

BIOCOPATIBILITY AND BIODEGRADABILITY OF ELECTROSPUN PHEA-PLA SCAFFOLDS: OUR PRELIMINARY EXPERIENCE IN A MURINE ANIMAL MODEL

A. I. LO MONTE^{a,b,e,g,h*}, M. LICCIARDI^{c,h}, M. BELLAVIA^{a,g,h}, G. DAMIANO^{e,h}, V. D. PALUMBO^{e,h}, F. S. PALUMBO^{c,h}, ALIDA ABBRUZZO^{g,h}, CALOGERO FIORICA^{c,h}, GIOVANNA PITARRESI^{c,f,h}, F. CACCIABAUDO^{b,h}, C. TRIPODO^{d,e,h}, B. BELMONTE^{d,e,h}, G. SPINELLI^{e,h}, R. ALTOMARE^{g,h}, M. C. GIOVIALE^{g,h}, G. CASSATAⁱ, A. SAMMARTANO^{b,h}, P. GENOVA^a, A. SALINA^j, G. BUSCEMI^{a,b,e,h,j}, G. GIAMMONA^{c,f,h}

^a*DiChirOn Department, bPhD Research in Surgical Biotechnology and Regenerative Medicine in Organ Failure, cDepartment of Scienze e Tecnologie Molecolari e Biomolecolari (STEMBIO), dScience and Health Promotion Department, eSchool of Medicine, fIBF-CNR, gSchool of Biotechnology, hUniversity of Palermo, iIstituto Zooprofilattico Sperimentale della Sicilia "A. Mirri", jConsorzio InterUniversitario per I Trapianti d'Organo – Rome, Italy*

We obtained a nano-fibrillar scaffold starting from a polymeric solution which, through electrospinning, gave a biodegradable material with optimal mechanical features and the capacity to allow cell adhesion. In this paper we report the in-vivo application on a murine animal model of two electrospun biodegradable materials, specifically designed to create tubular structures. In one case PHEA-PLA was co-spun with silk fibroin (Fibro-PHEA-PLA) by a parallel electrospinning process to obtain a scaffold with two different polymeric fibers. In the other case, PHEA-PLA was mixed with polycaprolactone (PCL-PHEA-PLA) to obtain a hybrid fibers scaffold. The in-vitro assay showed fibroblast colonization in both materials. The scaffolds were implanted in the dorsal fascial pouch of rats to evaluate their in-vivo biocompatibility and tissue integration. Histopathological findings showed that after implantation a neutrophilic reaction associated to colliquative necrosis was predominant, particularly for PCL-PHEA-PLA. Fibro-PCL-PHEA caused a non organized stromal reaction. Cell adhesion was confirmed at SEM scan. Both materials were totally absorbed after 40 days with an inflammatory reaction. This preliminary study showed that biocompatibility of the scaffolds needs further investigation. The capability of the materials to be functionalized could allow us to modulate the inflammatory host response.

(Received December 15, 2011; Accepted February 29, 2012)

Keywords: electrospinning; three-dimensional scaffolds, PHEA-PLA

1. Introduction

The organ failure appears as one of the major unsolved problems of medicine, given that a great number of patients are on waiting list for a transplant each year. The percentage of patients needing a transplant has increased exponentially over the last ten years and many of them die before receiving the organ. In addition, the costs of interventions, medical care and hospital stay are very high. The alternative use of artificial prostheses, on the other hand, cannot prevent a progressive deterioration of the patient's condition as they are not able to perform all the functions of a single organ.

* Corresponding author: attilioignazio.lomonte@unipa.it

Regenerative medicine is an emerging interdisciplinary field of research and clinical applications focuses on the repair, replacement, regeneration of cells, tissues or organs whose functions are damaged by congenital defects, diseases or trauma [1-6]. In particular tissue engineering, a specific branch of regenerative medicine which implies use of three-dimensional scaffolds, has emerged as versatile approach for the repair/regeneration of damaged tissues through the use of new biocompatible materials. Different approaches can be chosen in scaffold-based tissue engineering: the seeding of cells on an implanted scaffold, the implantation of tissues grown *in vitro* on a scaffold or the cell-free system of scaffolds to support tissue regeneration *in situ*. In particular the adhesion, proliferation and differentiation of cells are strongly influenced by the microenvironment associated with the scaffold as well as the size, geometry, density of pores, and superficial properties [7]. For all these approaches, the scaffold must provide a three-dimensional structure that will support the growth of new tissue inside with properties similar to replaced tissue [8].

Three classes of biomaterials are usually used in tissue engineering: materials of natural origin as collagen and fibroin alginate, acellular tissue matrices (submucosa of the bladder and small intestine), synthetic polymers (polyglycolic acid, polylactic acid) [9-12].

