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Electrospun polyurethane-based vascular grafts: physicochemical properties and functioning in vivo

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Abstract

General physicochemical properties of the vascular grafts (VGs) produced from the solutions of Tecoflex (Tec) with gelatin (GL) and bivalirudin (BV) by electrospinning are studied. The electrospun VGs of Tec-GL-BV and expanded polytetrafluoroethylene (e-PTFE) implanted in the abdominal aorta of 36 Wistar rats have been observed over different time intervals up to 24 weeks. A comparison shows that 94.5% of the Tec-GL-BV VGs and only 66.6% of e-PTFE VGs (p = 0.0438) are free of occlusions after a 6 month implantation. At the intermediate observation points, Tec-GL-BV VGs demonstrate severe neovascularization of the VG neoadventitial layer as compared with e-PTFE grafts. A histological examination demonstrates a small thickness of the neointima layer and a low level of calcification in Tec-GL-BV VGs as compared with the control grafts. Thus, polyurethane-based protein-enriched VGs have certain advantages over e-PTFE VGs, suggesting their utility in clinical studies.

1. Introduction

The overall population aging, imbalanced diet, low physical activity, and many other factors enhance the wide prevalence of atherosclerosis and the associated need for revascularization of the arteries affected by this disease [1]. Although balloon plastics and vascular stents are widely used, the 3 year primary patency in the stented arterial segments below the inguinal fold does not exceed 19% [2, 3], which demands that the affected vessels are replaced, i.e. open surgery is required. The replacement of such vessels in the current surgical practice utilizes autologous vessels (veins or arteries) [4], decellularized vascular grafts (VGs) [5], or synthetic VGs manufactured from expanded polytetrafluoroethylene (e-PTFE) [6] or polyethylene terephthalate (Dacron) [7]. Note that autologous vessels are not always available, whereas decellularized xenogenic VGs are prone to aneurismal degradation in the long term [8]. Synthetic VGs have shown good performance for replacing large-size vessels; however, the VGs of e-PTFE used for the replacement of small vessels (with a diameter of <6 mm) frequently develop stenosis [9]. This results from an insufficient compliance and biocompatibility of the material and eventually leads to a decrease in the vessel lumen at the site of anastomosis because of clotting in the early post-surgery period or hyperplasia of the neointima at extended observation time points [10].

Correspondingly, the design of synthetic small-diameter VGs (SDVGs) that would display an elevated mechanical compatibility, biocompatibility, and hemocompatibility and would not be prone to develop neointimal hyperplasia is a relevant challenge.

Biodegradable or stable synthetic polymers, such as lactide-co-glycolide [11], polycaprolactone [12], PTFE [13], and polyurethane [14], have been proposed for manufacturing SDVGs. Note that despite the theoretical advantages of biodegradable SDVGs, their practical use is considerably limited by the soundness requirements. Indeed, the rupture of a VG wall may well be a lethal event, while an increase in the wall strength synchronous to its degradation at the expense of the newly formed tissue may depend on the patient’s state (age, comorbidities, and regenerative capacity). Correspondingly, the polymers stable in biological media are regarded as the most promising
materials for SDVGs. One of the most promising classes of polymers is polyurethanes (PUs) thanks to their superb mechanical properties [15], high biocompatibility, and, depending on the structure of their soft and hard segments, different stabilities in biological media [16]. The thermoplastic PUs used in the biomedical industry (Chronoflex, Tecoflex, Elastane, and Pellethane polymers) display a characteristic increased stability in biological systems. Although *in vivo* biodegradation of such PUs is reported from time to time [17–19], the studies of electrospun VGs have not detected any signs of biodegradation in either *in vitro* and *in vivo* experiments [14, 20, 21].

Several methods are applicable to manufacturing VGs, including different variants of phase separation [22], freeze-drying [23], molding [24], and extrusion [25], including electrospinning (ES) [26]. The ES technique makes it possible to produce fibrous tubular scaffolds of synthetic or natural polymers, their mixtures, and mixtures of polymers with low molecular weight substances (various drugs, antibiotics, etc.) [27]. In their structure, the produced 3D matrices resemble biological tissues, and the introduction of extracellular matrix proteins (gelatin, collagen, elastin, and fibronectin) into the fibers makes these synthetic 3D matrices biocompatible. These matrices not only mimic the native tissues, but also differ from the matrices produced from purely synthetic polymers in their mechanical characteristics [28–32]. The introduction of direct thrombin inhibitors (for example, bivalirudin) into the fibers forming the inner VG layer can increase the hemocompatibility of the VG surface contacting blood [33]. Thus, ES is a promising technique for fabricating VGs.

