Poly(styrenesulfonate)/Poly(allylamine) Multilayers: A Route To Favor Endothelial Cell Growth on Expanded Poly(tetrafluoroethylene) Vascular Grafts

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Small-diameter synthetic vascular grafts of expanded poly(tetrafluoroethylene) (ePTFE) polymer concern one of the most common alternatives for the replacement of diseased vessels. However, high failure rates arise especially due to the lack of endothelial cells (ECs). EC seeding was developed to build a monolayer on the luminal surface. Because ECs show little or no adhesion on synthetic prostheses, it is necessary to promote their retention. On ePTFE surfaces we successfully deposited polyelectrolyte multilayer films (PMFs) consisting of poly(ethylenimine) (PEI), poly(sodium 4-styrenesulfonate) (PSS), and poly(allylamine hydrochloride) (PAH) to obtain PEI−(PSS−PAH)$_3$ films. EC adhesion and spreading on modified ePTFE were assessed by scanning electron and confocal microscopies. Cell viability was evaluated by Alamar Blue assay. After 7 days of culture, the ePTFE modified with PMF exhibited improvements of EC viability as compared to that of the controls (nonmodified ePTFE) or even ePTFE coated by a PAH monolayer ($p < 0.05$). Moreover, the spreading of ECs was largely enhanced compared to that of the same controls, resulting in a healthy confluent cell monolayer formation. Positive staining for the von Willebrand factor confirmed the EC phenotype. Promoting EC attachment and function on ePTFE modified with PMFs could become in the future a promising treatment for synthetic small-diameter vascular grafts.

1. Introduction

Cardiovascular diseases, including coronary artery and peripheral vascular diseases, constitute major causes of mortality in both the United States and Europe. Their current treatment is bypass surgery, allowing the replacement of atherosclerotic domains of blood vessels. Autologous vascular grafts are the ideal vascular substitutes for the replacement of small-diameter vessels (internal diameter smaller than 5 mm).[1] However, often they cannot be used because of poor quality or inadequate size or length.[2−3] For this reason, expanded poly(tetrafluoroethylene) (ePTFE) grafts have been developed as a synthetic alternative. However, such grafts are associated with high occlusion rates,[4−5] in part due to the thrombogenicity of the graft inner surface resulting from a lack of a functional endothelial cell (EC) monolayer.[2−4] This problem limits their use in current practice. To improve their long-term biocompatibility and hemocompatibility, it is necessary to recreate an internal environment similar to that found in native vessels.[6−9] For such an objective, EC seeding and tissue engineering techniques have been previously developed.[10] However, EC seeding on ePTFE has limited success due to the existence of electrostatic repulsive interactions between the negatively charged ECs and the negatively charged graft material. For this reason, before cell seeding, the luminal surface of the graft has to be modified to promote EC adhesion and spreading[11−12] on vascular prostheses. Because they are easy to carry out, biological coating techniques which use adhesion proteins were the first surface modifications prompted. Proteins (for example, collagen,[13] heparin,[1] fibronectin,[14] and fibrin glue[8,10]) were chosen for their potentials to promote favorable interactions with the physiological environment and with the human body[15] and to attract ECs. Nevertheless, such coatings led to limited improvements because the protein amount adsorbed on unmodified ePTFE is low. They can be desorbed and eventually lead to negative reactions (such as formation of thrombi in blood-contacting devices) and finally to clinical complications.[16] Moreover, the use of proteins, especially those of xeno- and allogenic origin, is associated with the risk of pathogen transfer and immune reactions.[17] Other approaches used an RGD peptide sequence[18] or RGD-streptavidin[3] to create specific interactions with integrin cell-surface receptors. The use of non-ligand-based techniques is another approach which has been proposed, such as photodischarge[19] and plasma chemical vapor deposition.[20] It permits deposition of reactive groups onto polymer surfaces or a decrease of the protein adsorption (such as fibrinogen) on the surface. However, these techniques show a lack of specificity and poor control over protein orientation, and they alter the porosity and texture of

