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Wet chemical modification of PTFE implant surfaces with a specific cell adhesion molecule

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The surface of PTFE based implant material could be covalently functionalised with adhesion molecules for improved bio-adhesion by wet chemical oxidation to introduce hydroxy groups followed by cross-linking with cyanuric chloride.

In vascular surgery, the insertion of artificial blood vessels (prosthetic grafts) is a common method, but it is limited to specific medical applications. Long term studies on PTFE based material have revealed that unspecific adhesion of fibrin and collagen to the implant surface leads to thrombus formation and occlusion, even though PTFE is extremely inert and hydrophobic. Therefore, the use of vascular grafts with an internal diameter less than 6 mm is currently not realistic in vascular and cardio-vascular surgery. The concept of endothelial cell seeding on vascular implant surfaces was developed to mimic physiological blood vessels, and to further reduce unspecific adhesion.[1–3] PTFE, however, is a hydrophobic matrix that is not suitable for optimal cell growth. Adhesive proteinaqueous coatings on the implant surface have shown only insufficient performance under shear stress conditions.[4] The covalent attachment of specific adhesion molecules may provide a means to achieve a strong shear stress resistant cell binding.

In the present study, we modified a customary vascular graft made of PTFE (Atrium Medical Cooperation) by a chemical method in combination with a biochemical method. The mechanical properties of the polymer were to be retained, and only the biological properties of the inner surface changed. Therefore, we evaluated a three step mechanism to selectively modify the polymer surface only. In the first step, the inner surface of the hydrophobic PTFE has to be modified with reactive groups, which then can covalently bind to a crosslinker for further attachment of an adhesion molecule. Different methods generating reactive surface groups (hydroxy, amino, carbonate) on formerly inert surfaces are available including physical treatment (plasma etching or ablation) and chemical methods (treatment with H_2O_2/H_2SO_4, chromate, permanganate).[5–7] For modification of PTFE tubes, the wet chemical method with H_2O_2/H_2SO_4 was the first choice, because it resulted in a sufficient number of free functional groups without damaging the structural integrity of the polymer (Fig. 1).

In the first reaction step, the surface was treated with H_2O_2/H_2SO_4 (1:1) at room temperature for 20 min. Variation in incubation time and temperature influenced the number of reactive hydroxy groups. Oxidation could be observed to occur to a depth of 10 nm by SEM. The presence of free hydroxy groups was verified by FTIR on a Perkin Elmer System 2000. The analysis was performed with an internal reflection element (45° incidence angle) with 100 scans and a resolution of 4 cm^{-1}. PTFE showed characteristic peaks between 1000 and 1400 cm^{-1} (CF_2 and CF_3 groups); additionally, a wide peak between 3300–3500 cm^{-1} (OH groups) and some shape peaks around 1650 cm^{-1} (C=O) could be observed. The course of oxidation was also observed by measurement of the equilibrium contact angle against water. A significant decrease of the equilibrium contact angle against water (milli Q, pH 5.5, 18.2 MΩ) from 125° to 88°–115° was noticed depending on reaction parameters. Specimens were taken time-dependently. No visible change of the gross surface morphology could be observed with SEM and AFM. The mean roughness of the surface does not change significantly during the oxidation procedure (untreated material: RMS 7.31 ± 0.22 nm/z-range 95.5 ± 5.3 nm 1μm scan range and RMS 42.7 ± 3.1 nm/z-range 470.5 ± 45.3 nm scan range 10 μm; oxidised material: RMS 7.58 ± 0.34 nm/z-range 89.1 ± 4.3 nm 1 μm scan range and RMS 44.3 ± 8.5 nm/z-range 501.7 ± 41.3 nm 10 μm scan range). The elasticity of the different materials are identical (obtained from the force curves).‡

In the second step, the introduced hydroxy groups were reacted with cyanuric chloride under the release of HCl. Incubation with a freshly filtered 10^{-3} M solution of cyanuric chloride in chloroform for 1 h was followed by intensive washing with chloroform to remove excessive cyanuric chloride. The surface morphology of PTFE remained unchanged. Stability and elasticity of PTFE was unaffected. The process of the surface modification could be followed with FTIR. The spectra showed some additional peaks at 2450 cm^{-1}, 1698 cm^{-1},1457 cm^{-1}, and 875 cm^{-1} characteristic of C=N bonds. The contact angle increased after from 88° (oxidized PTFE) to 110° (cyanuric chloride activated PTFE).