Among the natural polymers, silk fibroin provides an important group of biomaterials and scaffolds for options in biomedical applications because of its high tensile strength, controlled biodegradability, haemostatic properties, non-cytotoxicity, low antigenicity, and non-inflammatory features [13-15]. The advantages of implants consisting of polymeric biomaterials are: easiness of manufacturing in various forms (solid, film, viscoelastic materials), possibility of modulation of the chemical, physical and mechanical properties, absence of corrosion unlike metals, density of the polymer similar to that of natural tissues (1 g/cm^2), possibility to incorporate other substances (e.g. heparin) by direct linkage.

The disadvantages are: the low elastic modulus, their rapid biodegradability, the necessity of additives such as antioxidants or plasticizers.

The biocompatibility and biodegradability of polylactic acid (PLA) and polylactic-co-glycolic acid (PGLA) are extensively studied. Shive and Anderson reported, for example, that local or systemic administration of microspheres made of PLA and PGLA containing insulin does not give rise to any adverse reaction *in vivo* [16]. Over the past two decades, biodegradable polymers as PLA, PGA and poly- ϵ -caprolactone (PCL) have emerged as a class of biomaterials of growing interest in surgical applications, the drug delivery and tissue engineering (for example, sutures for wound healing, internal fixation devices for bone structures, carriers for the release of bioactive molecules, scaffolds for the regeneration of tissues or organs).

Each polymer has its own peculiarities so the development of composite scaffolds, based on a combination of two or more types of materials with selected properties, seems the best choice in tissue engineering [17].

The purpose of this work is to study the biocompatibility and biodegradability of electrospun PHEA-PLA polymeric scaffolds implanted in a murine animal model.

2. Materials and methods

2.1 Materials

The tested polymeric scaffolds were prepared in the Laboratory of Biocompatible Polymers, Department of Molecular and Biomolecular Sciences and Technologies (STEMBIO) at University of Palermo, and are made of biocompatible polymers based on synthetic and natural compounds. The starting polymer, used for the production of electro-spinnable copolymers is the α,β -poly(N-2-hydroxyethyl)-D,L-aspartamide (PHEA) [18-20]. This macromolecule has been used in the Laboratory of Biocompatible Polymers as starting material for the synthesis of the copolymer suitable for the construction of the scaffolds used in this study, i.e. the copolymer PHEA-PLA which was obtained by the chemical binding of PHEA with the biodegradable polymer PLA [18].

The electro-spinnable PHEA-PLA was mixed with two other biodegradable polymers, PCL and fibroin. The two three-dimensional scaffolds (PCL-PHEA-PLA and Fibro-PHEA-PLA) made in the form of leaflets microfibrils, were then tested *in vivo* by subfascial implantation on rats. In particular, thanks to the existing agreement between the University of Palermo and Experimental Zooprophylactic Institute of Sicily "A. Mirri" we used 6 Winstar breed male rats whose average weight was 350 g.

Surgical procedure

The animals were sedated with intramuscular injection of midazolam; anesthesia was maintained with isoflurane and oxygen gas mixture administered with a mask. With the animal in prone position and the four limbs attached to the operating table, it was carried out the trichotomy of the back and the disinfection of the surgical field with povidone iodine 10%. Using a scalpel blade 15 two longitudinal incisions were created, about 1 cm in length and, using scissors, it was reached the subcutaneous fascia surrounding the muscles of the back, with blunt dissection.

Opened the fascia, the PCL-PHEA-PLA scaffolds was hosted on the left. In the subfascial pocket on the right side it was hosted the Fibro-PHEA-PLA scaffold. During these procedures, care was taken to ensure adequate hemostasis, in order to house the scaffold in a bloodless field. To mark the implant site an intradermal injection of methylene blue along the edges of incision was made.

The skin was drawn close with stitches of silk 2/0. At intervals of 7, 15 and 40 days animals were sacrificed to remove the scaffold together with the adjacent tissue and, by histopathological analysis, to evaluate the biological response to implantation in terms of anatomomorphological changes. For sedation of the animals we used midazolan intramuscularly followed by intracardiac injection of 0.5 ml of enbutramide/mebezonio iodide/tetracaine for cardiac arrest. With the animal in the prone position it was removed the whole region containing the scaffold. More precisely, after severing the spinal column to the extreme, the side of the region from the adjacent flat was detached with scissors, taking care to avoid damaging during the dissection of intra-abdominal organs.

Scaffolds microscopy

Electro-spun fibers, prior to implantation *in vivo* and after explantation, were viewed by scanning electron microscopy (SEM) (ESEM FEI QUANTA 200F) with an accelerated voltage of 15 kV. After removal, the leaves of scaffolds were washed with phosphate-buffered isotonic saline and adherent cells component fixed in formaldehyde solution at 4% v/v. Subsequently, the samples were dehydrated by treatment with ethanol 30%, 50%, 70%, 90% and pure ethanol and finally, after treatment with hexamethyldisilazane, freeze-dried. The morphology of the electro-spun fibers and the biological adhesion after the removal of the materials were evaluated.

