

Electronic Supplementary Information

Bioengineering a pre-vascularized pouch for subsequent islet transplantation using VEGF-loaded polylactide capsules.

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Experimental Details

1. Materials

Hexamethylenediamine (HMDA), heparin sodium salt from porcine intestinal mucosa, toluidine blue O, fluorescein isothiocyanate isomer I (FITC) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich, Czech Republic. Recombinant Human VEGF 165 protein and Human VEGF Quantikine enzyme linked immunosorbent assay (ELISA) Kit were purchased from R&D Systems, Czech Republic. All other reagents were obtained from P-Lab a.s. and Lach-Ner s.r.o., Czech Republic, and used without further processing. High molecular weight poly(L-lactide-co- ϵ -caprolactone) (PLCL, $M_w = 316\ 000$ g/mol and $M_n = 120\ 000$ g/mol, 14 % wt of ϵ -CL) was synthesized according to the protocol described by Kubies et al.¹

2. Fabrication of samples

Macroporous 3D samples: The PLCL capsule-shaped scaffolds were prepared using a thermally induced phase separation (TIPS) approach according to the protocol reported earlier by our group.^{2,3} The protocol is schematically depicted in **Figure 1a**. Briefly, the metal template (with a diameter of 4 mm) connected to the cooling platform was dipped into a PLCL solution (5% w/v in 1,4-dioxane). The system was cooled to -80 °C and the phase separation proceeded for 30 s; the template with the frozen polymer-solvent phases was then removed from the PLCL solution and allowed to coarsen for 30 s. Subsequently, the template with the sample was dipped into 1,4-dioxane for another 30 s to avoid any skin effect, left to coarsen for 30 s and finally subjected to freeze-drying. The open capsule shaped porous scaffolds were then collected from the template and stored at -20 °C until further use.

Model 2D samples: To properly analyse the surface properties such as topography and the surface elemental composition of PLCL surfaces before and after functionalization, model PLCL films were used instead of the porous scaffolds. The model PLCL films were prepared by the solvent casting procedure as follows: 5 ml of a 10% PLCL solution (w/v in 1,4-dioxane) was poured onto a clean 5-cm² glass slide and allowed to evaporate for 6 h in a laminar flow box at laboratory temperature. The samples were then dried in a vacuum at 40 °C overnight. The glass

slide with the film was exposed to a water/ethanol mixture to lift the film off the glass surface. The obtained film was cut into 1-cm² pieces and stored at -20 °C until further use.

3. Functionalization of samples

3.1 Introduction of amine groups

The optimization study was performed using PLCL scaffolds that were cut in half to create 1-cm-long tubular pieces or on flat model films. The samples were saturated in 1.5 ml of isopropanol for 30 min; during this step, it was necessary to apply a slight vacuum to remove air trapped within the pores of the 3D scaffolds. The isopropanol was then exchanged with 1.5 ml of a freshly prepared HMDA solution (5% w/v in isopropanol) for varying incubation times (5, 15, 30 and 60 min) at two different temperatures (22 and 40 °C). During the reaction, the vials were gently shaken to maintain the homogeneity in the reaction mixture. After aminolysis, the samples were rinsed with ultrapure water for 4 h with at least 10× intermittent water exchanges to remove excess HMDA. If not used immediately in a subsequent modification step, the 3D samples were freeze-dried and stored under dry conditions at -20 °C until further analysis.

3.2 Immobilization of heparin

The optimization study was performed using the PLCL scaffolds or on the flat PLCL films, both aminolysed with HMDA (5% w/v in isopropanol) at 22 °C for 30 min. The aminolysed scaffolds were saturated in 1.5 ml of MES buffer (0.1 M, pH 5.5) for 30 min; during this step, a slight vacuum was applied to remove the trapped air. For the physical adsorption of heparin, each sample was immersed in 1.5 ml of a freshly prepared heparin solution (0.25% w/v in 0.1 M MES buffer pH 5.5) under constant shaking at 200 rpm at room temperature for 15 h. For the covalent crosslinking of heparin, the sample was immersed in 1.5 ml of a freshly prepared solution of heparin (0.25 % w/v in 0.1 M MES buffer pH 5.5) that was activated by 0.5 M EDC followed by 0.5 M NHS for 10 min each. The reaction proceeded under constant mild shaking at 200 rpm at room temperature for 1, 6 or 15 h. After physical adsorption or covalent

crosslinking, the samples were thoroughly rinsed with ultrapure water for 1 h with at least 10 intermittent water exchanges to remove any excess reactants.

3.3 VEGF loading and *in vitro* release

The screening study evaluating a VEGF loading and release was performed using the PLCL scaffolds (an average weight of 25 ± 1.5 mg) that had been aminolysed with HMDA at 22 °C for 30 min and contained a covalently immobilized heparin layer (0.25% w/v in MES, using EDC-NHS, 6 h).

VEGF loading for subsequent release studies was performed as follows: 100 μ l of a VEGF solution in PBS (pH 7.4) supplemented with 0.1% w/v BSA was applied directly onto the heparinized scaffolds. The tested VEGF concentrations were 0.08 and 0.4 μ g VEGF per mg of the scaffold (i.e., 2 and 10 μ g per scaffold, respectively). The samples were denoted as heparin/VEGF_2 and heparin/VEGF_10. The samples were allowed to dry in a laminar flow box for 30 min at room temperature and were then incubated at 4 °C for overnight. The unloaded VEGF was collected by gently washing each scaffold in 500 μ l of PBS supplemented with BSA for 5 min. The washing step was repeated twice, and the washes were pooled and stored at -20 °C until ELISA evaluation. The original loading solutions were analysed for comparison. To calculate the amount of VEGF loaded onto the capsule, the residual VEGF concentration was subtracted from the initial loading concentration.

The release kinetics of VEGF from the scaffolds were investigated by the full immersion method. The VEGF-loaded scaffold was immersed in 1 ml of PBS supplemented with BSA and incubated at 37 °C. At regular time intervals, i.e. 1, 2, 3, 6, 12, 24, 48, 72, 120, 168, 336, 508 and 672 h, 250 μ l of the buffer solution was collected, and an equal volume of fresh BSA-supplemented PBS was added to the vial. All release collections were stored at -20 °C until further analysis. During the entire study, the VEGF solutions were prepared and handled in low-protein-binding microcentrifuge tubes to minimize protein loss.

4. Characterization studies

4.1 Common methods

Morphological properties: The surface morphology of the pristine and functionalised dry samples was assessed by scanning electron microscopy (SEM, Vega, Tescan). Prior to the analysis, all samples were coated with platinum under vacuum in a sputter coating unit (SCD050, Leica Microsystems).

Topographical properties: The surface topology of the pristine and functionalized samples was assessed using an atomic force microscope in tapping mode (AFM, Nanoscope IIIa, Digital Instruments). Images with a scan area of $1 \times 1 \mu\text{m}^2$ were obtained at a scan rate of 0.6 Hz using standard silicon cantilevers (OTESPA7, Veeco Instruments, with a spring constant of 42 N/m).

Surface wettability: The wettability of the pristine and functionalized samples in terms of the static water-in-air contact angle (CA) was determined by the sessile drop method at room temperature using a video camera-assisted computerized goniometer system (OCA 20, Dataphysics). The obtained data were processed using the Young–Laplace equation.⁴

Surface chemistry: The pristine and functionalized samples were analysed by X-ray photoelectron spectroscopy (XPS) employing Al K α excitation radiation and by Fourier transform infrared spectroscopy in attenuated total reflectance mode (ATR-FTIR).

For SEM and ATR-FTIR and CA analysis the porous scaffolds were used, while for AFM, and XPS analysis, the model flat films were used.

