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Novel electrospun polyurethane/gelatin composite meshes for vascular grafts

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Abstract Novel polymeric micro-nanostructure meshes as blood vessels substitute have been developed and investigated as a potential solution to the lack of functional synthetic small diameter vascular prosthesis. A commercial elastomeric polyurethane (Tecoflex® EG-80A) and a natural biopolymer (gelatin) were successfully co-electrospun from different spinnerets on a rotating mandrel to obtain composite meshes benefiting from the mechanical characteristics of the polyurethane and the natural biopolymer cytocompatibility. Morphological analysis showed a uniform integration of micrometric (Tecoflex®) and nanometric (gelatin) fibers. Exposure of the composite meshes to vapors of aqueous glutaraldehyde solution was carried out, to stabilize the gelatin fibers in an aqueous environment. Uniaxial tensile testing in wet conditions demonstrated that the analyzed Tecoflex®–Gelatin specimens possessed higher extensibility and lower elastic modulus than conventional synthetic grafts, providing a closer matching to native vessels. Biological evaluation highlighted that, as compared with meshes spun from Tecoflex® alone, the electrospun composite constructs enhanced endothelial cells adhesion and proliferation, both in terms of cell number and morphology. Results suggest that composite Tecoflex®–Gelatin meshes could be promising alternatives to conventional vascular grafts, deserving of further studies on both their mechanical behaviour and smooth muscle cell compatibility.

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1 Introduction

Conventional vascular prostheses constructed from poly(ethylene terephthalate) (PET-Dacron) and polytetrafluoroethylene (PTFE-GoreTex) are functional for the replacement of medium and large calibre blood vessels (diameter >5 mm) but when employed as small diameter grafts they suffer from thrombogenicity and compliance mismatch problems [1–5]. Recently, tissue engineering has emerged as a promising approach to address the shortcomings of current vascular graft therapies and several opportunities for scaffold production have arisen due to the development of novel production techniques. In particular, electrospun polymeric meshes are emerging as a potential graft/scaffold material because of their interconnected, three dimensional porous structure, which mimics the randomly oriented, nano-fibred structure of the extracellular matrix [6–8].

Polyurethanes (PURs) are a broad family of polymers that have been used in a large number of medical applications since the 1960s [9] and are extremely interesting because their properties can be tailored according to the specific application. Particularly important for blood vessels grafts is that PURs can exhibit elastomeric behavior depending on the choice of the soft and hard segments [10, 11]. Electrospun elastomeric polyurethanes may represent an ideal class of materials for blood vessel replacement because of their porosity, strength and very high extensibility which could provide consistent matching with the mechanical properties of native vessels [12, 13].

Tecoflex® thermoplastic polyurethanes (TPUs) are a family of medical-grade, aliphatic, polyether-based TPUs synthesized from methylene bis(cyclohexyl)diisocyanate, poly (tetramethylene ether glycol) and 1,4-butanediol. In particular, Tecoflex® EG-80A is already used in medical tubing, it has good haemocompatibility, it does not have thrombogenic properties and has been further investigated to be employed as implantable membrane [14–17]. Furthermore, its non-(or slow) biodegradability could be considered positive in case of the replacement of tissues, such as blood vessels, which require substantial mechanical stability, because of the very high risks for the patient associated to a non optimal balance in the rate ratio of scaffold degradation and new extracellular matrix production. Some studies of Tecoflex® cytocompatibility show that, despite relatively good cell viability, Tecoflex® surfaces provide poor adhesion and proliferation of endothelial cells (ECs) [14, 17–19] which have a key role in giving the luminal surface of a blood vessel its antithrombogenic properties [20, 21].

Among the substrates which provide good EC adhesion and proliferation, gelatin is certainly the most well known. Gelatin is a natural biopolymer prepared by the thermal denaturation of collagen and is commonly used as substrate for culturing vascular endothelial cells [22–27]. Interestingly, it has been successfully electrospun and crosslinked resulting in a fine fibrous network that can hold its morphology in aqueous environment [28–31].