The fact that VGs produced by this method from polycarbonate PUs and PU urea elastomers by Nicast (Israel) [34] and Bard (United States) [35] are already present in the market demonstrates the high potential of VGs of this type. However, the VGs produced by Nicast and Bard are recommended for hemodialysis vascular access only, which indirectly suggests the need for improvement of their mechanical and biological properties.

In this paper, we describe the ES fabrication of SDVGs from protein-filled PU and their characterization. In addition, we have examined and compared the performance of such SDVGs and the VGs of e-PTFE implanted into the rat abdominal aorta for a period of 1 week to 6 months.

### 2. Materials and methods

#### 2.1. Preparation of VGs

The ES solutions were prepared in 1,1,1,3,3,3-hexafluoroisopropanol using stock solutions of the polymers (Sigma, USA): 10% PU Tecoflex-80A (Tec; Lubrizol Advanced Materials, EC), 5% gelatin (GL) solution, and 1.5% bivalirudin (BV) solution (Sigma, USA). The GL and BV concentrations in grafts are given as mass percentages of PU Tec-80A (wt/wt) [33]. The ES solutions contain 3% Tec and 15% GL or 3% Tec, 15% GL, and 1.5% BV.

VGs were fabricated using an NF-103 ES device (MECC, Japan) with cylindrical steel collectors (diameter, 2.7 mm and length, 190 mm). The VGs containing anticoagulant (wall thickness, 120–130 μm) were produced using the following parameters: feed rate, 1.0 ml h⁻¹; voltage, 19 kV; collector rotation speed, 300 rpm; and distance between drum collector and spinneret, 19 cm. The VGs comprised inner and outer layers (with and without 1.5% BV, respectively) with a thickness of 25–30 and 100–105 μm, respectively, successively applied on a cylindrical collector.

The grafts and 3D matrices were treated with glutaraldehyde to crosslink the protein in the fiber as earlier described [28]. The VGs were sterilized with 25 kGy electron beam irradiation using an ILU-6 accelerator (Institute of Nuclear Physics, Siberian Branch, Russian Academy of Sciences Russia).

#### 2.2. Analysis of surface structure and properties

The microstructure of the matrix surface was examined using scanning electron microscopy (SEM) as earlier described [36]. The fiber diameter and pore size were assessed from SEM images according to ISO 7198:1998. The VG porosity was calculated from the pore area and matrix area as \( \text{[pore area (matrix area + pore area)] \times 100\%} \). The contact angle was determined with a Drop Shape Analyzer DSA25 (Kruss GmbH, Germany) using water as a solvent. The drop volume was set to 1 μl and the camera speed to 160 frames per second as recommended by the manufacturer. The contact angle was calculated using four replicates for each VG type.

#### 2.3. Examination of VG mechanical properties

The tensile strength and suture retention ‘strength’ were measured with a Z100 (Zwick/Roell, Germany) tensile testing machine as earlier described using a pulling speed of 10 mm min⁻¹ and sewing the vessel at a distance of 2 mm from the sample specimen edge with a monofilament polypropylene thread with a diameter of 150 μm (needle diameter, 170 μm) [28].

The VG elasticity/compliance was tested using a bench for simulating the pressure drops and rises equipped with a noncontact thickness gauge (46 measurements/s). Systolic/diastolic pressures (120/80 mm Hg) were emulated using two containers with physiological saline solution with input/output electromagnetic valves (controlled according to a pre-defined program) and a VG specimen fixed in between. The loading cycle comprised pressure application (500 ms), containment (100 ms), relief (200 ms), and a time gap between cycles (50 ms). The changes in diameter of the electrospun VGs and the VGs of e-PTFE were plotted; fresh Wistar rat
abdominal aorta was used as the control. Compliance, the elastic component of the graft wall, was calculated as $C_{\text{compliance}} = \frac{\Delta d}{10^5/d_{\text{dia}} \times \Delta P}$, where $\Delta d$ is the change in diameter over the cardiac cycle; $\Delta P$, the difference between systolic and diastolic pressures; and $d_{\text{dia}}$ is the diastolic diameter. The parameter is shown as mm mm$^{-1}$ 

For measuring the burst pressure of VGs, we used an angioplasty balloon with a matching diameter (8.0 mm). The balloon was emptied and inserted into the graft; the pressure was slowly increased (0.5 MPa min$^{-1}$) to 2 MPa (20 atm) or until the tested graft burst. The parameter is shown as mm Hg.

