Effects of structure, morphology and heparin(-like) coatings on the tissue reaction to poly(ethylene terephthalate)
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CHAPTER 5

ReGeneraTing Agent (RGTA) improves the foreign body reaction against implanted poly(ethylene terephthalate) in rats

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ABSTRACT
Implantation of a medical device activates biological cascades such as inflammation, affecting the performance of the medical device over time. One way to modulate the inflammation resulting from the implantation of medical devices is to modify their surface e.g. with a heparin coating. This application is only partly effective, because the body contains heparinases, which degrade heparin. Regenerating agent (RGTA) is a synthetic polysaccharide that acts as a heparinase-resistant analog of heparin. Similar to heparin, RGTA binds and stabilizes a plethora of immunomodulatory factors, such as TGFβ and VEGF, thereby improving (wound) healing in various animal models. Unlike heparin, RGTA does not influence coagulation. We hypothesized that RGTA could act as a beneficial substance for long-term implanted medical devices, either as a solute in the tissue surrounding the implant, or applied as a coating to the implant surface. We tested our hypothesis through assessment of the FBR to poly(ethylene terephthalate) (PET) in the presence of injected or coated RGTA, or a combination thereof. We showed that RGTA coating inhibits the presence of inflammatory giant cells and cell influx in general, delays blood vessel growth and prevents the build-up of collagen. Injection of RGTA increases the number of blood vessels during the first 10 days. The combination of coated and injected RGTA results in larger, seemingly more permanent, blood vessels than the other test conditions. RGTA is also more potent in augmenting the foreign body reaction than heparin and saline. These characteristics make RGTA an appropriate substance for improving the FBR to PET.

INTRODUCTION
Implantation of a medical device activates biological cascades such as inflammation, affecting the performance of the device over time. In a previous study, we reported a chronic inflammatory reaction against knitted PET, as it is used in sewing rings of heart valves and annuloplasty devices.78 In order to improve biocompatibility it has been proposed to chemically alter the material surface such that it has immunomodulatory properties. Heparin enhances the activity of a large number of chemotactic cytokines and growth factors.125,126 Heparin surface modifications have therefore been shown to improve the acute tissue reaction to medical materials, including poly(ethylene terephthalate) (PET), poly(tetrafluoro ethylene) (PTFE) and poly(vinyl chloride) (PVC).127,128 This makes it suitable as a modification in tubing and other parts of extracorporeal circulation systems129-131, where it reduces complement activation and inflammatory response during cardiopulmonary bypass.108 In addition, heparin coatings are used on intraocular lenses.132,133 Because heparin is degraded by naturally present heparinase, we speculated that heparin surface modifications only acutely enhance the function of heparin-binding proteins.78 Regenerating agent (RGTA) is a heparin-like, synthetic polysaccharide that is resistant to heparinase degradation and it does not influence coagulation.134,135 It does, however, enhance the function of cytokines and growth factors.136 RGTA has been shown to improve healing of crushed skeletal muscle in rats137,138, infarcted myocardium in pigs139, colonic anastomosis in rats140,141, skull defects in rats and rabbits142-144, periodontitis in hamsters145,146, gastric and colic ulceration in rats147 and skin burn wounds in rats.148 We hypothesize that, in the presence of implanted PET, RGTA will improve the tissue reaction against the implant, thereby increasing vascularization, reducing inflammation and reducing the formation of a fibrous capsule. For this purpose, we coated RGTA onto knitted PET and implanted it subcutaneously in the back of rats. Smooth PET disks were used as a control. In order to mimic an RGTA-releasing system, we also co-injected RGTA with the implanted (coated or uncoated) PET samples and compared this to heparin co-injection. The effect of the RGTA on inflammation and vascularization was evaluated using digital histological analysis at 5, 10 and 21 days after implantation. The effect of RGTA on collagen deposition was measured by digital histological analysis. In addition, collagen crosslinks were characterized.

MATERIALS AND METHODS

RGTA
RGTA OTR4120 was prepared according to a previously described method.149 Briefly, a water-soluble dextran derivative was prepared from T40 dextran by carboxymethylation with monochloracetic acid treatment, followed by O-sulfonation with SO3-DMF complex in the
presence of 2-methyl-2-buten. Degrees of substitution, defined as the number of groups substituted by glucosidic units, were 0.50 for carboxymethyl groups and 1.30 for sulphate groups. This process generates reproducible batches of RGTA.