Electrospun composites that couple synthetic polymers, to give mechanical stability, and natural biopolymers, to promote cellular attachment and growth, have not been widely explored to date. Only a few studies describe the manufacturing of composite fibers by blended solutions of gelatin and some polyesters [32–35] while only one reported study investigates in detail the manufacturing of a synthetic/natural polymer composite membrane obtained by electrospinning from separate spinnerets of chitosan/polyvinyl alcohol blend and poly(lactic-*co*-glycolic) acid [36].

In the present article, Tecoflex® EG-80A and gelatin are electrospun from separate spinnerets to obtain a composite natural/synthetic polymer matrix. The possibility of employing the composite mesh as a vascular graft is investigated and discussed. The method for co-electrospinning of the polymers is also presented.

2 Materials and methods

2.1 Materials

Tecoflex® EG-80A (Velox GmbH, Germany) and gelatin from bovine skin, type B, cell culture tested (Sigma–Aldrich, MO) were used as received. Dimethylformamide (DMF), Tetrahydrofuran (THF), Acetic Acid (AA) and Ethyl Acetate (EA) were supplied by Sigma–Aldrich (Sigma–Aldrich, MO) and used without further purification.

2.2 Solutions preparation and single polymer electrospinning

Tecoflex® EG-80A was dissolved in a mixture of THF and DMF 70:30 at a concentration of 10% (w/v). Gelatin solutions at two different concentrations (10 and 15 wt%) were prepared by dissolving the polymer in a mixture of distilled water, AA and EA (22%, 44% and 34% (v/v), respectively).

Solutions were placed in 5 ml syringes equipped with 21 gauge blunt needles. Solution feeding rate was provided by infusion pumps (BSP-99M, Braintree Scientific Inc, MA and NE-1010, New Era System Inc., FL) and high voltage power supplies of positive and negative polarity (Spellman High Voltage, United Kingdom) were used to create an electric field between the tip of the needles and a rotating mandrel (4 cm in diameter) onto which a thin aluminum sheet was wrapped, to serve as a fibre collector.

Tecoflex meshes (T) were manufactured by electrospinning Tecoflex® solution employing a flow rate of 2 ml/h, a distance needle to collector of 29 cm and an electric field of 1 kV/cm by using two high voltage supplies of opposite polarity.

Gelatin fibrous mats (G) were obtained by providing a flow rate of 1 ml/h, keeping the distance between the needle and the collector equal to 11 cm and generating an electric field of 2.7 kV/cm by employing opposite polarity high voltage supplies.

To enhance the evaporation of DMF and water an infrared lamp (250 W) was positioned above the jet paths during process as heat source. After electrospinning, the thin aluminum sheet was taken off the mandrel and the spun meshes were left to dry under a fume extraction hood for 24 h, to facilitate mesh detachment, and kept under vacuum for 48 h to remove all residual solvents.

2.3 Composite sample electrospinning

Two types of electrospun Tecoflex®–Gelatin composite meshes were prepared:

- A gelatin layer was electrospun onto a previously prepared Tecoflex® mesh (TG). Each mesh was electrospun according to the parameters described in Sect. 2.2.
- Gelatin and Tecoflex® were co-electrospun (TGh) on the rotating mandrel (4 cm in diameter, 500 rpm) setting the parameters as described in Sect. 2.2. The two syringes hosting the solutions were kept at opposite

sides with respect to the mandrel to avoid electric fields mutual interferences and fibre repulsion due to equally charged jets (Fig. 1).

After removal from the thin aluminum sheet, both types of composite meshes were exposed to glutaraldehyde vapors, then kept under hood for 2 h and under vacuum for 48 h to remove all residual solvents and the residual glutaraldehyde [31].

2.4 Gelatin crosslinking

To stabilize gelatin fibers in aqueous environment, electrospun meshes were exposed to the vapours of 10 ml aqueous glutaraldehyde solution. Gelatin crosslinking kinetics was investigated by immersing samples in PBS after exposure at different crosslinking time intervals (20′, 30′, 45′, 90′, 120′, and 180′).

2.5 Scanning electron microscopy (SEM)

The morphology of the electrospun meshes was examined by SEM (Jeol LSM 5600LV, Japan) after they had been sputter coated with gold particles to reduce electron overcharging effects. SEM pictures were acquired at different magnifications using the signal from the secondary electron detector and fiber average diameter was determined from the SEM micrographs using Image J 1.36b software.