### 2.4. Graft implantation

All manipulations with animals complied with the European convention on the protection of laboratory animals [38].

In total, 36 Wistar rats with an average age of 6.8 ± 0.15 months and average body weight of 440 ± 40 g were used in the study. The animals were kept under stationary vivarium conditions with natural illumination, standard diet, and water ad libitum. For each observation period (1, 12, and 24 weeks), one specimen of either PU-based or e-PTFE (control) VG was implanted to the infrarenal aorta of six animals. Premedication (0.1% atropine at a dose of 0.1 mg kg$^{-1}$), anesthesia (Xylavet/Zoletil), and implantation procedures followed the earlier described scheme [12]; the anastomoses between the aorta and VGs were fixed with a Premilene 8.0 single thread and atraumatic needle using eight to ten interrupted stitches.

On completion of the surgical intervention and wakeup of animals, each individual was placed into a separate cage for 3 days. Clexane was subcutaneously injected (1 mg kg$^{-1}$) on a daily basis to prevent thrombosis, and the post-surgery sutures were treated with an antiseptic (10% Betadine). No drugs were administered in the remaining part of the observation period.

### 2.5. Post-implantation observation

An ultrasound examination was performed at the end of the observation period before explanation. The average linear blood flow velocity in the VGs was estimated using a DC7 (Mindray, China) ultrasound system at the region in front of the proximal anastomoses between the aorta and VG, VG middle part, and the region immediately after the distal anastomoses. After the ultrasound examination, the specimens were excised under anesthesia with 5 mm of the aorta adjacent to the anastomoses. The animals were euthanized immediately after excision of VGs (before awakening). The VGs were rinsed with physiological saline solution and placed into 4% formaldehyde solution (as is necessary for histological examination) to examine the specimens with a SteREO Discovery V12 (Carl Zeiss, Germany) stereo zoom microscope measuring the main parameters (outer and inner diameters and the thickness of neointima) with the help of AxioVision software. To assess the neointima, the VG wall was measured at four points (at the point where the thickness of the neointima was maximal and at three equidistant points). The average wall thickness (as the sum of four measurements divided by four) was used as an estimate.

### 2.6. Histological and immunohistochemical examination of VGs

Serial cryo cross-sections with a thickness of 10 μm were made using a Microm HM-550 (Carl Zeiss, Germany) cryomicrotome. Hematoxylin–eosin (Bio–vitrum, St. Petersburg, Russia) staining was used for histological examination.

Immunohistochemical staining was performed in a LabVision Autostainer 720 (Thermo Scientific, USA) according to the UltraVision Quanto HRP DAB protocol with primary monoclonal and polyclonal antibodies (Thermo Scientific, USA) to type IV collagen (clone PHM-12 + CIV22), α-SMA (clone 1A4/asm-1), and factor VIII (rabbit polyclonal) conjugated with horseradish peroxidase.

The morphometry comprised planimetric and stereological quantitative characteristics. The cellular and structural elements in the wall of experimental VGs and the neointima were assessed quantitatively and semiquantitatively. The calcified area was determined in each case by direct planimetry of the cross-sections stained with hematoxylin–eosin. For this purpose, the image of a VG cross-section was displayed on the screen using a light microscope camera (AxioCam ErC5s) to determine the area of the VG wall and the newly formed intima and, separately, the calcified area with the help of ZEN 2.3 imaging software. The relative calcification of cross-sections was calculated as a percentage of the total cross-section area.

The volume density (%) of immunohistochemically stained regions of the extracellular matrix was assessed with the ZEN 2.3 imaging software at a magnification of 400 × using a closed testing system of 100 points. All fields of view of the neointima (at least ten) were examined in each section to measure the area at five randomly selected positions of the ocular grid. The expression of type IV collagen was assessed semiquantitatively according to the volume density of the stained extracellular matrix as ‘pronounced’, 3 + (staining of over 50% of the extracellular matrix); ‘moderate’, 2 + (staining of 25%–50% of the extracellular matrix); and ‘weak’, 1 + (<25% staining) with a minus sign (−) denoting the absence of staining. The ASM expression was assessed according to the share of positively stained smooth muscle cells. The expression of factor VIII was also assessed according to the share of positively stained endotheliocyte cytoplasm. At least 100 cells were examined for each case.
2.7. Statistical analysis
The data were accumulated and initially sorted using Microsoft Excel 2010. The Statistica 10 software package in Windows 7 (StatSoft Inc., USA) was used for statistical data processing. The quantitative data are shown as the median with the interquartile range and the qualitative, as the total and the share (%). Statistical significance of the differences between groups in the quantitative data was determined using the Mann–Whitney U-test and in the qualitative data, the two-sided Fisher’s exact test. The deviation of the null hypothesis on the absence of differences between groups was set as $p < 0.05$.