Histopathological analysis

Histopathological analysis on tissue samples taken from areas of contact with the scaffold after 7, 15 and 40 days from implantation in rats was performed by the coloration with hematoxylin-eosin and trichrome.

3. Results

In the Laboratory of Biocompatible Polymers, Department of Molecular and Biomolecular Sciences and Technologies (STEMBIO) of the University of Palermo has been experimented a simple and reproducible methodology for the synthesis of the biocompatible copolymer PHEA-PLA [18]. This synthetic copolymer PHEA-PLA shows particular properties that make itself suitable for the production of biocompatible scaffolds: the first is insolubility in water (due to the high degree of functionalization in PLA that makes the copolymer hydrophobic enough to prevent its solubilisation in water), the second is that the polymer backbone still has 90% of free hydroxyl groups which can bind biologically active agents such as growth factors, chemotactic factors or drugs. Swelling studies of elettrospun PHEA-PLA-based scaffolds, (data not reported because elsewhere submitted) in a buffered phosphate solution at 37 ° C, to mimic physiological conditions, demonstrated that these fibers are able to incorporate water, swell and break up completely after 28 days of incubation. Interestingly, these degradation data in a physiological environment, observed for PHEA-PLA scaffolds, coincide with the time needed to dermal fibroblasts for the

synthesis of new ECM, making the absorbable material ideal for the production of new healthy tissue. On the other hand, cytocompatibility studies on fibroblasts (data not reported because elsewhere submitted), isolated from human dermis and putted in direct contact with the PHEA-PLA-based scaffolds *in vitro* demonstrated that PHEA-PLA scaffold does not cause a decrease in cell viability.

In the present study we evaluated the *in vivo* biocompatibility of electrospun scaffolds obtained by mixing PHEA-PLA copolymer alternatively with fibroin and PCL. The scaffolds obtained from the mixture Fibro-PHEA-PLA have fibers with a diameter between 600 nm and 1.9 microns (see Figures 1a and 1b). The fibers of scaffolds obtained from the mixture PCL-PHEA-PLA have instead a diameter between 500 nm and 1 μ m, as shown in Figure 1c and 1d. Although both scaffolds showed good mechanical macroscopic properties that enabled easy manipulation of implant for all experiments, the sample of PCL-PHEA-PLA had a better elasticity and strength, probably due to greater regularity and uniformity in size of its constituent fibers and the absence of fusion between them (which, on the contrary, partially characterizes the Fibro-PHEA-PLA sample). The homogeneity of the fibers and their disordered distribution give to both scaffolds morphological features similar to those of the native extra cellular matrix (ECM). The ability of the electro-spun scaffolds to attract and retain water is a very important feature in materials for the implant, given that retention of exudates in the wound bed promotes the recall of cells like fibroblasts and diffusion of growth factors needed for recovery of cell proliferation.

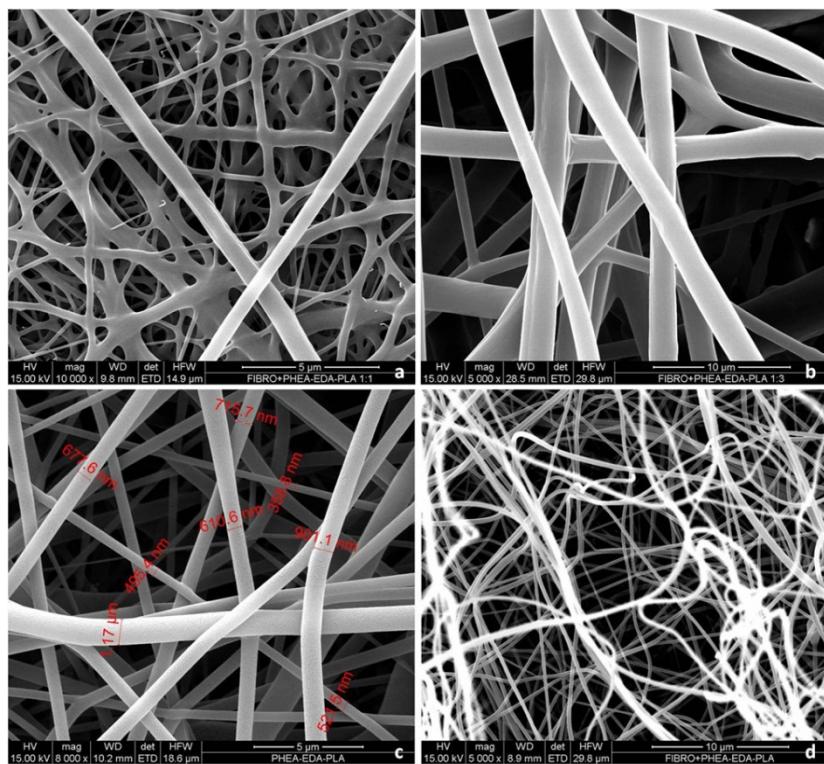


Fig. 1: SEM images of Fibro-PHEA-PLA electrospun fibers (images a and b) and PCL-PHEA-PLA electrospun fibers (images c and d). Magnification is 5000x for images a, b and d, and 8000x for image c.