2.6 Mechanical characterization

Stress–strain curves of electrospun Tecoflex® (T) and composite samples (TGh) were obtained in dry and wet conditions at room temperature by means of a tensile testing machine INSTRON 5564 (Instron, MA). Dogbone shaped samples for dry and wet testing (eight each) were cut from the same mesh and prepared according to the standard ASTM D-638. The uniaxial tensile test was performed at a constant elongation rate of 200 mm/min up to failure following ASTM D-882. To test mechanical properties of the wet scaffold, dogbone shaped samples were kept in a PBS bath for 24 h and the test was run immediately after sample removal from the PBS bath. After failure, elastic modulus, maximum stress and strain at break of each specimen were automatically determined and results expressed as average values (mean ± standard deviation). Due to the difficulties in determining the effective cross sectional area of electrospun meshes, apparent values of the mechanical properties were computed considering the mesh as compact. Comparison of mechanical properties between samples was made using Student's *t* test, to determine statistically significant differences.

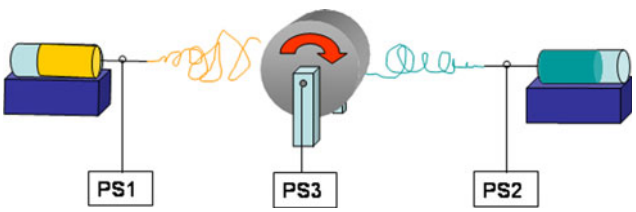

Fig. 1 Schematic of the co-electrospinning set-up. Electrospinning jets were positioned at opposite sides of the rotating mandrel. The voltage applied to the gelatin solution (PS1) and the Tecoflex® solution (PS2) were equal to 15 kV while the rotating mandrel power supply (PS3) provided a voltage of −15 kV

2.7 Biological evaluation

2.7.1 Cell culture

Human umbilical vein endothelial cells (HUVECs) were kindly provided by Dr. Rossella Di Stefano from the Cardiothoracic and Vascular Department, University of Pisa (Italy). Cells were isolated by digestion with 0.1% collagenase (specific activity: 316 U/ml, Gibco, Invitrogen) and grown to confluence at 37°C in a 5% CO_2 humidified incubator, on 25 cm^2 tissue culture flasks previously coated with 1% gelatin in supplemented culture medium (M199 with 10% heat inactivated foetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, 10 mM Hepes pH 7.4, 1% retinal derived growth factor, Sigma). Following trypsin treatment, the cells were detached from the flasks and monolayers were prepared by seeding HUVECs on gelatin-precoated culture plates and incubated for 24–48 h until confluent. HUVECs were identified by their typical cobblestone morphology and immunofluorescence staining by monoclonal antibodies against Von Willebrand Factor (Immunotech). Cells up to the fourth passage were used for all experiments.

The investigation conformed with the principles outlined in the declaration of Helsinki for the use of human tissue.

2.7.2 Cell viability

The viability of HUVECs seeded onto the prepared electrospun meshes was quantified by a bioluminescent measurement of ATP, which is present in all metabolically active cells, by using the commercial kit ViaLight HS (Lonza Rockland, Inc., USA). Briefly, T and TGh meshes were cut into 10 mm diameter discs, mounted onto CellCrown24 sample holder (Scaffdex, Finland), sterilized under UV light for 15 min, washed with culture media and seeded with 1.5×10^4 cells/well. Cells grown on gelatin

coated tissue culture polystyrene plates were used as controls. After six days of static culture, cells were incubated with the Nucleotide Releasing Reagent (NRR) and cell lysate was transferred to a luminescence compatible plate. Appropriate amounts of ATP Monitoring Reagent were added to the each well and the plate was analyzed by using a Victor 3 Multilabel Plate Reader (Perkin-Elmer). The recorded emitted light intensity (RLU, relative light units) is linearly related to ATP concentration and correlates with the number of viable cells present in the culture. Experiments were performed in triplicate. Data are expressed as mean ± standard deviation (SD). The data were compared using Student's t test and differences were considered significant at $P < 0.05$.