4.2 Specific characterization of aminolysis

The introduction of functional amine groups on the scaffolds was qualitatively verified by staining the sample with FITC (0.05% w/v in ethanol) for 4 h at room temperature followed by imaging using confocal laser scanning microscopy (CLSM, FV1200 IX83, Olympus; excitation wavelength = 490 nm and emission wavelength = 525 nm).

The effect of aminolysis on loss of physical mass from the scaffolds was assessed by determining difference in the weight of the sample before and after aminolysis. The change in the molecular weight of the polymer caused by aminolysis was analysed by gel permeation

chromatography with tetrahydrofuran and trifluoroacetic acid (99:1 volume ratio) as the mobile phase.

4.3 Specific characterization of heparinization

The efficiency of heparinization was assessed by toluidine blue assay. The heparinized scaffolds were immersed in 4 ml of 0.005% toluidine blue solution (w/v, in 0.01 N HCl and 0.25% w/v NaCl). Then, 1 ml of ultrapure water was added to bring the volume to 5 ml, and the samples were incubated at room temperature for 30 min with mild agitation. For qualitative assessment, the collected scaffolds were washed with 0.25% NaCl solution (w/v in ultrapure water; 5 times for 1 min each time), and visualized using a stereo zoom light microscope (Olympus Stereo Zoom Microscope SZ61). For quantitative assessment, the residual 5 ml of toluidine blue solution was mixed with 5 ml of n-hexane and vigorously vortexed to homogenize the mixture. The mixture separated into an upper n-hexane layer and a lower toluidine blue-water layer. A 200- μ l aliquot of the lower toluidine blue layer was placed in a 96-well plate, and its absorbance at 631 nm was read in a multi-well plate reader. A standard curve was prepared in a similar manner except that 1 ml of ultrapure water was replaced with 1 ml of heparin solution at a range of concentrations.

4.4 Specific characterization of VEGF loading and release

Qualitative assessment of VEGF loading onto the scaffolds was performed by immunohistochemical (IHC) staining with anti-VEGF antibody followed by imaging by phase contrast light microscopy. Quantitative determination of the amount of VEGF loaded onto the scaffold and the amount of VEGF released from the scaffolds over time was assessed by ELISA using a commercial kit (R&D Systems, U.S.A.). A standard curve of VEGF was prepared using the same kit according to the manufacturer's instructions.

To deduce the mode of VEGF release from the scaffolds, the release kinetics data were fitted to four mathematical models, viz., zero-order (equation 1), first-order (equation 2), Higuchi (equation 3) and power law (equation 4).⁵⁻⁸ The values of k_0 (zero-order constant), k_1 (first-order constant), k_H (Higuchi constant), k (Korsmeyer-Peppas constant) and n (release exponent)

were determined by fitting the release data to the respective equations shown below, where M_t/M_∞ is the fraction of a drug released at time t .

$$\text{Zero order model: } \frac{M_t}{M_\infty} = k_0 t \quad \text{Equation (1)}$$

$$\text{First order model: } \frac{M_t}{M_\infty} = 1 - \exp(-k_1 t) \quad \text{Equation (2)}$$

$$\text{Higuchi model: } \frac{M_t}{M_\infty} = k_H t^{1/2} \quad \text{Equation (3)}$$

$$\text{Power law model: } \frac{M_t}{M_\infty} = kt^n \quad \text{Equation (4)}$$

5. *In vivo* studies

5.1 Scaffold preparation for implantation

All scaffold modifications were performed under sterile conditions in a laminar flow box. The capsular PLCL scaffolds (2-cm-long, an average weight of $25 \pm 1,5$ mg) for *in vivo* evaluation were first sterilized in 70% ethanol for 1 h; a slight vacuum was applied to remove the trapped air from the scaffolds. The capsules were rinsed with ultrapure water and were immediately used in further modification steps. The capsules were subjected to aminolysis at 22 °C for 30 min (see Paragraph 3.1). After thorough washing with ultrapure water for 30 min and saturation in the MES buffer for 15 min, coating of the samples with a crosslinked heparin layer was performed for 6 h as described in Paragraph 3.2. The samples were then rinsed with PBS for 30 min to remove the excess reactants. Finally, the scaffolds were loaded with VEGF in PBS/0.1% w/v BSA as described in Paragraph 3.3. The doses used were as follows: 0.4, 2 or 4 μg per mg of the capsule, i.e., 10, 50 and 100 μg per capsule, respectively. For a better clarity, the samples were denoted as heparin/VEGF_10, heparin/VEGF_50, and heparin/VEGF_100. The VEGF-loaded scaffolds were placed in low-binding Eppendorf tubes and allowed to incubate for 3 h at laboratory temperature and then stored at 6 °C overnight until implantation into the animals. Heparinized scaffolds that had not been subjected to VEGF loading were used as control samples. The obtained functionalized scaffolds were placed separately in Eppendorfs and provided for *in vivo* evaluation.

5.2 Surgery and scaffold implantation

Experiments were conducted according to the guidelines for the Care and Use of Laboratory Animals of the Institute for Clinical and Experimental Medicine (ICEM), Prague, Czech Republic. The protocol was approved by the Animal Care Committee of the ICEM and the Ministry of Health, Czech Republic.

Lewis rats (230-270g, age 2-3 months, purchased from Charles River, Germany) were used in this study. All surgeries were performed in animals under general anaesthesia induced using 5% isoflurane and maintained using 1.5% isoflurane in the air. The analgesic effect of isoflurane was supported with intramuscularly injected butorphanol (2 mg/kg).

The PLCL scaffolds were implanted in the greater omentum. The surgery was performed using a previously described technique.⁹ Briefly, after midline laparotomy, the greater omentum was spread out on wet gauze square, and the scaffold combined with a Teflon bar was wrapped in the omentum and fixed in the site (lying transversely) using 7-0 Mersilk sutures. Abdominal wall was closed in two layers using 5-0 Vicryl sutures. Extreme attention was paid to pain and stress management to minimize the suffering of the experimental animals. The recovery of each animal after surgery was carefully monitored.

For the dose-optimization experiment, scaffolds supplemented with 10, 50, or 100 µg VEGF (the samples denoted as heparin/VEGF10, heparin/VEGF50 and heparin/VEGF100) were implanted into the greater omentum of the experimental animals for 2 weeks. For time-optimization experiments, scaffolds loaded with 50 µg VEGF (i.e., heparin/VEGF50) were implanted for 2, 3, or 4 weeks. After the selected time period, the abdominal cavity was again opened under general anaesthesia, and the scaffold with the surrounding tissues was excised and fixed; the animal was then euthanized.

5.3 Histochemical and immunohistochemical analysis

5.3.1 Tissue ingrowth through the scaffold wall

Each scaffold after surgery was fixed, and processed using standard techniques for histochemical and immunohistochemical staining. To this end, the samples were cleaned in PBS and fixed in 10% formaldehyde at 4 °C overnight. Each scaffold was divided transversely into

four pieces, routinely dehydrated, and embedded separately in paraffin. For subsequent microscopical examination, the samples were cut into 4- μ m thick slices. The sections were then deparaffinized, rehydrated in a graded series of ethanol and stained with the following stains according to standard protocols: haematoxylin and eosin to identify a basic structure of the tissue, Masson's trichrome to display extracellular matrix components, particularly collagen, and anti-CD31 antibody (Cat. # NB100-2284, Novus Biologicals, USA; rabbit polyclonal, Acris Antibodies GmbH, Germany) The primary antibodies were applied overnight at 4° C. The CD31 antibody was detected by biotinylated goat anti-rabbit IgG (H + L) (Vector Laboratories, USA). Then the sections were incubated with R.T.U. Vectastain Elite ABC Reagent (Vector Laboratories, USA) for 30 min to highlight endothelial cells.