The Fibro-PHEA-PLA scaffold was implanted in rats in order to assess its biocompatibility *in vivo*. 7 and 15 days after implantation it was removed and analyzed by SEM. The analysis revealed that after 7 days the scaffold underwent a cellular colonization which gave rise also to formation of a new tissue (Fig. 2).

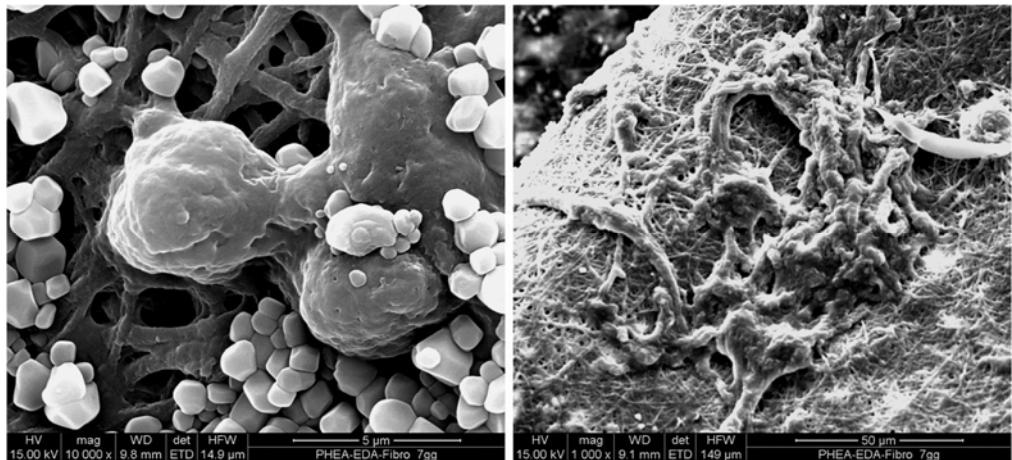


Fig. 2: SEM images of Fibro-PHEA-PLA scaffold after 7 days of implantation in rat at magnification 10000x and 1000x.

After 15 days, instead, the fibers of the scaffold fused superficially, probably due to metabolic degradation processes, forming a compact wall of about 30 μm (Figure 3a). The material is then partially degraded and its surface has a higher number of adherent cells compared to 7 days (Figure 3b).

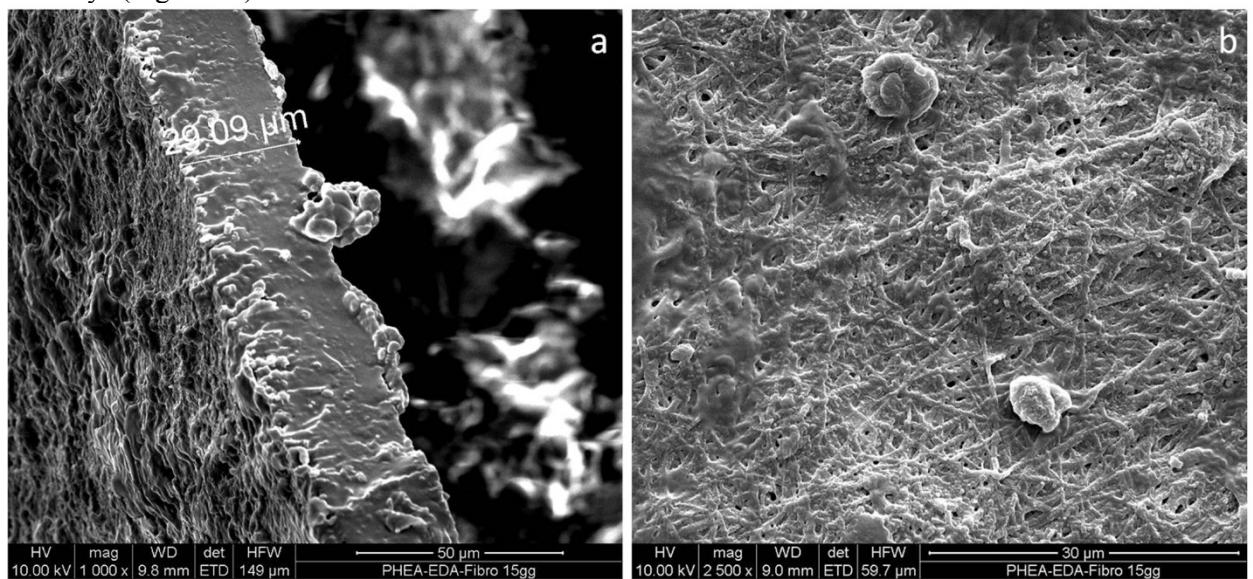


Fig. 3: SEM images of Fibro-PHEA-PLA scaffold after 15 days of implantation in rat at magnification 10000x (b) and 1000x (a).

