INTRODUCTION

It is not always possible to replace tissue defects with autologous material. Engineering of artificial tissue for functional tissue replacement after trauma or tumor resection holds great promise. One of the great advances in tissue engineering is the development of scaffolds from synthetic polymers (1). Although the variety of available synthetic biodegradable polymers continues to expand, the development of complex 3D scaffold construct with controlled...
internal pore architecture and predictable microstructure still faces significant challenges. It has been demonstrated that the physical and biochemical surface characteristics of scaffold biomaterials directly influence tissue responses (2, 3).

The main obstacle encountered by manufactured tissue substitutes is reduced or delayed vascularization. The ingrowth of vessels and the creation of a sufficient microcirculation are necessary for the survival of transplanted and adjacent tissue, but the ongoing search for substitutes, however, reflects the difficulty in achieving this (4-6). Although the effect of pore size on vascularization has been studied in many systems, scaffolds do not often possess the highly interconnected macro- and micro-pore structures to facilitate cell adhesion and proliferation, and to allow for rapid vascular ingrowth (7).

While the neovascularization of synthetic scaffold constructs can be supported by means of coordinated application of pro-angiogenic factors (8, 9), co-implantation of endothelial cells, in situ pre-vascularization (10), and biomimetic surface coatings (8, 11), the 3-dimensional structure is a factor of decisive importance that guides the growth of blood vessels throughout the scaffold. The ε-caprolactone terpolymer matrix used in the present study has not been investigated for vascular response until now. PEGT/PBT block copolymer, developed for dermis, cartilage and bone replacement, is another matrix material sharing some similarities in structural composition (12, 13). The aim of this study was to analyze the neoangiogenic potential of the new ε-caprolactone terpolymer matrix in comparison to PEGT/PBT block copolymer, particularly with regard to the early microvascular response in an in vivo model.

MATERIALS AND METHODS

Preparation of the dorsal skinfold chamber

The experiment was conducted on a total of 20 female balb/c mice (weight 18-22 g; Charles River, Sulzfeld, Germany). The research protocol complied with all regulations related to animal use and other federal statutes. It was conducted in compliance with the Guide for the Care and Use of Laboratory Animals from the German Animal Welfare Act, which conforms to the provisions of the 1995 Declaration of Helsinki. The titanium chamber frames were implanted to sandwich the stretched skinfold of the animal's back. The prepared skin layer containing the striated skin muscle was covered with the second titanium frame, incorporating a removable coverslip. The implantation procedure of the dorsal skinfold chamber was performed as reported previously (11).

ε-caprolactone terpolymer matrix

The ε-caprolactone terpolymer matrix (Supratel Plus®; Institute of Textile and Process Engineering, Denkendorf, Germany) consists of a synthetic copolymer mainly based on DL-lactide (>70%). The additional components are trimethylene carbonate and ε-caprolactone. The monomers were polymerized by a melting procedure. In a second step they were dissolved in organic solvents. The material was subsequently processed by means of a modified phase inversion technique and freeze-drying technique. The final product, a 3-dimensional scaffold matrix, has a symmetrical interconnected pore structure with a porosity of 85-93% and a pore size of 50 µm to 400 µm (Fig. 1a).

PEGT/PBT-block-copolymer matrix (control)

PEGT/PBT-block-copolymer (Polyactive™; IsoTis NV, Bilthoven, Netherlands) is a segmented block copolymer composed of alternating soft hydrophilic polyethylene glycol terephtalate and hard hydrophobic polybutylene terephtalate segments. A copolymer with a PEGT/PBT weight ratio of 55/45 and PEGT MW of 300 Da (300PEGT-55PBT45) was used in this study (Fig. 1b). Copolymer matrices with a pore size of 250 µm to 300 µm were utilized.