**RGTA coating**

RGTA was coated onto PET in order to lessen this reaction. Knitted PET fabric (M04301; C.R. Bard, Inc, Tempe, AZ) was treated with plasma-polymerized ethylene to which an acrylamide/acrylic acid graft was coupled using ammonium cerium(IV) nitrate. Poly(ethylene imine) was bound to the poly(acrylamide) graft using carbodiimide. After washing with water, RGTA was covalently coupled to the polyamine layer using NaCNBH₃ (Figure 1a and 1b) and washed with water again. From the RGTA-coated PET sheet, several strips were randomly cut out and stained with toluidine blue (binds to the negatively charged polysaccharide molecules) in order to confirm homogeneity of the coating.

During this study, we tested uncoated knitted PET, RGTA-coated knitted PET and uncoated smooth PET. These materials will be referred to as BARE, RGTA and Control respectively (Table 1).

**Rat study**

Male, 8 - 10-week-old Albino Oxford rats were anesthetized with a halothane-N₂O-O₂ mix. For the animal study, eight-mm disks were generated for the following test groups: uncoated, knitted PET, RGTA-coated, knitted PET and uncoated, smooth PET. All samples were EtO sterilized prior to implantation. On either flank of the back of the rat, three mid-line incisions were made to generate a total of six subcutaneous pockets. In each pocket, a single disk was placed. For each material, two disks were implanted per rat.

The subcutaneous pockets of the rats were co-injected with either 200 μl saline, 200 μl heparin (100 μg/ml) or 200 μl RGTA (100 μg/ml). This was done about 10 minutes after creating the pockets in order to allow initiation of the acute inflammatory response (Table 1).

At day 5, 10 and 21 after implantation, three rats were sacrificed for each of the co-injection conditions and the implanted disks, including the surrounding tissue were retrieved. For each rat, one explant of each material was fixed in 2% glutaraldehyde in phosphate-buffered saline (pH 7.4) for at least 24 h at 4°C, dehydrated in increasing ethanol concentrations (50–70–96–100%), and embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany). The second explant of each material was fixed in formalin for at least 24 hours. These samples were analyzed for hydroxylysyl pyridinoline content, as described later.

**Staining of tissue sections**

Two-micrometer sections of the Technovit 7100 embedded explants were generated for staining with toluidine blue in order to quantify blood vessels and cellular nuclei. These slides were incubated in toluidine blue solution for 10 minutes after which they were washed with tap water and mounted. Additionally, four-micrometer-thick sections were stained with alcian-picrosirius

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**Table 1: Experimental setup**

<table>
<thead>
<tr>
<th>Sample i.d.</th>
<th>Material / coating</th>
<th>Type of subcutaneous co-injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare-S</td>
<td>Uncoated, knitted PET</td>
<td>Saline (200 μl) n = 3 per timepoint</td>
</tr>
<tr>
<td>RGTA-S</td>
<td>RGTA coated, knitted PET</td>
<td></td>
</tr>
<tr>
<td>Control-S</td>
<td>Uncoated, smooth PET</td>
<td></td>
</tr>
<tr>
<td>Bare-R</td>
<td>Uncoated, knitted PET</td>
<td>RGTA (200 μl; 100 μg/ml) n = 3 per timepoint</td>
</tr>
<tr>
<td>RGTA-R</td>
<td>RGTA coated, knitted PET</td>
<td></td>
</tr>
<tr>
<td>Control-R</td>
<td>Uncoated, smooth PET</td>
<td></td>
</tr>
<tr>
<td>Bare-H</td>
<td>Uncoated, knitted PET</td>
<td>Heparin (200 μl; 100 μg/ml) n = 3 per timepoint</td>
</tr>
<tr>
<td>RGTA-H</td>
<td>RGTA coated, knitted PET</td>
<td></td>
</tr>
<tr>
<td>Control-H</td>
<td>Uncoated, smooth PET</td>
<td></td>
</tr>
</tbody>
</table>

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**Figure 1: RGTA coating of poly(ethylene terephthalate) (PET)**

A) After plasma treatment, acrylamide and acrylic acid are polymerized onto the PET, resulting in a poly(acrylamide) graft, providing amide and carboxyl groups for further treatment. B) Poly(ethylene imine) is coated onto the acrylamide graft, after which the RGTA is covalently bound through random ring opening chemistry.
red, which specifically stains collagen. These slides were incubated in 1% alcian blue 8GX (Sigma Aldrich) in 3% acetic acid at room temperature, for one hour. The slides were then successively washed with tap water, distilled water and incubated overnight in 96% ethanol, followed by air drying. After this, the slides were incubated for 24 hours at 50°C in a 0.1% solution of sirius red F3B (Direct Red 80, Sigma Aldrich) in saturated picric acid (Sigma Aldrich), after which they were again successively washed with tap water, distilled water and incubated for six hours in 96% ethanol. After drying, the slides were incubated for one hour at 50°C in Gill’s haematoxylin (Sigma). Lastly, the slides were successively washed with tap water and distilled water, dried and incubated in xylene (Sigma) for 10 minutes. All slides were mounted with DPX mountant (Fluka).

