Effects of structure, morphology and heparin(-like) coatings on the tissue reaction to poly(ethylene terephthalate)
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CHAPTER 2

Ongoing foreign body reaction to subcutaneous implanted (Heparin) modified Dacron in rats

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ABSTRACT
Dacron-containing heart valve repair devices trigger chronic inflammation characterized by the presence of activated macrophages, foreign body giant cells and capsule formation. Upon blood contact, pro-inflammatory proteins adsorb to the material and provide a substrate for monocyte binding and differentiation. Various heparin-coated polymers have been shown to reduce adsorption of pro-inflammatory proteins in vitro and in vivo. In this study, the effect of knitted, heparin-coated Dacron on the foreign body reaction was tested subcutaneously in rats. We hypothesized that the anti-inflammatory effect of heparin would reduce monocyte recruitment and differentiation and therefore limit the inflammatory reaction. An ongoing foreign body reaction, characterized by the presence of foreign body giant cells and high vascularization was observed in uncoated as well as (heparin-) coated Dacron up to 180 days of implantation. Also, a thin capsule was formed around each material up to this time-point. In conclusion, although heparin coatings might have an effect on the acute inflammatory response, we were not able to show any difference between heparin-coated and uncoated Dacron after 180 days implantation in rats. Further research needs to be conducted to assess the difference in pro-inflammatory protein adsorption between the tested materials and the effect this has on the long-term foreign body reaction.

INTRODUCTION
Heart valve regurgitation is a condition in which the cusps of a valve fail to close properly, resulting in backflow of blood. The left ventricular remodeling that is associated with mitral valve regurgitation can lead to heart failure. Current treatment options for these conditions include valve replacement (with mechanical, biological or donor valve prosthesis) or valve repair. The use of valve repair techniques using annuloplasty (AP) devices greatly reduced the occurrence of post-operative, valve replacement associated complications such as anticoagulant hemorrhage, thromboembolism, prosthesis valve endocarditis and structural valve degeneration. Sewing rings of valve prosthesis as well as AP devices are covered with elastic, multifilament, knitted poly(ethylene terephthalate) (Dacron®). This material has been reported to trigger chronic inflammation characterized by the presence of activated macrophages and foreign body giant cells. Proliferation of surrounding fibroblasts accompanies the chronic foreign body response and can eventually lead to excessive encapsulation, causing the device to fail. Upon implantation, fibrinogen, among other proteins, adsorbs to the material and provides a substrate for integrin mediated macrophage binding. Secretion of monocyte chemoattractant protein-1 (MCP-1) by adhering macrophages further contributes to the development of the foreign body reaction (FBR). Various heparin-coated polymers such as PVC and polystyrene have been shown to reduce adsorption of fibrinogen compared to uncoated polymers. Also, a reduced adsorption of fibronectin and C3 complement factor as well as a decreased activation of neutrophils, terminal complement complex and coagulation has been shown. This suggests that a heparin coating may reduce inflammation. In this study, the effect of heparin-coated Dacron on the FBR was tested. We hypothesized that the anti-inflammatory effect of heparin would reduce recruitment of macrophages and therefore limit the inflammatory reaction. Disks of heparin-coated and uncoated Dacron were subcutaneously implanted in the back of rats, and explants were microscopically reviewed at 5, 10, 21, 42, 90 and 180 days after implantation.

MATERIALS AND METHODS
Materials
Dacron® knitted fabric (M04301, C.R. Bard, Inc, Tempe, AZ. USA) was coated with a plasma-polymerized, carboxyl-group containing polyolefin layer, to which poly(ethylene imine) was bound using carbodiimide. After washing with water, NaIO-modified heparin was covalently coupled to the polyamine layer (Figure 1) using NaCNBH₃. Eight-mm disks were punched out of the untreated, the polyamine modified and the heparin-coated Dacron and ethylene oxide (EtO) sterilized. Uncoated, polyamine- and heparin-coated Dacron are further referred to as BARE, PEI and HEP respectively.
Animals
Male, 8 - 10-week old AO rats were anesthetized with a halothane-N₂O-O₂ mix. Subcutaneous pockets were created on both sides of three midline incisions on the back of each rat. One Dacron disk was implanted in each of the pockets. Two disks of each of the three materials were implanted in each rat. NIH-guidelines for the care and use of laboratory animals (NIH publication #85-23 rev. 1985) were observed.

Explants
At day 5, 10, 21, 42, 90 and 180 after implantation, three rats were sacrificed and the implanted disks, including the surrounding tissue, were retrieved. For each rat, one explant of each material was fixed in 2% glutaraldehyde in PBS (pH 7.4) for 24 hours at 4°C, dehydrated in increasing ethanol concentrations (50-70-96-100%) and embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany). Two-micrometer sections of these were stained with toluidine blue. The second explant of each material was snap-frozen in liquid nitrogen and stained immunohistochemically. A 1:400 dilution of ED1 (Instruchemie, Hilversum, The Netherlands) monoclonal antibodies were used to stain monocytes/macrophages. Vascularization was evaluated after staining with a 1:150 dilution anti-collagen IV monoclonal antibodies (ITK Diagnostics BV, Uithoorn, The Netherlands).77

Analysis
Light microscopic evaluation was carried out with a Leica DM-LB microscope (Leica, Rijswijk, The Netherlands) and photomicrographs were taken using Leica Qwin 2.3 software (Leica, Rijswijk, The Netherlands). Toluidine blue stained slides were used to determine the extent of encapsulation, macrophage/giant cells presence and vascularization. Encapsulation around each implant was determined by measuring the area of fibrous tissue in relation to the size of the tissue-material contact (Figure 2). Infiltration of macrophages and the presence of giant cells as well as the extent of vascularization in and around the implanted samples were scored on a scale from 1 (weakest reaction) to 10 (strongest reaction) by two people independently. Moreover, the immunohistochemically stained slides were used to verify the presence of macrophages, giant cells and blood vessels.

