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Small Diameter Vascular Grafts Coated with Gelatin

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In the field of tissue engineering, there is an increased demand for small diameter vascular grafts to treat peripheral vascular pathologies and ischemic heart diseases. The limited availability of suitable autogenous veins and the drawbacks related to the use of synthetic materials, such as polyethylene terephthalate (Dacron®) and expanded polytetrafluoroethylene (ePTFE), especially when they are used as substitutes for small diameter vessels, have attracted several investigators turning their attention toward the fabrication of alternative biocompatible grafts. In this study, small diameter tubular grafts (2 mm), made of poly (ε-caprolactone) (PCL) and poly (glycerol sebacate) (PGS) at a ratio of 1:1 (v/v) were obtained by electrospinning. With the aim to reduce water permeability, their surface was modified by dynamic coating of gelatin at 37 °C for 1 h, followed by UV-irradiation. Thickness, fiber diameters, porosity, mass loss, fluid uptake, water permeability, gelatin release, mechanical properties, cytotoxicity, and hemocompatibility of gelatin-coated electrospun scaffolds (GCS) were studied and compared with uncoated scaffolds (UCS). Scanning electron microscopy (SEM) images showed that the gelatin surface modification did not affect the 3D structure and pore interconnectivity of the scaffolds. A significant decrease in the water permeability was noticed when gelatin was used as coating agent. The results of this study highlighted the importance of a very low cost surface treatment with gelatin to improve the properties of PCL:PGS electrospun grafts. In conclusion, these gelatin-coated prostheses could be considered as a good candidate for vascular replacement in tissue engineering.

1. Introduction

The majority of vascular diseases are characterized by thickening of arterial wall with subsequent decrease in the arterial lumen (Boland et al., 2004). Once blood flow is compromised, vascular bypass is often the only solution to restore blood flow to tissues distal to the stenosis or occlusion site. The success of artificial vascular substitutes with diameter larger than 6 mm, realized in materials such as polyethylene terephthalate (Dacron®) or expanded polytetrafluoroethylene (ePTFE) is now consolidated (Greenwald and Berry, 2000). Although the introduction of these synthetic materials has been considered as a definitive solution, many drawbacks, especially when they are used as substitutes for small diameter vessels (< 5-6 mm), are noticed (Teebken and Haverich, 2002). One of their most important limitations is the absence of endothelization after implantation leading to thrombosis and intimal hyperplasia (De Visscher et al., 2012), post-surgical infections and reocclusion. The development of engineered blood vessel substitutes has driven much of the research in the tissue engineering field over the past 30 years. Different approaches were introduced to obtain high performing blood vessel replacements, such as hydrogel scaffolds (Liu and Chan-Park, 2009), cell-seeded biodegradable synthetic polymers (Pawlowski et al, 2004), decellularized bioprostheses (Schaner et al., 2004), electrospun constructs (Gaharwar et al., 2015), and bioprinted scaffolds (Norotte et al., 2009). The ideal vascular prosthesis could be biodegradable, acting as a structural framework, in order to reproduce a native artery, like vessel (Nojiri et al., 1995). It should hopefully exhibit resistance to thrombosis, infections, aneurysmal dilatation and ectopic calcification showing good suture retention, ease of handling, flexibility with kink resistance, predictable degradation kinetic, bio- and hemocompatibility. Poly (glycerol sebacate) (PGS)

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and FDA approved poly (ε-caprolactone) (PCL) represent two of the best-known polymers in vascular tissue engineering. Gelatin is a low cost natural biopolymer that derives from the denaturation with high temperature of the collagen. Due to its many good qualities, such as its biological origin, biodegradability, biocompatibility, and commercial availability at low cost, gelatin has been widely used in vascular medicine as sealant for vascular grafts (Zhang et al. 2004), carrier for drug delivery systems (Li et al., 1998), and dressings for wound healing (Ulubayram et al., 2001). Regarding fibrous scaffolds, one of the most important problem to bypass is represented by their excessive water permeability. In the last years, many methods of surface modifications have been proposed with the aim to improve scaffold biocompatibility (Ma et al., 2002) but the literature concerning minimized water permeability is yet poor. In this work, PCL:PGS electrospun scaffolds were coated with gelatin using a very simple and cheap protocol in order to decrease their water permeability. Gelatin-coated scaffolds (GCS) were studied in terms of thickness, fiber diameters, porosity, degradation, fluid uptake, water permeability, gelatin release profile, mechanical properties, cell viability, and hemocompatibility.