**Histological analysis**

All complete stained sections were digitally scanned (Nikon Coolscan) and analyzed with Visiopharm software (Visiopharm Denmark). In each image, muscle and fat tissue as well as the background was first excluded from further consideration. Of the toluidine-blue stained sections, blood vessels were manually defined after which all cellular nuclei were (automatically) defined. The total blood vessel area as well as the total area of the selected nuclei was related to the total tissue area (Figure 2). The relative blood vessel area around the knitted samples was corrected for the control samples in each rat. The resulting value was used as a measure for vascularization. Cellular density was used as a measure for inflammation. Significance of differences was calculated separately for each of these parameters at each time point using a one-way ANOVA with a Dunnet’s post test (GraphPad).

![Figure 2: Visiopharm-mediated histological analysis (Color figure: pg. 87)](image)

Slides were scanned and analyzed using Visiopharm software. Firstly, background (white areas), artifacts and the biomaterial (PET) were excluded from analysis. Then, blood vessels (BV) were defined, as shown in red. Dark blue cell nuclei were defined, shown in black. The relative surface area of both the blood vessels and cells was calculated. ‘A’ shows a representative section before digital analysis, ‘B’ shows the same section after analysis.

![Figure 3: Vascularization in the tissue surroundings at day 10](image)

Micrographs (20x) taken from toluidine blue stained slides of explants at day 10. Examples of blood vessels are indicated by arrows. Examples of PET fibers are indicated by double arrowheads. The total blood vessel area around the RGTA-coated samples was larger than around BARE. This was mainly caused by the larger diameter of the blood vessels surrounding RGTA-coated PET. The diameter of blood vessels around RGTA-R was larger than any of the other test conditions.
Of each alcian-picosirius red stained section, the red-colored area was calculated and divided by the total tissue area as a measure for total collagen. In addition, histopathological analysis of all sections was carried out to confirm the results of the digital analysis. Results of both the digital analysis as well as the histopathological analysis were confirmed blindly by a second, independent reviewer.

**HPLC analysis of collagen crosslinks**

The collagen that is present in fibrotic and scar tissue contains a larger number of hydroxylsyl pyridinoline (HP) and lysyl pyridinoline (LP) inter-molecule crosslinks that greatly increase the resistance of collagen to enzymatic degradation. As part of this study, we measured the number of HP and LP crosslinks surrounding the implanted PET. The analysis was performed at TNO Leiden, The Netherlands. Briefly, the formalin-fixed samples were hydrolyzed in 6 M HCl and used for amino acid and cross-link analysis. Hydroxyproline (Hyp) and hydroxylysine (Hyl) levels were determined in the acid hydrolysates by reverse-phase high-performance liquid chromatography (RP-HPLC) after derivatization with 9-fluorenylmethyl chloroformate (FMOC). Hydroxylsylpyridinoline (HP) and lysylpyridinoline (LP) cross-links were measured in the same acid hydrolysates as described previously. Pyridinoline and Hyl levels were expressed as total amount of residues per collagen molecule, assuming 300 Hyp residues per triple helix.

**RESULTS**

RGTA is a synthetic, heparin-like substance with anti-inflammatory and pro-healing properties. In this study, we tested whether immobilized and soluble, injected RGTA improves the foreign body reaction (FBR) against subcutaneously implanted PET in rats. This was compared with BARE PET and with co-injection of either saline or heparin.

**Vascularization around RGTA-coated PET versus bare PET**

Blood vessels around RGTA-coated PET generally had a larger diameter than uncoated PET samples at 10 days (Figure 3). This caused the total blood vessel area to be larger around RGTA-coated PET samples, compared to uncoated PET (Figure 4). In time, the RGTA-coated PET samples showed a different course of vascularization, compared to BARE (Figure 4). Around the RGTA-coated samples, a smaller total blood vessel area was measured at day 5 (p < 0.05). At day 1, however, a larger blood vessel area was measured around the RGTA-coated samples (p < 0.05). There was no difference between the tested conditions at 21 days. Around BARE, a larger blood vessel area was measured at day 5, followed by a decrease at day 10, indicating a delayed blood vessel growth around the RGTA-coated samples.
Vascularization in the surrounding tissue after RGTA co-injection