Intravital fluorescence microscopy

Discoid specimens of both scaffolding matrices had an outer dimension of 2 mm x 250 µm. They were placed in the center of each chamber with direct contact to the perfused striated skin muscle (Fig. 2). Contrast enhancement was achieved by i.v. injection of 0.05 mL of 5% fluorescein isothiocyanat labeled dextran (FITC-Dextran, mw 150,000; Sigma Chemicals Co., St. Louis, MO, USA) via tail vein. A 40x water immersion objective (Axiotech Vario 100 HD; Zeiss, Oberkochen, Germany) in conjunction with a Zeiss filter set (BP 450-490, FT 510, LP 520) was used to observe the microvasculature. Microcirculatory observations were performed on days 1, 5, and 10 after implantation.
The number of vessel sprouts and newly formed vessels per field of view were assessed. The neovascularized area was set in relation to the whole observational area (a total of 10 view fields per implant).

**Blood vessel profile counting**

Ten observation areas were selected along the border of the discoid implants for microcirculatory analyses. Each field of view for analysis was positioned to fall onto the implant material (Fig. 2). In addition, the functional vascular density (FVD) of the adjacent skin muscle tissue (n=5) was recorded. The images were captured using a charge coupled device video camera using the Axiovision 3.1. system (Carl Zeiss Vision GmbH, Wetzlar, Germany) for exact relocation of the observation areas. Quantitative off-line analysis of the digitalized images was performed using image analysis system CapImage® image analysis system (Zeintl, Heidelberg, Germany).

The following measurements were performed within each of the observation areas:

1. Functional vessel density (FVD), a parameter used as an index of tissue perfusion and tissue oxygenation (14), was measured as the length of perfused microvessels per observational area (mm/mm²).
2. Microvascular diameters (µm) were measured at a 90-degree angle to the capillary wall.
3. The red-blood cell velocity (RBCV) (µm/sec) was analyzed by the computer-assisted image analysis system using the line-shift method.
4. The microvascular permeability assessed as leakage of the plasma marker FITC-Dextran into the extravascular space was used to measure the endothelial integrity. This is determined by the ratio between the fluorescence intensity measured in and outside of a vessel lumen (le/li) (15).

5. The number of vessel sprouts and newly formed vessels per field of view were assessed. The neovascularized area was set in relation to the whole observational area (a total of 10 view fields per implant).

Fig. 1 - Scanning electron microscopy images show the pore-interconnected 3-dimensional structure of the ε-caprolactone terpolymer (a) and PEGT/PBT copolymer (b) scaffold matrix.

Fig. 2 - The scaffolds were transplanted onto the perfused skin muscle in the center of chamber’s observation window. Observation fields (n=10) were selected along the border of the discoid implants for microcirculatory analyses.

After 10 days, the scaffolds adhering to the adjacent skin muscle were removed en bloc, fixed in a 5.0% formaldehyde solution, and embedded in paraffin. The discoid scaffolds were divided in half. The specimens were sliced into
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RESULTS

Neoangiogenesis and the formation of a newly formed vascular network within the matrix material were noted throughout the experiment. The earliest signs of the angiogenesis were visible on day 1 in all ε-caprolactone terpolymer matrices. Morphologically, the capillaries at implants border began to dilate and to form vessel loops (Fig. 3a). The vessel loops were observed to generate vascular sprouts (Fig. 3b), which were directed towards scaffold’s center. The new developed vessels extended along the matrix’s border and interconnected to form a perfused microvascular network (Figs. 4a and c). Finally, the scaffolds were observed to be completely surrounded and infiltrated by newly developed vessels (Figs. 4b and d). A deep vascular infiltration by perfused new developed microvessels into the matrix till up to 600 µm from the edge was noted within 10 days of implantation period. In comparison, the

The intravital fluorescence microscopy images display the findings on day 1 (a) and 5 (b) post implantation of the ε-caprolactone terpolymer matrix. The capillaries became dilated and developed vessel loops (triangles). Vessel sprouts (VS) expanded from a vascular loop towards the matrix on day 5. Magnification 355x.

Fig. 3 - The intravital fluorescence microscopy images display the findings on day 1 (a) and 5 (b) post implantation of the ε-caprolactone terpolymer matrix. The capillaries became dilated and developed vessel loops (triangles). Vessel sprouts (VS) expanded from a vascular loop towards the matrix on day 5. Magnification 355x.
Onset and the intensity of new vessel development was markedly delayed and reduced in the group of PEGT/PBT copolymer. The blood vessel density per observation field was significantly lower in this group. Although the distribution of the newly formed vascular network corresponded to that one seen in the ε-caprolactone terpolymer matrices the extent of neovascularized areas was lower (day 1: 30% vs. 0%, day 5: 80% vs. 50%, day 10: 100% vs. 70%).