2. Materials and methods

2.1 Chemicals and spinning solution

Poly (ε-caprolactone) (PCL) (Mn = 80.000), glycerol, sebacic acid, gelatin from bovine and porcine bones, anhydrous chloroform and absolute ethanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Poly (glycerol sebacate) (PGS) (Mn = 2.673) was synthetized as described by Wang et al. (2002). PCL and PGS solutions (both 20 %, w/v) were prepared in a mixture of chloroform:ethanol (9:1, v/v) under magnetic stirring for at least 12 h at room temperature (25 ± 1°C). The spinning solution was obtained mixing PCL and PGS solutions at a volume ratio of 1:1.

2.2 Fabrication of the small diameter tubular scaffold via electrospinning and surface coating

We used an electrospinning apparatus from Spinbow (Bologna, Italy) It was made of a high voltage electric force supplier (PCM series, Spellman, NY, USA), an aluminium collector (2 mm outer diameter and 13 cm in length) and a syringe pump (KDS-100, KD Scientific, Holliston, MA, USA). Scaffolds were electrospun using 1.50 mL of polymeric solution with a flow rate of 2.20 mL h^{-1}, a driving voltage of 17.0 kV, a rotational speed of 500 rpm, a translation speed of 600 mm min^{-1}, and a distance of 18.0 cm between the needle and the cylindrical collector. At the end of the electrospinning process, scaffolds were placed in a desiccator overnight to allow the complete evaporation of the solvents. In order to obtain surface coated scaffolds, they were completely immersed in a gelatin solution (67 mg mL^{-1}, w/v) and maintained under mechanical agitation (agitator model Kirk 510, Bicasa, Monza Brianza, Italy) for 1 h at 37 ± 2°C. After that, gelatin-coated scaffolds were placed under an UV lamp for 1 h to allow gelatin cross-linking.

2.3 Scanning electron microscopy (SEM) analysis

Scaffold morphology was studied using SEM Hitachi 2500 (Tokyo, Japan). Fibers mean diameters were measured using image processing software (Image J, USA) in at least three different images. For each sample, the diameters of about 60 fibers were taken into account to have a meaningful statistical value.

2.4 Physico-chemical characterization of scaffolds

Electrospun scaffolds were characterized reporting their thickness, porosity, degradation during time, fluid uptake, water permeability, and gelatin release. The thickness of the samples was measured using a caliper (Series 209, Mitutoyo, USA). Tubular scaffolds of 3 cm in length were used for mercury intrusion porosimetry with Pascal 140 and Pascal 240 (ThermoQuest, Rodano, Italy). For degradation studies, samples were cut in small cylindrical pieces (5 x 2 mm), weighted and immersed in phosphate buffered saline (PBS) for a total period of 28 days in an incubator (Heraeus, Hanau, Germany) at 37 ± 2°C. After 1, 7, 14 and 28 days, samples were washed three times with deionized water and weighted again. Similarly, fluid uptake was performed weighting samples after 0.5, 1, 2, 4, 7 and 24 h of immersion in PBS at the same conditions reported above. The mass loss and the fluid uptake percentages were calculated as previously reported by Ferrari et al. (2017). Water permeability tests were performed as described by Madhavan et al. (2013), using pressurized water at 2×10^5 Pa (150 mmHg). To determine gelatin release from the scaffolds, they were cut into small pieces and incubated with 1.5 mL of PBS, as described above. At each time point (1, 2, 3, 4, 8, 24, 48 and 72 h), 150 μL of the supernatant was collected and replaced with an equal amount of fresh PBS. Released gelatin was quantified through the bicinchoninic acid assay (Novagen, EMD Chemicals Inc., CA, USA) according to manufacturer’s instructions.

2.5 Mechanical characterization

To perform mechanical loading tests in the axial direction, uniaxial Z0.5 test machine (Zwich Roell, Ulm, Germany) was used. The samples were prepared and the tests were performed as described in Ferrari et al., (2017) using both dry and wet samples, after immersion in a solution of NaCl 0.9 % (w/v) for 5 minutes.

2.6 Cell viability test

To determine possible cytotoxicity effects, scaffolds were put in contact with human endothelial cells EAhy926 following the same procedure described in Ferrari et al. (2017). Cell viability was tested by using the colorimetric assay CellTiter96® AQueous One Solution Cell Proliferation Assay (Promega, WI, USA) according to the manufacturer's instructions.