Co-injection of RGTA during implantation resulted in a larger blood vessel area in the tissue surrounding the PET implants, compared to saline or heparin co-injection (Figure 5). Around BARE, RGTA co-injection resulted in a higher blood vessel area than both heparin co-injection ($p < 0.05$) and saline co-injection ($p < 0.001$) at day 5. At day 10, RGTA co-injection only differed significantly from saline co-injection ($p < 0.05$). No significant differences between any of the test conditions were calculated at day 21 (Figure 5a). Around the RGTA-coated PET, co-injection of RGTA yields a larger blood vessel area compared to saline co-injection ($p < 0.05$) at day 5. At day 10, co-injection of RGTA yields a larger blood vessel area than co-injection of either heparin or saline ($p < 0.05$). No significant differences were calculated at day 21 (Figure 5b). Supporting micrographs from day 10 are displayed in Figure 3. Histopathological analysis of these showed that around BARE, the increase in total blood vessel area that we measured in the surrounding tissue of the RGTA-co-injected samples was mainly caused by an increase in the total number of blood vessels. Around the RGTA-coated samples, however, the additional RGTA-co-injection appeared to result in an increase in blood vessel size.

Collagen deposition around RGTA-coated PET

In contrast to the RGTA-coated PET, the BARE samples showed an increase in collagen content between day 5 and 10, resulting in a significant difference between the samples at day 10 ($p < 0.05$). An increase in collagen content around the RGTA-coated samples was measured between day 10 and 21 (Figure 6a). This suggested a delay in collagen build-up around the RGTA-coated samples. Supporting micrographs from day 10 (Figure 7) confirmed the difference in collagen deposition around BARE-S and RGTA-S.

Collagen deposition in the surrounding tissue after RGTA co-injection

Co-injection of either saline or heparin during implantation caused an increase in collagen content around BARE between day 5 and 10 (Figure 6b). Collagen build-up around the RGTA co-injected samples did, however, not increase until day 21. Collagen content around the RGTA co-injected samples was significantly lower compared to both the saline and heparin co-injected samples at day 10 ($p < 0.01$). Moreover, this showed a delayed build-up of collagen in the presence of injected RGTA in time. Representative micrographs of the picrosirius-stained slides (Figure 7) demonstrate the decreased amount of collagen around BARE-R, compared to BARE-H and BARE-S. It also shows a complete lack of collagen around RGTA-R, compared to RGTA-H and RGTA-S.
Figure 8: Collagen deposition between the PET fibers at day 21 (Color figure: pg. 89)
Micrographs (20x) taken from picrosirius red stained slides of explants at day 21. Examples of red-stained collagen sections are indicated by arrows. Examples of PET fibers are indicated by double arrowheads. RGTA-S, RGTA-H and RGTA-R showed less collagen between the PET fibers than any of the BARE samples. RGTA coating therefore prevents collagen deposition.

Figure 9: Cell influx at day 21
Micrographs (20x) taken from toluidine blue stained slides of explants at day 21. Examples of foreign body giant cells are indicated by arrows. Examples of PET fibers are indicated by double arrowheads. In contrast to the BARE samples, the fibers of RGTA-S, RGTA-H and RGTA-R are barely surrounded by giant cells. In addition, cellularity between the fibers is much lower between the fibers of RGTA-coated samples.
Collagen deposition between fibers of RGTA coated, knitted PET

Micrographs in Figure 8 show almost a complete lack of collagen build-up between the fibers of the RGTA-coated PET at day 21, although some collagen appears to be present closely around the fibers. This is in contrast to BARE, where the abundance of red color shows that collagen was formed between the PET fibers. Although RGTA coating appears to affect collagen formation inside the material, we did not see any effect of RGTA co-injection (Figure 6a). In addition to histological analysis the explanted samples were analyzed by reverse phase HPLC to determine the number of hydroxylysyl pyridinoline (HP) and lysyl pyridinoline (LP) crosslinks per collagen triple helix (TH). Although we detected a difference in collagen formation both in the surroundings and between the fibers of PET using histological analysis, none of the samples contained more than 0.05 HP + LP crosslinks per collagen triple helix in total. This was earlier shown to be a normal value for skin tissue.\(^\text{150}\)

Cellular influx and giant cell formation in RGTA-coated PET

The individual fibers of BARE are mostly surrounded by foreign body giant cells (FBGCs), as indicated by arrows in Figure 9. On average, each giant cell appeared to contain at least 10 active, large nuclei. This is in contrast to RGTA-coated PET. The number of giants cells present in this material was much lower. Also, the number of nuclei per giant cell was lower in RGTA and frequently had an apoptotic appearance. In addition to the low presence of FBGCs, general cellularity between the fibers of RGTA-coated PET was decreased. RGTA coating clearly inhibits the presence of inflammatory giant cells. Co-injection of RGTA does, however, not further minimize this aspect of the FBR.

