Improving diagnostic accuracy in aortic prosthetic graft infection
Saleem, Rani

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Chapter 8

Reduced bacterial counts in supernatant of vascular graft material after polymer brush coating

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Ben R. Saleem
Henny C. van der Mei
M. Reza Nejadnik
Ignace F. Tielliu
Jan J. van den Dungen
Willem Norde
Henk J. Busscher
Clark J. Zeebregt
Abstract

Background: Prosthetic vascular graft infection is a devastating complication. Recently, polyethylene oxide brush coatings (Pluronic F-127) have been introduced to prevent primary prosthetic infection. The aim of this study was to investigate the value of this coating against bacterial adhesion to commonly used vascular grafts.

Methods: Dacron® and PTFE segments were coated with Pluronic F-127. Coated and uncoated segments were exposed to three bacterial suspensions. After two hours, specimens were removed from the bacterial suspension, transferred into tube containing sterile buffer, and sonicated in order to detach the adhering bacteria. Subsequently, the numbers of bacteria remaining in the supernatant were determined.

Results: Univariate analysis showed a significant decrease in colony forming units (CFUs) per cm² between the uncoated and coated PTFE of 63.0±13.2% (P<0.001). Despite the reduction of 59.2±16.7% observed in Dacron® after coating, this difference was not significant (P= 0.069).

Conclusions: Polymer brush coating reduces adhesion of bacterial strains known to infect vascular prosthetic grafts and may be used as an additional layer to vascular prosthetic grafts to further reduce the number of infections.
Introduction

Prosthetic graft related infections after vascular reconstruction of aorto-iliac disease is a rare but devastating and potentially life-threatening complication. The incidence of graft related infection has been reported to range from 0.6% up to 3%, with a mortality rate varying from 25% up to 88% and with an amputation rate of 5% to 25%.\textsuperscript{1-5} Usually, considerable effort is undertaken to decrease the rate of contamination of prosthetic grafts with microorganisms during implantation. Although application of prophylactic antibiotics, avoidance of groin incisions, and adherence to sterility measures have reduced the incidence of prosthetic graft related infection over the last four decades, still a significant number of patients suffer from such infections as could also be concluded from our own series recently published in the \textit{American Journal of Surgery}.\textsuperscript{1-5} The most common bacteria cultured from infections related to grafts include coagulase-negative \textit{staphylococci} (CNS) and \textit{Staphylococcus aureus} (\textit{S. aureus}), which are believed to be the cause of infection in 50% of all cases.\textsuperscript{5} Other important but less frequent cultured bacteria are \textit{Escherichia coli} (\textit{E. coli}) and \textit{Pseudomonas} species.\textsuperscript{3-5} The two most commonly used synthetic materials for vascular reconstruction are polyester (Dacron\textsuperscript{®}) and polytetrafluoroethylene (PTFE) grafts. These prosthetic grafts have been impregnated with either rifampicin or silver. However, multiple studies have shown that there is no benefit in the routine use of rifampicin bonding in an attempt to reduce prosthetic graft related infections.\textsuperscript{6} Besides, \textit{in situ} antibiotic-soaked grafts may become recolonized, especially when placed in areas with purulent material.\textsuperscript{7} Associated high health care cost burdens for infection mitigation, patient discomfort, and not infrequently death, present motivation to provide new solutions to this problem. Recently, polyethylene oxide (PEO) brush coatings (e.g. coatings using Pluron-ic F-127) have been introduced as a tool to prevent primary infection related to prosthetic materials. The polymer brushes are end-tethered polymer chains, which are forced to stretch away from a surface into the adjacent solution due to a high density of chains per surface area unit.\textsuperscript{8} PEO coating is considered to be a promising method for preventing biomaterial-centered infections, as it can form a barrier for deposition of particles on a (biomaterial) surface.\textsuperscript{9} Different studies have used PEO brush coatings to prevent adsorption of globular proteins as well as bacterial adhesion.\textsuperscript{8-11} Brush coatings showed significant reduction in bacterial adhesion.\textsuperscript{10,12} The few bacteria that adhere to the brush are
weakly attached\textsuperscript{13,14} and they grow into biofilms that are more susceptible to antibiotics than biofilms on biomaterials without a brush coating. The thickness of the absorbed brush layer and the conformation of the molecules in the layer strongly depend on the hydrophobicity of the underlying surface. This can be tested by measuring the so-called water contact angle, which for the purpose of this study have also been determined on the surface of both Dacron\textsuperscript{®} and PTFE.\textsuperscript{15} In the current study we investigated the additional value of a polymer brush coating against adhesion of three bacterial strains, isolated from infected grafts, to commonly used Dacron\textsuperscript{®} and PTFE grafts in the field of vascular reconstruction.