2.7.3 *Cell morphology investigation by confocal laser scanning microscopy (CLSM)*

Morphology of HUVECs cells grown onto T and TGh electrospun meshes was investigated by means of CLSM. After six days of static culture cells were fixed with 3.8% paraformaldehyde in Phosphate Buffer Saline 0.01 M pH 7.4 (PBS 1X), permeabilized with a PBS 1X/Triton X-100 solution (0.2%) for 5 min and incubated with a solution of 4′-6-diamidino-2-phenylindole (DAPI, Invitrogen) and phalloidin-AlexaFluor488 (Invitrogen) in PBS for 45 min at room temperature. After dye incubation, samples were washed with PBS before mounting on a glass slide and sealing with resin for microscopic observation.

A Nikon Eclipse TE2000 inverted microscope equipped with a EZ-C1 confocal laser and Differential Interference Contrast (DIC) apparatus was used to analyze the samples (Nikon, Japan). A 405 nm laser diode (405 nm emission) and a Argon Ion Laser (488 nm emission) were used to excite respectively DAPI and FITC fluorophores. Images were captured with Nikon Ez-C1 software with identical settings for each sample. Images were further processed with GIMP (GNU Free Software Foundation) image manipulation software and merged with Nikon ACT-2U software.

3 Results and discussion

3.1 Single polymer electrospinning

3.1.1 *Tecoflex®*

As presented in Fig. 2a and b, electrospun Tecoflex® meshes (T) showed a typical fibrous morphology and had an average fibers diameter of few microns (3.2 ± 0.55 μm) and inter-fiber spaces also in the micrometre range.

3.1.2 *Gelatin*

Electrospun gelatin (G) at 10 wt% concentration caused beading of the fibres indicating a low concentration of the starting solution. By increasing the polymer concentration to 15 wt%, the fibrous morphology improved and defect-free meshes were obtained, whose fibers had average diameters of hundreds of nanometers (255 ± 41 nm) (Fig. 2c, d).

3.2 Tecoflex®–Gelatin electrospinning

Both types of composite mesh (TG and TGh) were successfully prepared by following the procedure described in Sect. 2. The double layer mesh (TG) showed however a weak cohesion at the interface of the two materials, rising concerns about its use as blood vessel replacement. The co-electrospun mesh (TGh) exhibited better mechanical consistency and presented an interesting fibrous morphology, characterized by a uniform co-presence of the two kinds of fiber. Some gelatin fibers, much thinner in diameter, seem to be partially fused with Tecoflex® ones in an anastomosis-like configuration, as visible in Fig. 2e and f, likely due to an incomplete removal of solvents before fibers landed on the collector.

3.3 Gelatin crosslinking

Crosslinking studies on electrospun gelatin meshes (G) demonstrated that gelatin was stabilized in an aqueous environment upon previous exposure of the fibrous network to glutaraldehyde vapors. Mesh morphology was preserved at all the crosslinking times analyzed showing that crosslinking treatment did not cause any detrimental effect on fibrous integrity (Fig. 3a, b). Twenty minutes was chosen as a suitable short treatment time to effectively crosslink electrospun gelatin fibers.

The effect of glutaraldehyde exposure on Tecoflex® fibers was also investigated, as it was intended to manufacture Tecoflex® meshes (T) associated with electrospun gelatin, with the need of crosslinking the composite mesh. Electrospun Tecoflex® (T) morphology was not influenced by glutaraldehyde vapors exposure either (Fig. 3c, d). This confirmed the suitability of the crosslinking treatment, to give global stability to the composite sample in physiological environment.

3.4 Mechanical characterization

Typical stress–strain curves of electrospun Tecoflex® (T) and co-electrospun Tecoflex®–Gelatin composite meshes (TGh) in wet and dry conditions are shown in Fig. 4. No anisotropy could be noticed in mesh morphology and

