



A Fibrillar Biodegradable Scaffold for Blood Vessels Tissue Engineering

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In recent years there has been a growing interest for the development of tubular scaffolds employed to assist the replacement of small blood vessels. Materials designed for this purpose need to be biodegradable, have good mechanical properties and improve cell adhesion, proliferation and differentiation. To obtain biomaterials with these properties, electrospinning seems to be one of the most useful technique. Several biodegradable synthetic polymers or constituents of the extracellular matrix (ECM) have been electrospun showing optimal mechanical properties and biodegradability. However, such polymers are lacking in versatile chemical structure affordable to immobilize growth factors or chemokines. The glycosaminoglycan heparin is able to bind several growth factors like vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) and, when grafted onto the scaffold surface it is able to attract cells thus improving their proliferation and differentiation. Aim of this research was the production and the preliminary in vitro biological characterization of a new biodegradable material, obtained by electrospun a polyaminoacid-graft-polyester copolymer. The electrospun biomaterial has been successfully grafted with heparin exploiting the better chemical reactivity of the polyaminoacid portions of the graft copolymer. Then its morphology has been investigated by scanning electron microscopy (SEM) and the potential biodegradability of the material has been studied until 60 days. Preliminary biological data in vitro, on human endothelial cells, show a good compatibility of the scaffold obtained by electrospinning, with regard to cell adhesion and proliferation. Experiments are in progress to evaluate the effects of heparin on cell differentiation.

1. Introduction

In the last years there has been a rising interest among tissue engineering community for the development of small-sized blood vessels substitutes (Sheridan et al., 2012; Pooyan et al., 2012; Nottelet et al., 2009). Each year there is a strong patient demand for vascular bypass graft due to atherosclerosis and other cardiovascular diseases that are still the leading cause of mortality in the western countries.

Several studies have been focused on the development of a biodegradable vascular graft able to substitute only temporary the blood vessel and allows its complete regeneration after a certain time. For this purpose, the gold standard material must be biodegradable, to show optimal mechanical

characteristics (to resist to the blood stream) and an optimal capacity to allow cell adhesion, differentiation and proliferation.

The biodegradation rate of the system should be adequate in order to allow, for the time necessary to the cells to produce the new tissue, the mechanical stability of the vascular graft, and its complete degradation after the new vessel is formed. To develop a biomaterial with these properties, electrospinning seems to be one of the most useful technique due to the possibility to obtain nano-fibrillar scaffolds starting from polymeric solution.

Peculiar properties of electrospun scaffolds make them as optimal candidates for several regenerative medicine applications. Actually, the size (ranging from 200 nm to 2 μ m), shape and geometrical distribution in the space of the fibers obtained from the electrospinning, are highly similar to the collagen fibrils present in the natural ECM. Also, the disordered arrangement of electrospun fibers, allows a distribution of the mechanical forces applied to the material resulting in an increase in scaffold mechanical resistance.

Several biodegradable synthetic polymers like poly(lactide acid) (PLA), polycaprolactone (PCL) (Vaz et al, 2005), and natural polymeric materials like collagen (Li et al., 2005) have been electrospun with the aim to develop a successful vascular graft. However, due to the lack of suitable mechanical properties, the unsuitable rate of biodegradation and the poor capacity to create an optimal microenvironment for cell adhesion and differentiation, none of these materials seems to be appropriate for further application in the human body.

Besides, another limitation of the current used polymers is the lack of a versatile chemical structure.

Due to a scarce number of derivatizable functions, these polymers cannot be exploited to link active molecules like growth factors, chemokines and heparin that allow the material to be recognized as "self" by the body offering an optimal substratum for cell attachment and proliferation.

Heparin is an anticoagulant sulfate glycosaminoglycan (Ishihara and Ono, 1998) physiologically produced in the body, its hydroxyl and carboxyl groups are the main functional groups contributing to its anticoagulant activity. Heparin is also able to bind growth factors like vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) (Jayson and Gallagher, 1997) (Soker et al, 1994) and could be exploited to improve the ability of a biomaterial to allow cell attachment and proliferation.

Here we report the production of a new biodegradable material, by the electrospinning of an innovative polyaminoacid- graft-polyester copolymer able to be functionalized with heparin, for a potential use in tubular structures for vascular regenerative medicine. The main aspect to be investigated in the realization of a biomaterial applicable for the production of a bioengineered blood vessel, is its ability to promote adhesion and proliferation of vascular endothelial cells.