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vascular prosthesis surfaces. Recently, a technique of surface modification was developed by the alternate deposition of oppositely charged polyelectrolytes[21] (layer-by-layer technique), which leads to the buildup of a polyelectrolyte multilayer film (PMF). This deposition method is simply based on electrostatic interactions between polyanions and polycations and on the excess of charges that appear on the top of the films (respectively positive and negative after polycation and polyanion deposition). The technique is of great simplicity and allows large possibilities in film property tuning such as film thickness, film structuration, surface roughness, wettability, and modification of viscoelastic properties.[22] Scaffolds with an irregular shape and an inner structure can be successfully modified with this technique, whereas traditional methods are generally unavailable or at least difficult to use. Murphy et al.[23] recently used the PMF approach to build a membrane-mimetic film produced by the polymerization of a phospholipid assembly deposited on the top of an alginate/polylysine assembly. This biomimetic film type showed interesting properties in terms of blood compatibility.[24] We previously demonstrated on glass surfaces that PMFs terminated by poly(allylamine hydrochloride) (PAH) possess interesting properties in terms of initial cell adhesion and viability of ECs and that an interesting modified surface in terms of adhesion, viability, and growth concerns the PMF corresponding to poly(ethylenimine) (PEI)−(poly(sodium 4-styrenesulfonate) (PSS)−PAH)$_3$.[25] Compared to a simple PAH layer, a film architecture constituted by three PSS/PAH layer pairs was found to increase importantly the adhesion properties of ECs. Moreover, such a number of PSS/PAH layer pairs appears to be sufficient to coat most of the underlying solid surface.[26]

Thus, with the focus to prepare suitable small-diameter blood vessel substitutes, the aim of the present study was first to demonstrate the possibility of building up such a multilayered film on ePTFE and in particular inside an ePTFE tube. Second, we also wanted to investigate whether such a film improves the endothelialization of the inner ePTFE vascular graft and whether the cells on these surfaces keep their phenotype. This last point is of primary importance for future applications of such systems. Human umbilical vein endothelial cells (HUVECs) were used as a model for the endothelium. The cells were seeded on nonmodified ePTFE, on ePTFE modified with a PAH monolayer, and on ePTFE modified with a PEI−(PSS−PAH)$_3$ multilayer. Seeded ECs were characterized by von Willebrand factor (vWF) staining, scanning electron microscopy (SEM), and viability evaluation.

2. Materials and Methods

2.1. Modification of a Vascular Graft Polymer with a Polyelectrolyte Multilayer Film. Homemade patches of 9 mm diameter were prepared from thin-walled ePTFE graft polymer tubes (6 mm inner diameter, 25 μm fibril length) and were glued in 48-well plates. Then, PMFs were constructed directly on the ePTFE within the wells. Thus, different polyelectrolyte solutions from Sigma-Aldrich (France) were prepared in 1 M NaCl solution (pH 6.5): at a concentration of 10 mg/mL for PEI (cationic, MW = 750 000) and 5 mg/mL for PSS (anionic, MW = 70 000) and PAH (cationic, MW = 70 000). The different polyelectrolyte solutions were incubated alternatively, and each individual polyelectrolyte adsorption was followed by three ultrapure water rinses. The alternated deposition procedure was pursued until a PEI−(PSS−PAH)$_3$ architecture as previously described[25] was obtained.

2.2. Visualization of the Polyelectrolyte Film by Confocal Laser Scanning Microscopy (CLSM). To visualize the buildup of the multilayer film on the ePTFE surface, PAH covalently coupled to rhodamine ($\lambda_{excitation}$ = 541 nm, $\lambda_{emission}$ = 572 nm; ICS, UPR 22 CNRS, Strasbourg, France) was used for the deposition of the outermost film layer. The ePTFE patches, modified with this film, were observed using an SP2-AOBS Leica microscope (objective 40×, NA = 1.25; Germany).

2.3. Cell Culture and Seeding. HUVECs were isolated from fresh umbilical cords according to the method of Jaffe et al.[27] The cells were cultured at 37 °C in 5% CO_2 in a 25 cm^2 tissue-culture-treated flask in complete medium. The medium consisted of an equal mixture of medium M199 and medium RPMI 1640, supplemented with 20% pooled human serum AB, 2 mM L-glutamin, 20 mM HEPES, 100 IU/mL penicillin, 100 mg/mL streptomycin, and 2.5 mg/mL Fungizon. Confluent cultures at the second passage were used, and the cells were seeded at a density of 5 × 10^4 cellules/cm^2 on ePTFE modified with PEI−(PSS−PAH)$_3$ PMF, on ePTFE modified with a PAH monolayer, on ePTFE, and on a tissue culture polystyrene surface (TCPS) used as a positive control. The media were replaced every 3 days.