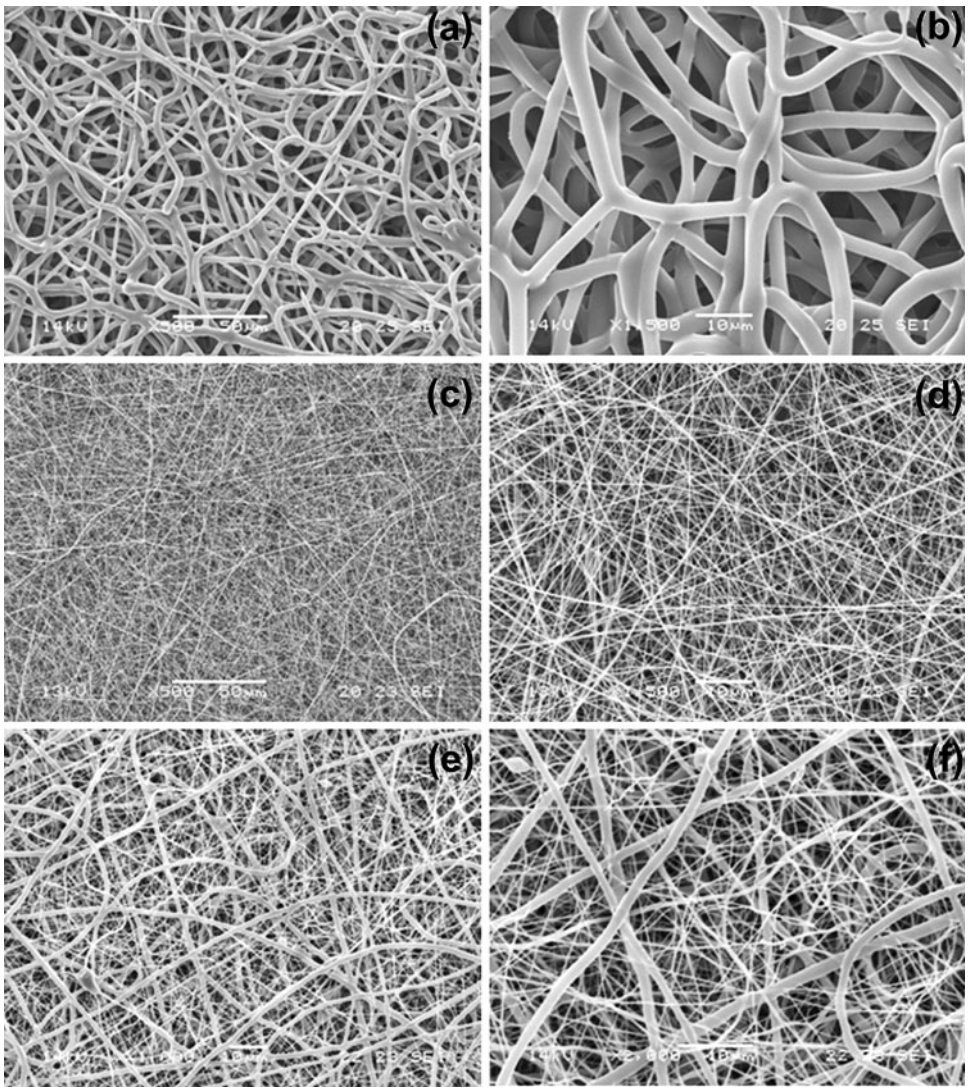

Fig. 2 SEM micrographs of Tecoflex® (**a** and **b**), gelatin (**c** and **d**) and composite mesh (TGh) obtained by co-electrospinning (**e** and **f**) at low and high magnifications. In the case of the composite, gelatin fibers (*smaller*) are visible together with Tecoflex® fibres (*larger*) creating an integrated network

mechanical properties, and for this reason, results deriving from samples cut in the circumferential and longitudinal direction were joined. There were no statistically significant differences in mechanical properties between Tecoflex® (T) samples maintained in dry or wet environments, because of the hydrophobic nature of the polymer. In both conditions, Tecoflex® (T) exhibited good mechanical properties for the proposed application in terms of stress and strain to failure (7.5 ± 1 MPa and 400 ± 38%, respectively). The elastic modulus was very low (0.8 ± 0.15 MPa) compared to other reported electrospun polymeric systems [8, 37–40], indicating the T electrospun meshes have better distensibility. Furthermore, T samples showed stress–strain curves typical of elastomeric, rubber-like materials [41, 42], i.e. a nonlinear elastic stress–strain response with large deformations.