The scaffold of PCL-PHEA-PLA, unlike that of Fibro-PHEA-PLA has a more linear fibrillar structure with fibers orderly arranged and separated by larger spaces (Figure 1c and 1d). This type of structure should promote cell adhesion allowing them to penetrate and arrange in layers.

The microscopic analysis at 7 days, on samples explanted from rats, showed that even this type of scaffold was colonized by cells (Figure 4a and 4b), still maintaining a fibrillar structure in depth. After 15 days, however, it could be observed an increase in the number of adherent cells and the formation of new biological tissue (Figure 4c and 4d).

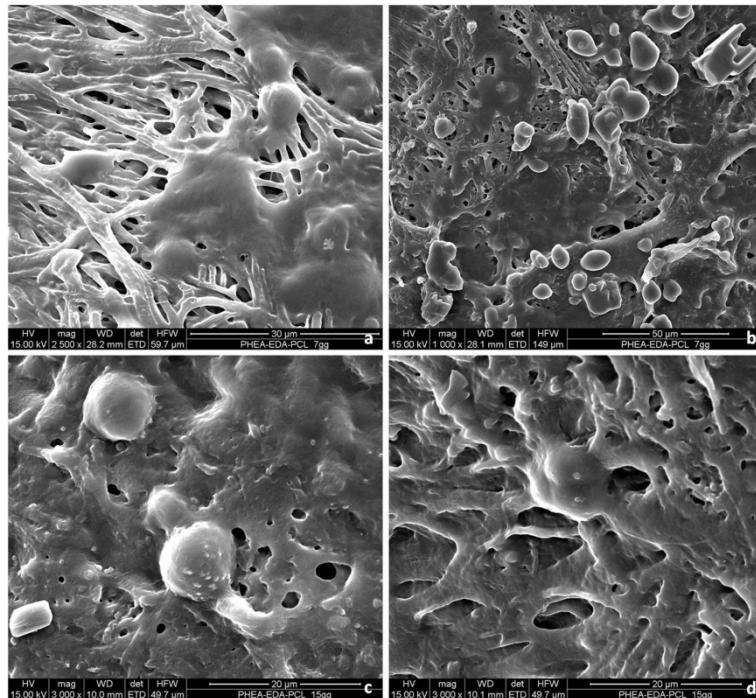


Fig. 4. SEM images of PCL-PHEA-PLA scaffold after 7 (images a and b) and 15 (images c and d) days of implantation in rat, at magnification 2500x (a), 1000x (b) and 3000x (c and d).

In the second phase of the study we carried out also an anatomo-morphological analysis of portions of the tissue surrounding the explanted scaffolds after 7, 15 and 40 days.

In particular, sections of tissue in which scaffolds (PCL-PHEA-PLA or Fibro-PHEA-PLA) were implanted, were carefully examined at a microscopic level. Tissue sections in correspondence to the Fibro-PHEA-PLA scaffold explanted after 7 days showed an eosinophilic amorphous material, arranged in a helicoidal trend, associated with necrosis and the presence of an intense neutrophilic granulocyte infiltration and a strong stromal reaction, and a low level of fibrosis (Figure 5a and 5b). The analysis and evaluation of PCL-PHEA-PLA samples showed that after 7 days there was an eosinophilic amorphous material with some areas of necrosis and also an intense inflammatory neutrophil granulocyte infiltrated with intense stromal reaction. Fibrosis was absent while inflammation was intense (Figure 5c and 5d).