In the third step, the activated PTFE surface was coupled with functional molecules to achieve targeted adhesion and spreading of endothelial cells. High affinity interaction of these molecules with physiological receptor molecules on the cells were exploited. Candidate adhesion molecules are constituents of the extracellular matrix (collagen, fibronectin, laminin, vitronectin), or other peptides and proteins containing the RGD-motif or adhesion proteins of microorganisms like the invasin A of *Yersinia sp*. and fragments thereof. These adhesion proteins

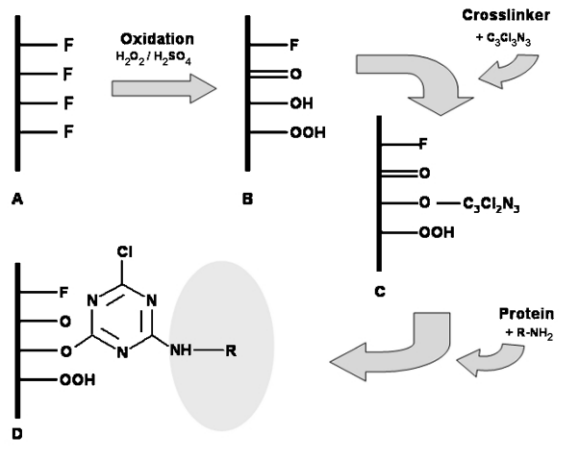

Fig. 1 Reaction scheme of the wet chemical modification of PTFE; A) unmodified PTFE; B) oxidized PTFE with possible formed reactive groups; C) binding of the crosslinker cyanuric chloride; D) covalent binding of adhesion protein to PTFE.

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Table 1 Quantification of endothelial cell adhesion after 24 h to PTFE modified with collagen, fibronectin, gelatin and invasin fragment Min3

	Pure	Gelatine	Min3	Fibronectin	Collagen
Adherent cells [%]	4.5 ± 2.24	11 ± 4.52	22.5 ± 3.45	14.5 ± 4.62	9.5 ± 5.74

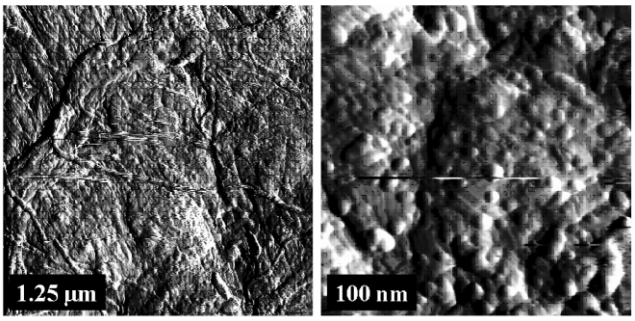

Fig. 2 Atomic force microscopy of PTFE film surface modified with parts of the bacterial invasin protein (Min3).