2.7 Hemocompatibility

Hemocompatibility represents a crucial factor to consider a vascular prosthesis as implantable. For hemocompatibility assay, blood was collected from a healthy volunteer by venipuncture and maintained into ethylenediaminetetraacetic acid (EDTA) vacutainer tubes at 4°C until using. For hemolysis test, samples were prepared and treated as previously described by Ferrari et al. (2017). In order to exclude red blood cells disruption not directly induced by the tested materials, at the same time, 0.1mL of blood was mixed with 5 mL of deionized water and of NaCl 0.9 %, as a positive and negative controls, respectively. At the end of incubation, samples were centrifuged at 1000 × g for 5 min (centrifuge Hettich Rotina 35 R, Tuttlingen, Germany), the supernatants were collected and read at 540 nm using the spectrophotometer Lambda 25 (PerkinElmer, Milan, Italy). The hemolysis rate (HR) was calculated following Eq(1):

$$HR\ (\%)=\frac{A_s - A_n}{A_p - A_n} \times 100 \quad (1)$$

in which A_s, A_n and A_p are the absorbances of the sample, the negative, and the positive controls, respectively.

2.8 Statistical analysis

Data are expressed as means ± standard deviations (SD) of experiments performed in triplicate. Statistical analysis was done using ANOVA Tukey's post hoc test with the Statistica v 8.0 software (StatSoft, Tulsa, OK, USA). Multiple comparison of the mean values was performed by the least significant difference test at $p < 0.05$.

3. Results and Discussion

3.1 Morphology of gelatin coated scaffolds (GCS)

Figure 1 shows the SEM micrographs of gelatin coated external (A) and internal (B) surfaces of scaffolds. Fibers appeared randomly distributed and they were bead-free. The presence of gelatin did not affect the structure of the electrospun scaffolds in comparison with uncoated PCL:PGS (data not shown).

3.2 Physico-chemical properties

The thickness of GCS and UCS was measured and resulted to be 0.46 ± 0.11 and 0.43 ± 0.11 mm, respectively (Table 1). Data suggest that surface modification did not increase scaffold thickness and therefore the gelatin layer can be considered at a sub-millimeter level. Both the inner and the outer surfaces of GCS exhibited microfibers, without any differences between GCS and UCS. In general, porosity of biomaterials plays a pivotal role due to its influence to favour cellular colonization and growth on the prosthesis. In the case of GCS, porosity was significantly lower (43.49 ± 10.85 %) in respect of UCS (68.19 ± 0.02 %) (Table 1). This fact can be attributed to the formation of a uniform layer during gelatin coating that is able to occlude the pores of the scaffolds. Degradation rate of biomaterials is another key factor that has to be taken into account when considering tissue architecture reorganization and integration. In fact, during a fast degradation, scaffolds do not represent a good substrate for cell attachment and growth while, during a slow degradation, biomaterials go under a process of calcification and hardening, losing their mechanical properties. As can be seen from Figure 2 A, mass loss was more evident with GCS. In fact, during the first period of degradation, also gelatin is released from the scaffolds, lowering their weights. Mass loss, in this case, is the sum of two different processes, degradation of the polymers and release of gelatin. The degradation profile was very similar between the GCS and UCS and therefore, the observed differences are due only to the released gelatin and not to a faster degradation of the biomaterial. Figure 2 B shows the fluid uptake profile of the studied materials during 24 h. The presence of gelatin caused an increase of fluid uptake

during the first hours. Gelatin induced a peak of fluid uptake after 2 h (248 %) and after 24 h this parameter decreased (144 %) as a consequence of a concomitant gelatin release. As shown in Figure 2 C, the majority of gelatin (58.96 %) is released within the 8 h and the total amount of released gelatin was 70.64 % after 72 h. The release of gelatin from the scaffolds is responsible of the different behaviour between GCS and UCS in degradation rate and fluid uptake profiles. Water permeability was greatly decreased by gelatin surface modification. In fact, in the case of UCS, first water drops were already registered at 0.2 atm, while working with GCS there was not water leakage until 0.7 atm was reached.