5.3.2 Islet graft structure

Tissues were fixed in 10% formaldehyde overnight and embedded in paraffin. Four μ m thick sections were deparaffinized in xylene and rehydrated in a graded ethanol series. Sections were pretreated using a heat mediated antigen retrieval with a sodium citrate buffer (anti-insulin and anti glucagon staining) or a TRIS buffer (antiCD31 staining) for 20min. Endogenous peroxidase was blocked by 3% H₂O₂ dissolved in methanol. To prevent nonspecific binding, the samples were preincubated with a 10% normal goat serum. The sections were then incubated with a primary antibody overnight at 4°C (anti-insulin antibody: rabbit monoclonal ab181547, dilution 1/64 000, Abcam; anti-glucagon antibody: mouse monoclonal ab10988, dilution 1/1000, Abcam; and anti-CD31 antibody: rabbit monoclonal ab182981, dilution 1/2000, Abcam). A goat anti-rabbit biotinylated secondary antibody (IgG (H+L) BP-9100, RTU, Vector) was used to detect the anti-CD31 primary antibody. For detection, Streptavidin HRP was used (Vector, SA-5004, 1 ug/ml) for 10 min at room temperature. A goat anti-mouse alkaline phosphatase preadsorbed secondary antibody (IgG (H+L) ab7069, 1/300, Abcam) and a goat anti-rabbit peroxidase preabsorbed secondary antibody (IgG PI-1000, 1/300, Vector) were used for detection of anti-insulin and anti-glucagon primary antibodies. DAB or StayRed were used as chromogens (Dako, K3467, 1/50; Abcam, ab103741, 1/150), and incubated for 5 min at room temperature. Sections were then counterstained with Mayer's hematoxylin and mounted with

Pertex. The inset negative control image is taken from an identical assay without the primary antibody.

5.4 Pancreatic islet isolation

Pancreatic islets were isolated from Lewis male rats (300 - 400 g) according to the standard protocol.¹⁰ After intraductal collagenase (1 mg/ml, Sigma Aldrich, USA) injection, donor pancreata (2 – 3 donor rats per recipient) were excised and digested at 37°C for 20min. Islets were then separated from exocrine tissue using Ficoll (Sigma Aldrich, USA) discontinuous gradient and handpicked. The isolated islets were cultured overnight at 37°C and 5% CO₂ in CMRL-1066 medium (Sigma-Aldrich, USA) supplemented with a 5% HEPES buffer, a 10% fetal bovine serum, and 1% penicillin/streptomycin.

5.5. Diabetes induction and islet transplantation and monitoring of graft survival

Male Lewis rats (n=8) were injected with 65 mg/kg of streptozotocine (Sigma Aldrich, USA) intraperitoneally. Animals with confirmed diabetes mellitus (a minimum of 21 mmol/l blood glucose measured in 3 consecutive days) were considered as recipients (n=6) of pancreatic islets (10 PIs/kg of recipients bodyweight).

The scaffolds loaded with 50 µg VEGF (i.e., heparin/VEGF_50) were immersed in platelet enriched plasma and were implanted within the greater omentum of recipients as previously described (see Par. 5.2). Insulin pellets were implanted subcutaneously to control blood glucose levels prior to islet transplantation.

Islets were manually counted (10 PIs/g of recipients bodyweight) using a dissection microscope and collected in a small plastic tube connected to a Hamilton syringe prior to transplantation. Four weeks after the scaffold implantation, a cranial midline laparotomy was performed and the scaffold was placed on a wet gauze. A small incision in the omentum was performed to allow a teflon bar removal and islets were then injected into the created cavity. Omental incision was closed using 7-0 Mersilk sutures and abdominal wall was closed in two layers using Vicryl 5-0 sutures. Two weeks after islet transplantation, insulin pellets were removed.

Blood glucose level was monitored twice a week and body weight once a week for 50 days. Glycemia under 10 mmol/l was considered as a sign of a functional graft, glycemia over 11.1 measured at least three times was considered a marker of a graft failure. Intravenous glucose tolerance test (0,5 g of glucose per kg) was performed 40 days after islet transplantation. At the end of experiment each scaffold with the with the islet graft was harvested, fixed and provided for immunohistological analysis.

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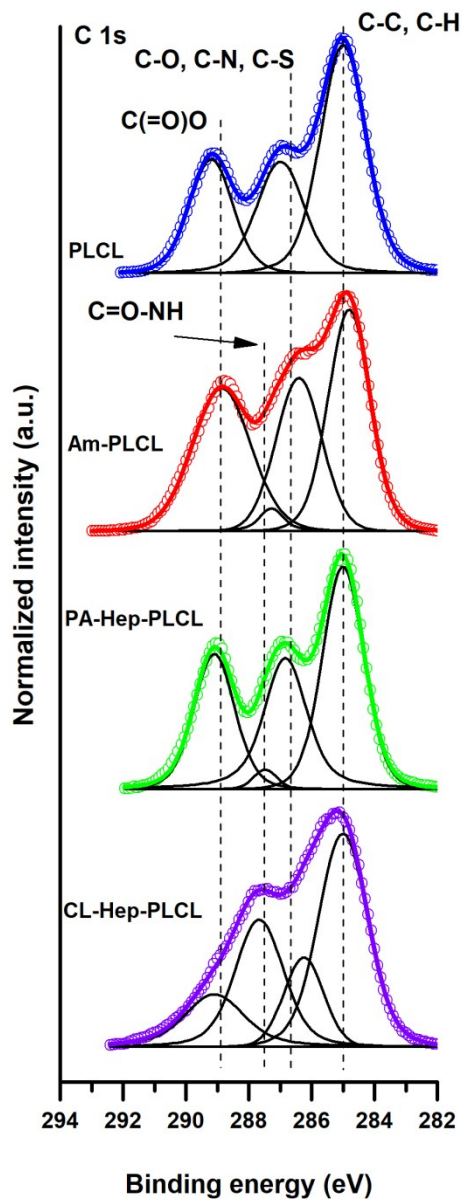


Figure SI 1 Representative high resolution C 1s XPS spectra of the pristine PLCL, aminolyzed Am-PLCL, physically adsorbed heparin PA-Hep-PLCL and chemically crosslinked heparin CL-Hep-PLCL coated surfaces.