**Materials and methods**

**Design of the study**
This *in vitro* study was designed to evaluate the effects of a polymer brush coating on knitted Dacron\textsuperscript{®} and PTFE on bacterial adhesion. Coated and pristine (uncoated) Dacron\textsuperscript{®} and coated and uncoated PTFE were tested with CNS, *S. aureus* and *E. coli* as the most common cause of infection in vascular graft surgery. Pluronic F-127 (polyethylene oxide (PEO)-polypropylene oxide (PPO)-polyethylene oxide block co-polymer with an average molecular structure of $\text{PEO}_{99}\text{PPO}_{65}\text{PEO}_{99}$ and a molecular weight of 12600) was purchased from Sigma-Aldrich (St. Louis MO, USA). Ethanol of analytical grade was purchased from Merck (KGaA, Darmstadt, Germany) and Dacron\textsuperscript{®} and PTFE from Le-Maitre Vascular (Sulzbach/Ts, Germany) and B. Braun Medical (Oss, the Netherlands). Pluronic F-127 adsorbs with its central PPO block attached to hydrophobic surfaces whereas the terminal PEO parts reach out in the solution to form a polymer brush.\textsuperscript{15}

**Contact angle measurements**
The contact angle is the angle at which a liquid interface meets the solid surface. The contact angle is specific for any given material and is determined by the interactions across the interfaces. Most often the concept is illustrated with a small liquid droplet resting on a flat horizontal solid surface. If the liquid is strongly attracted to the solid surface (for example water on a strongly hydrophilic solid) the droplet will spread out on the solid surface and the contact angle will be close to 0°. If the solid surface is hydrophobic, the contact angle will be around or larger than 90°.
Measurement of the water contact angles on the surface of Dacron® and PTFE is necessary as the proposed coating method results in a proper brush coating only when the substratum surface is hydrophobic with a water contact angle around 80 degrees or higher. In this study, prior to the experiment, the hydrophobic features of Dacron® and PTFE were measured at room temperature (25°C) with a homemade contour monitor using the sessile drop technique. In our experiments on each sample, three ultrapure water droplets were placed on different spots and their average contact angles were determined.

**Material preparation and coating**

Segments of one and a half cm in length and 6 mm in diameter of Dacron® and PTFE, respectively, were used for this experiment. Dacron® and PTFE were washed with RBS 35 (detergent), rinsed with water, ethanol (Merck, Darmstadt, Germany) and repeatedly with sterile demineralized water to remove contamination and fingerprints. Subsequently the Dacron® and PTFE pieces were exposed to a solution of 0.5 g l⁻¹ Pluronic F-127 (PEO₉₉-PPO₆₅-PEO₉₉, Sigma-Aldrich, USA) in demineralized water while shaking moderately for 24 h at room temperature. Tests to check whether coating was adequate or complete could not be performed. The coating is an ultrathin coating with the thickness of 6-9 nm depending on the hydrophobicity of the substratum surface. Imaging of the brush coating with scanning electron microscope (SEM) was not feasible, as the drying step needed for SEM sample preparation would destroy the physiosorbed brush coating.

**Bacterial strains and culturing**

CNS 08093, *S. aureus* 08091 and *E. coli* 08095 isolated from infected vascular grafts were used in this study. The CNS 08093 and the *E. coli* 08095 were isolated from a prosthetic graft obtained during an initial operation, in which an aorto-biiliac bypass using a Dacron® graft was inserted. The *S. aureus* 08091 was isolated from a PTFE graft after explantation in the femoro-popliteal region, due to infection. We found it to be most interesting to use bacterial strains obtained from clinical vascular grafts infections knowing their background.

All strains were first grown overnight at 37°C on a blood agar plate from a frozen stock. The plates were kept at 4°C, never longer than one week. One colony was used to inoculate 10 ml of tryptone soya broth (TSB, OXOID, Basingstoke, England) and incubated at 37°C in an aerobic incubator for 24 h. This preculture was used to inoculate a second culture of 200 ml that was grown for
16 h under the same conditions. The bacteria were harvested by centrifugation for 5 min at 5000 × g and washed twice with demineralized water. To break bacterial aggregates, bacteria were sonicated for 3 times during 10 s at 30 W (Sonics & Materials INC, Danbury Connecticut, USA). Finally, bacteria were suspended in phosphate-buffered saline (PBS) solution (PBS: 10 mM potassium phosphate, 150 mM NaCl, pH 6.8) to a concentration of 3 x 10^8 per ml for all experiments.

