w group. In fact, regeneration was mainly present on the lateral side of the bone, near the ulna, leaving the central and medial areas of scaffolds macroscopically observable. Histological data further supported these findings by showing a newly formed bone tissue that progressively invaded the scaffold on the side closer to the ulna. At 24 weeks, the growing tissue had only partially colonized the scaffold section being infiltrated in a few fibrous layers. It is, therefore, evident that the bone regeneration process requires longer experimental time to be completely accomplished and that a fibrous scaffold structure is still needed 24 weeks after implantation to guide tissue growth and fill the defect gap.

The paraffin embedding process required the use of xylene that, as reported in the literature,<sup>52,53</sup> completely dissolves PCL and other biodegradable polyesters, leaving behind white spots in the histological sections corresponding to the original scaffold structure. However, in thisthistological images, the \*PCL fibres are still visible, even if in some cases they appear partially dissolved. The reason for this is not clear, but it might be related to different factors, such as the slow solubility of \*PCL in comparison to linear PCL due to its relatively high molecular weight (189,000 g/mol) and star molecular structure.

Mallory trichrome staining allowed assessing that the regenerated bone was mainly evident on the lateral site, near the ulna, where a periosteal activation which leads to join the newly formed bone with the ulna was detected. Similar results were collected in other studies about bone regeneration in a radial rabbit model and could be related to the surgical technique or the presence of the cambium cells that induce osteogenesis.<sup>33,35,36</sup> Gentle drilling under saline

cooling, permanence of periosteum and interosseus membrane could accelerate bone regeneration as scratching of the ulnar bone surface during their removal.<sup>54–56</sup> O’Driscoll<sup>57</sup> and Simon et al.<sup>58</sup> highlighted the importance of the cambium layer which is finely connected to fibrous tissue and can be easily stimulated during periosteal removal. The cambium cells, stimulated by surgery osteotomy, are more active in young animals<sup>59</sup> and can penetrate the scaffold and start the process of osteogenesis. The bone experimental model used in this study may have influenced results because of the close proximity between radius and ulna. A large number of studies were performed using other bone segments, such as femur, tibia, maxilla, iliac crest or calvarian bones.<sup>34,60,61</sup> More accurate data about regeneration capacity of scaffolds tested seem to be obtained from these models in the absence of influence of periosteum activity of neighbouring bones. However, in these models, a large number of complications in intra- and post-operative periods can occur.<sup>34</sup> Thus, radial diaphysis osteotomy represents a validate model to study bone regeneration process in critical size defect.<sup>33–35,62</sup> Furthermore, the rabbit model is the most used in experimental studies about the evaluation of different types of biomaterials for bone implants.

## **Conclusion**

This study has shown that the computer-aided wet-spinning technique can be employed for the AM of tailored 3D \*PCL-based scaffolds with an external shape modelled on the anatomical shape of a critical size rabbit’s radius defect. The optimization of the processing parameters allowed to develop a predefined scaffold microstructure composed by layers of aligned \*PCL or \*PCL-HA fibres with a highly porous morphology. No signs of macroscopic and microscopic inflammatory reactions were detected during in vivo study, and a regenerated bone tissue penetrating into the scaffolds structure validates the osteoconductivity of both tested scaffolds. However, HA loading into the polymeric matrix did not lead to any significant change in scaffold osteoconduction properties. Although further evaluations are necessary regarding the ability of osteointegration and osteoinduction of scaffolds, also using other animal models, the scaffolds analysed in this study represent an interesting alternative to standard bone grafts for the treatment of bone critical size defects.

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