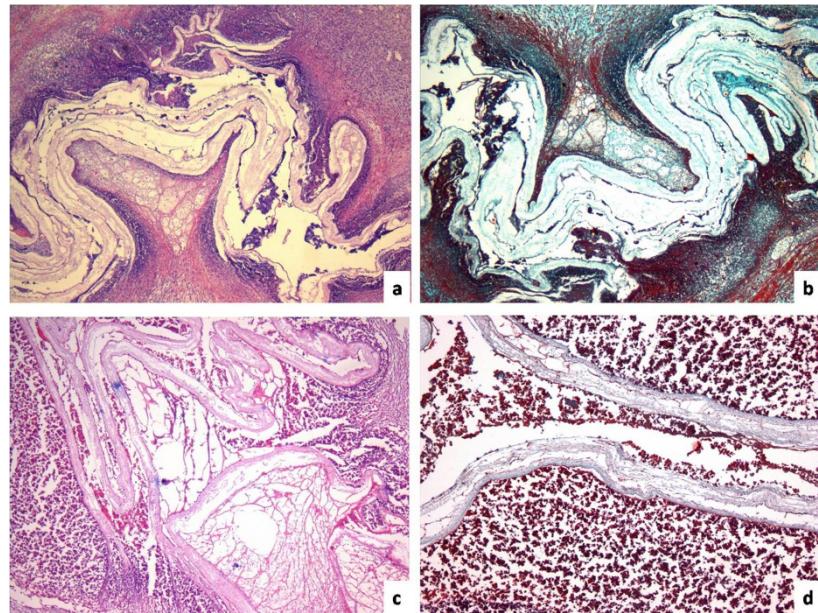


Fig. 5. Histopathological microscopy on tissue samples taken from areas of contact with the scaffolds Fibro-PHEA-PLA (images a and b) and PCL-PHEA-PLA (images c and d) after 7 days from implantation in rats. Samples were colored with hematoxylin-eosin (a, c) and trichrome (b, d)

A similar result was observed for Fibro-PHEA-PLA samples taken after 15 days from implantation, but in these cases fibrosis was increased (Figure 6a and 6b). In the case of PCL-PHEA-PLA after 15 days it was found an amorphous eosinophilic material associated with an intense inflammatory neutrophil granulocyte infiltrated and an intense stromal reaction; these data were comparable to those obtained by the analysis of the sections containing the material of Fibro-PHEA-PLA (Figures 6c and 6d) type.

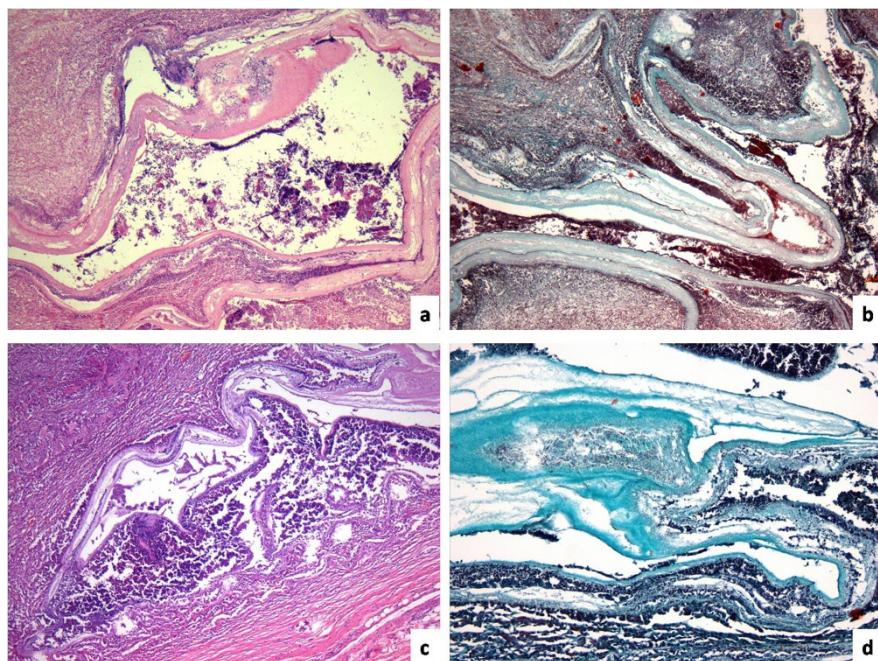


Fig. 6. histopathological microscopy on tissue samples taken from areas of contact with the scaffolds Fibro-PHEA-PLA (images a and b) and PCL-PHEA-PLA (images c and d) after 15 days from implantation in rats. Samples were colored with hematoxylin-eosin (a, c) and trichrome (b, d).

Finally, after 40 days, Fibro-PHEA-PLA histological samples showed however the presence of a partially reabsorbed eosinophilic amorphous material associated with modest lymphohistiocytic infiltrated and the presence of foreign body-associated giant cells. The degree of fibrosis was significantly increased compared with previous samples (Figure 7a and 7b).