Indeed, the formation of an endothelial layer on a synthetic substrate would avoid the direct contact between the polymeric material and the circulating blood. This layer would also prevent the risk of developing blood clots by decreasing the gap between synthetic material and living tissue. For this reason, in this work, besides the construction and characterization of polymeric biomaterials and the scaffold, we have also determined the biocompatibility towards human endothelial cells ECV304.

2. Experimental

Electrospinning polymeric solution was prepared in a mixture dimethylformamide/dichlorometane 50:50 v/v. The solution was loaded into a 5 ml plastic syringe fitted with a needle having a diameter of 0.8 mm. Electrospinning process was carried out horizontally with an accelerating voltage of 20 kV supplied by a high voltage power supply. A syringe pump was used to feed the polymeric solution into the needle tip at a constant rate of 2 mL/h.

The electrospun scaffold was collected on an aluminium foil (15 cm by 10 cm) wrapped around an earthed rotating collector (200 rpm) 25 cm from the tip of the needle.

The resulting fibrillar scaffold was dried under vacuum at room temperature for 3–4 days.

The morphology of the scaffold was evaluated by scanning electron microscopy (SEM).

The heparinization of the scaffold was performed in 2-(N-morpholino)ethanesulfonic acid medium (MES), having a pH of 5.5 at 37 °C.

The scaffold was washed with different saline solutions and then with water to eliminate the unreacted heparin and the excess of salt, respectively. The degradation of the scaffold was investigated by incubating the samples in phosphate buffer pH 7.4 at 37 °C. At scheduled times, samples were washed several times with distilled water, freeze dried and weighted. The degradation was calculated comparing the initial and the final weight of samples and it was expressed as loss of weight %.

A toluidine blue colorimetric assay was used to determine the amount of heparin chemically linked to scaffold surface. Briefly, heparin treated scaffold was incubated for 4 h at 37 °C with a toluidine blue 0.4% w/v in 0.01 M HCl aqueous solution containing 0.2 % w/v NaCl. The excess of dye was then removed by exhaustive washing with distilled water. The scaffold was then solubilized with a mixture of ethanol/ 0.1 M NaOH (4:1 v/v) and the absorbance at 530 nm of the obtained solution was evaluated by UV spectrophotometer. Similar treatment was carried out on non-heparinized scaffold to have a negative control. A calibration curve was obtained by incubating a known amount of heparin in MES buffer with an excess of a solution of toluidine blue. The complex given by the electrostatic interaction between heparin and the dye becomes insoluble in aqueous solution and it was isolated by centrifugation. This precipitate was washed with 0.01 M HCl containing 0.2 % w/v NaCl, and then solubilized with 2 mL of mixture ethanol per 0.1 M NaOH (4:1). From this stock solution, more dilute solutions were obtained and analyzed to obtain the calibration points.

Cell culture. For biological assay, ECV304 human endothelial cells, from ECACC (European Collection of Cell Cultures), cultured in Medium199 added with 1 % antibiotic, 1 % glutamine and 10 % Fetal Calf Serum (FCS) were used.

Cell proliferation assay by MTS test. The ECV304 cells were seeded on scaffolds in 12 well plate, at density of 20,000 cells/well in MEM199 complete medium and incubated respectively for 24 h and 48 h at 37 °C in a humidified 5 % CO₂ atmosphere. Control cells were seeded on plate.

After the designated time in culture, cell viability was determined by the MTS assay; 200µL/mL of Cell Titer 96 AQueous One Solution Cell Proliferation (Promega), in complete culture medium was added to each well and the plates were incubated for 3 h at 37 °C. The MTS tetrazolium compound is bio-reduced by cells into a colored formazan product that is soluble in culture medium. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. 100 µL/well of each sample was transferred in 96 well plate, so each sample was read 5 times. The quantity of formazan product, as measured by the absorbance at 490 nm, is directly proportional to the number of living cells in culture. The absorbance was recorded by a Microplate Reader Model 680-BIO RAD.

3. Discussion

A very important outset in tissue engineering is the development of a biomaterial able not only to allow in vitro and in vivo cell adhesion, differentiation and spreading, but also to be recognized as “self” by the organism when it comes in contact with native tissues. In other words, the bioengineered material must be able to interact with the host tissues both mechanically and biochemically.