2.4. Cell Viability. To measure cell viability, Alamar Blue redox assay (Serotec Ltd., Kidlington, Oxford, England) was used. ECs were seeded on the surface for 1, 3, or 7 days. These periods of time were chosen because we investigated endothelial growth on PMFs deposited on glass at identical contact times. Moreover, it appeared in the same study that the cell growth became less important after 7 days of culture.[28] At the set time, the medium was removed and the ECs were washed with DMEM (Dulbecco's medium without red phenol, Gibco BRL, France). Then, Alamar Blue reagent dissolved in DMEM at 10% was added to each well. After 3 h of incubation at 37 °C, the solution was removed and the color change, due to the mitochondrial activity, was measured by monitoring the absorbance with a spectrophotometer (Beckman DU640) at wavelengths of 570 and 630 nm. The optical density difference $OD = OD_{570\ nm} - OD_{630\ nm}$ was calculated to estimate the metabolic activity of the cells.[29] Wells without cells were used as a reference.

2.5. Cell Morphology. The morphology of ECs was analyzed after 7 days of culture by SEM. Thus, the seeded grafts were fixed by immersion in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 overnight at 4 °C. After a short rinse in 30% ethanol (Sigma, France), the specimens went through a series of ethanol solution (30%, 50%, and 70%) at 10 min intervals for dehydration. The dehydrated specimens were treated in hexamethyldisilazane (Sigma, France) and dried under the hood. They were fixed on aluminum pieces with colloidal carbon and subsequently coated with a 5−10 nm layer of gold−palladium in a Polaron SC 7640 sputter coater. Finally, the specimens were examined with a scanning electron microscope (Autoscan S 240, Cambridge, U.K.) at 20 kV, at six magnifications (112×, 149×, 169×, 503×, 508×, and 513×), to evaluate the characteristics of the graft surface, such as the cell distribution, the cell density, and cell spreading.

2.6. Characterization of Endothelial Cells. The phenotype of the ECs was evaluated by the expression of the vWF by confocal microscopy. To visualize each cell, the nuclei were stained with propidium iodide (PI).

After 7 days of culture, the ECs were washed with DMEM at 37 °C. The cells were immediately fixed with 1% paraformaldehyde (PAF; w/v in PBS (phosphate-buffered saline)) at 37 °C, then permeabilized in the presence of 0.5% Triton X-100 (v/v in PBS, Sigma, France), and incubated for 45 min with 1/50 diluted monoclonal mouse anti-human vWF (clone F8/86, Dako, Trappes, France) in 0.1% Triton. The cells were washed with DMEM to remove the excess of antibody and were incubated for 30 min at room temperature with 1/100 diluted polyclonal goat anti-mouse IgG conjugated with Alexa Fluor 488 (Molecular Probes, Oregon) in DMEM. An isotype control was prepared in the same conditions. The cells were incubated for 30 min with 1/100 diluted PI (Molecular Probes) in DMEM. Fluorescently labeled cells were visualized with a confocal laser scanning microscope using a 40× objective and a He−Ne laser for 543 nm excitation (PI) and an Ar laser for 488 nm excitation (vWF).

2.7. Statistical Analysis. All data are presented in this study as the mean ± standard error of the mean (SEM) for each substrate (n = 9).

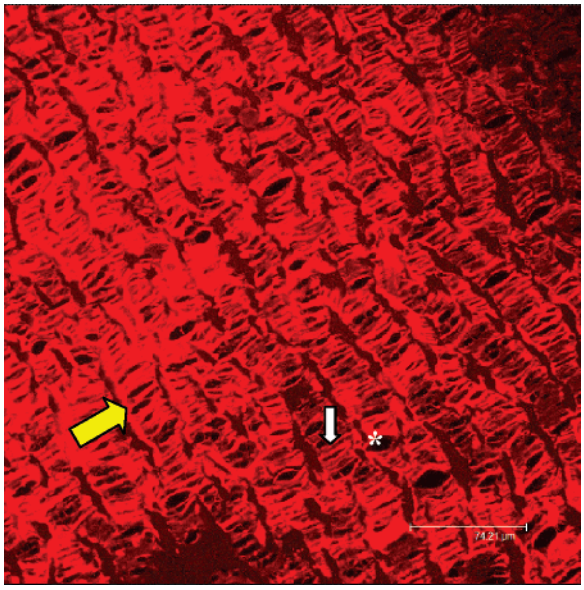

Figure 1. Visualization of the PMF deposited on ePTFE patches by using PAH covalently coupled to Rhodamine (PAH^{Rho}). The observed architecture corresponds to PEI−$(PSS-PAH)_2$−PSS−PAH^{Rho}. Scale bar 74.21 μm. The white arrow corresponds to the microfibrils or to the distance between two nodes and the yellow arrow to the nodes. The pore size is expressed as the length of the microfibrils. The asterisk corresponds to the pore.