The FVD was found to increase progressively within the observation areas for both scaffolds. However, the statistical analysis showed significant elevated values for FVD from day 1 to day 10 for ε-caprolactone terpolymer scaffolds. A 1.3-fold increase compared to the PEGT/PBT copolymer scaffolds was noted on day 10.

The vessel diameters increased over time accompanied by a gradual amplification of RBCV. The analysis of the microvascular permeability revealed rising values displaying a slightly increase of extravasation within the border zones from the start of the experiment (Tab. I). There was no compromise in microperfusion in both groups. The FVD adjacent to the implant appeared to be stable and was

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**Fig. 4** - New developed blood vessels (triangles) extended along the ε-caprolactone terpolymer (a, b) and PEGT/PBT copolymer (c, d) scaffolds centripetal from the border towards the center of the matrix and anastomosed to form a perfused microvascular network. Both scaffolds induced a strong angiogenic response. However, a higher vascular density was noted in ε-caprolactone terpolymer scaffolds. Magnification: 88x.
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Additionally, in contrast to ε-caprolactone terpolymer scaffolds, a few foreign body giant cells were observed within the PEGT/PBT copolymer matrix.

150±8.2 on day 1 and 154±7.8 on day 10, on average, for the ε-caprolactone terpolymer scaffolds and 147±9.3 on day 1 and 154±9.5 on day 10 for the PEGT/PBT copolymer scaffolds, respectively.

The SEM scan showed an intense infiltration of matrix pores by fibrovascular tissue in both scaffolds (Figs. 5a and b). However, the amount of blood vessel profiles evaluated within the observation fields of ε-caprolactone terpolymer matrix exceeded that of PEGT/PBT copolymer matrix significantly (4.3±0.71 vs. 2.8±0.67, Mann-Whitney, p<0.05).

Additionally, in contrast to ε-caprolactone terpolymer scaffolds, a few foreign body giant cells were observed within the PEGT/PBT copolymer matrix.

### TABLE I - MICROCIRCULATORY PARAMETERS MEASURED IN PEGT/PBT COPOLYMER AND IN ε-CAPROLACTONE TERPOLYMER (LCTP) MATRICES

<table>
<thead>
<tr>
<th>Observation time</th>
<th>day 1</th>
<th>day 5</th>
<th>day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PEGT/PBT</td>
<td>LCTP</td>
<td>PEGT/PBT</td>
</tr>
<tr>
<td>Functional Vessel Density [cm/cm²]</td>
<td>0</td>
<td>18.12 ± 3.0</td>
<td>37.37 ± 4.36</td>
</tr>
<tr>
<td>Microvascular Diameter [µm]</td>
<td>0</td>
<td>7.2 ± 0.08</td>
<td>7.1 ± 0.06</td>
</tr>
<tr>
<td>Red-Blood-Cell Velocity [mm/s]</td>
<td>0</td>
<td>0.029 ± 0.004</td>
<td>0.021 ± 0.003</td>
</tr>
<tr>
<td>Microvascular Permeability [Ie/Ii]</td>
<td>0</td>
<td>0.762 ± 0.004</td>
<td>0.759 ± 0.008</td>
</tr>
</tbody>
</table>

The data are given as mean ± SEM. Statistically significant differences (*Mann-Whitney, # ANOVA on Ranks, P<0.05) are displayed.

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**Fig. 5** - The electron microscopic scans show an intense infiltration of ε-caprolactone terpolymer (a) and PEGT/PBT copolymer (b) matrices by new developed vascularized tissue (asterisks) in magnification: 400x.
DISCUSSION

An improvement of artificial scaffold for reconstruction of lost tissue is still necessary to enhance the ingrowth of blood vessels. Structural characteristics such as porosity, pore size, and pore interconnectivity are crucial. Although the vascularization can be improved by various biomimetic surface modifications, great attention should above all be given to an adequate 3D structure of the scaffolding matrix.