For this reason, the chemical versatility of the starting material offers an interesting opportunity to obtain a scaffold bearing bioactive molecules onto its surface able to minimize the physiological gap between native tissues and the bioengineered one. Still, the fabrication technique of the scaffold could be crucial in obtaining a biomaterial morphologically similar to the tissue that must be replaced.

Here, with the aim to produce a new biomaterial potentially suitable for the production of a bioengineered blood vessel, we have electrospun a highly functionalizable polyaminoacid-graft-polyester copolymer.

The obtained scaffold combines morphological characteristics similar to those of natural extracellular matrix, a suitable rate of degradation in simulated physiological medium (after 60 days 50 % about of the scaffold is degraded), ability to be easily functionalizable and to allow endothelial cell adhesion and proliferation. Figure 1 shows a SEM image of the obtained scaffold.

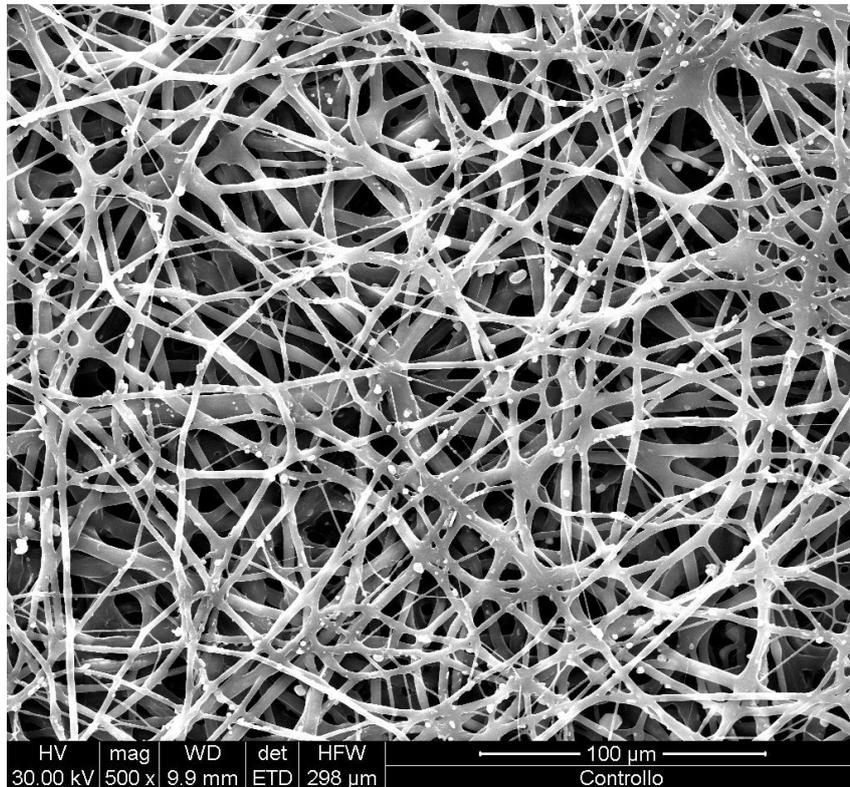


Figure 1. SEM image of obtained fibrillar scaffold

Heparin has been covalently linked to the electrospun scaffold through a simple and reproducible chemistry. The amount of linked heparin, determined by a colorimetric assay, resulted to be suitable for carrying out its biological function. To test our hypothesis, cell proliferation tests by MTS solution were performed on scaffold containing or not heparin.

As shown in Figure 2, the absorbance data, which are directly proportional to the number of metabolically active cells present in various samples, show the viability over time of the cells grown on the polymeric electrospun scaffold (Figure 2 a-b) containing or not heparin (EPA). Even if the initial number of cells (20,000 cells/well) was the same in all experiments, the final number of cells was lower on both types of scaffolds compared to the control on the plate; this is due to a certain amount of cells that initially slide on the bottom of the well. Nevertheless, it is possible to show a slight cell proliferation over time, on the scaffold containing heparin (Figure 2-a) than without heparin (Figure 2-b).

4. Conclusions

A fibrillar scaffold able to be functionalized with bioactive molecules, potentially employed for the regeneration of vascular structure was successfully produced by the technique of the electrospinning.

By SEM analysis it was possible to demonstrate that the produced scaffold is similar to the native ECM from the morphological point of view, since its fibrils have diameter in the nanometers range.