3. Results

3.1. Fabrication and characterization of VGs
Table 1 lists the mechanical properties of the Tecoflex EG-80A-based VGs with GL and BV (Tec-GL-BV) and e-PTFE VGs. According to SEM, all matrices consisted of fibers with a diameter of about $1.81 \pm 0.31 \mu m$ and had pores of $2.28 \pm 0.92 \mu m$ (table 1; figure 1, panel 1).

The tensile strength of the Tec-GL-BV VGs was close to that of the e-PTFE ones (table 1). The electrospun Tec-GL-BV VGs were almost threefold more resistant to the suture retention test ($470 \pm 35.0 \text{ g versus } 170 \pm 5.7 \text{ g for e-PTFE}$). Note that these mechanical characteristics considerably exceed those of the native arteries (tensile strength, approximately 2 MPa [39]).

The burst pressure of our Tec-GL-BV VGs was $19.6 \pm 0.4 \text{ atm (14 896 mm Hg)}$. This was at least 11-fold more resistant as compared to e-PTFE VGs (table 1). The Young’s modulus of the Tec-GL-BV VGs was fourfold lower than that of the e-PTFE VGs ($0.84 \text{ versus } 3.98 \text{ MPa}$).

The typical size of the produced VGs was a length of 60 mm, inner diameter of 2.0 mm, and wall thickness of 130–150 mm. Before implantation, pieces of the required length were cut off from a VG with a razor blade. The VGs of PU as well as of e-PTFE did not show any trend of fiber separation.

The VG compliance was tested in a pulsating phosphate-buffered saline (PBS) flow with a pressure drop of 120/80 mm Hg with monitoring (measurement) of the change in the VG diameter. As has been shown, the rat abdominal artery under this pressure drop changes its diameter by 13%–17% (figure 1, panel 2A). $C_{\text{compliance}} = 29.9 \pm 0.13\% / \text{mm Hg}^{-1} \times 10^{-2}$. The electrospun Tec-GL-BV VGs are approximately twice less compliant and change their diameter by 6%–8% (figure 1, panel 2C). $C_{\text{compliance}} = 18.6 \pm 0.11\% / \text{mm Hg}^{-1} \times 10^{-2}$. The e-PTFE VGs did not respond to application of either a 120/80 pressure drop or other pressure drop up to 200 mm Hg (figure 1, panel 2B). Note that application of pressure of 80 mm Hg increases the VG diameter from 2.00 to 2.30 mm.

These data and the data on bio- and hemocompatibility of the earlier obtained material [33] demonstrate both the good properties of Tec-GL-BV VGs and the need for in vivo studies to evaluate their potential for vascular replacement.

3.2. In vivo animal study
Animals of the same age, weight, sex, and diameter of abdominal aorta were studied (table 2). The groups almost did not differ in the dose of used anesthetics (table 2). Assessment of the quality of fabricated VGs during the surgery demonstrated the absence of fiber separation/dissociation and a high ability to retain their cylindrical shape during all stages of surgery. The VG edges did not roll when forming anastomoses and a tight contact was formed between the graft and the native artery.

In the group with e-PTFE VGs, the time of local hemostasis (the time between the release of the clamps from the aorta and cessation of bleeding from the anastomoses and needle perforations) was 24.5 [23.0; 25.0] min versus 18.5 [17.0; 20.0] min for the group of experimental VGs (Tec-GL-BV inner layer and Tec-GL outer layer). Indeed, the e-PTFE VGs are less elastic/compliant, co-opt less with the aorta edges, and have worse propensity to heal the needle perforations, which leads to a longer bleeding at the perforations (table 2).

Table 3 consolidates the data on animal survival rate and the causes of death. Over the observation period, only one animal (5.5%) died in the group with implanted electrospun VGs versus five animals (27.7%) in the group with e-PTFE VGs. The main cause of death for both VG types was thrombosis/VG occlusion at different observation time points. VG occlusion was observed in six animals (33.3%) with e-PTFE VGs: three in the early post-surgery period (days 3–5 after surgery) and three in the extended observation period (days 41, 65, and 130, respectively). VG occlusion was observed in only one animal (5.5%) on day 125 of observation.

Thus, the implanted VGs were free of occlusion over the observation period in 94.5% of the Tec-GL-BV grafts and 66.6% of the control e-PTFE variants ($p = 0.04$).