Most early approaches in tissue engineering relied on blood vessel ingrowth into tissue-engineered structures from the host to achieve long-term vascularization. This method has been successful for some tissues. One of the first attempts to solve this problem involved designing a complex 3-dimensional synthetic biodegradable polymer scaffold with an intrinsic network of interconnected branching channels. The limitation of this technology is that it lacks the resolution needed to form templates containing the microcirculation (the capillaries), which are on the order of 10 microns in diameter. Polylactide based implants have already shown to induce a marked angiogenic response, revealing a high density of newly formed microvessels and adequate engraftment. A positive correlation between the pore size and tissue neoformation has been shown for poly-L-lactic acid (PLLA) implants by Wake and colleagues (16). The adhesion and proliferation of endothelial cells seeded on elastic biodegradable PCL scaffolds implanted in mice were increased with increasing pore size. Higher porous scaffolds (pore size 200-250 micron) exhibited more cell adhesion and proliferation compared to lower porous ones (50-100 micron) (8, 10).

In the current study the microcirculatory changes within the border of implanted matrices as well as vessel ingrowth into the matrix were quantified and amelioration of the microvascular network was observed. The vascular density within the scaffolds was observed to increase over time, thus confirming the potential of both synthetic matrices to induce angiogenesis and the ability to vascularize. However, the most significant increase was seen for the ε-caprolactone terpolymer scaffold matrix. Here, the quantification of vessel diameter showed improved values over time, thus indicating the angiogenic potential of the scaffold matrix. The high values for red-blood-cell velocity within new developed vascular network illustrate the augmented perfusion throughout the matrices and demonstrate the high angiogenic induction rate of the scaffolding matrices.

There was no evidence of toxic tissue effects or severe inflammation as measured by microcirculatory parameters such as a massive increase of FITC-dextran leakage from the microvasculature into the extravascular space, or decrease of functional vessel density. Unlike in PEGT/PBT copolymer matrix, no infiltration of macrophages or foreign body giant cells was observed within the ε-caprolactone terpolymer matrix. This may be a sign for a more balanced inflammatory reaction necessary for the physiological process of neoangiogenesis as the formation of new functional blood vessels depends on precise regulation of the molecular effectors that promote the different processes involved. The foreign body reaction seen in the PEGT/PBT copolymer matrix group may have delayed the vascularization process of this scaffold.

The results of the current study confirm our earlier findings that the 3-dimensional structure of artificial constructs is of great importance for their neovascularization. The 3-dimensional structure has been shown to have highest influence aside from the material chemistry and degradation behavior of the synthetic matrix (17, 18).

The dorsal skinfold chamber model in mice is an established tool for in vivo microcirculation research. The neovascularization of biomaterials is especially amenable to study under standardized conditions (17-19). Of course, the difficulties regarding vascularization of tissue-engineered matrices escalates as the volume of the implanted matrix increases. However, in this experimental setting using the dorsal skinfold chamber in mice, the small size of the implant offers the possibility to investigate and visualize the microcirculation within the adjacent tissue and the ingrowth of vessels in the matrix. Certainly, the size and thickness of implants is limited to the construction of the chamber. However, when using thicker implants (>250 µm), the deep crater developing around the implant cannot be penetrated by intravital microscopy as the bordering skin muscle tissue is depressed by the overlying thick implant. Perhaps other methods like subcutaneous implantation of scaffolds and subsequent histological evaluations could offer alternative approaches, but it would then be impossible to observe the dynamics of angiogenesis and vascular integration and the pathophysiology of the microcirculation. The purpose of the experiment was not to reveal the complete vascular infiltration of the matrix; our intention, rather, was to analyze the dynamics of the vascular response of the adjacent host tissue and the initial phase of vessel growth and ingrowth in the matrices by intravital
microscopy at an early stage, namely, within the first 10 days (20).

In conclusion, both matrices were observed to support and guide vascular tissue ingrowth. However, with regard to the onset of neoangiogenesis and the intensity of vascularization, the ε-caprolactone terpolymer matrix was found to be superior to PEGT/PBT-block-copolymer. The results suggest that the ε-caprolactone terpolymer matrix may become a promising biomaterial for the generation of tissue-engineering constructs.

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