The heparinization of the scaffold was conducted by a simple and reproducible chemistry.

The amount of linked heparin, calculated via colorimetric assay, could allow its biological function both in vitro and in vivo.

We demonstrated that the obtained scaffold allows the viability and a slight proliferation of ECV304 human endothelial cells at least for 48 h.

These results are in our opinion encouraging for further studies concerning the development of tubular electrospun structures that could be exploited as scaffolds for the culture of both endothelial and smooth muscle cells.

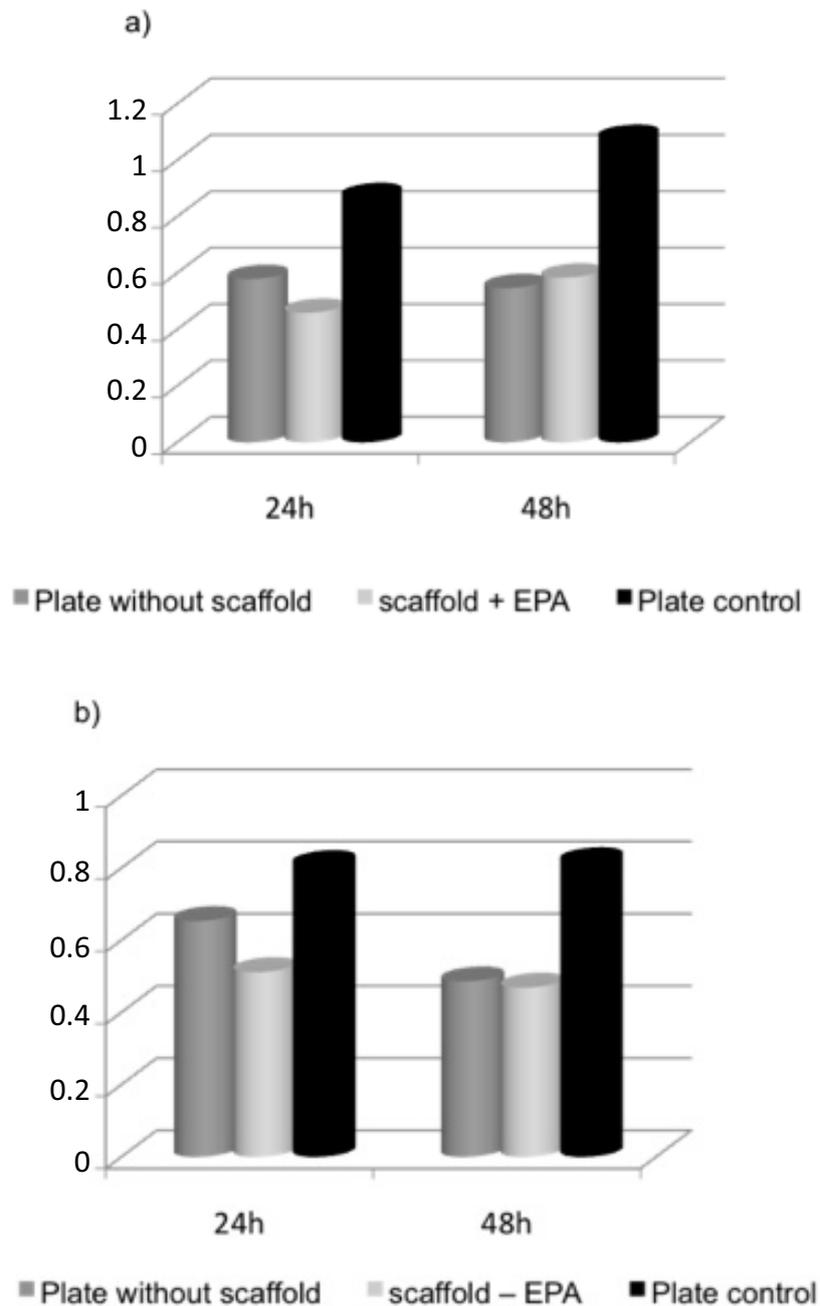


Figure 2. Evaluation of cell viability by MTS assay as absorbance values as a function of time. The test was carried out on scaffolds with and without heparin (EPA). The MTS assay was carried out in the well after removing the scaffold and on the scaffold alone put in a new plate. Cell growth directly on plate was used as a control.

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