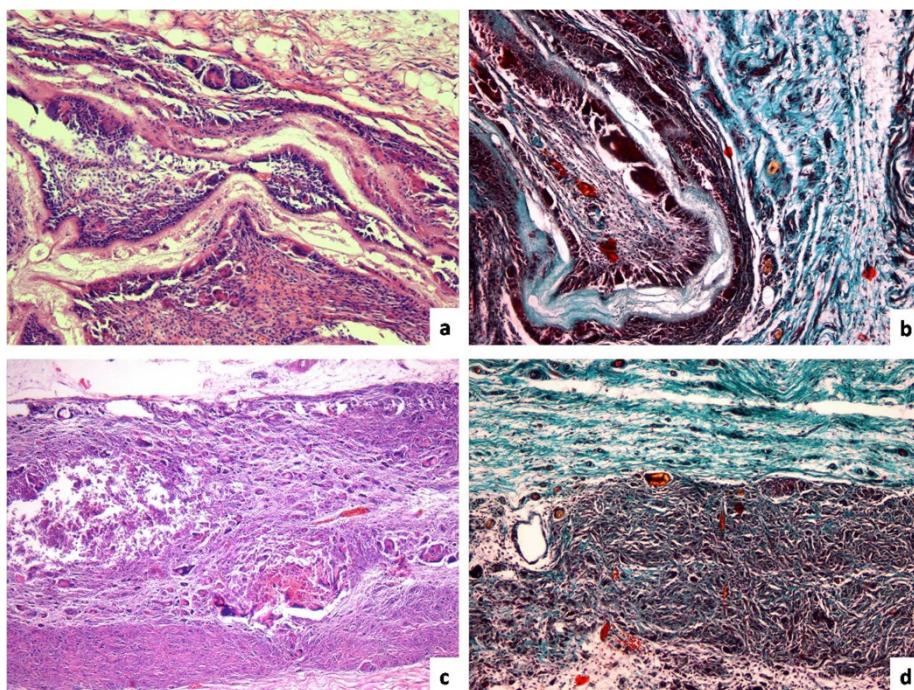


Fig. 7. histopathological microscopy on tissue samples taken from areas of contact with the scaffolds Fibro-PHEA-PLA (images a and b) and PCL-PHEA-PLA (images c and d) after 40 days from implantation in rats. Samples were colored with hematoxylin-eosin (a, c) and trichrome (b, d).

Differently, PCL-PHEA-PLA histological samples, after 40 days, showed a near complete absorption of the material; it was still present an intense inflammatory lymphohistiocytic infiltrated with the presence of giant cells and an intense stromal reaction. Both inflammation and fibrosis were still present (Figure 7c and 7d).

4. Discussion

Different methods to produce polymeric nanofibers or submicrofibers are reported in literature, using liquid-liquid phase separation, the self-assembly and electrospinning [21]. Electrospinning is a methodology to create continuous solid fibers of material with a very small diameter (from micro to nanometers) through the use of electric fields [22]. This technique offers some unique advantages as high surface to volume ratio, the porosity of adaptive structures and the possibility of electro-spun in different shapes and sizes [22].

The technique has been widely used to fabricate fibers for various applications; among them the biomedical scaffolds represent the greatest challenge as they mimic the structure of natural extracellular matrix [23].

Several biodegradable synthetic polymers are used to produce micro/nanofibers for biomedical applications in the field of tissue regeneration (skin, blood vessels, cartilage), including polyglycolic acid, polylactic acid, the poly (lactic acid-co-glycolic acid) and poly ϵ -caprolactone

[23]. Since each polymer has its own peculiarities, the development of composite scaffolds, based on a combination of two or more materials with selected properties, is an advantageous approach.

We decided to electrospin the PHEA-PLA in combination with the PCL (PCL-PHEA-PLA) or with fibroin (Fibro-PHEA-PLA), in order to obtain scaffolds presenting the ideal combination of the proper features of these materials.

The scaffolds were obtained in the form of micro-fibrillar leaflets of about 1 mm thickness (see Figure 8).

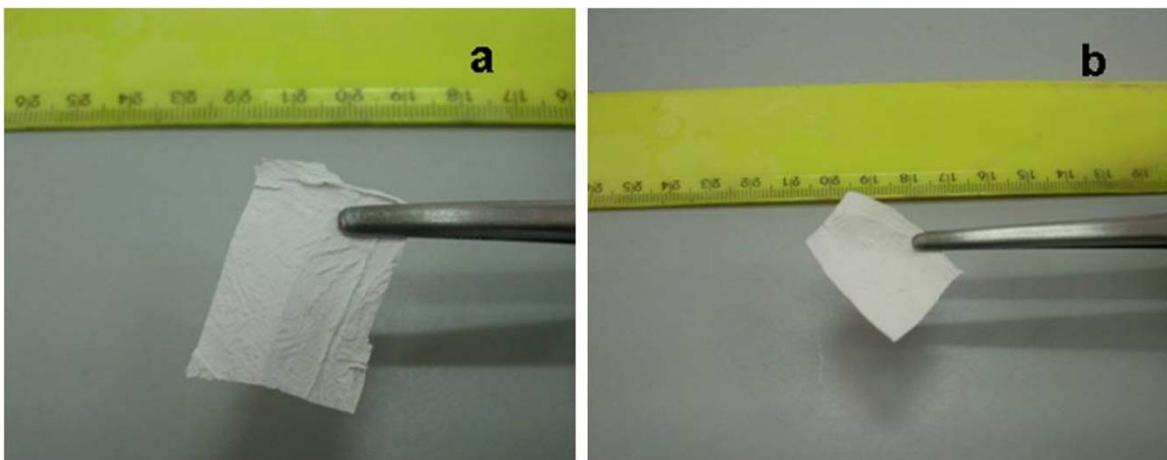


Fig. 8: Photos of scaffolds obtained by electrospinning of Fibro-PHEA-PLA (a) and PCL-PHEA-PLA (b) in the form of micro-fibrillar leaflets of about 1 mm thickness.