3.2.1. Intraoperative examination of VGs and microscopic survey
Figure 2 shows the VGs during explantation at different observation time points. A certain deformation of the e-PTFE VG wall was observed at week 24; this was presumably caused by calcification and tight intergrowth of VG and the adjacent tissues. Note that this was completely unobservable in the Tec-GL-BV VGs. Both groups displayed no signs of any infection, inflammation, or hematomas in the region of the graft and adjacent tissues.
| VG composition | Fiber diameter, μm | Pore size, μm | Contact angle, ° | Tensile strength, MPa | Suture retention strength, g | Young’s modulus (MPa) | Burst pressure (mm Hg) |
|----------------|-------------------|---------------|-----------------|------------------------|---------------------------|----------------------|-----------------------|
| 3% Tec, 15% GL, 1.5% BV | 1.81 ± 0.31 | 2.28 ± 0.92 | 87.10 ± 2.20 | 15.60 ± 0.80 | 470.00 ± 35.00 | 0.84 ± 0.20 | 14 896 ± 304 |
| e-PTFE | — | — | 125.00 ± 3.10 | 16.00 ± 0.90 | 170.00 ± 5.70 | 3.98 ± 0.37 | 1327 ± 289 |

The data are shown as the mean ± error of the mean.
Figure 1. Structure and mechanical properties of VGs. **Panel 1.** SEM of electrospun VGs. (A) cross-section of the VG wall; (B) a side view of the VG inner surface; and (C) a perpendicular view of the VG inner surface. **Panel 2.** Compliance test of VGs. The diameters of VG and rat abdominal aorta were monitored in a pulsing liquid flow with a pressure drop of 120/80 mm Hg: (A) rat abdominal aorta; (B) e-PTFE VG; and (C) electrospun Tec-GL-BV VG. The x-axis represents time in ms and the y-axis represents the VG outer diameter in mm.

Table 2. Characterization of animals and surgery conditions.

| Parameter                              | Tecoflex group, n = 18               | e-PTFE, n = 18                  | p    |
|----------------------------------------|--------------------------------------|---------------------------------|------|
| Weight (g)                             | 481.0 [470.0; 510.0]                 | 491.5 [472.0; 510.0]            | 0.59 |
| Males, n (%)                           | 18 (100)                             | 18 (100)                       | 1.00 |
| Age (months)                           | 7.0 [6.0; 7.0]                       | 7.0 [7.0; 7.0]                 | 0.38 |
| Zolletil amount used for anesthesia (mg)| 9.6 [9.4; 10.2]                     | 9.8 [9.4; 10.2]                | 0.60 |
| Xylazine amount used for anesthesia (mg)| 1.9 [1.8; 2.0]                      | 1.9 [1.8; 2.0]                 | 0.59 |
| Rat aorta diameter (mm)                | 2.0 [1.8; 2.0]                       | 2.0 [1.9; 2.0]                 | 0.72 |
| Inner VG diameter (mm)                 | 2.0 [2.0; 2.0]                       | 2.0 [2.0; 2.0]                 | 1.00 |
| Length of implanted region (mm)        | 20.0 [18.0; 20.0]                    | 20.0 [18.0; 20.0]              | 1.00 |
| Total time of surgery (min)            | 101.0 [95.0; 110.0]                  | 108.0 [98.0; 113.0]            | 0.0004 |
| Aorta occlusion time (min)             | 40.0 [37.0; 45.0]                    | 40.0 [40.0; 42.0]              | 1.0000 |
| Hemostasis time (min)                  | 18.5 [14.0; 20.0]                    | 24.5 [20.0; 28.0]              | 0.0000 |
A microscopic survey of the VG cross-sections and an ultrasound examination (figure 2, table 5) show that both examined groups had the trend of an increase in the thickness of the neointima with time. This trend is more pronounced in the e-PTFE VGs but the thickness of the neointima in the considered groups does not differ in a statistically significant manner. However, an ultrasound examination of the intervention region demonstrates a statistically significant increase in the blood flow velocity in the e-PTFE group as compared with the Tec-GL-BV group at weeks 12 and 24 (table 4), whereas the velocity in the latter group did not significantly differ from the intact (without surgery) animals, additionally examined by ultrasound. These data suggest a decrease in the efficient section of e-PTFE VGs. Note also that the walls of e-PTFE VGs were infiltrated with erythrocytes as early as week 1 after grafting. In addition, this group displayed a more pronounced growth of the neoadventitial layer, especially 24 weeks after the implantation.