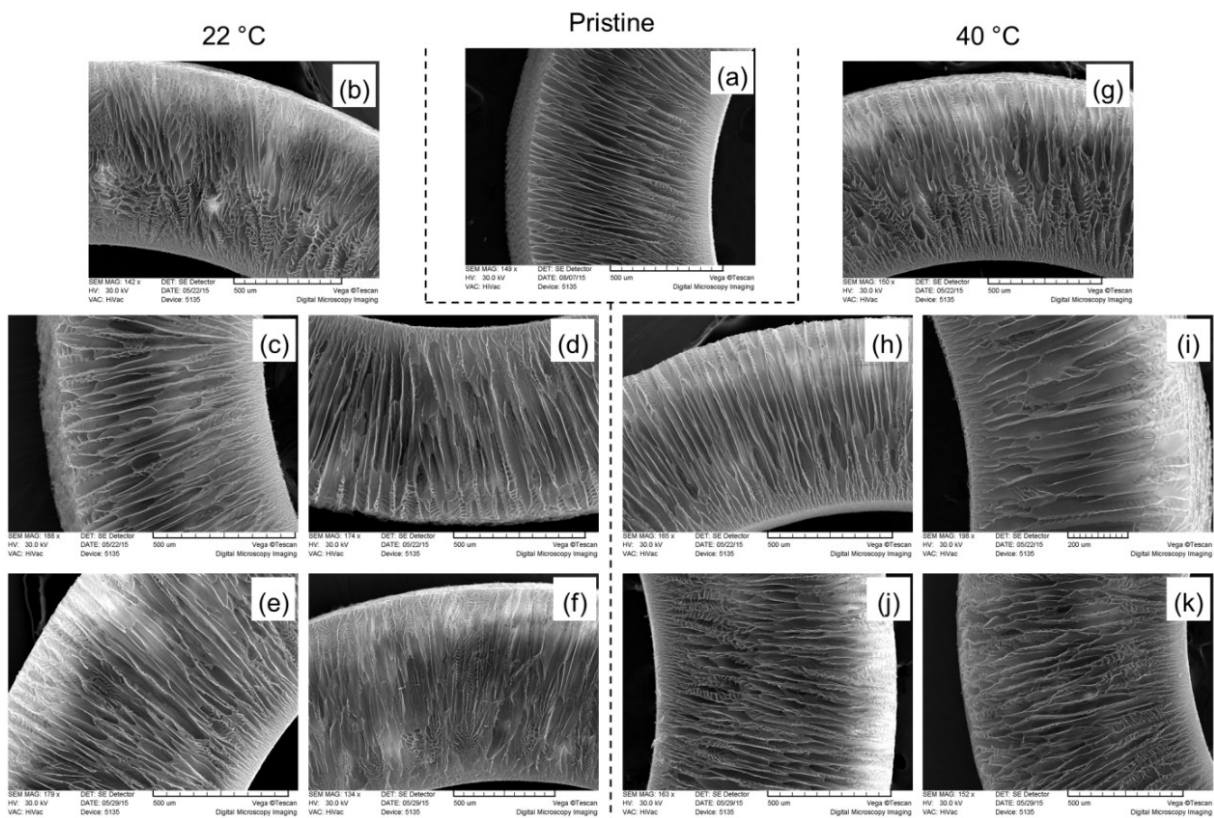


Figure SI 2 Morphological changes after aminolysis: SEM images of pristine and aminolyzed samples suggested that the gross morphological appearance remained largely unaltered after aminolysis reaction. Sample details: a) pristine sample, b-f) samples aminolyzed at 22 °C for 5, 10, 15, 30 and 60 min respectively, and g-k) samples aminolyzed at 40 °C for 5, 10, 15, 30 and 60 min respectively.

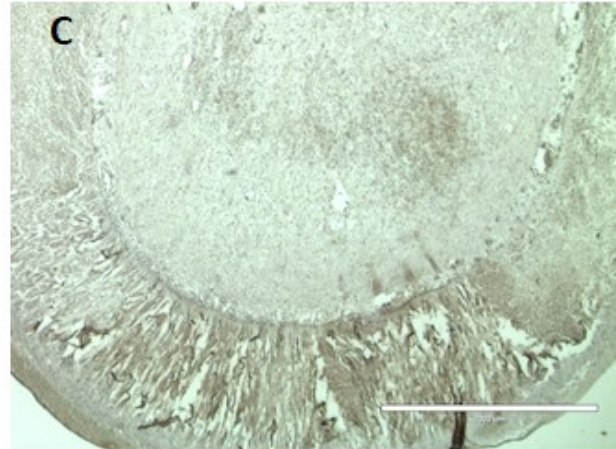
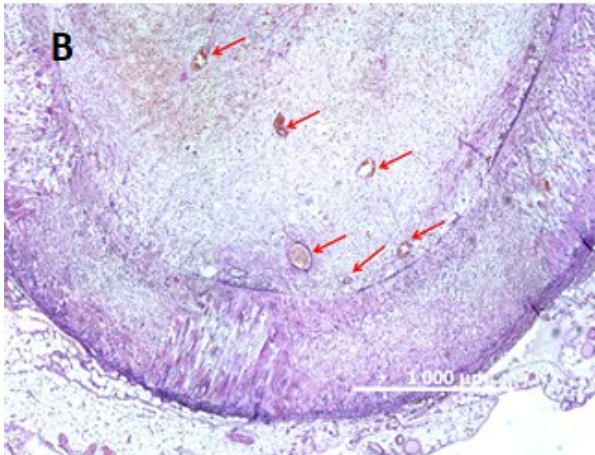
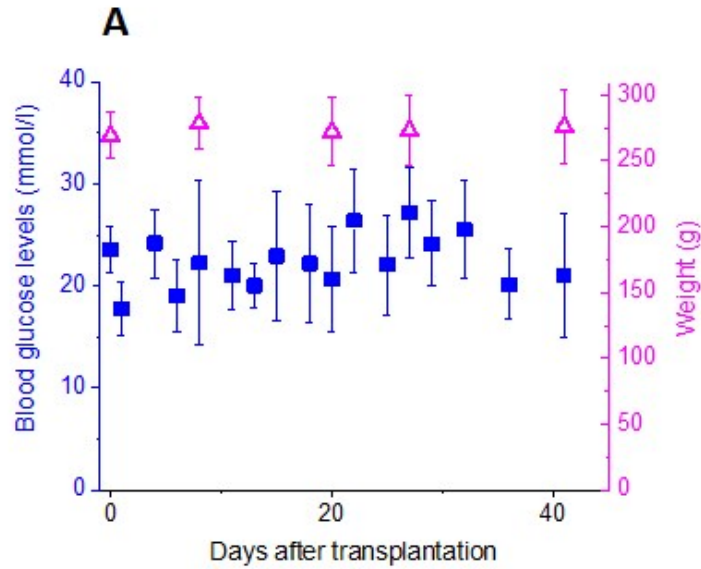


Figure SI 3 Evaluation of islets survival in a pouch prepared using the pristine PLCL scaffold (4-week preimplantation, six animals in the study). **(A)** *Blood glucose levels* (a left y-axis) in streptozotocin diabetic rats after transplantation of pancreatic islets. Blood glucose levels ranged around the value of 20 mmol/l within the time of the experiment, i.e., 41 days. The body weight (a right y-axis) of all animals remained constant within the time of the experiment. **(B)** *The H&E staining* showed that the cavity was fully filled with connective tissue seven weeks after transplantation and some vessels were detected as well (red arrows). **(C)** *The anti-insulin staining* of the graft (no brown color objects observed) showed no presence of beta-cells in the lumen of the cavity (in the case of the examined cuts) seven weeks after islets transplantation. Bar - 1000 μm .