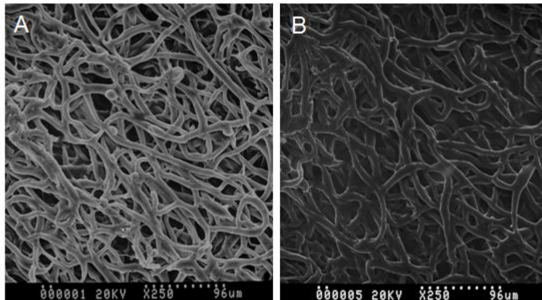

Figure 1: SEM images of external (A) and internal (B) surfaces of GCS.

Table 1: Thickness, fiber diameters and porosity of GCS and UCS

Sample	Thickness (mm)	Fiber diameters (µm)	Porosity (%)
PCL:PGS 1:1	0.46 ± 0.11[a*]	5.29 ± 1.31[a*]	68.19 ± 0.02[a*]
PCL:PGS 1:1 + gelatin	0.43 ± 0.11[a]	5.50 ± 1.34[a]	43.49 ± 10.85[b]

Results are mean of three measurements ± SD. Different letters do refer to statistically significant differences among results for each column ($p < 0.05$, ANOVA with Tukey's multiple comparison test). [*] Data previously presented in Ferrari et al., 2017.

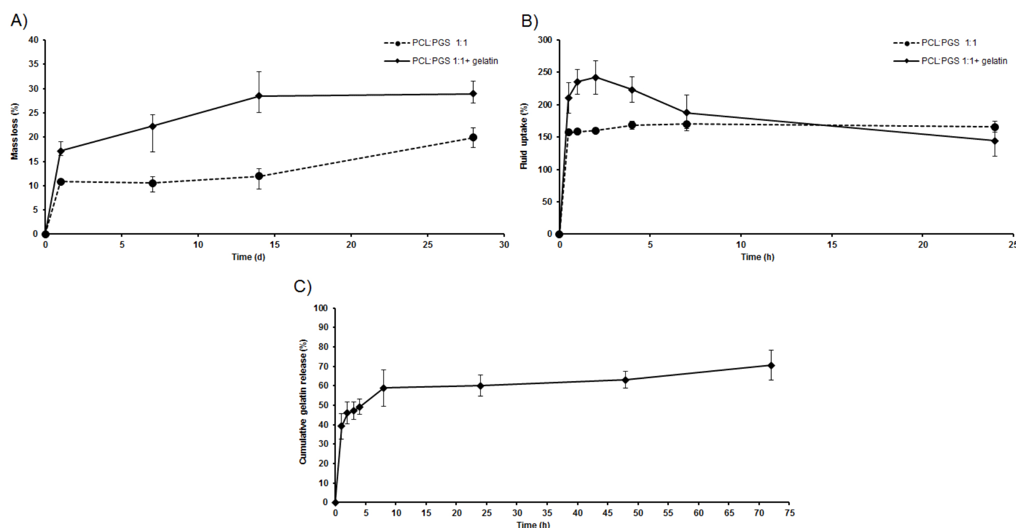

Figure 2: Mass loss (A), fluid uptake (B) of GCS and UCS and cumulative release of gelatin (C) from GCS. Data presented in Figure 2 (A) and (B) related to the PCL:PGS 1:1 are from Ferrari et al., 2017.

3.3 Mechanical properties

To evaluate mechanical properties of GCS and UCS, Young's modulus, tensile strength and elongation were calculated (Table 2). Dry GCS presented very different behaviour under a mechanical point of view in comparison with all the other samples. Considering Young's modulus, statistically significant differences ($p < 0.05$) were reported only in the case of dry GCS that presented a modulus approximately nine times higher in

respect with UCS. This hardening property was overcome immersing GCS in a NaCl solution (0.9 %, w/v). Surprisingly, all the tested samples showed a comparable tensile strength without any relevant differences (*p* < 0.05). The hardening of the polymeric samples caused by the surface modification with gelatin can be the main parameter affecting low values in elongation. Following vascular surgery procedures, GCS and UCS scaffolds were immersed in a NaCl solution and their mechanical responses were investigated. Wet samples were considered to correspond better than dry samples to the final application of the prostheses, and therefore, taken together, all these data suggested that the coating of PCL:PGS scaffolds with gelatin did not modify the mechanical properties of native scaffolds. In particular, the condition of hydration facilitated the sliding of polymers chain under mechanical deformation, both in the case of GCS and UCS (Kharaziha et al., 2013).