Figure 2. (A), (B), (E), (F), (I), and (J) VGs at different observation time points (OPMI Pico surgical microscope) and (C), (D), (G), (H), (K), and (L) cross-sections of explanted VGs (Stereo Discovery V12 stereomicroscope). Scale bar is 500 μm.

Table 3. Significant events in the studied groups during the observation period.

| Parameter                     | Tec-GL-BV group, n = 18 | e-PTFE group, n = 18 | p     |
|-------------------------------|-------------------------|----------------------|-------|
| Mortality rate, n (%)         | 1.0 (5.5)               | 5.0 (27.7)           | 0.09  |
| Anastomosis aneurism, n (%)   | 1.0 (5.5)               | 0.0 (0.0)            | 0.89  |
| Graft occlusion, n (%); Of them, | 1.0 (5.5)          | 6.0 (33.3)           | 0.04  |
| Early occlusion (to day 7), n (%) | 0.0 (0.0)         | 3.0 (16.7)           | 0.25  |
| Delayed occlusion (after day 7), n (%) | 1.0 (8.3)       | 3.0 (16.7)           | 0.29  |

Table 4. Blood flow velocity in VGs (intravital ultrasound examination) and the thickness of the neointima in explanted VGs (microscopic survey) at different observation time points.

| VG type       | 1 week | 12 weeks | 24 weeks |
|---------------|--------|----------|----------|
|                | Thickness of neointima, μm | Blood flow velocity, m s⁻¹ | Thickness of neointima, μm | Blood flow velocity, m s⁻¹ | Thickness of neointima, μm | Blood flow velocity, m s⁻¹ |
| Tec-GL-BV     | 5.18 ± 2.26 | 0.93 ± 0.10 | 31.29 ± 8.32 | 1.03 ± 0.18 | 47.71 ± 7.46 | 1.16 ± 0.09 |
| e-PTFE        | 6.29 ± 3.32 | 1.07 ± 0.11 | 44.57 ± 14.84 | 1.72 ± 0.1 | 59.86 ± 12.22 | 1.74 ± 0.08 |
| p             | 0.532 | 0.401 | 0.578 | 0.014 | 0.128 | 0.012 |
3.3. Histology of explanted VGs

Histological data demonstrate that fibrin/erythrocyte thrombi are formed on the VG inner surface 1 week after implantation in both Tec-GL-BV and e-PTFE grafts. These thrombi in the latter VGs are more voluminous and loose in their structure (which may result from their lower compliance). In addition, the wall of e-PTFE VGs swells and is infiltrated by erythrocytes. The wall of the Tec-GL-BV grafts retained its integrity and structure. There are no signs of any formation of neointimal and adventitial layers (figure 4). The neointima is formed in all VGs 3 and 6 months after their grafting. This layer in general matches the normal intima in its structure (figures 3 and 4). This is confirmed by the presence of the endothelial lining with detectable positive factor VIII expression (figure 4(A)) and type IV collagen within both the basal membrane and the formed intima (figure 4(B)). Note that any α-SMA expression in the formed neointima was undetectable (figure 4(C)).

Calcification of different degrees mainly of a dust-like type was observed in the VG walls (figure 3). The intensity of calcium accumulation differs between both the graft types and individual VGs implanted for different periods. In particular, none of the VGs of any type explanted on day 7 displayed any signs of calcification. The e-PTFE grafts explanted after 12 weeks contain tenfold more calcified regions as compared with the Tec-GL-BV grafts implanted for the same period (table 6). After 24 weeks, the implanted e-PTFE VGs are considerably more calcified: the calcified area is 4.5-fold larger as compared with the Tec-GL-BV VGs (table 5, figure 3). The area of the calcified region increases approximately fourfold in the Tec-GL-BV group and doubles in the e-PTFE group from weeks 12 to 24 after grafting.

The formation of fibrous tissue of different densities with sinusoidal (capillary)-type vessels and focal mononuclear infiltration was observable in the neoadventitial layer of all VGs (figure 3).

Figure 3. Cross-sections of VGs at different observation time points (hematoxylin–eosin staining; AxioCam Erc5s light microscope with ZEN 2.3 software). Scale bar is 200 μm. The inner lumen of VGs is at the top of the images (marked with an asterisk).
Analysis of the cell composition of the explanted VGs shows a significantly higher cell density in the walls of e-PTFE grafts as compared with the Tec-GL-BV grafts at both time points—weeks 12 and 24 of their performance in the rat infrarenal aorta (table 6). The observed differences are explainable with an increased permeability of the e-PTFE VGs for the blood and its cells, which is also confirmed by the increased erythrocyte infiltration at week 1 after grafting (figure 3).