DISCUSSION

Synthetic heparin-like RGTA has been shown to modulate wound healing in different animal models. In this study, we showed that RGTA coating on implanted PET inhibits the presence of inflammatory giant cells and cell influx in general, delays blood vessel growth and prevents the build-up of collagen for up to 21 days. Injection of RGTA around implanted PET triggers an increase in the number of blood vessels during the first 10 days after implantation, compared to both heparin and saline co-injection. The combination of RGTA co-injection around an RGTA-coated PET disk results in larger, seemingly more permanent, blood vessels. Heparin-binding cytokines and angiogenic proteins are important regulators in the onset and progression of the FBR.\(^\text{128}\) The presence of a heparin (-like) substance in an environment where these factors are likely to accumulate could cause them to become immobilized, thereby preventing them from exerting their function. RGTA coating clearly impairs the FBR. We speculate that, in time, the inflammatory reaction possibly causes coating degradation. Phagocytes that are present at the implant site have been shown to generate oxidants that will affect the chemistry of the coating.\(^\text{156}\) Gradual oxidative or hydrolytic breakdown of the coating after implantation could then cause (the delayed) release of the heparin-binding, angiogenic proteins at later time points. Although the histological evaluation showed a collagen increase around the saline and heparin co-injected samples at day 10 and 21, a commensurate increase in collagen HP and LP crosslinks per TH was not observed. The stable HP and LP crosslinks that are common in cartilage, tendon and scar tissue prevent enzymatic degradation. In an earlier study we have already reported that a capsule builds up around PET implants during the first 10 days after subcutaneous implantation in rats.\(^\text{78}\) At later time points, however, this capsule disappeared. The remodeling that takes place after 10 days suggests that the deposited collagen is susceptible to enzymatic degradation. Although we show that collagen build-up around PET is delayed in the presence of both soluble and coated RGTA, none of the samples caused the formation of a stable capsule until 21 days, as demonstrated by the absence of HP and LP crosslinks.

Co-injection of RGTA results in a larger blood vessel area at 5 and 10 days compared to both injection of heparin and saline. In situ injection of RGTA was shown by Desgranges et al. to enhance neovascularization after skeletal muscular ischemia in rats.\(^\text{156}\) Others have shown that RGTA sequesters heparin-binding proteins among which VEGF, TGF\(\beta\) and fibroblast growth factors (FGFs), thereby augmenting the formation and growth of blood vessels.\(^\text{157-159}\) It appears that RGTA has similar pro-angiogenic properties in the presence of a foreign body-mediated inflammatory response. Co-injection of either heparin or RGTA into the subcutaneous pockets causes an increase in the blood vessel area. However, RGTA shows a larger increase at least until 10 days after implantation, indicating it to have a higher potency than heparin. A second property, which does not exclude the first, is that RGTA is resistant to heparinase degradation, causing it to act longer than heparin.

In contrast to BARE, collagen deposition was minimal between the fibers of RGTA-coated PET. The presence of some collagen closely around the fibers of RGTA-coated PET might indicate that heparin-binding, pro-fibrotic proteins are indeed immobilized and draw the fibrotic response away from the tissue that is more distant from the immediate material-tissue interface. As a result, the individual fibers contain a very thin capsule that possibly also shields the fibers from a long-term inflammatory response. Our observation that there is an absence of FBGCs inside RGTA-coated PET after 21 days supports this argument. We earlier reported that a chronic inflammatory reaction, characterized by the presence of giant cells, persists inside subcutaneously implanted, knitted PET.\(^\text{78}\)

In summary, immobilized RGTA delays blood vessel growth, delays collagen deposition and prevents giant cell formation up to three weeks after implantation. Injected, soluble RGTA increases growth of blood vessels and prevents collagen deposition, compared to both saline and heparin. Having these
properties, RGTA could be used to acutely improve blood vessel formation around implanted devices, making it an excellent substance for improving survival of cells and tissues in devices that contain tissue-engineered components or living cells. In addition, we conclude that RGTA could be used to improve the performance of PET-containing implantable devices such as the earlier mentioned sewing rings of artificial heart valves and annuloplasty devices.