The electro-spinning technique allows also to produce tubular scaffolds with a diameter of about 6 mm (see Figure 9), which may be investigated in future for application in vascular regeneration.



Fig. 9. Representation of tubular scaffold that can be produced by the used electro-spining technique.

The electrospinning methodology has demonstrated to be very useful as it allowed us to create biocompatible porous fibers able to mimic the structure of the ECM. Both scaffolds described in this paper (PCL-PHEA-PLA and Fibro-PHEA-PLA), showed good mechanical properties, which allowed an easy handling for all experiments we performed. Although both scaffolds obtained showed good mechanical properties, PCL-PHEA-PLA scaffold had a better elasticity and strength. In vivo biocompatibility assays of both implanted scaffolds in rats showed a high level of cellular adhesion on fibers but an associated inflammation and fibrosis of the host tissue. Interestingly, 40 days after scaffold implantation both materials were almost completely degraded and reabsorbed.

5. Conclusions

In conclusion, the data obtained from experiments reported in our paper show that the two materials can easily be colonized by cells *in vivo* and they have a good biocompatibility *in vitro*, but their implantation *in vivo* causes also an intense inflammatory response associated to fibrosis. Obviously this adverse reaction should be taken under control in future experiments, through adding, for example, anti-inflammatory molecules to specific chemical groups of the material.

Once modulation of inflammation/fibrosis will be achieved, we believe that the scaffolds described in this work, thanks to the possibility to acquire a tubular shape, could be investigated for their potential in vascular regeneration.

References

- [1] Haseltine WA. Regenerative Medicine **6**, 431 (2011).
- [2] Daar AS. Gutmann T, Daar AS, Sells RA, Land W, editors. Munich: PABST Publishers (2005).
- [3] Baron F, Storb R, Polak J, Mantalaris S, Harding SE, editors. London: Imperial College Press (2008).
- [4] Damiano G, Gioviale MC, Lombardo C, Lo Monte AI. Transplant Proc **41**, 1116 (2009)
- [5] Gioviale MC, Damiano G, Montaldo G, Buscemi G, Romano M, Lo Monte AI. Transplant Proc **41**, 1363 (2009)
- [6] Gioviale MC, Damiano G, Cacciabuado F, Palumbo VD, Bellavia M, Cassata G, Spinelli G, Buscemi G, Lo Monte AI. Transplant Proc **43**, 1173 (2011)
- [7] Choi SW, Zhang Y, Xia Y. Langmuir **26**, 19001 (2010)
- [8] Day RM, Boccaccini AR, Maquet V, Shurey S, Forbes A, Gabe SM, Jerome R. Journal of Materials Science: Materials in Medicine **15**, 729 (2004)
- [9] Pariente JL, Kim BS, Atala A. J Urol **167**, 1867 (2002)
- [10] Li ST. In: The biomedical engineering handbook edited by Brozino JD, Boca Raton FL, editors. CRC Press (1995).
- [11] Smidsrod O, Skjak-Brekk G. Trends Biotechnol **8**, 71 (1990)
- [12] Takasu Y, Yamada H, Tsubouchi K. Biosci Biotechnol Biochem **66**, 2715 (2002)
- [13] Murugan R, Ramakrishna S. Tissue Eng **12**, 435 (2006)
- [14] Stitzel J, Liu J, Lee SJ, Kamura M, Berrya J, Sokerc S, Lime G, Dyke MV, Richard C, James JY. Biomaterials **27**, 1088 (2006)
- [15] Jun S, Hong Y, Imamura H, Ha BY, Bechhoefer J, Chen P. Biophysical J **87**, 1249 (2004)
- [16] Shive MS, Anderson JM. Adv Drug Deliv Rev **28**, 5 (1997)
- [17] Guarino V, Causa F, Taddei P, Di Foggia M, Ciapetti G, Martini D, Fagnano C, Baldini N, Ambrosio L. Biomaterials **29**, 3662 (2008).
- [18] Pitarresi G, Palumbo FS, Fiorica C, Calascibetta F, Giammona G. European Polymer Journal **46**, 181 (2010)
- [19] Craparo EF, Teresi G, Bondì ML, Licciardi M, Cavallaro G. Int J Pharmaceutics **406**, 135 (2011).

- [20] Licciardi M, Cavallaro G, Di Stefano M, Fiorica C, Giammona G. *Macromol Biosci* **11**, 445 (2011)
- [21] Garg K, Bowlin GL. Electrospinning jets and nanofibrous structures. *Biomicrofluidics* **5**, 13403 (2011)
- [22] Bhardwaj N, Kundu SC. *Biotechnol Adv* **28**, 325 (2010)
- [23] Timnak A, Yousefi Gharebaghi F, Shariati Pajoum R, Bahrami SH, Javadian S, Emami Sh H, Shokrgozar MA. *J Mater Sci Mater Med* **22**, 1555 (2011)