Table 5. Calcification (%) of the studied VG walls.

| VG type | 12 weeks | 24 weeks | \( p \) (12, 24) |
|---------|----------|----------|-----------------|
| Tec-GL-BV | 0.6 [0.30; 1.10] | 2.85 [1.10; 5.20] | 0.028 |
| e-PTFE | 6.55 [5.80; 6.60] | 13.65 [12.20; 15.50] | 0.027 |
| \( p \) | 0.005 | 0.005 |

* Horizontal comparison: \( p \) (12, 24) for the parameters within one group at weeks 12 and 24 of observation.
Morphometry of the neointima and VG walls has detected a number of differences between the control and experimental grafts. The cell density in the neointima of Tec-GL-BV VGs 12 weeks after implantation was 1.5-fold higher as compared with the e-PTFE grafts. This difference somewhat decreased 24 weeks after grafting but the general trend still remained. The VG intima is composed of small spindle-like cells with different cytoplasmic processes as well as cells with small monomorphic nuclei and poorly detectable cytoplasm (figure 3, table 6). The endothelial marker (factor VIII) is distinctly and densely expressed on the surface of the neoendothelial layer in Tec-GL-BV VGs versus the e-PTFE grafts with its ‘looser’ diffuse expression. Type IV collagen expression was present in all VG types but was denser immediately under the neoendothelial layer in the Tec-GL-BV group versus the e-PTFE grafts, where it was more diffusely distributed over the neointima. α-SMA expression in the neointima of both VG types was almost absent.

### 4. Discussion

Numerous variants of VG ES of synthetic polymers have been described in the literature. Both more and less stable (for example, biostable PUs [20, 40–42]) and biodegradable (such as PCL [43, 44], PLGA [45, 46], and TMC [47, 48],) polymers have been used for this purpose. Concurrently, VG modification has been attempted by loading endothelial growth factors [49, 50] that would enhance cell adhesion [51–53], various drugs [54, 55], and so on. Multilayered grafts have been proposed to improve their biomechanics [56, 57]. For example, the earlier designed tissue-engineered grafts of GL-loaded polycaprolactone and a poorly permeable inner layer have shown both good mechanical properties and biocompatibility [12, 58]. However, the biostability of such VGs in the extended post-surgery period is still vague [59, 60]. A high biodegradation rate and mechanical characteristics of the polymers with ester bonds between their segments, such as polyglycolide, polyactide, and their copolymers, limit their use for VG fabrication [61, 62]. A team from the University of Liverpool was among the first to electrospin an artificial VG of a segmented PU. In a series of biological and biomechanical tests, they demonstrated that such VGs were applicable in artery replacement [63, 64]. Currently, PUs are the biomaterials widely used for the manufacture of various grafted items, including artificial heart valves and VGs. The PUs used in medicine are biocompatible polymers that do not induce any tissue inflammatory responses [65–67]. The mechanical and biological properties of PUs are determined by the structure of their soft and hard segments, which allows for the selection of polymers which are close in their properties to natural tissues [68–70] in the manufacture of the grafts.

According to the current view, the main reasons underlying VG stenosing are the misfit of the VG and blood vessel mechanical properties and insufficient endothelialization of the VG inner surface [71, 72].

Modification of the VG surface with the ligands of cell adhesion receptors has been proposed to improve the endothelialization [73]. However, it is known that the expression of such ligands may also lead to an increased binding of immunocompetent cells, which eventually enhances stenosis [74].

In our study, we tested GL—an inexpensive lyase of collagens, frequently used to increase adhesion of endotheliocytes [75–77]—to increase the binding efficiency of endothelial cells and their precursors. GL was added to the ES solution because we have earlier demonstrated that GL in this case is not only exposed on the fiber surface for a long time and considerably improves the adhesion of endotheliocytes, but also makes the matrices more stable [28]. The GL cross-linking by glutaraldehyde significantly improves the adhesion of primary endotheliocytes, which may be associated with a decreased mobility of the GL exposed on the surface [28]. In order to decrease the binding to the GL exposed on the blood cell surface, platelets included, the ES solution was additionally supplemented with BV [33]; to decrease the BV spending, this BV-containing solution was used to make only the inner VG layer. As we have shown earlier, the surface of these types is readily populated by HUVEC cells and thus is good for neoendothelialization and low adhesion of platelets in vitro [33].