The composite specimen (TGh), in dry conditions, had a higher elastic modulus (11.5 ± 2.4 MPa) compared to the Tecoflex® mesh (T) alone ($P < 0.01$), indicating that the stiffer gelatin fibers increased the overall rigidity of the sample, taking most of the load in the initial stage of deformation until their rupture. After this point, Tecoflex® fibers alone bore the load and the curve showed a large "plateau" until macroscopic failure of the specimen. The composite mesh (TGh) in dry conditions showed lower maximum tensile stress (2.5 ± 0.35 MPa) and lower strain at break (275 ± 45%) as compared to Tecoflex® samples (T) ($P < 0.01$ in both cases), probably due to the early rupture of gelatin fibers which reduced the effective loaded (stressed) area.

Mechanical behavior of the composite samples (TGh) in wet conditions was more similar to that of electrospun Tecoflex® (T), their stress–strain curves being superimposable at low strains. This suggests that gelatin fibers absorbed water, behaving like a hydrogel, which did not contribute to the overall properties of the sample. This would have the effect of reducing the area capable of bearing the load and lowering stress and strain at break

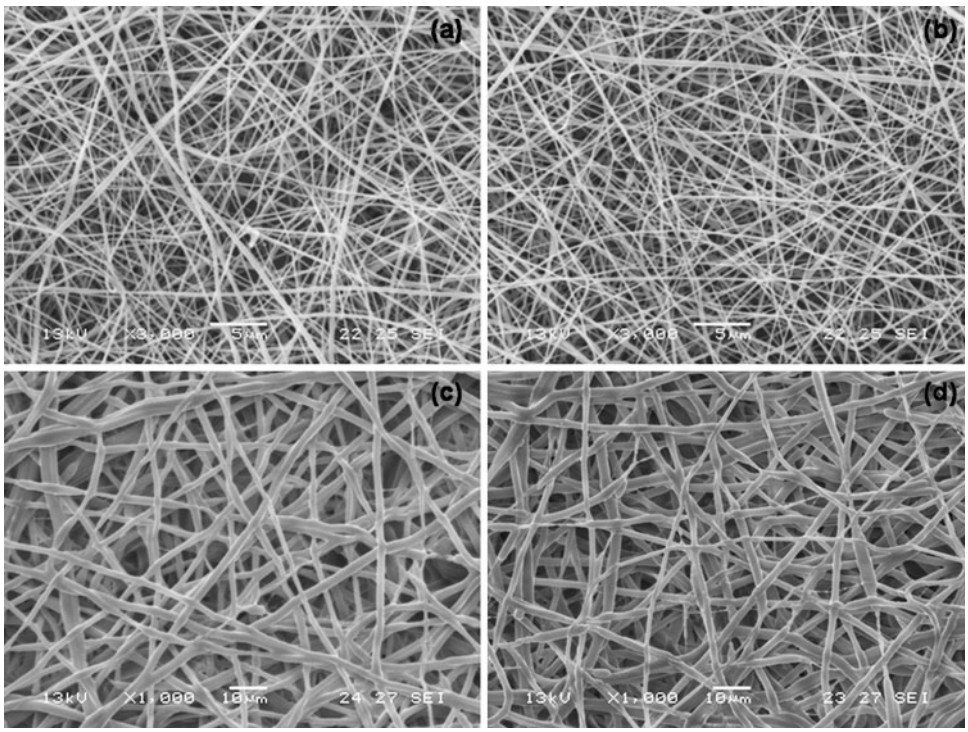

Fig. 3 SEM analysis of crosslinked meshes after different exposure times to glutaraldehyde. Gelatin: **a** 20 min, **b** 90 min; Tecoflex®: **c** 20 min, **d** 90 min

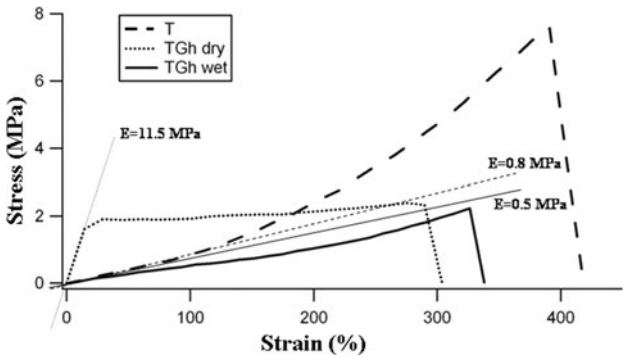

Fig. 4 Typical stress–strain curves of electrospun Tecoflex® (T) and composite Tecoflex®–Gelatin (TGh) dogbone shaped samples, dry and after wetting. The elastic modulus (E) of the samples was calculated as the angular coefficient of the linear regression curves, tangent to the first linear tract of the graph