One-factor ANOVA with PLSD Fisher correction (Statview IVs, Abacus Concepts Inc., Berkley, CA) was used for data analysis. A value of $p < 0.05$ was considered significant.

3. Results and Discussion

ePTFE vascular grafts are the most common small-diameter vascular synthetic prostheses used nowadays.[30] They are constituted by expanded PTFE polymer, which is chemically inert, and present a unique microporous wall structure (as we can see in Figure 1). They also possess highly flexible mechanical properties. However, these prostheses have a hydrophobic and negative surface that limits negatively charged EC adhesion and finally often leads to graft failure. For this reason, several investigators tried to improve the patency of this synthetic vascular substitute through the development of an EC monolayer on the lumen of the grafts before implantation. In the current study, we evaluated the possibility of modifying ePTFE with a PMF to build a biocompatible surface for ECs and thus confer antithrombogenicity to the vascular grafts. Indeed, the PMFs present interesting properties in terms of adhesion and viability of ECs as previously demonstrated on glass surfaces.[31] However, the properties found on glass cannot simply be extrapolated to ePTFE due to the difficulty of modifying such surfaces. The chosen PMF buildup is easy to carry out and does not need initiators or an energy supply to start the deposition process. The selected polyelectrolytes are also water-soluble, and the physicochemical properties of the PMF can be modified as needed by changing the preparation conditions (number of layers deposited, polyelectrolytes used, ionic strength, pH, ...).[32] PEI was chosen as the first initiating layer because of its ability to strongly adsorb to a substrate surface such as ePTFE and to render the structure of the films more uniform.[33] The regular and homogeneous deposition of the PMF on the luminal surface of ePTFE is an important parameter to consider for cell adhesion. The PEI−$(PSS-PAH)_3$ PMF has been well characterized on glass surfaces by different techniques (quartz microbalance, optical waveguide light-mode spectroscopy, or atomic force microscopy).[25] The techniques usually used to characterize the PMF apply only for flat surfaces and cannot be used in the present case due to the porosity, opacity, shape, and texture of the polymer. The PMF deposited on the vascular graft luminal surface was thus characterized by means of confocal microscopy. This was possible by ending the film with a Rhodamine-labeled PAH (PAH^{Rho}) layer instead of a PAH layer. Figure 1 shows a typical image of a film of PEI−$(PSS-PAH)_2$−PSS−PAH^{Rho}. The fluorescence was distributed in a homogeneous manner over the entire surface, suggesting that the film was deposited uniformly (at least at the optical scale) over the whole luminal synthetic surface. Moreover, PMFs, a few tens of nanometers thick, do not reduce the cross-section of vascular grafts contrary to the fibrin glue coating, which presents fibrin deposits in some cases up to 0.8 mm thick.[11]

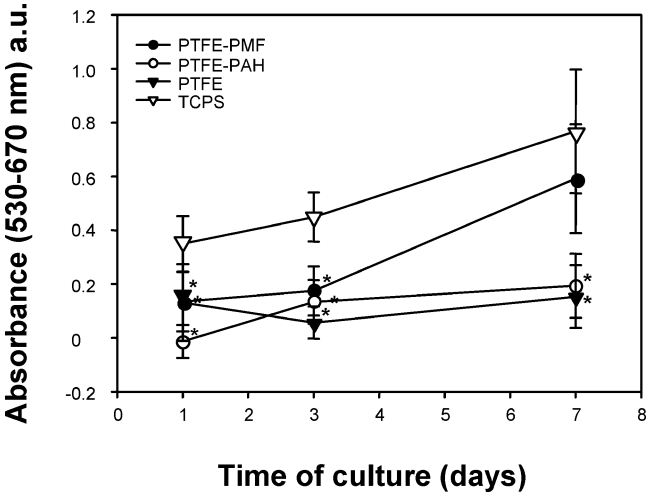

Figure 2. Viability of HUVECs seeded on ePTFE−PEI−$(PSS-PAH)_3$, ePTFE−PAH, ePTFE, and TCPS, after 1, 3, and 7 days of culture, tested by Alamar Blue assay (incubation time 3 h). An asterisk indicates $p < 0.05$ versus TCPS, $n = 9$.