target specific receptors on the cell surface; especially, β1- and β3-integrins. In earlier experiments, we observed a very high cell adhesion rate after 24 hours using the *Yersinia enterocolitica* invasin fragment (denoted Min3) compared to collagen or fibronectin (Table 1). This adhesion is mediated by a high affinity binding to β1-integrins, especially α3-, α4- and α5-β1 (VLA-3/4/5).[8] The association constant is approximately three magnitudes higher than the commonly used extracellular matrix proteins (collagen, fibronectin) and RGD-peptides.[9] Invasin fragment Min3 was produced as fusion protein with *E. coli* maltose binding protein (MBP), and purified by maltose-binding affinity chromatography and anion-exchange chromatography to apparent homogeneity. An aqueous solution of the tested adhesion protein (borate-buffer pH 8.4) was contacted for 12 hours to allow reaction of surface bound cyanuric chloride residues with amino groups of the protein. All modification steps from oxidation to the attachment of the protein were followed by the contact angle method, but an exact quantification is difficult. The oxidation yielded sufficient reactive groups like hydroxys as well as ketones, aldehydes and chain-breaks. As a single protein molecule covers an area of 60–100 nm^2, the process of surface activation required for efficient protein coupling can be very low. For comparison, the diameter of the cyanuric chloride molecule is in the range of 1 nm, hydroxy groups are 0.2 nm. Theoretically, one protein molecule could cover approximately 200 molecules of cyanuric chloride.

The surface morphology during modification can be followed by AFM. All investigations were performed on a Digital Nanoscope IIIa Dimension 5000 (Digital Instruments, Santa Barbara, CA) as described elsewhere.[10] The results were visualised either in height or in amplitude mode. No changes in the overall surface morphology were detectable after oxidation. Fig. 2 shows the surface of a Min3 modified PTFE film. Using the high resolution of the AFM technique, it was possible to visualise single protein molecules attached to the surface. These appeared as round shaped structures with a diameter of 8 to 10 nm. This size corresponds well with the relative molecular mass of Min3 (117000). Such structures were not detected on the corresponding control surfaces (data not shown). This indicates a good selectivity by this method. It was also possible to quantify the degree of modification: There were 408 ± 28 proteins per square μm on a total analysed area of 100 μm^2.

The surface modification was further investigated by FTIR spectroscopy. Typical protein peaks were detected at 1639 cm^{-1} (amide I), 1548 and 1122 cm^{-1} (amide II) and further peaks at 3415 and 3214 cm^{-1}.

Cell seeding experiments were performed after functionalisation of the PTFE surface with various proteins to test the biological adhesiveness. In addition, shear stress was applied for 72 h to investigate the stability of the endothelial cell lining.

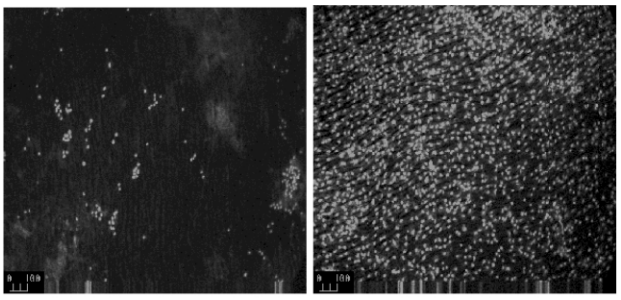

Fig. 3 Cell adhesion experiment (72 h): confocal laser scanning microscopy of endothelial cells seeded onto PTFE modified with inactive control protein (left) and the invasin A fragment Min3 (right) after perfusion. Cell nuclei were stained with propidium iodide.

As shown in Fig. 3, only endothelial cells cultivated on PTFE modified covalently with Min3 could withstand the shear stress and remained on the surface. Approximately 90% of the total implant surface was covered with cells. Endothelial cells seeded on PTFE modified with maltose binding protein, an inactive control, were lost.

Absorptive coating of PTFE with laminin, collagen and fibronectin (ECM proteins) has been described in the literature to improve the growth of endothelial cells on vascular implants. However, this method did not show satisfactory results in small vessels as the cell lining was lost under the shear stress encountered. Here, we report the development of a new strategy for bioadhesive modification of PTFE, which was able to establish a strong shear stress resistant cell binding *via* a fragment of the bacterial adhesion protein Invasin A. The improved performance of this modification can be attributed to both its higher receptor affinity compared to ECM proteins and its covalent binding. Especially, the latter will be important to guarantee the resistance against shear stress as encountered in the blood circulation.

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Notes and references

‡ The elasticity was not calculated, but the estimation was drawn by comparison.

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