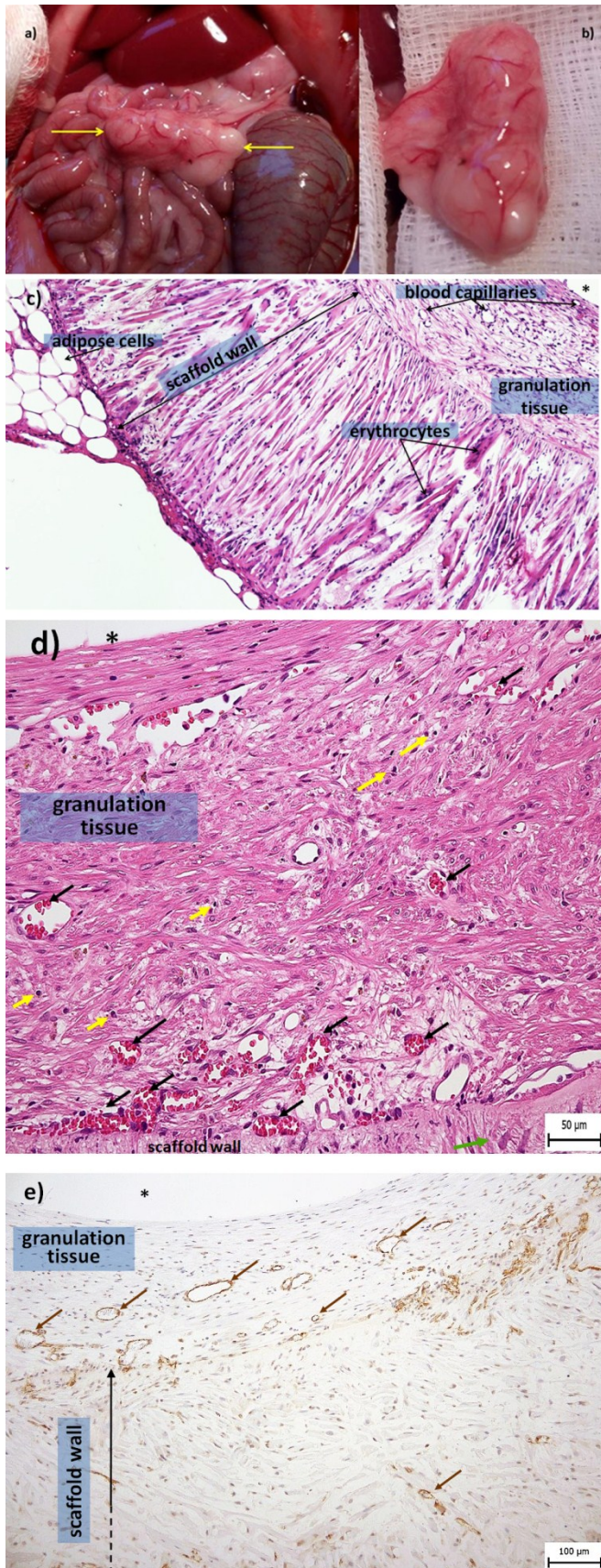


Figure SI 4 Heparin/VEGF50 PLCL scaffolds four weeks after implantation into the greater omentum. Macroscopic overview of the implanted scaffold *in situ* (a) – yellow arrows indicate the engrafted scaffold; The scaffold out of the abdominal cavity placed over the wet gauze (b); Hematoxylin/Eosin (H&E) staining (c) presenting an illustrative cell ingrowth through the scaffold wall and formation of granulation tissue within the internal cavity of the capsule; H&E (d) and immunohistological anti-CD31 (e) staining at higher magnification demonstrated granulation tissue rich in vascular structures close to the inner surface of the scaffold as well as inside of the cavity (red cells – black arrows, lymphocytes and plasmocytes – yellow arrows, histocyte – green arrow (d); formed vessels – brown arrows (e)); *- indicates the internal cavity of the capsule

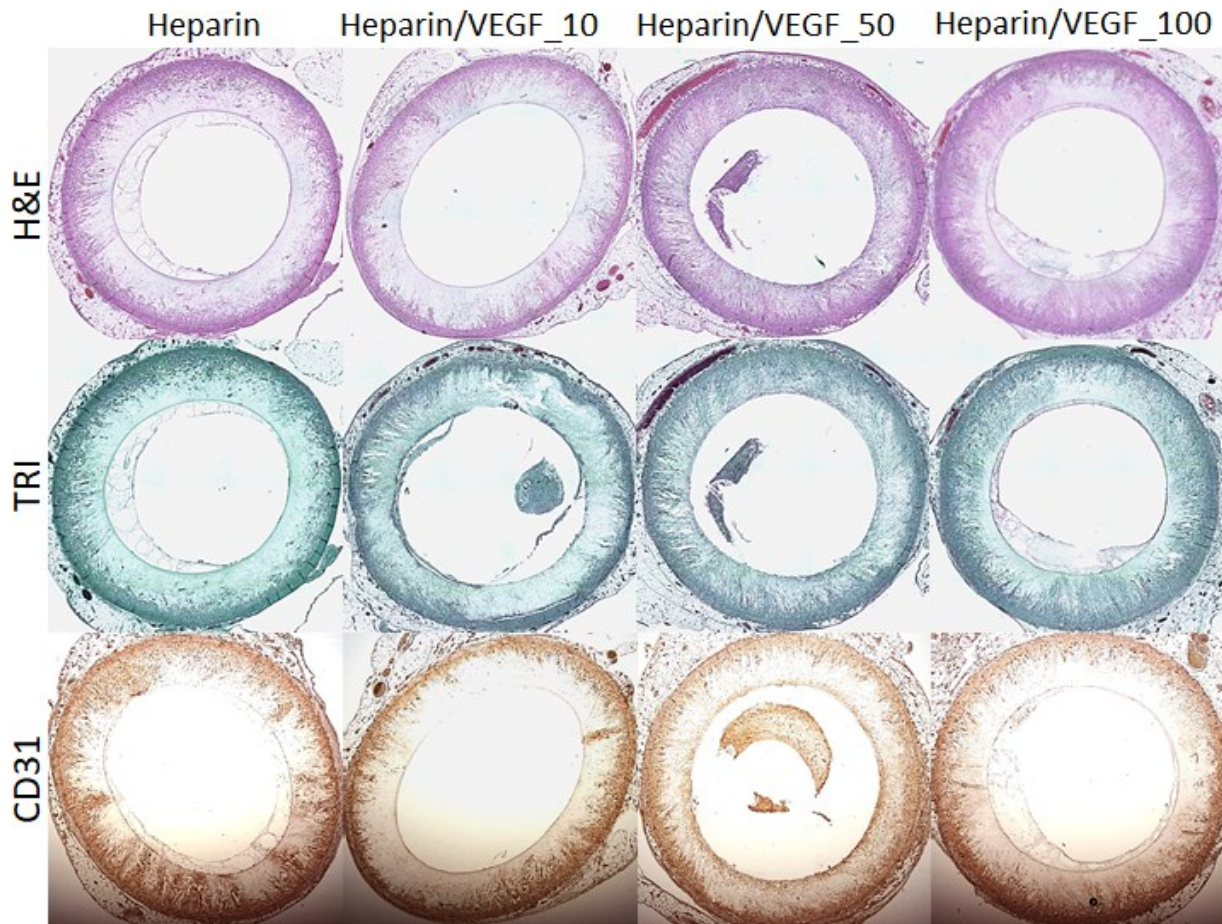


Figure SI 5 Dose-dependent experiment: Overview images of cross-sections of heparin- and heparin/VEGF PLCL capsules loaded with 0.4 μg VEGF/mg scaffold (heparin/VEGF_10), 2 μg VEGF/mg scaffold (heparin/VEGF_50) and 4 μg VEGF/mg scaffold (heparin/VEGF_100) two weeks after implantation in greater omentum. (H&E, Masson's Trichrome and anti-CD31 stainings, original magnification $\times 20$).