It is also essential that the ECs adhere and remain viable on the polymer surface. Indeed, ECs seeded on nonmodified ePTFE cannot adhere due to its highly hydrophobic nature and negative charge.[34] The viability of cells seeded on tissue culture polystyrene surfaces, ePTFE, and ePTFE modified with a PMF or a PAH monolayer is presented in Figure 2. After 1 day of culture on the ePTFE surfaces, no real detectable metabolic activity was observed whatever the tested surface. After 3 days of culture, a significant increase of cell metabolic activity was measured only on the tissue culture polystyrene surface (0.45 ± 0.09, $p < 0.0001$). On ePTFE surfaces, the metabolic activity remained equal to the value of day 1. After 7 days of culture, the values of the cell metabolic activity observed on ePTFE modified with PMF (0.59 ± 0.20) were not different from those on tissue culture polystyrene surfaces (0.76 ± 0.23, $p = 0.0808$). On the contrary, for the same culture time, the values of the metabolic activity of cells on nonmodified ePTFE or on ePTFE modified with a PAH monolayer were significantly smaller than those for ePTFE modified with PMF (0.15 ± 0.11, $p < 0.0001$, and 0.19 ± 0.11, $p = 0.0003$, respectively). It appears, thus, that the cells started to proliferate on ePTFE modified with PMF after 3 days of culture and grew to a dense confluent cell layer within 7 days. It can be equally noted that only a low density of cells (5×10^4 cells/cm^2) was required to obtain the formation of a confluent cell monolayer. On the contrary, it was found that the modification of ePTFE with only a PAH monolayer is

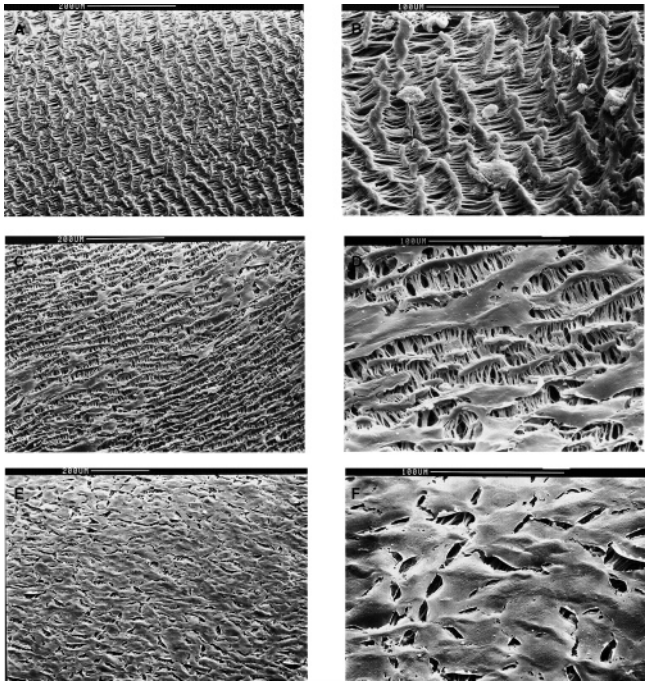

Figure 3. Scanning electron micrographs of the luminal surface of nonmodified ePTFE patches (A, D) and ePTFE patches modified with a PAH monolayer (B, E) and a PEI−(PSS−PAH)$_3$ PMF (C, F). All the surfaces were cultivated for 7 days with HUVECs (original magnification 169× (A), 508× (B), 149× (C), 503× (D), 112× (E), and 513× (F)).

not sufficient to reach a similar cell viability after an identical culture period. A comparable observation was made for a PAH monolayer deposited on glass.[31] This also illustrates the efficiency of PMF coatings on the ePTFE surface.