**Bacterial adhesion**

Uncoated and brush coated Dacron® and PTFE pieces were separately exposed to 100 ml bacterial suspensions in 100 ml Erlenmeyer flasks for 2 h while shaking at 37°C. To compare coatings and surfaces for their performance during a fixed surgical period, it was chosen to fix the time for bacterial adhesion and not to contaminate all surfaces with the same number of organism. In our *in vitro* model, bacteria were allowed to adhere for 2 h prior to cell adhesion and spreading, which is considered to mimic the clinical situation where implants become contaminated prior to implantation. After 2 h Dacron® and PTFE pieces were removed from the PBS and rinsed mildly with sterile demineralized water in order to remove bacterial suspension and loosely adhering bacteria. Finally, all Dacron® and PTFE pieces were separately put in a test tube holding 5 ml sterile buffer and sonicated for 5 min (Omnilabo International BV, Breda, The Netherlands) to detach bacteria from the surfaces. This bacterial suspension was diluted and 100 μl of the dilutions were plated on blood agar. The agar plates were kept in the incubator overnight at 37°C, and the bacterial colony forming units (CFU) were counted the next day manually (Figure 1). All experiments were repeated three times with separately cultured bacteria, coated and uncoated pieces of Dacron® and PTFE. Eighteen segments of one and a half cm in length and 6 mm in diameter of Dacron® and PTFE, respectively, were used for this experiment.

**Statistical analyses**

Statistical analysis was performed with SPSS for Windows (SPSS 17-0, SPSS Inc., Chicago, Illinois, USA). Data are presented as mean ± standard deviation. Differences between variables were tested with univariate analysis (ANOVA) and t-test. Statistical significance was set at P < 0.05.
Figures 1. Blood agar plates showing colony forming units after incubating overnight with *E. coli* 08095, from coated PTFE on the left and uncoated PTFE on the right.

**Results**

The average advancing water contact angle measured on Dacron® was 75 ± 8 degrees and on PTFE 118 ± 2 degrees. Fewer colonies were counted the night after detachment of bacteria from brush coated Dacron® and PTFE compared to uncoated prosthesis. The adhesion of CNS 08093, *E. coli* 08095 and *S. aureus* 08091 was respectively $2.06 \times 10^6$, $1.06 \times 10^6$ and $1.54 \times 10^6$ on uncoated PTFE compared to $1.37 \times 10^6$, $0.32 \times 10^6$ and $0.51 \times 10^6$ CFUs per cm$^2$ on brush coated PTFE. Thus, cumulatively, the CFUs per cm$^2$ of these bacteria were decreased by 63.0% (SD 13.2%, SEM 4.4%) on brush coated PTFE. Univariate analysis showed the difference to be highly significant (P<0.001). In the individual bacterial strains, between uncoated and coated PTFE, a significant difference was found in the adherence of *S. aureus* 08091 (P=0.030). No significant differences were found in the adherence of CNS 08093 (P=0.093) and *E. coli* 08095 (P=0.083) (Figure 2).

On Dacron® the adhesion of CNS 08093, *E. coli* 08095 and *S. aureus* 08091 was respectively $1.63 \times 10^6$, $3.56 \times 10^6$ and $1.01 \times 10^6$ on uncoated Dacron® compared to $0.73 \times 10^6$, $0.15 \times 10^6$ and $0.34 \times 10^6$ on coated Dacron®. Similarly, a cumulative decrease of 59.2% (SD 16.7%, SEM 5.6%) in CFUs per cm$^2$ was observed in Dacron® after coating. Univariate analysis showed that the difference in bacterial adhesion between the uncoated and brush coated Dacron® was not statistically significant (P=0.069). In the individual bacterial
strains, a significant difference was found in the adherence of *E. coli* 08095 (P=0.018). No significant differences were found in the adherence of CNS 08093 (P=0.32) and *S. aureus* 08091 (P=0.27) (Figure 3).