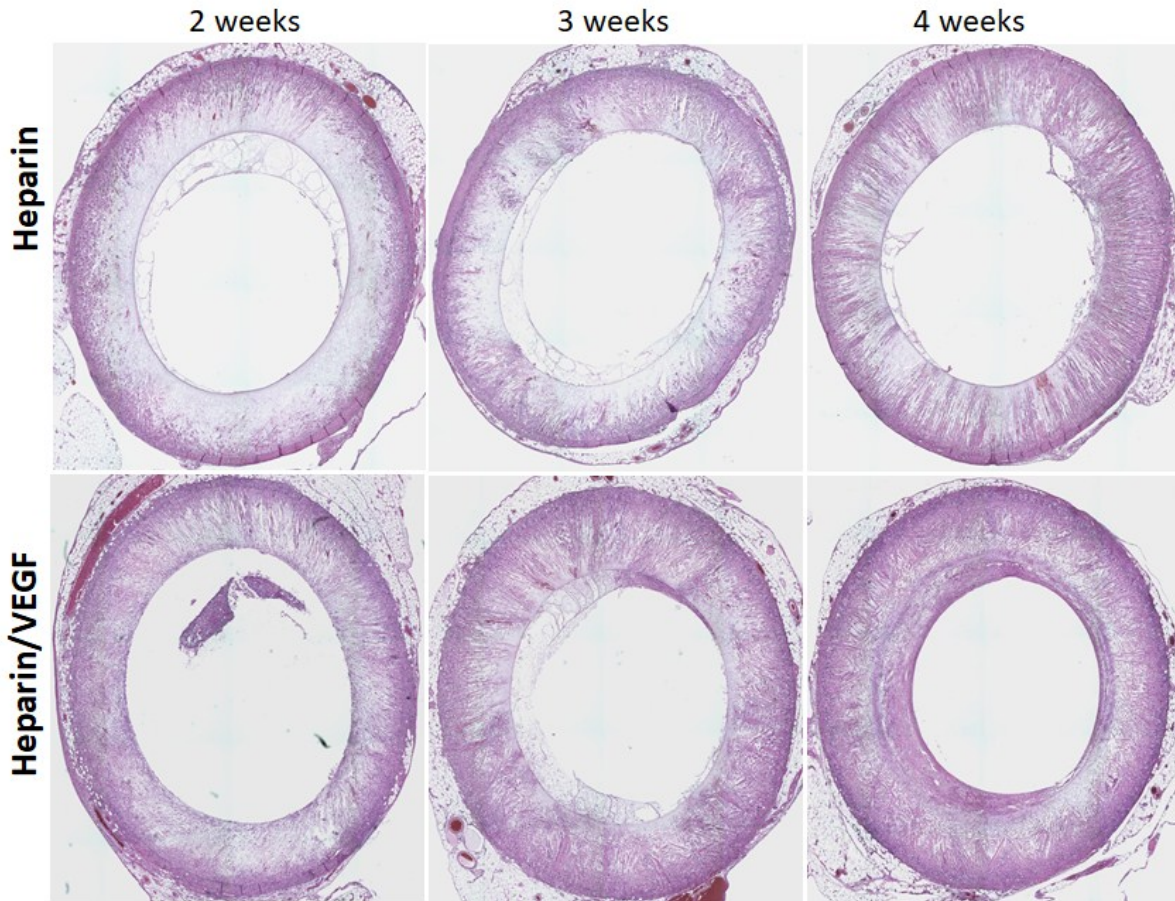


Figure SI 6 Time-dependent experiment (evaluation of the time required for sufficient ingrowth of the tissue and formation of new vessels on the inner surface of the capsule), H&E staining: Overview images of cross-sections of the heparin- and heparin/VEGF₅₀ capsules (2 μ g VEGF/mg scaffold) 2, 3 and 4-week after implantation in Lewis rats in the greater omentum (original magnification $\times 20$).

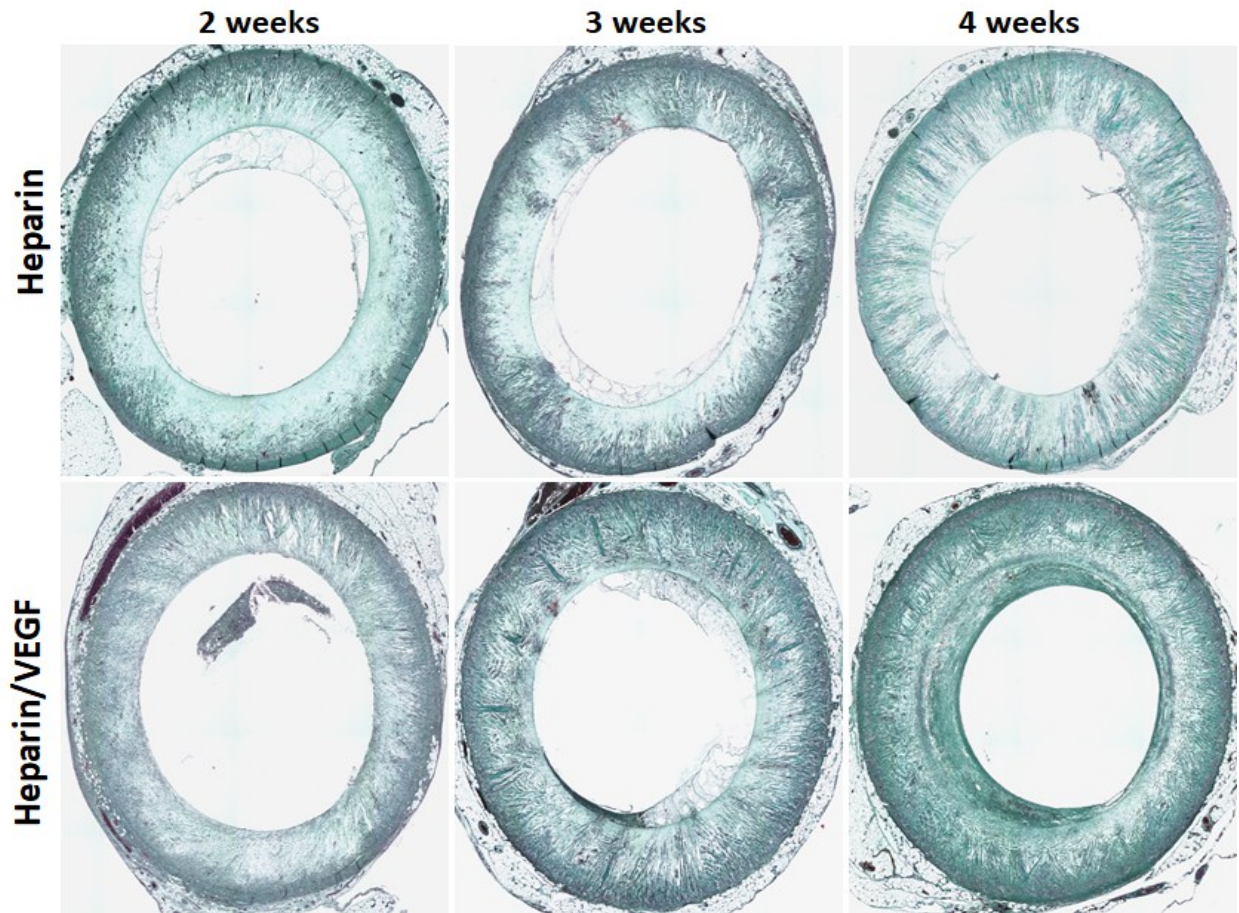


Figure SI 7 Time-dependent experiment (evaluation of the time required for sufficient ingrowth of the tissue and formation of new vessels on the inner surface of the capsule), Masson's Trichrome staining: Overview images of cross-sections of the heparin- and heparin/VEGF₅₀ capsules (2 μ g VEGF/mg scaffold) 2, 3 and 4-week after implantation in Lewis rats in the greater omentum. (Original magnification $\times 20$).