Table 2: Mechanical properties of GCS and UCS

Sample	Young's modulus (MPa)	Tensile strength (MPa)	Elongation (%)
PCL:PGS (1:1) (dry)	6.21 ± 2.13[a]	2.33 ± 0.75[a]	482.34 ± 193.44[a]
PCL:PGS (1:1) + gelatin (dry)	62.02 ± 8.81[b]	4.00 ± 1.21[a]	12.79 ± 4.44[b]
PCL:PGS (1:1) (wet)	8.05 ± 0.86[a]	2.89 ± 0.81[a]	516.66 ± 63.13[a]
PCL:PGS (1:1) + gelatin (wet)	8.63 ± 2.02[a]	3.27 ± 1.44[a]	327.56 ± 74.36[a]

Results are mean of three measurements ± SD. Different letters do refer to statistically significant differences among results for each column (p < 0.05, ANOVA with Tukey's multiple comparison test).

3.4 Cell viability

Different studies have suggested that gelatin is able to enhance biocompatibility of biomaterials promoting cell adhesion and proliferation (Ghasemi-Mobarakeh et al., 2008). In our case, only the external surface of the scaffold was coated with gelatin with the scope of reducing water permeability. In order to study whether our scaffolds were responsible to release toxic molecules, they were put in Transwell-clear multiwell inserts and the EAhy 926 cell viability after 1, 2, 3 and 7 days was measured through MTS assay. The results expressed as percentage values with respect to the control are shown in Figure 3 A. After 1 and 2 days, a significant increase in cell viability was noticed. No statistically significant differences (*p* < 0.05) were found between the cells grown on a tissue culture plate as control and the cells grown in presence of GCS and UCS after a week.

3.5 Hemocompatibility

Hemocompatibility of our electrospun scaffolds was evaluated registering their inability to induce erythrocytes disruption. Figure 3 B shows that GCS diminished dramatically the hemolysis percentage, probably because of the gelatin coating, also reducing the porosity of the scaffolds, caused a decrease of the roughness of PCL:PGS scaffolds. Considering that, for both GCS and UCS this parameter was always < 5 and we can conclude that they are highly hemocompatible (Sternberg et al; 2015).

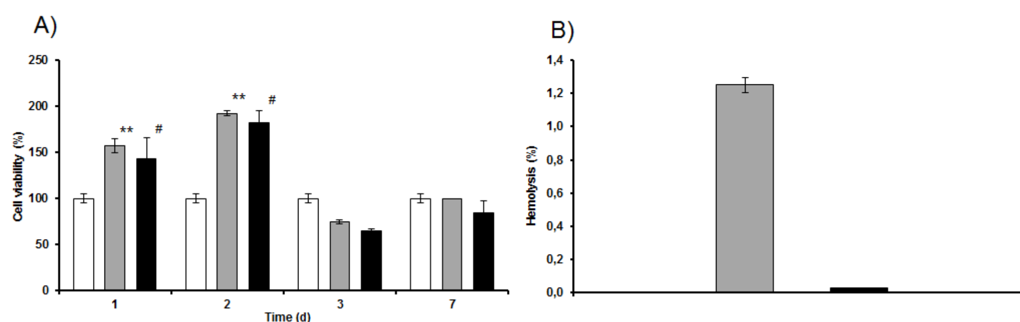

*Figure 3: Cell viability (A) of GCS and UCS by MTS assay. Results are mean of three measurements ± SD. Different symbols (** and #) do refer to statistically significant differences among results (p < 0.05, ANOVA with Tukey's multiple comparison test). Hemolysis percentage (B).* □ *control,* ■ *uncoated scaffolds,* ■ *gelatin coated scaffolds.*

4. Conclusions

In this work, we fabricated small diameter tubular grafts as vascular prostheses by electrospinning. Fibrous, degradable, porous, bio- and hemocompatible scaffolds of PCL:PGS 1:1 showed good mechanical properties. Oppositely, these scaffolds exhibited an excessive water permeability even at low pressure. In order to overcome this limitation, our scaffolds were coated with gelatin. Gelatin presence did not influence degradation rate and fluid uptake of the studied PCL:PGS blended polymer. Furthermore, the surface modification with gelatin did not alter thickness, fiber diameters, mechanical properties, when they are wet, biocompatibility, and hemocompatibility of PCL:PGS scaffolds, reducing the water permeability. These gelatin coated electrospun scaffolds could be used to be surgically implanted in animal model as arterial graft.

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