values as compared with electrospun Tecoflex® alone (T). The maximum tensile stress of the wetted composite samples (TGh) was comparable to that of the dry samples (2.55 ± 0.6 MPa) while strain at break was slightly higher (330 ± 28%, $P < 0.05$). The most remarkable difference was the large reduction in elastic modulus (0.5 ± 0.18 MPa) as compared to the dry composite mesh, likely due to the aforementioned behavior of gelatin fibers in an aqueous environment.

In conclusion, the ultimate tensile strength and strain to failure of the investigated Tecoflex® and Tecoflex®–Gelatin meshes are indeed higher than those of native human coronary arteries [43] while elastic modulus is comparable to the coronary arteries modulus determined (for different age groups) in a study by Ozolanta et al. [44]. This suggests, if confirmed by further mechanical characterization (such as compliance and burst pressure test), that the investigated meshes could be promising alternative to conventional vascular grafts with respect to their mechanical properties.

3.5 Biological evaluation

Preliminary biological investigations were performed in order to assess the suitability of the prepared electrospun meshes to sustain human endothelial cell adhesion and proliferation on the inner layer. Experiments were carried out on the T and TGh samples. Quantitative data regarding HUVEC viability and proliferation after 6 days of static culture onto the prepared meshes revealed significant differences between the plain Tecoflex® mesh (T) and the composite Tecoflex®–Gelatin (TGh) samples. Cell growth on the T mesh was fairly poor, after 6 days the total number of viable cells was in the range of 20% of controls (cells grown onto gelatin coated culture plates), while, interestingly, cell growth on the composite TGh mesh resulted in much higher viable cell numbers (70% in respect to the control) thus indicating the importance of the gelatin component in allowing good cell adhesion and proliferation (Fig. 5).

The qualitative investigation of cell morphology carried out by CLSM confirmed the quantitative data. There was a

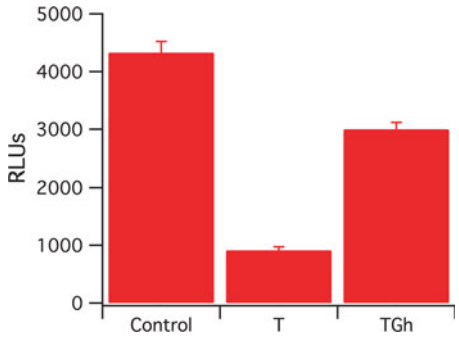

Fig. 5 HUVECs cell viability after 6 days of culture on gelatin coated polystyrene plates (control), Tecoflex® electrospun meshes (T) and composite Tecoflex®–Gelatin electrospun meshes (TGh)

marked difference both in terms of cell number and morphology between the T and TGh electrospun meshes (Fig. 6). In particular HUVECs grown onto TGh (Fig. 6b, d) tend to form a monolayer that almost reaches confluence and appears to be similar to the physiological morphology of endothelial cells, with a cobble-stone like arrangement. On the contrary it appears clear that cells seeded onto the T mesh (Fig. 6a, c) are not able to undergo appropriate spreading and proliferation, thus displaying a rounded morphology.

On the basis of these preliminary promising results, further biological investigation will be devoted to the assessment of the production of von Willebrand factor (vWF) as a marker of endothelial phenotype and appropriate endothelial cells extracellular matrix components such as laminin 8 and 10. Interaction of the composite sample with smooth muscle cells will also be examined.