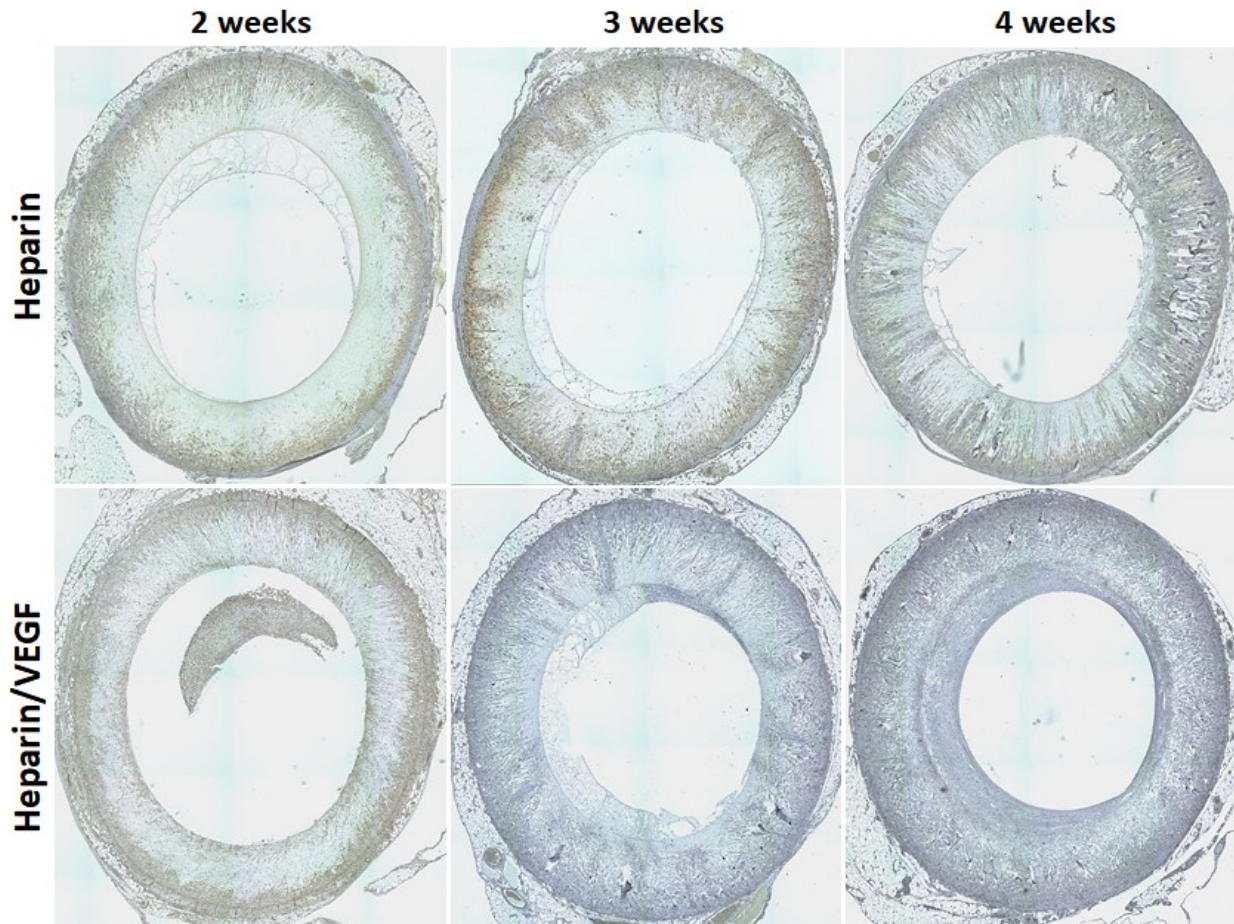


Figure SI 8 Time-dependent experiment (evaluation of the time required for sufficient ingrowth of the tissue and formation of new vessels on the inner surface of the capsule), CD-31 staining: Overview images of cross-sections of the heparin- and heparin/VEGF₅₀ capsules (2 μ g VEGF/mg scaffold) 2, 3 and 4-week after implantation in Lewis rats in the greater omentum. (original magnification $\times 20$).

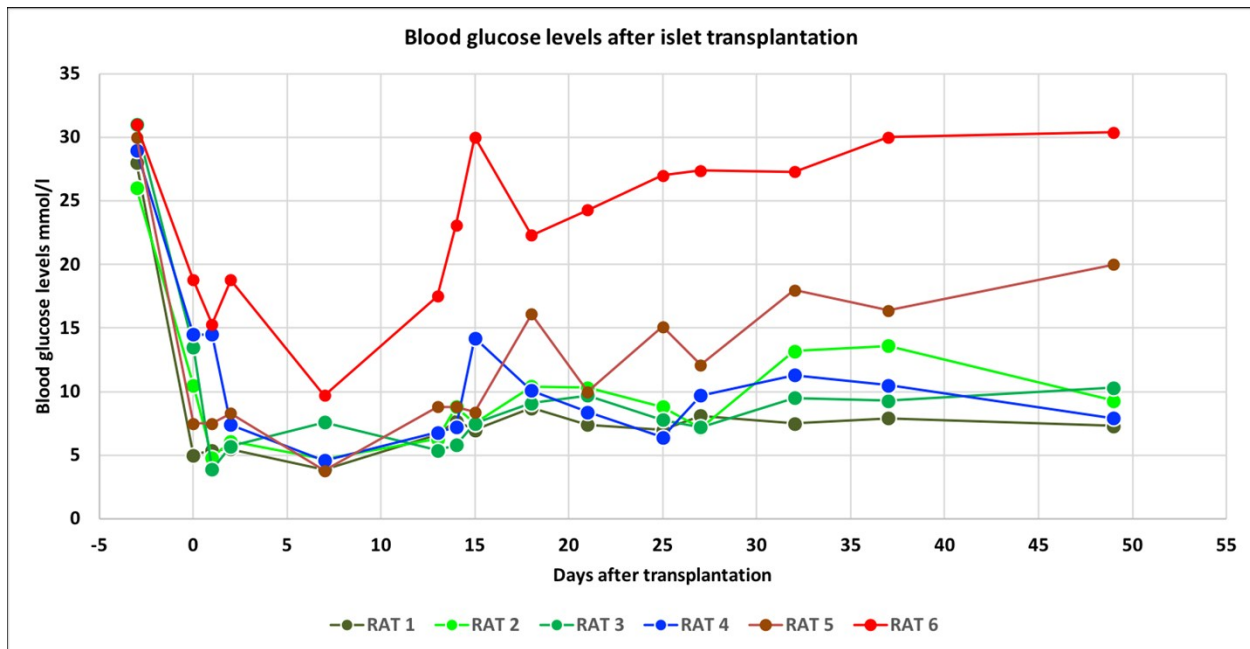


Figure SI 9: Blood glucose levels in streptozotocin diabetic rats after transplantation of pancreatic islets into the cavity formed by the VEGF-loaded PLCL capsular scaffold (Heparin/VEGF50). Blood glucose levels in five of six animals dropped down to physiological range within three days after transplantation. The graft did not start to function in one animal (red curve), and the function failure appeared in the second animal three weeks after transplantation (brown curve). In four animals, the function of the graft was detected until the end of the study on Day 50 after transplantation.