**Figure 2.** Comparison of colony forming units (CFU) between coated and uncoated PTFE.

**Figure 3.** Comparison of colony forming units (CFU) between coated and uncoated Dacron®.
Discussion

Different synthetic materials and methods have been used for treating prosthetic graft infection, albeit with little success. In the best of our knowledge, this study is the first to demonstrate a reduction in bacterial adhesion on prosthetic graft material (PTFE and Dacron®) with polymer brush coating (Pluronic F-127). The bacterial adhesion was reduced by 63% on the PTFE group and by 59.2% on the Dacron® group after coating with a polymer brush. Bacterial adhesion to surfaces is influenced by physicochemical properties of the surface. Previous studies have shown a slower formation and a higher viability of biofilms on brush coatings. Bacteria adhering to a polymer brush coated surface are also more susceptible to antibiotics when compared to the uncoated material. These findings are clinically relevant and may give antibiotics a better chance to eliminate bacteria that have adhered to a brush coated implant surface during surgery and prior to the formation of a more mature and more resistant biofilm. Moreover, polymer brush coatings have been demonstrated to be stable during and after biofilm removal and after prolonged exposure to physiological fluids, such as saliva or urine.

Physisorbed polymer brushes are highly hydrated, as has been confirmed by quartz crystal microbalance with dissipation (QCM-D) analysis of these brush coatings. Brush formation of Pluronic F-127 only succeeds if preferably attaches its PPO block (rather than its PEO blocks) to the surface and this occurs when the substratum surface is sufficiently hydrophobic with a water contact angle of above 80 degrees. The water contact angle on the surfaces of PTFE measured 118 ± 2 degrees. This is sufficient for the formation of brush surface. However, the water contact angle on Dacron® is 75 ± 8 degrees, which is at the limit of hydrophobicity needed for formation of adsorbed Pluronic F-127 brush. Therefore, Pluronic F-127 adsorbed to Dacron® will not always be in the anti-adhesive brush-like configuration, but probably also in a more mushroom-like configuration. This may be the reason that the reduction in bacterial adhesion found on coated Dacron® could not be demonstrated to be statistically significant, i.e. large standard deviations were found over the coatings due to their potentially heterogeneous nature. Furthermore, Dacron® is manufactured either as a woven or knitted polyethylene with various porosity and velour.

In spite of the small sample significant lower adherence of the individual bacterial strain S. aureus 08091 (P=0.030) in coated PTFE was found as well as a significant lower adherence of E. coli 08095 (P=0.018) in coated Dacron®.
this effect in lower adherence in the coated materials is most probable due to the coating. However, it is unclear why *S. aureus* (spherical, Gram-positive bacterial strain) did not show a lower adherence in coated Dacron® and vice versa for *E. coli* (rod-shaped, Gram-negative bacterial strain) in coated PTFE. Roosjen et al. investigated the effects of a brush on adhesion of seven very different microbial strains; they concluded that the form of the bacteria and composition of the cell membrane are not of a major influence on adhesion to the brush. As previously stated there is a clear difference between the properties of PTFE and Dacron® in general, it can be hypothesized that these properties are of influence on bacterial adhesion.

It has to be noted that the presented results exhibit the number of bacteria that were removed from the uncoated and brush coated Dacron® and PTFE pieces during the sonication step. Although sonication is a common method for removing different sorts of adhering particles from solid surfaces, the possibility that some bacteria remain attached to the surfaces, even after sonication, cannot be ruled out. Previous studies, however, indicate that brush coating weakens the adhesion force between bacteria and the substratum surfaces, meaning that removal of the bacteria by sonication would be more efficient for the brush coated than the uncoated surfaces. Consequently, the possibility that some bacteria remain attached to the surfaces after sonication being existent, would make the conclusions of this study only stronger.

There are some limitations to this study. Despite a CFU reduction of 59.2% seen in the Dacron® coated material, this difference in bacterial adhesion was not significant between the uncoated and coated Dacron® (P= 0.069). This can be due to the small size of the experiment and a type-II statistical error. It is important to test the value of polymer brush coating after imitating blood circulation in Dacron® and PTFE. When placed in the blood stream, a protein layer is formed and attached to the prosthesis. The protein layer would affect the bacterial adhesion.

However, there is a great chance that polymer brush coatings remain functional after exposure to blood, knowing the stability in physiological fluids. Obviously, the question raises into what extent the effects of coating (i.e. reduction of biofilm formation and higher susceptibility for antibiotics) are of importance in the reduction of clinical manifest infections.
Conclusion

This study shows that a PEO polymer brush coating strongly reduces adhesion of bacterial strains known to infect prosthetic grafts commonly used in the field of vascular surgery. More research is needed to improve this promising technique for routine clinical use.
References