4 Conclusions

The ideal tissue engineered blood vessel substitute, possessing mechanical and biological properties close to those of native vessels, has not been developed yet, because most attempts have focused on just one of the two aspects, mechanical performance (especially compliance) or cytocompatibility, both crucial in determining the clinical outcome of tubular constructs. The aim of our work was to

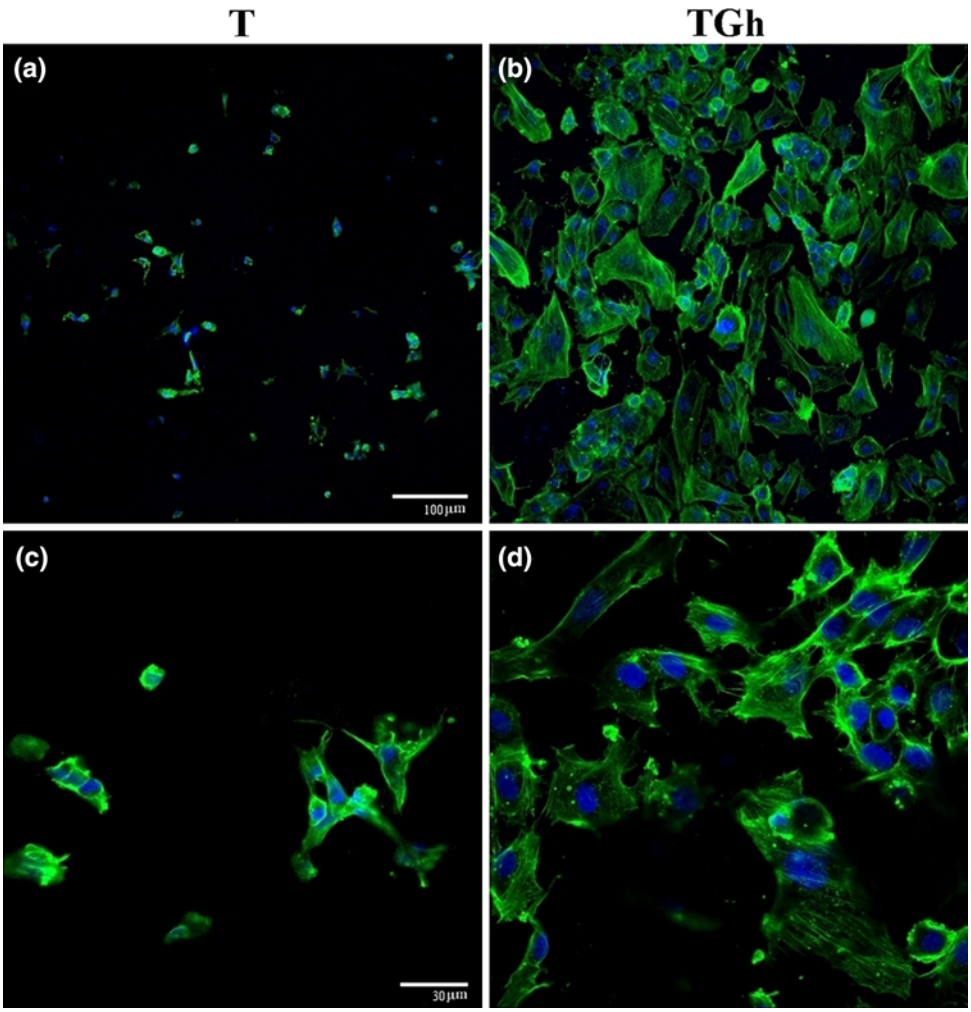

Fig. 6 CLSM micrographs of HUVECs morphology after 6 days of culture on Tecoflex® (**a** and **c**) and Tecoflex®–Gelatin (**b** and **d**) electrospun meshes

develop a composite vascular graft by simultaneous electrospinning of a synthetic biomedical elastomer (Tecoflex®) and a natural biopolymer (gelatin) in order to produce a construct that combines the mechanical characteristics of the synthetic polymer with the favorable cell interaction features of the biopolymer fibers. The successfully electrospun composite construct possessed an integrated network of synthetic and natural polymer fibers, showing also adequate strength, low elastic modulus and good extensibility. Gelatin fibers contributed to the overall mechanical properties of the composite mesh, as compared to plain Tecoflex® meshes (control), by increasing the rigidity of dry samples but having little effect on the elastic modulus after samples were immersed in buffer solution. Furthermore, endothelial cells cultured onto the composite electrospun substrate attached, survived and proliferated significantly better compared to cells on meshes made from Tecoflex® alone, confirming the advantages offered by a composite synthetic/natural matrix in providing appropriate mechanical properties combined with good cytocompatibility.

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