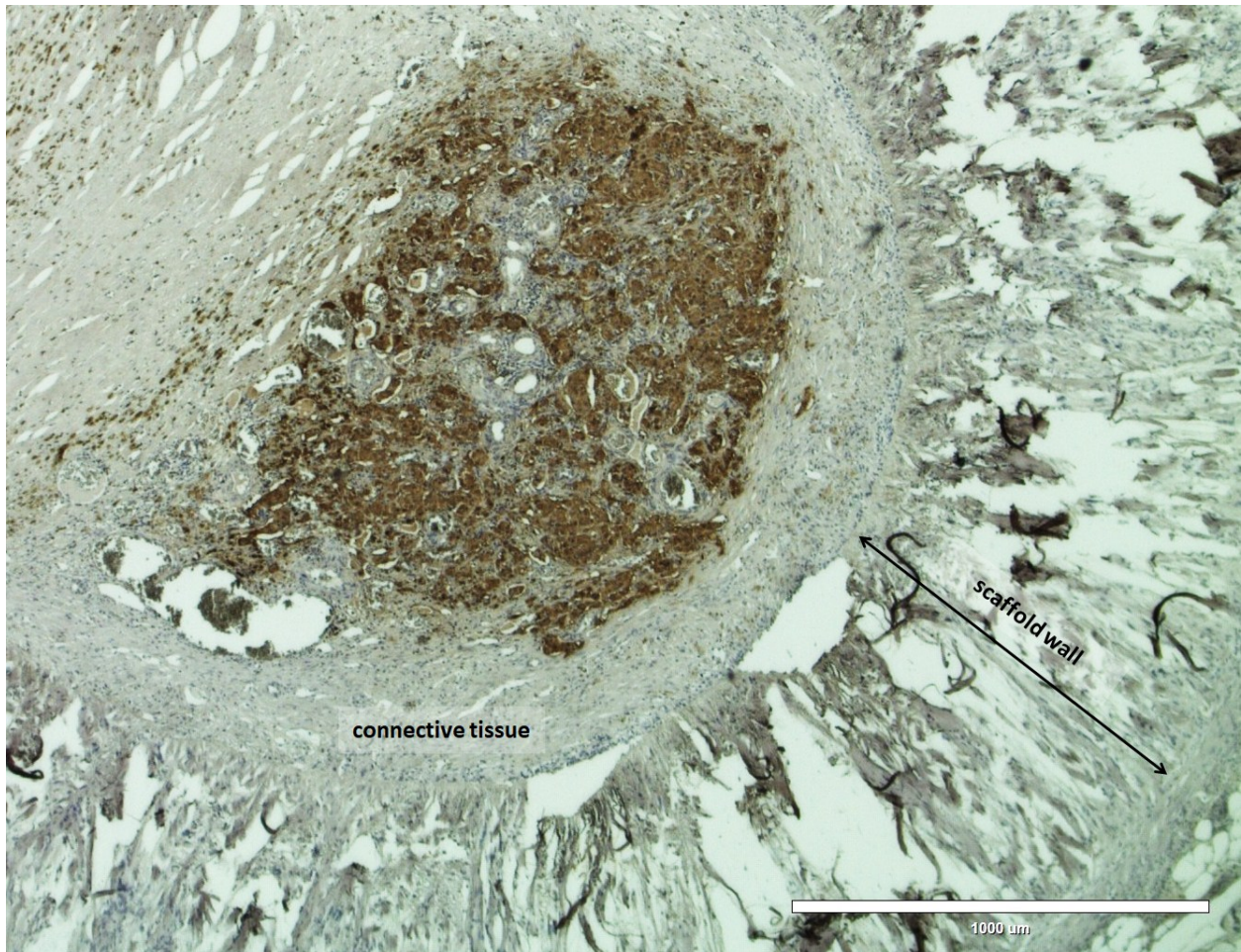


Figure SI 10 Immunohistology of the graft seven weeks after transplantation: Anti-insulin staining proved the presence of beta-cells within engrafted islets (brown color). *- indicates the lumen of the capsule filled with tissue. The defects in the wall of the polymer scaffold as well as in the capsule lumen observed as white holes were caused by staining procedure. Bar-1000 μm.

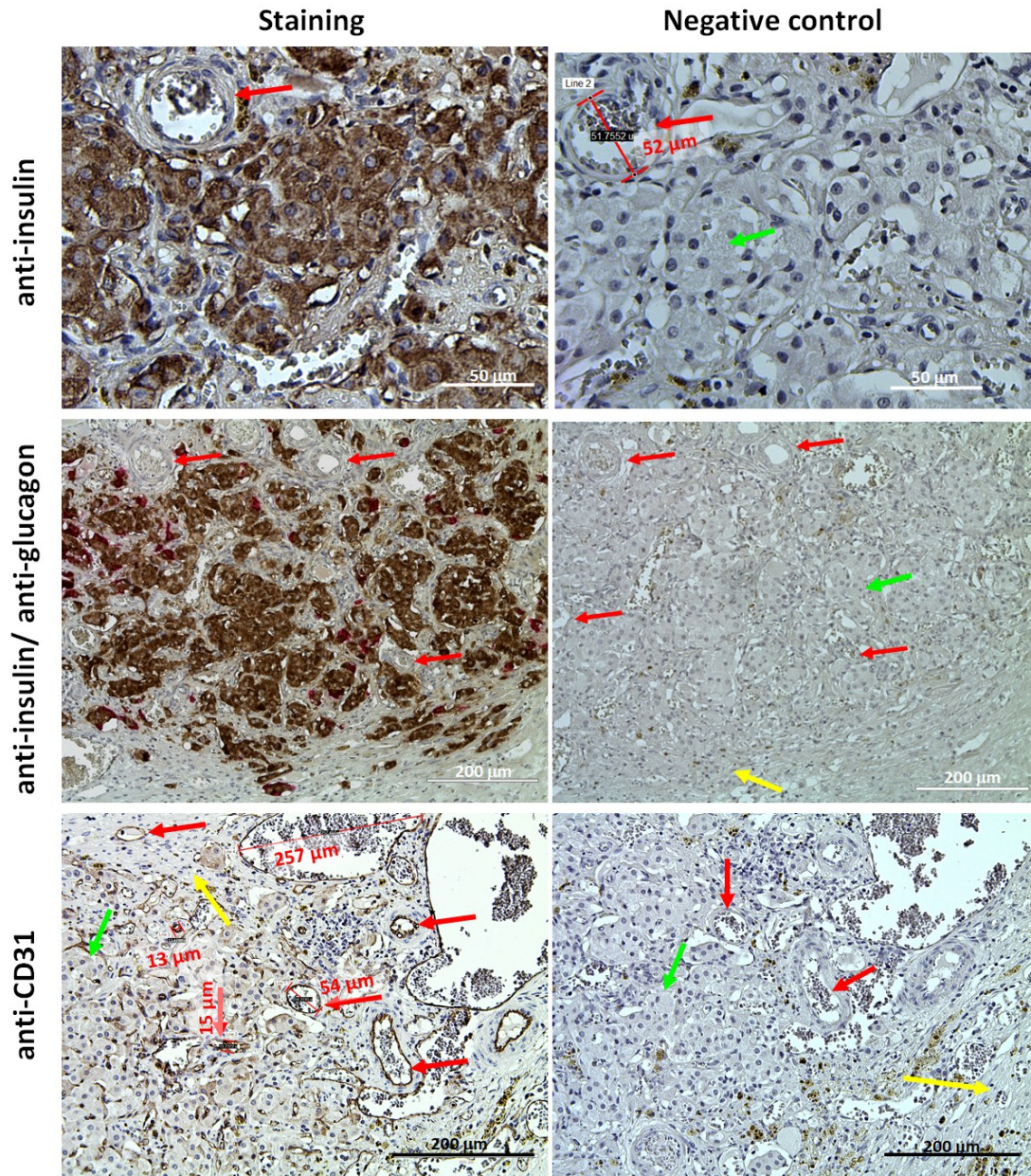


Figure SI 11 – Immunohistology of the graft seven weeks after transplantation: Anti-insulin (brown color) and anti-glucagon (red color) staining proved the presence of beta-cells and alfa-cells within engrafted islets. Anti-CD31 staining proved endothelial cells in the graft and surrounding tissues. The numbers are diameters of detected formed vessels. Negative controls of the same samples without primary antibody are presented as well. Fibrotic tissue is indicated by yellow arrows, endothelial cells by red arrows and endocrine cells of islets by green arrows.