The scanning electron micrographs taken of the luminal surface of ePTFE vascular prosthesis samples after a 7 day EC culture period exhibited a typical node−fibril organization, indicating that the surface modification does not seem to alter the ePTFE microstructure as confirmed by confocal microscopy. The observations of nonmodified samples (control grafts) revealed that only a few cells were present, but with a rounded aspect, a sign of cell suffering (Figure 3A,B). In the same way, ePTFE modified with a PAH monolayer presented some rare flattened cells (Figure 3C,D). On the contrary, the samples modified with PMF showed a great number of cells which adhered and spread on the ePTFE surface after 7 days of culture. They constitute a confluent cell monolayer (Figure 3E). The cell density was sometimes so high that it was difficult to differentiate the cells from one another, as previously described by Bowlin et al.[35] However, the cells were in general homogeneously distributed and presented a cobblestone shape (Figure 3F), which is a typical morphology of healthy endothelial cells. The cell densities observed by scanning electron microscopy also confirmed the viability values previously determined with Alamar Blue assay.

In addition, it is known that the adhesion surface can influence the cell phenotype after several days of culture. The vWF, which is an adhesive glycoprotein synthesized exclusively in vascular ECs and megakaryocytes, is widely used as a phenotype marker of ECs.[36] The balance of hemostasis and thrombosis in the cardiovascular system is directly affected by the EC function. We observed by confocal microscopy positive fluorescent labeling of ECs with an antibody specific for vWF on ePTFE modified with PMF (Figure 4). In this case, all cells expressed vWF and then exhibited this endothelial characteristic after 7 days of culture. No cells were detected for the expression of vWF on nonmodified ePTFE or ePTFE modified with a PAH monolayer (data not shown).

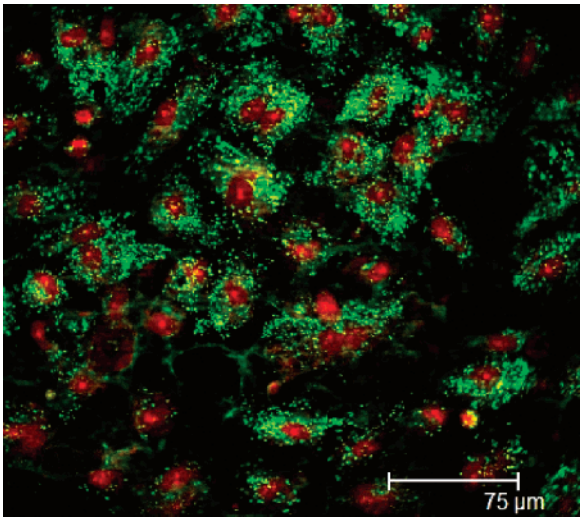

Figure 4. Visualization of the von Willebrand factor (green) and nuclei (red) of HUVECs seeded on ePTFE patches modified with PEI−(PSS−PAH)$_3$ after 7 days of culture. The von Willebrand factor was visualized using Alexa Fluor 488 fluorochrome (λ_{ex} = 494 nm, λ_{em} = 517 nm). Nuclei were labeled with propidium iodide (λ_{ex} = 536 nm, λ_{em} = 617 nm). Scale bar 75 μm, objective 40×.

The confocal microscopy observations prove the absence of dedifferentiation. This suggests that ECs seeded on ePTFE modified with a PMF keep their endothelial functionality, which is essential for the use of this type of material for vascular grafts. The persistence of differentiation and functionality of EC cells seeded on ePTFE modified with PMF is in accordance with results obtained with such a PMF type deposited on glass.[31] More sophisticated architectures could be built to get biofunctionalized films by means of peptide,[37] drug,[38] growth factor,[39] or DNA[40] embedding. Even if the functionalization is often realized with films made of hyaluronic acid or polyglutamic acid and polylysine, which are in general weakly adherent for cells, their adhering properties can be improved by capping such films by a few PSS/PAH layers. Such a capping allows functionalization and good cellular adherence.[41] Finally, all these architectures can be seeded by ECs and represent an extremely promising route to achieve antithrombogenic vascular prostheses.

4. Conclusion

This study demonstrates for the first time to our knowledge that the luminal surface of expanded poly(tetrafluoroethylene) vascular grafts can be modified with a PMF, made of PSS and PAH, to improve the adhesion and viability of human ECs. Rapidly, ECs form a confluent cell monolayer when they are seeded on ePTFE modified with a PEI−(PSS−PAH)$_3$ PMF. Moreover, these cells keep their endothelial phenotype after several days of culture. These results confirm the versatility of our system, which can be used to modify ePTFE vascular grafts. The concept would probably also hold more generally for other biomaterial surfaces.

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