The BV exposure in the inner layer of Tec-GL-BV VGs, as well as the compliance close to a natural artery (as is shown in figure 2) allowed the number of clotting and occlusion cases to be decreased at both early observation points (no cases in the experimental group versus three cases in the control group) and later stages (one case in the experimental group versus three
cases in the control group). Note that the occlusion cases in the e-PTFE group were not associated with any defects in surgery or with infections.

The VG intima contained two types of cells: small spindle-like cells with different cytoplasmic processes and cells with small monomorphous nuclei and poorly detectable cytoplasm. Note that the ratio of these cells in the e-PTFE and Tec-GL-BV VG groups was different. The neointima of both VG types after 12 weeks of performance contained four- to fivefold more cells than after 24 weeks. However, the Tec-GL-BV VGs contained 1.5-fold more cells as compared with the e-PTFE grafts. This suggests that the VG composition and mechanical properties influence the duration of the fibroelastic phase of inflammation. The number of cells decreases faster in the neointima of compliant Tec-GL-BV VGs. This may reflect the trend of the decrease in hyperplasia development in the VGs walls changing their linear sizes in response to a pulse wave. A small pore diameter in the Tec-GL-BV VGs and their compliance is likely to result in a less intensive development of the adventitial layer and ingrowth into the adjacent tissues. Note here that this has no effect on the formation of the vas vasorum, which develops in the VGs of both types in a similar manner.

An immunohistochemical examination demonstrated that the Tec-GL-BV VG inner surface is more densely populated by endotheliocytes, as suggested by the all-round expression of factor VIII. The expression of type IV collagen between the cells and the VG wall suggests formation of the neobasal membrane, i.e. formation of a structure capable of supporting the normal function of the newly forming endothelium.

The compliance of VGs and the abdominal aorta is of special importance. The viscoelastic properties of the material and the small pore diameter prevent blood infiltration of the Tec-GL-BV VGs and prevent the blood cells from entering the VG wall. An excessive infiltration of the VG walls with blood cells is an additional risk factor for clotting and calcification [78, 79]. This is also confirmed by histological examination, which has made it possible to detect a more pronounced calcification of the a-PTFE grafts as compared with the Tec-GL-BV VGs.

The in vivo studies of tissue-engineered PU VGs are so far rather few. Several studies demonstrate the feasibility of in vivo biodegradation of such PUs [17–19]. However, an Austrian research team comprehensively studying thermoplastic PU grafts produced by ES has not found any signs of their biodegradation in their in vitro and in vivo experiments [14, 20, 21]. The VGs of this kind fabricated from thermoplastic PU Pellethane™ have shown perfect mechanical properties and biocompatibility with a high resistance to biodegradation. The patency of these VGs was comparable to our data. These results are also confirmed by several other long-term in vivo studies with an observation period of up to 580 days [63, 80]. Recently, a UK team implanted polyhedral oligomeric silsesquioxane poly(carbonate-urea) urethane grafts with an inner diameter of 5 mm fabricated by an electrospray technique with phase inversion into the carotid artery of 12 sheep [37]. The observation period in this study was 9 months. The patency rate of VGs was 64% (which is considerably smaller as compared with our Tec-GL-BV VGs) yet these grafts also did not display any hyperplasia of the intima, calcification, or aneurysms of anastomoses. These grafts displayed performance characteristics comparable to native arteries. Presumably, their low patency was associated with the absence of a bifunctional hemocompatible inner layer loaded with anticoagulant (as in our Tec-GL-BV VGs), with another animal model, or some other reasons requiring further studies. Thus, the data on the physical properties of Tec-GL-BV VGs, their biological characteristics, manipulation comfort when implanting the grafts, and absence of stenosis and aneurysms demonstrate their utility for clinical practice. Further studies of these Tec-GL-BV VGs in larger animals will demonstrate whether they can be clinically used for the replacement of small-diameter blood vessels in human subjects.

5. Conclusions

Two-layered VGs of the thermoplastic PU Tecoflex EG-80A loaded with 15% GL and 1.5% BV (in their inner layer) have shown a high durability, exceeding seven–eightfold that of natural arteries with a similar diameter, as well as mechanical characteristics (elasticity and compliance) comparable to the native vessels. The tested VGs have shown high patency (94.5%) over the observation period of up to 24 weeks; the linear blood flow velocity in the experimental VGs did not significantly differ from the normal arteries at all observation points. A histological examination of the explanted VGs demonstrated their high biostability, low calcification of their walls, and a good cell population on their inner surface, contacting with the blood flow. An immunohistochemical examination showed the formation of a functionally sound neoendothelial layer with neobasal membrane, which explains the good patency at all observation points. Altogether, these properties suggest that these VGs should be further studied from a clinical standpoint.

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