RESULTS
Encapsulation
Encapsulation by fibroblasts was observed from day 5 on (Figure 3). In all samples, an increase in capsule thickness was evident during the first 10 days following implantation. Around each of the materials, the thickest capsule was present at day 10. At later explantation times, the capsule thickness had decreased; measurements made at 42, 90 and 180 days showed that barely any capsule remained around any of the different materials. Over time, PEI had the thinnest capsule whereas BARE had the thickest. Furthermore, the number of fibroblasts between the fibers of the implant was generally lowest in HEP and highest in BARE.

Macrophages / Giant cells
At all explantation time points, the number of macrophages in the surroundings and between the fibers of BARE and PEI was comparable (Figure 3). At day 5, macrophages were detected mainly in the tissue surrounding each of the

Figure 1: Heparin coating
Dacron fabric was coated with a plasma-polymerized, carboxyl group-containing polyolefin layer, to which poly(ethylene imine) was bound using carbodiimide. After washing with water, NaO₄-modified heparin was covalently coupled to the polycationic layer using NaCNBH₃.

Figure 2: Capsule measurement.
D = Dacron; C = fibrous capsule area; L = tissue – Dacron contact; S = surrounding tissue. Fibrous capsule was determined by dividing the total capsule area C by the length of the dashed line L.

Figure 3: Aspects of the FBR against (modified) Dacron samples in time.
different materials. At days 5 and 10 larger numbers of macrophages were detected between the fibers of HEP than in either BARE or PEI (Figure 4). A decreasing number of macrophages was detected from day 21 on (Figure 3). Associated with the decrease in macrophage number, the number of giant cells surrounding the individual fibers of each material increased over time. Giant cells were observed as early as on day 5. A comparable number of giant cells was observed in both BARE and PEI throughout the study. The number of giant cells between the fibers of the three different materials increased over time and was similar in the different materials at day 21. From day 42 on, the number of giant cells between the fibers exceeded the number of unfused macrophages around BARE, PEI and HEP. At this time point, HEP contained the lowest number of giant cells, compared with BARE and PEI. At 90 days, both BARE and PEI contained higher numbers of giant cells than HEP. The highest number of giant cells surrounding the fibers of HEP was detected at day 180, whereas in BARE and PEI the highest number was already observed at 90 days. Generally, the size of the giant cells increased over time from typically three nuclei at day 5, to seven nuclei at day 180 (Figure 4). The presence, location and abundance of blood vessels was confirmed by staining of collagen IV.

**Vascularization**

Around all three materials, more blood vessels were detected on day 10 than on day 5, indicating angiogenesis during the first 10 days after implantation. In the surrounding tissue of both BARE and PEI at days 5 and 10 post-implantation, blood vessels were activated as suggested by the rounded shape of endothelial cells and the adherence of leukocytes to the blood vessel wall. Formation of blood vessels between the fibers of all three different materials was first observed at 10 days (Figure 4). The number and diameter of blood vessels within and surrounding the implants increased in time (Figure 3). From day 42 on, blood vessel size ranged from capillaries, typically inside the implants, to large venules and arterioles within the surrounding tissue. Over time, fewer blood vessels and less blood vessel activation was detected in the tissue surrounding and between the fibers of HEP, compared to BARE and PEI (Figure 4). No clear difference was detected between BARE and PEI, either within the implant or the surrounding tissue, in particular at 90 and 180 days. The presence, location and abundance of blood vessels was confirmed by staining of collagen IV.

**DISCUSSION**

In this study, capsule thickness, phagocyte infiltration and vascularization in, and around bare, poly(ethylene imine) and heparin-coated Dacron was assessed at 5, 10, 21, 42, 90 and 180 days after subcutaneous implantation in rats. We hypothesized that the anti-inflammatory effect of heparin would reduce recruitment of cytokine-producing macrophages and therefore limit capsule thickness and phagocyte infiltration.
formation of a fibrous capsule. However, we did not see a clear difference in any of the measured aspects between heparin-coated and bare Dacron, 180 days after implantation. A decreased adsorption of pro-inflammatory proteins such as fibrinogen, fibronectin and C3 on different heparin-coated polymers after contact with blood or plasma has been reported in literature. In sheep, an anti-inflammatory effect was reported up to 5 days after blood exposure to a heparin-coated extracorporeal circulation. In addition, it has been shown that the extent of adsorption of denatured fibrinogen on implanted Dacron is directly related to the severity of inflammation. Since a decreased inflammation was not detected at 180 days of subcutaneous implantation, it remains to be determined whether there is indeed a decreased inflammatory protein adsorption on heparin-coated Dacron and whether this acute difference contributes to an improved long term performance. Although the described heparin coating has anticoagulant properties (unpublished results), it is chemically distinct from the coatings described in the aforesaid studies. The chemical stability in the presence of macrophage-derived reactive oxygen species has not been investigated. A potential alteration of the coating would have an effect on the protein-adsorption characteristics of the surface. Additionally one shall bear in mind that when applied clinically, the heparin-coated Dacron will be in continuous contact with blood where the acute response of coagulation factors and blood cells plays a significant role in the chronic performance. In the present model, specifically, the presence of platelets that potentially accumulate and secrete wound-healing mediators, such as growth factors and remodeling factors, has not been accounted for. In conclusion, the chemical properties of heparin-coated Dacron that were thought to determine the acute protein adsorption and tissue response seem to play a smaller role with respect to long term performance. Further research needs to be done to understand the mechanisms that govern the ongoing FBR to this undegradable biomaterial and to elucidate the effect of heparin coating.